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Afferent Spontaneous Electrical Activity Promotes the Survival of Target Cells in the Developing Retinotectal System of the Rat

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This study was undertaken to investigate the role of afferent spontaneous electrical activity in regulating death of target cells in the developing mammalian visual system. We show here that naturally occurring cell death in the rat superior colliculus is greatly augmented when the spontaneous firing of retinal ganglion cells is transiently blocked with TTX. An increased number of dying cells is already observed after 1 hr of afferent blockade. A 50% increase of cell death is reached after 3 hr of blockade, an effect that closely parallels increased cell death caused by eye enucleation after similar intervals of time. These results suggest that, during development, input cells exert a trophic action on target cells, which is prevented by silencing input electrical activity. A likely explanation of this effect is that the spontaneous firing of input cells causes the release by afferent fibers of a trophic agent promoting the survival of target cells.

[Key words: cell death, spontaneous activity, TTX, superior colliculus, visual system, development]

The mature nervous system is traversed by a continuous flow of electrical impulses. Sensory signals and neural commands to different body parts travel encoded in trains of electrical impulses along neural processes. On this flow, the life of the organism relies. Indeed, most natural lethal compounds act blocking or altering neural electrical communication.

In the early stages of its formation, the nervous system does not sustain the same vital role. Neural pathways cannot yet convey sensory information, or guide the functions they will control in adulthood. Yet, neurons start producing electrical impulses as an early aspect of their developmental program. In the mammalian visual system, for example, cells are electrically active long before they have formed mature connections and even before many of them have been eliminated in the natural process of cell death, which leads many newly formed neurons to degenerate quickly and disappear (Galli and Maffei, 1988; Maffei and Galli-Resta, 1990; Meister et al., 1991).

When developing neurons destined to connect, first come into contact, trophic exchanges rather than electrical communication seem to dominate their interactions. Developing neurons need and exchange trophic substances to promote their survival and to differentiate further (reviewed in Purves, 1988). Different trophic substances are known to be present in the developing nervous system, each vital for subpopulations of neurons (Walicke, 1988; Thoenen, 1991).

The study of trophic interactions has revealed that target cells are important for the survival of input cells and that, in turn, input cells are necessary to promote a normal development of their target. A number of studies have shown that the early removal of the main afferent system leads to massive degeneration in the target (reviewed in Oppenheim, 1981, 1991). It is natural to wonder how input cells may be crucial in shaping their developing target. The early presence of spontaneous electrical activity suggests that it could be a main component of the input action on target during development. The following experiments were planned to investigate whether, in the developing mammalian visual system, input spontaneous activity may modulate cell death in the target, thereby controlling one of the major developmental events that shape the target structure. The study has been focused on the rat retinotectal system, where retinal activity was transiently blocked by the use of TTX, and the effects of such blockade on cell death in the superior colliculus, the major retinal target, were observed.

Materials and Methods

Long–Evans rats were mated in the laboratory colony. Most of the experiments were performed on animals of 6 d of age (P6). Neonates of 2 d of age (P2) as well as fetuses of 20 d of gestation (E20) were also studied. The experimental procedure consisted of transiently blocking retinal spontaneous electrical activity in the right eye by the use of TTX, a sodium channel blocker. Since retinal projections are already nearly completely crossed at the ages studied (Bunt et al., 1983), our procedure leads to a block of the major input activity to the left side of the colliculus, contralateral to the injected eye. We will refer to this side as the treated side. The right side of the colliculus, innervated by the left eye where spontaneous activity was left unaltered, was always used as an internal control for the normal density of dying cells. The correctness of this assumption is documented in the results.

Intraocular TTX injections. Rat pups were anesthetized with ether, the right eye lid was gently opened, and 100 ± 25 nl of 1.6×10^{-3} M solution of TTX (Sigma Chemicals, St. Louis, MO) in citrate buffer (0.1 M, pH 4.8) was injected with a fine glass micropipette, right behind the temporal ridge of the ora serrata. The injection was performed under a surgical stereomicroscope with a hydraulic pressure injector. After the injection, the eye was washed with sterile saline and an antibiotic collyrium was applied. The pups were returned to the mother soon after complete recovery from the anesthesia. As a control, some animals were injected with citrate buffer using the same procedure. In eight animals, we enucleated the right eye under ether anesthesia.

Electrophysiological controls. In 14 P6 animals, we tested electrophysiologically the effectiveness and the duration of the blockade induced by the doses of TTX used. Electrophysiological recordings were performed in rat pups anesthetized with urethane (1.25 gm/kg body weight) before TTX injection, since young animals badly tolerate multiple anesthetic administrations. Heart rate and body temperature were continuously monitored. The body temperature was kept constant using

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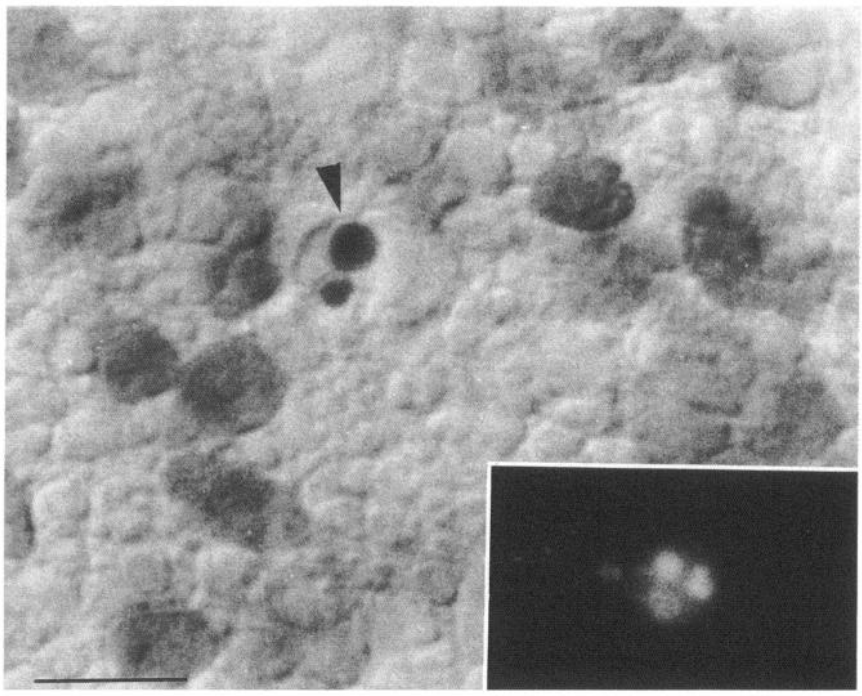

