Naturally-Occurring Neuron Death in the Ciliary Ganglion of the Chick Embryo Following Removal of Preganglionic Input: Evidence for the Role of Afferents in Ganglion Cell Survival

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With only a few exceptions, most investigations of the mechanisms involved in naturally-occurring neuron death have focused on interactions between neurons and their targets, with much less attention having been paid to the possible role of the afferent inputs in this phenomenon. This is true of the avian ciliary ganglion (CG), which is composed of a population of peripheral autonomic neurons that project to smooth and striated musculature in the eye and which receive afferents from a single source, the accessory oculomotor nucleus (AON), which is the avian homolog of the Edinger-Westphal nucleus. Although several lines of evidence strongly support the important role of targets in regulating the death and survival of CG neurons, the role of afferents has not yet been systematically examined.

Following the destruction of the AON on embryonic day (E) 4, which is several days before the onset of normal cell death in the CG, we have found that by the end of the normal cell death period (E14–E15), 85–90% of the CG neurons degenerate and die, compared to 50% in controls. This is comparable to the amount of induced cell loss that occurs following removal of the optic vesicle containing the CG targets. The neurons surviving after deafferentation appear to be sustained by some influence from their targets since combined deafferentation and eye removal results in the loss of virtually all neurons in the CG. Following deafferentation of the CG on E4, the ganglion develops normally up to about E10, after which a precipitous loss of cells occurs. Based on several kinds of evidence (e.g., axon counts, silver stain, retrograde labeling of the CG), we conclude that the deafferented neurons project to and innervate their muscular targets in the eye. Therefore, the increased cell death following deafferentation cannot be due to the failure of deafferented neurons to contact their targets. The deafferented neurons undergo a normal sequence of initial ultrastructural differentiation. When they do begin to degenerate, the type of fine structural changes they exhibit appears indistinguishable from the degenerative changes observed in control embryos. Neurons in deafferented ganglia were occasionally observed to receive synaptic contacts, which we attribute

to aberrant intraganglionic connections induced by deafferentation. These contacts probably play little, if any, role in the maintenance of neurons since, as noted above, following combined deafferentation and target deletion virtually all neurons degenerate and die.

These results indicate that neurons in the avian ciliary ganglion may be critically dependent on influences from both targets and afferents for their survival during a period of naturally-occurring cell loss. The possible role of trophic factors and physiological activity ("function") in regulating afferent and target influences on neuronal death and survival are discussed.

Studies of naturally-occurring neuronal death have tended to emphasize almost exclusively the role of neuron–target interactions in the competitive events that result in the survival of a proportion of a pre-cell death population of neurons and the degeneration and death of the remainder. The reason for this restricted focus on targets, of course, is that a large number of studies examining different types of neurons in a variety of species have shown that increases or decreases in the size, volume, or number or type of targets are associated with increased or decreased survival of those neurons that innervate the altered targets (Jacobson, 1978; Oppenheim, 1981). Consequently, there is a consensus among workers in this field that, for many populations of neurons, targets are exceedingly important in regulating how many neurons will survive the cell death period. However, there is also a growing recognition that afferent influences may be involved in regulating cell death (Cunningham, 1982; Okado and Oppenheim, 1984; Clarke, 1985). Ideally, in order to undertake a critical examination of the relative contributions of afferent and target influences on neuronal survival and death, it would be advantageous to focus on a neuronal population that receives a single source of afferent input and projects to a single target region, and in which the afferent and target cells can be independently and systematically perturbed at developmental stages prior to the onset of naturally-occurring cell death. The ciliary ganglion (CG) of the chick embryo is one of the few neuronal populations that meets these criteria.