Figure 1. Dying cell in the superficial layers of the superior colliculus of a normal P6 rat (Nomarski optics). Dying cells can be recognized by the presence of several darkly stained masses of chromatin in their cell body (*arrowhead*). Some dying cells were neurons projecting outside the superior colliculus. A retrograde fluorescent tracer injected at P4 in the LP, a brain region to which the superior colliculus projects, can be visualized at P6 inside dying cells of the colliculus (cell in the *inset*). Scale bar, 10 μm.

an infrared lamp driven by a temperature control device. At the times after the TTX injection when we wished to control the blockade, anesthesia was supplemented if necessary, and then a small cannula was inserted in the trachea. The animals were then paralyzed with Pavulon (pancuronium bromide, 0.01–0.03 cc, injected i.m.) and artificially ventilated. Good physiological conditions are critical for recording in neonatal rats, and neuronal activity deteriorates considerably if the heart rate goes below 350 beats/min (see Fitzgerald, 1985). After the animals had been paralyzed, good physiological conditions were usually maintained for about 2 hr. A detailed description of the recording procedure can be found in Galli and Maffei (1988) and Maffei and Galli-Resta (1990). Two animals injected with TTX under urethane anesthesia were analyzed for the density of dying cells in the superior colliculus. No difference in the effect was found with respect to what observed in animals injected with TTX under ether anesthesia.

Histological processing. At various times after intraocular injection, animals were anesthetized with chloral hydrate (4.2 gm/kg body weight) and perfused with a 4% solution of paraformaldehyde in phosphate buffer (0.1 M, pH 7.4). The region of the brain containing the superior colliculus was postfixed for 10 d and included in paraffin. Serial coronal sections 10 μm thick were cut throughout the extent of the superior colliculus, and stained with a Feulgen reaction that enhanced the detectability of pyknotic cells (Wyllie et al., 1980). Retinas were routinely whole-mounted to control for lesions as well as for control counts. Only animals with no significant lesions in the retinas were considered in this study.

Injection of fluorescent tracers. Under ether anesthesia, 4 P4 and 12 P2 animals were stereotaxically injected with 100–200 nl of a 4% saline solution of Fluorogold (Fluorochrome Inc.) in the lateral posterior nucleus (LP), a brain region to which the superior colliculus projects, forming multiple maps (Sefton and Dreher, 1985). At P6, the animals were killed and perfused with paraformaldehyde in phosphate buffer; the brains were kept in 15% sucrose in phosphate buffer overnight and cut with a cryostat in 10 μm serial sections.

Staining for macrophages. Three P6 animals were injected with TTX as described above and killed 5 hr later to be analyzed for macrophage distribution. A periodate-lysine-paraformaldehyde fixative was used to improve the reaction. OX42 (a kind gift of Dr. Hugh Perry, University of Oxford), an antibody recognizing the CD3 surface antigen of macrophages, was used to stain macrophage selectively with a standard immunocytochemical procedure using the ABC method performed on serial coronal cryostat sections cut throughout the extent of the superior colliculus. Macrophages were counted with a procedure similar to that described below for dying cells. Only cells having their cell body in the sampling field were considered.

Counting procedure and statistical analysis. Dying cells were counted in every fourth serial section under a light microscope with a 100× immersion objective in six sampling regions (100 × 100 μm²), equally spaced from medial to lateral in the superficial layers on each side of the superior colliculus. This resulted in about 400 samples per specimen. The counts were sometimes performed by two independent observers, and in all cases the experimenter never knew which was the treated side of the specimen before counting. Samples of total cell density were taken in four equally spaced sections of the specimen, drawing under a camera lucida the cells found in each of six sampling regions (90 × 90 μm²) equally spaced from medial to lateral in the superficial layers on each side of the superior colliculus. The density of pyknotic cells was measured as density for 1000 living cells before the ratio between the densities of pyknotic cells in the treated and the normal side was computed. This procedure makes the results independent of any asymmetrical shrinkage of the tissue induced by histological treatment. Counts of fluorescent cells after LP injection were performed on a lower number of sections. Data from the treated and the normal side of the colliculus were compared using a block comparison test contrasting data from homologous locations in the two sides of the superior colliculus (see, e.g., Armitage, 1971).

Results

Normal density and distribution of dying cells in the rat superior colliculus during development

Dying cells are observed in the optic layers of the rat superior colliculus during the first 2 postnatal weeks (Giordano et al., 1980) and at least in the last few days of gestation (L. Galli-Resta, M. Ensini, E. Fusco, A. Gravina, and B. Margheritti, unpublished observations). Dying cells can be recognized by the presence of several darkly stained masses of chromatin in their cell bodies (Fig. 1). Due to their appearance, degenerating cells are often referred to as pyknotic cells.

Many of the pyknotic cells that we observed in the rat superior colliculus were neurons already projecting out of the colliculus. A fluorescent dye injected in the LP, a nucleus to which the superior colliculus projects, can be seen inside pyknotic colli-

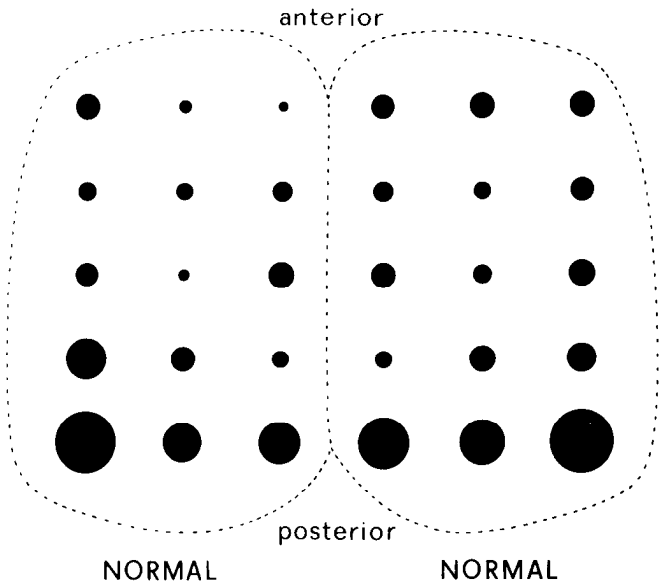

Figure 2. Normal distribution of dying cells in the superior colliculus of P6 rats. The superior colliculus is represented as seen from above. The *diameter of each circle* is proportional to the density of pyknotic cells observed in the corresponding location of the superior colliculus, and represents the average density of pyknotic cells observed in 12 sampling fields. No significant difference in the distribution or in the average density of dying cells was observed between the two sides of the colliculus.