The chick CG is composed of 2 homogeneous populations of neurons: the small choroid cells that innervate the smooth musculature of the choroidal coat, and the larger ciliary cells that innervate the striated iris and ciliary muscles. Despite these and several other anatomical and physiological differences between the ciliary and choroid cells (Landmesser and Pilar, 1978), both cell types are known to be derived from the cranial neural crest (Hammond and Yntema, 1958; Narayanan and Narayanan,

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Figure 1. Transverse section of the midbrain in control embryos at the level of the oculomotor nucleus (arrowheads, ON) and nerve (arrow) on E4 (stage 25), at the time when the nucleus was lesioned (*A*) and on E7 (stage 33) when the AON comprises a distinct subnuclear group (*B*). Thionin stain was used in *A*. Bar, 200 μ m. Reduced silver with thionin counterstain was used in *B*. Bar, 50 μ m. *C*, Cerebellum; *V*, ventricle; *EC*, schema of electrocautery tip used for lesioning the AON (not to scale); *FLM*, fasciculus longitudinalis medialis; *DM*, dorsomedial ON; *DL*, dorsolateral ON; *VM*, ventromedial ON.

Figure 2. Schematic drawing of E2 (stage 11) embryo at the time of unilateral removal of the optic vesicle (OV). Lines on right side indicate the approximate area removed.

1978a). Beginning at stages 8–9 (Hamburger and Hamilton, 1951), the neural crest precursors migrate from the dorsal-lateral surface of the mesencephalon to a site behind the eye, close to the emerging optic nerve. Aggregation of the ganglion precursors is completed by about E5 (stage 25), and by E6–E7 (stages 31–33), the maximum pre-cell death population of neurons is attained. Efferent projections to the iris are present by E5–E6, and afferent projections to the ganglion from the accessory oculomotor nucleus (AON), the avian homolog of the mammalian Edinger-Westphal nucleus, are present by E3–E4 (Tello, 1922; S. Furber, R. W. Oppenheim, and D. Prevette, unpublished observations). Between E7 and E15 approximately 50% of the original population of neurons in the ganglion undergo naturally-occurring degeneration, resulting in a final population size of about 3000 cells (Landmesser and Pilar, 1974a); the ciliary and choroid populations appear to be about equally de-

pleted by this process of cell death. Removal of the optic vesicle on E2 (target deletion), prior to efferent innervation, results in a greatly enhanced cell loss in the CG (Amprino, 1943; Levi-Montalcini and Amprino, 1947; Cowan and Wenger, 1968; Landmesser and Pilar, 1974b), whereas transplantation of a supernumerary optic vesicle on E2 or a reduction in competition at the target rescues a significant number of ganglion cells from neuronal death (Narayanan and Narayanan, 1978b; Pilar et al., 1980). Finally, tissue culture media “conditioned” by heart or skeletal muscle cells have also been shown to support the survival of virtually the entire pre-cell death population of CG neurons *in vitro* (Nishi and Berg, 1979). Collectively, these various lines of evidence argue strongly for the important, perhaps even exclusive, role of targets and target-derived survival factors in regulating CG cell survival. Yet other populations of neurons for which an equally strong case can be made for the primary role of targets have recently been shown to be significantly affected (i.e., increased cell death) by the deletion of afferents (e.g., Okado and Oppenheim, 1984; Clarke, 1985). Consequently, it seems plausible that despite the obviously important role of targets in ciliary ganglion cell survival, afferents may also be involved.

Materials and Methods

Fertilized eggs (Hubbard Farms, Statesville, NC) were maintained in a turning incubator at a temperature of 37°C and a relative humidity of 60–70%.

Embryonic surgery: AON removal. Initially, AON removal was attempted by bilateral removal of the dorsal mesencephalon in the region containing the prospective oculomotor complex on E2 (stages 11–12), a stage when, according to earlier reports (Hammond and Yntema, 1958), all of the neural crest progenitor cells of the CG were believed to have completed their migration from this region. However, an examination of several of these embryos on E6–E7, which is prior to the initiation of normal cell death in the CG, revealed that cell number was already severely depleted, indicating that the operation on E2 must have destroyed a significant number of ganglion precursors.