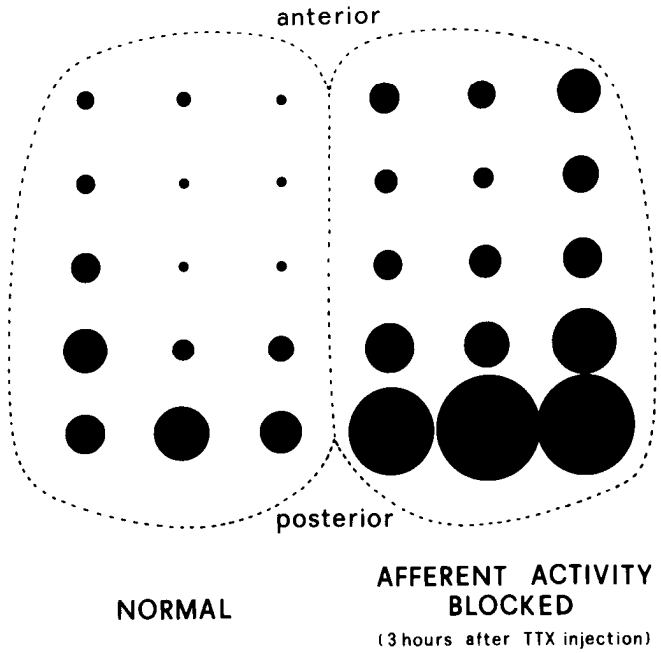

Figure 3. Distribution of dying cells in the superior colliculus of a P6 rat after retinal afferents to one side have been electrically blocked for 3 hr. The *diameter of each circle* is proportional to the density of pyknotic cells observed in the corresponding location of the superior colliculus, which is represented as seen from above. A much higher density of dying cells is observed throughout the treated side of the colliculus, innervated by retinal afferents silenced for 3 hr. The average difference in the total density of dying cells with respect to the normal control side of the superior colliculus is 50%.

cular cells 2 d later, suggesting that these cells picked up the dye when previously projecting to the LP nucleus (inset in Fig. 1).

As in many other neural populations few pyknotic cells are observed at each time, although where assessed, the total number of dying cells is remarkable. This is due to the rapid removal of pyknotic cells, which are estimated to last a few hours (see Purves and Lichtman, 1985). The normal distribution of pyknotic cells in the superior colliculus of P6 rats is illustrated in Figure 2, where the colliculus is shown as seen from above. The size of each circle is proportional to the density of dying cells found in the corresponding location. More pyknotic cells are observed at this stage in the posterior regions of the colliculus. In normal P6 animals, no differences in the distribution and average density of dying cells are observed between the right and left side of the colliculus. The normal average density is 16.9 ± 0.4 pyknotic cells/1000 live cells ($N = 4$). Intraocular injection of citrate buffer does not affect the distribution of pyknotic cells in the superior colliculus, leading to an average density of 16.8 ± 0.2 pyknotic cells/1000 live cells ($N = 4$). In a sample of six normal P6 rats and 4 P6 animals injected with citrate buffer in one eye, we found a maximal difference of 5.4% in the density of pyknotic cells between the two sides of the colliculus, with an average difference in absolute value of 2.5 ± 2.8%. Similar results were obtained in P2 animals (2.7 ± 2%; $N = 3$) and E20 animals (2.8 ± 1.9%; $N = 3$).

Afferent activity blockade induces an increase in the number of dying cells in the target

Blockade of retinal spontaneous electrical activity by TTX induces a rapid increase in the density of pyknotic cells in the superficial layers of the colliculus, to which retinal afferents project. An example is shown in Figure 3, where the diameter of each circle is proportional to the density of dying cells in the corresponding area of the colliculus. The increase in the density of pyknotic cells in the side of the colliculus innervated by the retina electrically silenced is 50% on average, illustrating a case observed after 3 hr of retinal electrical activity blockade.

Single-unit recordings in the retina revealed that, at the doses used, intraocular TTX injection induces a total blockade of retinal ganglion cell impulse activity in a few minutes (Fig. 4*A,B*). No activity was recorded 2 and 3 hr after the injection. Occasional spikes evoked by antidromic electrical stimulation could be recorded after 4 hr (Fig. 4*C*), while 5 hr after the TTX injection both spontaneous and antidromically evoked activity of ganglion cells was reestablished in four out of five recorded animals. Eight hours after TTX injection, activity recorded in the retina was indistinguishable from that recorded in normal littermates (Fig. 4*D,E*).

An increased density of dying cells is observed in the optic layers of the superior colliculus as early as 1 hr after retinal electrical blockade. The effect is maximal after 3 hr of blockade, starts to decline 5 hr after the TTX injection (when some retinal ganglion cells may have resumed their firing), and is over by 8–12 hr (Fig. 5). While retinal blockade lasts about 4.5–5.5 hr, the increase in pyknotic cell density is not over so quickly, and is observed until 8–12 hr after TTX injection. This delay is likely to reflect the time necessary for pyknotic cells to be cleared. From these results we can estimate that, in the superior colliculus, pyknotic cells are cleared in about 2.5–7.5 hr.

The effect of retinal blockade is even more striking on the population of neurons labeled by injection of a retrograde fluorescent tracer in the LP. After 3 hr of retinal afferent blockade, we observed an average increase of 100% in the density of

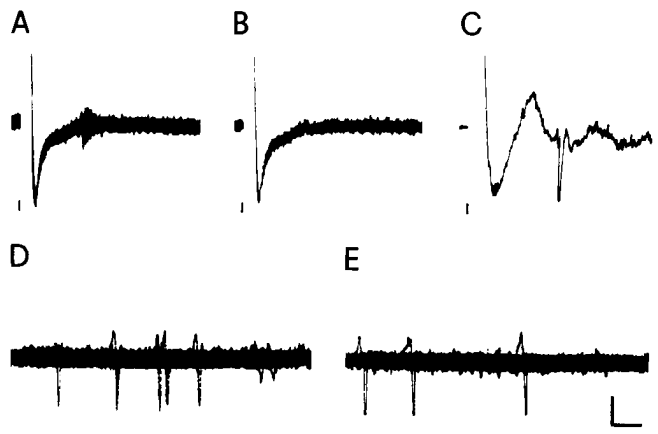

Figure 4. Electrophysiological controls of TTX blockade duration. At the dose used, a single intraocular injection of TTX causes a nearly immediate blockade of retinal ganglion cell impulse activity. In *A*, several superimposed sweeps show the decreasing amplitude of the spikes evoked by antidromical stimulation soon after TTX injection. Five minutes after TTX injection, spontaneous and evoked spikes have completely disappeared (*B*). Occasional antidromically activated spikes could be recorded after 4 hr (*C*), but only 5 hr after TTX injection, activity had recovered in four out of five recorded animals. Eight hours after TTX injection, activity has completely recovered (*E*), and is indistinguishable from normal (*D*). All traces but *C* show several superimposed sweeps. The *vertical bars* below traces *A–C* indicate the time of electrical stimulation. At this age, spikes are larger in amplitude than the mature spikes, an effect possibly due to the immaturity of the Na^+ channels (Wollner et al., 1988). Calibration: 300 μV; 20 msec (*A, B*) or 5 msec (*C–E*).

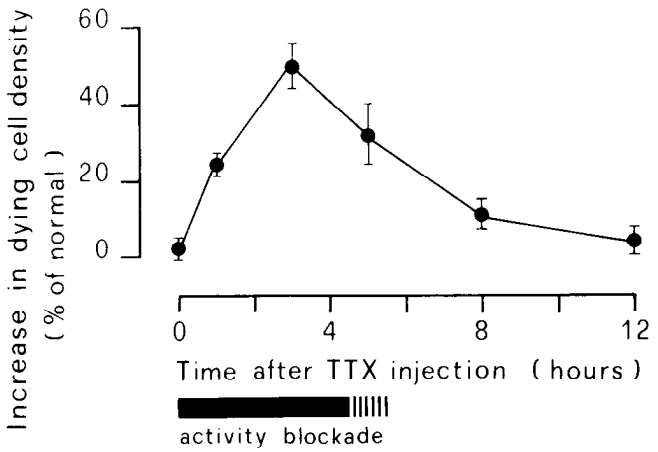

Figure 5. Time course of the increase in the density of dying cells in the superior colliculus induced by electrical blockade of retinal afferents. Electrical blockade of retinal afferents causes an increase in the density of dying cells in the superior colliculus, which is already observable after 1 hr of blockade. The effect is maximal after 3 hr of blockade and starts to decline 5 hr after the TTX injection, when most retinal ganglion cells have already resumed their firing. Control electrophysiological recordings, performed in a separate set of animals, have shown that retinal activity recovers in about 4.5–5.5 hr from the blockade induced by the dose of TTX used. This estimated duration of blockade is represented by the *horizontal bar* below the graph, the portion between 4.5 and 5.5 hr being interrupted. The increase in pyknotic cell density induced by the blockade of retinal afferents is not over as soon as activity resumes, since some time is required to clear pyknotic cells. A total of 30 cases were analyzed between 1 and 12 hr after TTX injection, with a range of three to eight cases per time point.

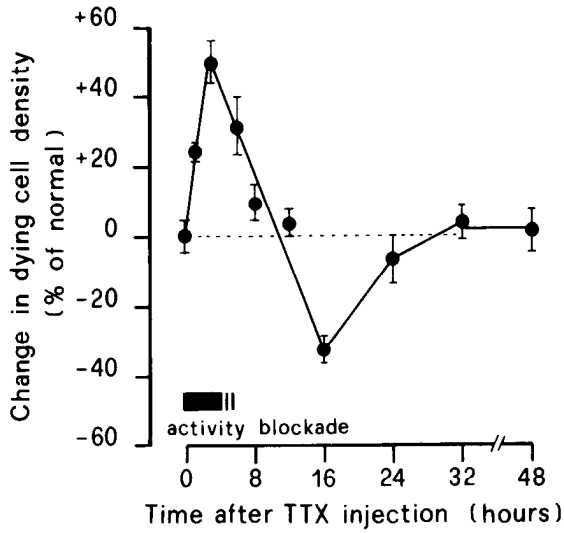

Figure 6. A transient decrease in the density of dying cells in the superior colliculus follows the end of the increase in pyknotic cell density induced by electrical blockade of retinal afferents. The *polygonal line* traced in the graph is only meant to stress the biphasic behavior observed, with higher amounts of cell death in the superior colliculus while retina activity is blocked and a following transient decrease in dying cell density. A total of 45 cases were analyzed for this time curve, ranging from a minimum of two cases to a maximum of eight cases per time point.

labeled dying neurons (120 ± 31 labeled dying neurons/1000 labeled cells in the treated side, 65 ± 16 labeled dying neurons/1000 labeled cells in the normal side; $N = 9$). In three normal animals, we observed no difference in the density of pyknotic cells between the two sides of the colliculus. The error in this value ranges around 30% due to the difficulty of counting fluorescently labeled cells.

Recovery from blockade

After the increase in dying cell density induced by blockade of afferent activity is over, a normal rate of cell death is not reestablished right away in the superior colliculus. Rather, the phase of increased dying cell density is followed by a transient period in which the density of dying cells in the superficial layers of the colliculus is decreased with respect to normal (Fig. 6). The decrease is maximal around 16 hr after TTX injection (11.5–10.5 hr after activity has recovered). The amount of cell death is close to normal 24 hr after the TTX injection, and counts at 32 and 48 hr do not show any difference between the treated and the normal side of the superior colliculus.