Consequently, in all the subsequent experiments reported here a different procedure was used. Embryos were exposed via a lateral window on E4 (stages 24–25), a small opening was made in the amnion over the head of the embryo and, using a glass probe and forceps, the dorsal mesencephalic region was exposed and stabilized under this opening. Using a small battery-operated electrocautery (Storz Instruments, St. Louis) with a specially prepared fine tip, the dorsal midline region of the mesencephalon containing the AON was briefly cauterized (Fig. 1), the head of the embryo was then replaced within the amniotic sac, the egg opening sealed, and the egg replaced in the incubator. Approximately 30–50% of such operated embryos (total, 325) survived to between E8 and E16.

In another group of embryos, the right optic vesicle (eye primordium) was removed on E2 (stages 11–12) using forceps and a vibrating needle (Wenger, 1968; see Fig. 2). In approximately half of these eye-removal embryos the AON was also destroyed bilaterally on E4 as described above. Sham control operations for both AON destruction and eye removal involved all of the same procedures used for experimental embryos except for actual cauterization or eye removal.

HRP injections. In a few embryos with bilateral AON removal, HRP was injected into the choroidal coat, ciliary body, and iris of the eye on E8 in order to retrogradely label neurons in the CG. A drop of a 50% solution of HRP (Type VI, Sigma) was recrystallized on the tip of Minutens pins and placed into the appropriate eye structures for 30–60 sec. Three to 4 pins were used per eye. The eye, eye musculature, and attached CG were isolated in an oxygenated organ bath, injected with HRP, and allowed to survive for 6–7 hr at 33°C. The CG were then removed and fixed by immersion in a solution of 2% glutaraldehyde in a 0.1 M phosphate buffer. The ganglion was embedded in a gelatin-yolk mixture and sectioned at 60–80 μ m on a microslicer (Dosaka EM). All sections were reacted with diaminobenzidine (DAB); in some cases the DAB reaction was intensified by the heavy-metal procedure of Adams (1981). The brains of these animals were also removed following iso-

Figure 3. Schematic drawing of midbrain, oculomotor nerve (OMv), ciliary ganglion, and eye. Dotted lines indicate the smallest and greatest extent of the lesion following electrocautery of the middorsal midbrain on E4 (stage 25). AON, Accessory oculomotor nucleus; EW, Edinger-Westphal nucleus; ICo, nucleus intercollicularis; Imc, nucleus isthmi, pars magnocellularis; Ipc, nucleus isthmi pars parvocellularis; TPC, nucleus tegmenti pedunculopontinus pars compacta. Drawing of ciliary ganglion and eye modified from Landmesser and Pilar (1978).

lation of the eye and CG in the organ bath, fixed (see above), embedded in paraffin, sectioned at 15 μ m, stained with thionin, and examined for verification of AON deletion.

Histologic and morphometric procedures. Following surgery, experimental and sham-control embryos were allowed to survive to various ages, ranging from E4.5 (stage 25) to E18 (stage 44). For embryos younger than E10, the dorsal skull was opened and the entire head and eyes fixed by immersion in Carnoy's solution, processed for paraffin histology, sectioned at 8 μ m, and stained with thionin. For embryos older than E10, the CG was removed bilaterally and the ganglia and brain fixed separately by immersion and processed for paraffin histology as described above. Both the brain and ganglia were sectioned at 12–15 μ m and stained with thionin. In a few cases the entire head and eyes of control and experimental embryos on E3 to E8 were processed for silver impregnation using either a modification of the Cajal-De Castro reduced silver method (Levi-Montalcini, 1949), or a thionin counterstained-reduced silver technique (Gross, 1981), in order to examine the development of pre- and postganglionic fibers. Embryos in the groups with eye removal (with or without AON deletion) were usually sacrificed on E12 (stage 38) and the entire head with CG left *in situ* processed for paraffin histologic examination as described above. A few embryos in these groups were allowed to develop to E15 (stage 41).