The occurrence of a transient phase of lower pyknotic cell density requires the recovery of spontaneous afferent activity, as we found making two consecutive injections of TTX, at an 8 hr interval. The second TTX injection silences retinal afferents at times when the increase in pyknotic cells induced by the previous blockade is nearly over, and the transient phase of lower density of dying cells would normally follow. As illustrated in Figure 7, a second blockade of retinal afferents induces an immediate increase in the density of dying cells. This increase lasts throughout the blockade and is nearly over 8 hr after the last TTX injection (16 hr after the beginning of the first blockade). Twenty-four hours after the first TTX injection, when afferent activity has recovered from the last blockade by about 10.5–11.5 hr, a lower than normal density of pyknotic cells is

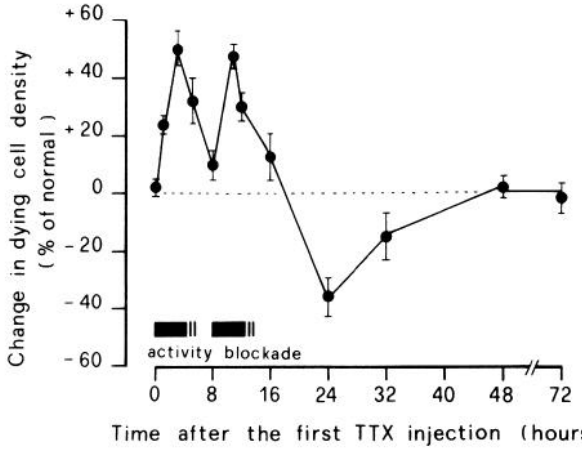

Figure 7. The transient decrease in dying cell density requires recovery of afferent spontaneous activity from previous blockade. Two consecutive injections of TTX, the second given 8 hr after the first, caused two consecutive blocks of afferent activity, as illustrated by the *horizontal bars* below the graph. An increased amount of dying cells is observed throughout the first phase of afferent blockade and is still present 8 hr after the injection since some hours are required to clear pyknotic cells. The second TTX injection induces an immediate increase in the density of dying cells, at times when the effect of previous blockade is nearly over and a transient phase of lower density of dying cells would normally follow. This increase lasts throughout the blockade and is nearly over 8 hr after the last TTX injection (16 hr after the beginning of the first blockade). Twenty-four hours after the first TTX injection, when afferent activity has recovered from the last blockade by about 10.5–11.5 hr, a lower than normal density of pyknotic cells is observed in the superior colliculus. Difference from normal is no longer observed 48 and 72 hr following the first injection. A total of 49 cases were analyzed for this time curve, ranging from a minimum of two cases to a maximum of eight cases per time point.

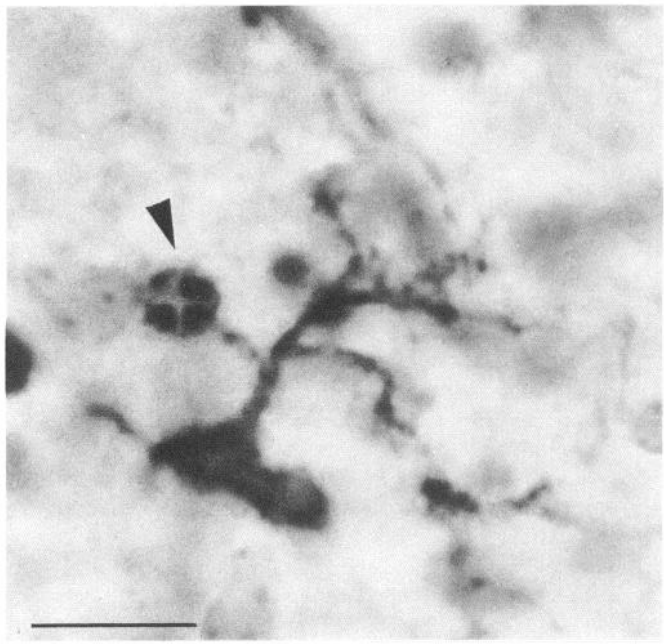

Figure 8. Macrophage in the process of engulfing a dying cell in the superficial layers of the superior colliculus of a P6 rat. Macrophages were selectively stained with an antibody against the CD3 surface antigen, and the tissue was then counterstained with cresyl violet to stain pyknotic nuclei. The darkly stained vacuoli of the dying cell are clearly visible in the picture (*arrowhead*). The dark rim surrounding the dying cell as well as the branch reaching it are processes of the macrophage, positive for CD3. Scale bar, 10 μm.

observed in the superior colliculus. Difference from normal is no longer observed 48 and 72 hr following the first injection. Therefore, the effect of the second TTX injection mimics the effect of the first. While afferent electrical activity is prevented, a higher number of cells die in the colliculus. A transient phase of lower than normal density of dying cells follows only when afferent activity has resumed from previous blockade.

Experiments at different ages

The effects of retinal activity blockade on cell death in the superior colliculus here described were analyzed in details in P6 animals, an age when the normal density of dying cells in the superior colliculus is maximal (Giordano et al., 1980). The same qualitative effects were observed in E20 ($N = 12$) and P2 ($N = 10$) animals although with a slower time course. At both ages the density of dying cells was increased after 3 hr of afferent blockade ($+51 \pm 12\%$ at E20, $N = 6$; $+46 \pm 10\%$ at P2, $N = 5$), while a lower density of dying cells is observed 24 hr after the intraocular TTX injection ($-70 \pm 20\%$ at E20, $N = 6$; $-61 \pm 18\%$ at P2, $N = 5$). No dying cells are observed in the superior colliculus of adult normal rats, and cell death is not induced by retinal blockade in adult animals ($N = 5$).

Electrical blockade of retinal afferents simulates the early effects of eye enucleation

Previous studies of the mammalian visual system have shown that the removal of one eye during development induces degeneration of many target cells (Guillery, 1973; Kalil, 1980). In P6 rats, we found $13 \pm 3\%$ less cells in the deafferented side of the superior colliculus as early as 16 hr after enucleation and a $220 \pm 20\%$ increase in the density of pyknotic cells. To compare the effect of enucleation with that of afferent electrical blockade, we analyzed the density of dying cells in the first hour following enucleation. Four hours after enucleation, we found a $47 \pm 23\%$ increase in dying cell density in the superior colliculus of P6 animals ($N = 5$), an effect very similar to that induced after similar intervals of retinal afferent activity blockade.

The variation in dying cell density reflects a change in the total number of dying cells

More pyknotic cells than normal can result from an actual variation in the number of dying cells, or simply from a change in the rate at which degenerating cells are cleared. The most direct way to address this question would be to count the total number of cells present in the superior colliculus, since if more cells die, less cells should be left. However, although we observed up to a 50% increase in the density of pyknotic cells when retinal afferents were blocked, this effect lasted only 8 hr. Dying cells are observed in the superior colliculus at least from E20 to P14, and the time course of their density has been previously described (Giordano et al., 1980). Assuming that pyknotic cell clearance requires between 2.5 and 7.5 hr, as we derived above, we can estimate that between 40% and 80% of the cells in the superior colliculus undergo cell death; that is, an average of 2.5–5% of the initial number of cells die per day. Even if we assume that at P6 half of the cells have already died, a 50% increase lasting 8 hr would correspond to about 2% fewer live cells than normal ($0.50 \cdot 8/24 \cdot 0.05/0.50 = 1.68\%$), a difference hidden in the normal difference observed between the two sides

of the colliculus (6 ± 5%; $N = 10$). Indeed, the differences in the absolute density of cells between the two sides of the colliculus of treated animals are within normal limits.

During the period of cell death, pyknotic cells rapidly disappear and pyknotic debris are engulfed by macrophages, as shown in Figure 8 (for a review, see Perry and Gordon, 1991). We selectively stained macrophages in the superior colliculus using an antibody against a macrophage surface antigen. After 5 hr of input firing blockade, a time when a significant increase is induced in the density of dying cells in the colliculus, no significant difference is observed in the density of macrophages between the treated (146 ± 34 macrophages/mm^2) and the normal side of the colliculus (144 ± 37 macrophages/mm^2) ($N = 3$), nor was any gross morphological difference observed between the macrophages of the normal side of the colliculus and those observed in the treated side. These results suggest that the variations in the density of dying cells induced by the blockade of input electrical activity cannot simply reflect a change in the rate of clearance by macrophages. This conclusion is strengthened by the recent observations of Andersson et al. (1991), who showed that lesions in the rat brain induce a rapid synchronous neuronal death but only recruit macrophages after about 2 d. Therefore, the variations in the density of dying cells induced by blockade of input activity are very likely to reflect real changes in the number of dying cells. This conclusion is also supported by the observation that in a short time, retinal electrical blockade induces an increase in dying cell density that closely parallels the increase following eye enucleation, which is known to lead eventually to a conspicuous decrease in the number of cells in the superior colliculus.

No change in the density of pyknotic cells is observed in the retina during electrical blockade

A question that we considered is whether TTX blockade directly affects cell death in the retina. Specifically, we studied whether at times when a considerable increase in the number of dying cells is observed in the superior colliculus, any variation is observed in the number of dying cells in the retina silenced with TTX. Previous studies (O'Leary et al., 1986a,b; Friedman and Shatz, 1990) have already addressed the problem, showing that impulse blockade prevents the selective elimination of wrongly projecting cells but does not alter the number of dying cells. We confirmed these results for the time period analyzed. Counts of pyknotic cells in the ganglion cell layer of retinas injected with TTX did not show any significant difference with respect to normal retinas in the 24 hr following the injection. The average difference in the density of pyknotic cells between the two retinas of TTX injected animals was 5.3 ± 3.4%. Therefore, the rapid increase in the number of dying cells observed in the colliculus during retinal spontaneous firing blockade is not a consequence of a change in the number of dying cells in the retina. Rather, the higher amount of cell death in the superior colliculus directly relates to the silencing of retinal input electrical activity.

The untreated side of the superior colliculus has the same density of dying cells observed in the superior colliculus of normal animals

In P6 rats, retinal projections to the superior colliculus are already largely crossed, each retina innervating nearly exclusively the contralateral side of the superior colliculus (Bunt et al., 1983). In our experimental protocol, we always injected TTX in one eye, while leaving the other eye untouched or injecting it with citrate buffer in a few cases. This corresponds to blockade of retinal input activity to one side of the colliculus while leaving unaltered the spontaneous firing of the afferents to the other side of the colliculus. The density of dying cells in the untreated side has been used as a normal control since no difference is observed in the density of pyknotic cells when comparing, among littermates, the untreated collicular side of TTX-injected animals (17.1 ± 0.3 pyknotic cells/1000 live cells; $N = 4$), both colliculi of normal animals (16.9 ± 0.4 pyknotic cells/1000 living cells; $N = 4$), and the colliculi innervated by eyes injected with citrate buffer (16.8 ± 0.2 pyknotic cells/1000 live cells; $N = 4$). Both TTX-injected and citrate buffer–injected animals were analyzed 5 hr after the injections. We already mentioned that the difference in total cell density between the two sides of the colliculus is within the normal limits even in treated animals. This makes the measure of the density of pyknotic cells per 1000 live cells a reliable measure of the total number of pyknotic cells.

Discussion

We have shown here that, during the period of naturally occurring cell death, a rapid and massive increase in the number of dying cells is induced in the optic layers of the superior colliculus, when the spontaneous electrical activity of retinal ganglion cells is transiently blocked by the use of TTX.