Cell counts of CG neurons were carried out according to previously established procedures (Landmesser and Pilar, 1974a; Furber and Oppenheim, 1984). Briefly, all neurons containing a large distinct clump of nucleolar material were counted in every fifth section through the entire ganglion at a primary magnification of 400 $\times$. No attempt was made to count ciliary and choroidal cells separately. Raw counts were corrected by the Abercrombie (1946) method for split nucleoli. The number of neurons containing a pyknotic nucleus (i.e., degenerating cells) were counted in every fifth section through the ganglion at a primary magnification of 500 $\times$; care was taken to exclude glial cells and cells undergoing mitosis from these counts. Cell size was estimated by measuring nuclear diameter in 2 dimensions at a primary magnification of 1200 $\times$; nuclear measurements were taken from cells in all regions of the ganglia and thus include both ciliary and choroidal populations.

Electron microscopy. For ultrastructural examination, the CG of con-

trol and AON removal embryos (E12 and E15) were dissected free while the eye was continuously drenched in 2.5% phosphate-buffered glutaraldehyde. Following dissection the ganglia and postganglionic nerves were immersed in the glutaraldehyde solution for 2 hr and post-fixed in 1% osmium tetroxide for 4 hr, after which they were dehydrated and embedded in Epon 812. Blocks were sectioned on a Sorvall MT2-B ultramicrotome and stained with lead citrate and uranyl acetate. Sections through various regions of the ganglion were examined and photographed at a variety of different magnifications using a Phillips 400 EM. The CG of 3–4 control and experimental cases were examined on E12 and E15. For examination of postganglionic nerves (E12 only), successive thick sections were made through the region of the block just proximal to the exit of the nerves until sections were attained that only contained transverse sections of postganglionic nerves without the presence of neuronal cell bodies. Semithin transverse sections were then taken through this region for a quantitative analysis of postganglionic axon numbers. Sections through the entire cross section of the nerve were photographed at a magnification of 1000–1200 $\times$ and their areas measured. Approximately 10% of this area was then photographed in the EM, enlarged, and the number of axons counted. An estimation of total axon number was derived by extrapolation from this sample area. Landmesser and Pilar (1976) have previously shown that this sampling procedure provides an accurate estimate ($\pm 5\%$) of total axon number. Two to 3 control and experimental embryos were used for the quantitative assessment of postganglionic axons.

Results

All embryos utilized in the results presented below were confirmed to have a complete uni- or bilateral destruction of the entire oculomotor complex (Figs. 3, 4). Further confirmation of the success of the AON deletion was the absence of the oculomotor nerve and of preganglionic nerves in the CG (Fig. 5). Furthermore, only embryos with completely normal development of the eyes as determined by gross inspection and examination of the histology were retained for further analysis.

Figure 4. Transverse sections of control (*A*) and lesioned (*B*) oculomotor complex on E14. In *B* the right oculomotor complex, including the AON, is absent, whereas the left side is only partially deleted. Arrow indicates midline; *V*, ventricle. Bar, 100 μ m.

Figure 5. Transverse section of control (*A*) and deafferented (*B*) ciliary ganglion in the same approximate region of the ganglion on E8 (stage 34). Note the absence of the oculomotor nerve (*arrowhead, A*) in *B* and the presence of the postganglionic nerve (*arrows*) in *A* and *B*. Silver with thionin counterstain. *E*, eye. Bar, 30 μ m.

Figure 6. Number of ciliary ganglion cells in control and deafferented embryos. *, $p < 0.05$.