A number of studies have already suggested that input cells are necessary for the normal development of their target. In many cases, ablation of a main input during development causes a considerable decrease in the number of target cells, an effect known as transneural degeneration (Levi Montalcini, 1949; Guillery, 1973; Kalil, 1980; Okado and Oppenheim, 1984; Furber et al., 1987; Hashisaki and Rubel, 1989; Rubel et al., 1990; for a more complete list, see Oppenheim, 1991). More recent experiments have shown that, in a number of cases, prolonged blockade of input electrical activity can mimic the effects of input removal on target development. Long-term blockade of excitatory synaptic transmission decreases the number of skeletal muscle fibers (Ross et al., 1987), chronic blockade of ganglionic transmission reduces the number of cells surviving death in the ciliary and sympathetic ganglia (Wright, 1981; Maderdrut et al., 1988), and electrical blockade of the auditory nerve for 2 d causes the death of one-third of the target neurons (reviewed in Rubel et al., 1990). The study of the immediate effects caused silencing input electrical activity may help in understanding the mechanism by which input cells influence target development. In the developing auditory system of gerbils and chickens, blocking presynaptic activity with a pellet releasing TTX on the eighth nerve causes, within an hour, decreased protein synthesis in the target (Born and Rubel, 1988), a reversible proliferation of glial processes (Canady and Rubel, 1992), and a destruction of ribosomes (Canady and Rubel, 1991). These early effects parallel those caused by removal of the cochlear afferents (reviewed in Rubel et al., 1990). In the developing visual system, as we have shown here, the absence of the main input spontaneous activity induces an immediate increase in the number of dying cells in the target, suggesting that, through their spontaneous activity, input cells normally promote the survival of target cells during the period of naturally occurring cell death. Spontaneous activity is likely to play an important role in the trophic action of input cells, since the effects of a transient afferent blockade on cell death are indistinguishable from the early effects of enucleation. Besides blocking Na^+ channels, TTX has been shown to slow

down transport of glucosamine-labeled material in the goldfish retinotectal system (Edwards and Grafstein, 1984). However, while the first effect is nearly immediate, the latter is first observed after 12 hr. Therefore, it is most likely that the phenomena described above, occurring shortly after TTX application, are only due to blockade of afferent electrical activity.

The mechanisms through which input activity promotes cell survival in the target

Two not mutually exclusive hypotheses can be considered to explain how input spontaneous activity may promote target cell survival. Depolarization induced by input firing could promote cell survival in the target, or something could be released when input cells fire that is trophic for target cells.

Chronic membrane depolarization has been shown to promote cell survival in culture (Scott, 1971; Lasher and Zagon, 1972; Nishi and Berg, 1981; Gallo et al., 1987; Wakade et al., 1988). Membrane depolarization could be a frequent event in the developing nervous system. Significant increases in extracellular potassium concentrations after cell firing are reported in a number of developing neural structures, including the rat optic nerve (Connors et al., 1982). The spontaneous firing of retinal ganglion cells could induce local transient accumulations of extracellular potassium in the superficial layers of the colliculus, where retinal afferents project. This could be sufficient to depolarize the membrane of collicular cells, long before mature synapses are formed in the colliculus.

Electrical impulses traveling along retinal axons could also cause the release, at the nerve endings, of a substance that promotes the survival of collicular cells. Spontaneous and spike-evoked release of neurotransmitter from growth cones has been observed, even before developing neurons have established contacts with other cells (Hume et al., 1983; Young and Poo, 1983; Sun and Poo, 1987). Appositions between retinal terminals and collicular cells are observed at this stage of development in rats. In many cases the presynaptic ending already contains vesicles (Lund and Lund, 1971). Although these are not mature synapses, they could already allow the release from the presynaptic part of substances picked up by the postsynaptic cell. This release could be triggered by the arrival of spikes in the presynaptic terminal as in mature synapses. This second hypothesis is also in agreement with the observation that, *in vitro,* TTX promotes death of spontaneously active neonatal retinal ganglion cells, but this effect is antagonized by cultured medium preconditioned in cultures of retinal cells (Lipton, 1986).

When input activity recovers, a transient period occurs, during which a lower than normal number of cells die in the colliculus. This result seems difficult to reconcile with the idea that input activity promotes target cell survival simply through membrane depolarization. If a normal firing rate is resumed, a normal rate of depolarization should be expected, and a normal rate of cell death. On the contrary, if input spontaneous activity promotes the survival of collicular cells causing the release of a substance that is trophic for target cells, one could explain why a transient period of less cell death is observed in the colliculus after retinal activity recovers. During TTX blockade, impulses do not travel along the optic nerve and little, if any, trophic agent may be unaffected by TTX treatment, leading to an accumulation since none is released at the terminals in the lack of impulse activity. When activity resumes, a transient increase in the amount of trophic agent released following each impulse would rapidly bring the cell back to the equilibrium concentration, inducing in the meantime a transient decrease in cell death in the colliculus, since more trophic agent is available in this interval of time. The possibility should also be considered that input blockade induces a sensitization of the deprived target cells to the afferent supplied factor.

The nature of dying cells

In this study, we could not determine the relative contribution of neural and glial cells to the population of cells undergoing naturally occurring cell death in the superior colliculus. We have shown that at least some of the dying cells were neurons, and that afferent blockade greatly augmented their number. It is interesting to consider that, in normal animals, the ratio between dying and live cells in the population of neurons projecting to the LP is more than three times the ratio determined for the entire population of cells forming the superior colliculus. This observation would suggest that most cells undergoing naturally occurring cell death in the superior colliculus are neurons.

References

Andersson PB, Perry VH, Gordon S (1991) The kinetics and morphological characteristics of the macrophage-microglial response to kainic acid-induced neuronal degeneration. Neuroscience 42:201–214.

Armitage P (1971) Statistical methods in medical research. Oxford: Blackwell.

Born DE, Rubel EW (1988) Afferent influences on brain stem auditory nuclei of the chicken: presynaptic action potentials regulate protein synthesis in nucleus magnocellularis neurons. J Neurosci 8:901–919.

Bunt SM, Lund RD, Land PW (1983) Prenatal development of the optic projection in albino and hooded rats. Dev Brain Res 6:149–168.

Canady KS, Rubel EW (1991) Activity-dependent regulation of ribosomal RNA. Soc Neurosci Abstr 17:229.

Canady KS, Rubel EW (1992) Rapid and reversible astrocytic reaction to afferent activity blockade in chick cochlear nucleus. J Neurosci 12: 1001–1009.

Connors BW, Ransom BR, Kunis DM, Gutinick MJ (1982) Activity-dependent K^+ accumulation in the developing rat optic nerve. Science 216:1341–1343.

Edwards DL, Grafstein B (1984) Intraocular injection of TTX in goldfish decreases fast axonal transport of [H^3]glucosamine-labelled materials in optic axons. Brain Res 299:190–194.

Fitzgerald M (1985) The post-natal development of cutaneous afferent fibre input and receptive field organization in the rat dorsal horn. J Physiol (Lond) 364:1–18.

Friedman S, Shatz CJ (1990) The effects of prenatal intracranial infusion of tetrodotoxin on naturally occurring retinal ganglion cell death and optic nerve ultrastructure. Eur J Neurosci 2:243–253.

Furber S, Oppenheim RW, Prevette D (1987) Naturally occurring neuronal death in the ciliary ganglion of the chick embryo following removal of preganglionic input: evidence for the role of afferents in ganglion cell survival. J Neurosci 7:1816–1832.

Galli L, Maffei L (1988) Spontaneous impulse activity of rat retinal ganglion cells in prenatal life. Science 242:90–91.

Gallo V, Kingsbury R, Balazs R, Jorgesen RO (1987) The role of depolarization in the survival and differentiation of cerebellar granule cells in culture. J Neurosci 7:2203-2213.

Giordano DL, Murray M, Cunningham TJ (1980) Naturally occurring neuronal death in the optic layers of the superior colliculus of the postnatal rat. J Neurocytol 9:603–614.

Guillery RW (1973) Quantitative studies of the transneuronal atrophy in the dorsal lateral geniculate nucleus of cats and kittens. J Comp Neurol 149:423–438.

Hashisaki GT, Rubel EW (1989) Effects of unilateral cochlea removal on anteroventral cochlear nucleus neurons in developing gerbils. J Comp Neurol 283:465–473.

Hume RI, Role LW, Fishbach GD (1983) Acetylcholine release from growth cones detected with patches of acetylcholine receptor-rich membranes. Nature 305:632–634.

Kalil R (1980) A quantitative study of the effects of monocular enu-

cleation and deprivation on cell growth in the dorsal lateral geniculate nucleus of the cat. J Comp Neurol 189:483–524.
Lasher RS, Zagon IS (1972) The effect of potassium on neuronal differentiation in cultures of dissociated newborn cerebellum. Brain Res 41:482–488.
Levi Montalcini R (1949) The development of the acoustic-vestibular centers in the chick embryo in the absence of the afferent root fibers and of descending fiber tracts. J Comp Neurol 91:209–241.
Lipton SA (1986) Blockade of electrical activity promotes the death of mammalian retinal ganglion cells in culture. Proc Natl Acad Sci USA 83:9774–9778.
Lund RD, Lund JS (1971) Modifications of synaptic patterns in the superior colliculus of the rat during development and following deafferentation. Vision Res 3:281–298.
Maderdrut JL, Oppenheim RW, Prevette D (1988) Enhancement of naturally occurring cell death in the sympathetic and parasympathetic ganglia of the chicken embryo following blockade of ganglionic transmission. Brain Res 444:189–194.
Maffei L, Galli-Resta L (1990) Correlation in the discharge of neighboring rat retinal ganglion cells during prenatal life. Proc Natl Acad Sci USA 87:2861–2864.
Meister M, Wong ROL, Baylor DA, Shatz CJ (1991) Synchronous bursts of action potentials in ganglion cells of the developing mammalian retina. Science 252:939–942.
Nishi R, Berg DK (1981) Effects of high K^+ concentrations on the growth and development of ciliary ganglion neurons in cell culture. Dev Biol 87:301–307.
Okado N, Oppenheim RW (1984) Cell death of motoneurons in the chick embryo spinal cord. IX. The loss of motoneurons following removal of afferent inputs. J Neurosci 4:1639–1652.
O'Leary DDM, Crespo D, Fawcett JW, Cowan WM (1986a) The effect of intraocular tetrodotoxin on the postnatal reduction in the numbers of optic nerve axons in the rat. Dev Brain Res 30:96–103.
O'Leary DDM, Fawcett JW, Cowan WM (1986b) Topographic targeting errors in the retinocollicular projection and their elimination by selective ganglion cell death. J Neurosci 6:3692–3705.
Oppenheim RW (1981) Neuronal cell death and some related regressive phenomena during neurogenesis: a selective historical review and progress report. In: Studies in developmental neurobiology (Cowan WM, ed), pp 74–133. New York: Oxford UP.
Oppenheim RW (1991) Cell death during development of the nervous system. Annu Rev Neurosci 14:453–501.
Perry VH, Gordon S (1991) Macrophages and the nervous system. Int Rev Cytol 125:203–244.
Purves D (1988) Body and brain. A trophic theory of neural connections. Cambridge, MA: Harvard UP.
Purves D, Lichtman JW (1985) Principles of neural development. Sunderland, MA: Sinauer.
Ross JJ, Duxson MJ, Harris AJ (1987) Neural determination of muscle fiber numbers in embryonic rat lumbrical muscles. Development 100: 383–394.
Rubel EW, Hyson RL, Durham D (1990) Afferent regulation of neurons in the brain stem. J Neurobiol 21:169–196.
Scott BS (1971) Effects of potassium on neural survival in cultures of dissociated human nerve tissue. Exp Neurol 30:297–308.
Sefton AJ, Dreher B (1985) Visual system In: The rat nervous system (Paxinos G, ed), pp 169–221. Sydney: Academic.
Sun Y-a, Poo M-m (1987) Evoked release of acetylcholine from the growing embryonic neuron. Proc Natl Acad Sci USA 84:2540–2544.
Thoenen H (1991) The changing scene of neurotrophic factors. Trends Neurosci 14:165–170.
Wakade AR, Wakade RK, Malhorta RK, Bhave SV (1988) Excess K^+ phorbol ester activate protein kinase C and support the survival of chick sympathetic neurons in culture. J Neurochem 451:975–983.
Walicke PA (1989) Novel neurotrophic factors, receptors and oncogenes. Annu Rev Neurosci 12:103–126.
Wollner DA, Scheinman R, Catterall WA (1988) Sodium channel expression and assembly during development of retinal ganglion cells. Neuron 1:727–737.
Wright L (1981) Cell survival in chick embryo ciliary ganglion is reduced by chronic ganglionic blockade. Dev Brain Res 1:283–286.
Wyllie AH, Kerr JFR, Currie AR (1980) Cell death: the significance of apoptosis. Int Rev Cytol 68:251–306.
Young SH, Poo M-m (1983) Spontaneous release of neurotransmitter from growth cones of embryonic neurons. Nature 305:634–637.