Development following deafferentation

Deletion of the AON, the single source of preganglionic afferents to the CG, on E4, which is several days prior to the onset of naturally-occurring cell death in the ganglion did not have any noticeable effect on the ganglion up to E8. From the cell counts (Fig. 6) and histology (Figs. 5, 7) it seems likely that the migration, proliferation, aggregation, and axonal outgrowth of the deafferented CG have progressed normally in the absence of afferents (cf. Levi-Montalcini and Amprino, 1947). By E6–E7, control and embryonic ganglia contain equal numbers of neurons. By contrast, between E7 and E15, the control ganglia lose approximately 50% of their neurons (6000 to 2700), whereas the number of neurons in the deafferented ganglia falls from a peak of about 6200 to 900 neurons, a loss of approximately 85%. Thus, there is an enhanced cell loss in the deafferented ganglia of 35%. Most of this increased loss occurs after E10, when cell number in the control ganglia goes from 4000 to 2700 (33% loss) compared to a 75% loss (3500 to 900) in the deafferented ganglia. Although it is conceivable that this enhanced cell loss in the deafferented ganglia is due to factors other than increased cell death (e.g., see Clarke, 1985), the data on dying neurons (i.e., cells with pyknotic nuclei) make this possibility exceedingly unlikely. There were significantly more degenerating neurons (Fig. 7) in the deafferented than in the control ganglia on E10 (57 ± 8 vs. 10 ± 3 pyknotic neurons per 1000 healthy cells, $n = 5$ per group, $p < 0.01$).

The mean nuclear diameter of healthy appearing neurons in the CG of control and deafferented embryos on E15 did not differ (8.69 ± 0.91 vs. $8.81 \pm 0.63 \mu\text{m}$). However, a χ^2 test of the distribution of cell sizes (Fig. 8) shows that the deafferented ganglia were significantly different from controls ($p < 0.05$), exhibiting more cells in the middle size range (8–9.5 μm) and fewer larger cells ($> 10.0 \mu\text{m}$) than controls.

Development following eye removal and deafferentation

Consistent with prior reports (Amprino, 1943; Cowan and Wenger, 1968; Landmesser and Pilar, 1974b), we have found that deletion of the postsynaptic targets (eye removal) of CG neurons on E2, which is several days prior to both cell death and target innervation, results in a marked reduction in the number of CG neurons at later stages. Although we have not systematically examined earlier or later stages, on E12, embryos with a complete eye removal (Fig. 2) have approximately 55% fewer CG cells than controls (Landmesser and Pilar, 1974b), a value similar to that of E12 embryos following deafferentation (Fig. 9).

Two embryos with eye removal that survived to E15 had 540 and 515 surviving CG neurons (i.e., 81% less than controls). By contrast, embryos with a combined eye removal and deafferentation had a considerably greater reduction in cell number on E12 than following either operation alone (93% fewer neurons than controls and 84% fewer than either eye or AON removal embryos, Fig. 9); one embryo in this group that survived to E16 had few, if any, discernible surviving ganglion cells on the side of the brain ipsilateral to the eye removal.

Postganglionic nerve and target innervation following deafferentation

Examination of the CG in thionin-stained and silver-impregnated preparations following deafferentation indicates that the deprived ganglion cells send axons towards the eye (Fig. 5). Furthermore, postganglionic nerves could be seen running across the surface of the eye towards the ciliary musculature as in control embryos. To confirm that all surviving ganglion cells contribute axons to the postganglionic nerves and actually innervate target structures in the eye, we have used 2 strategies: axon counts in the postganglionic nerve immediately distal to the ganglion and retrograde labeling of CG neurons following HRP injection into the target regions of the eye. Both approaches have confirmed our initial impression that the deafferented ganglion cells project axons to the eye. Estimates of axon numbers show that virtually all of the surviving neurons have at least one axonal process in the postganglionic nerve on E12: control, 4336.3 axons ($n = 2$); deafferented, 2967.3 ($n = 2$). The deafferented embryos have a 32% reduction in axon number. The cross-sectional area of the deafferented postganglionic nerve was reduced by about one-half on E12 (control, $2929.9 \mu\text{m}^2$; deafferented, $1611.1 \mu\text{m}^2$). Ultrastructurally, the postganglionic axons of control and deafferented CG neurons appeared similar (Fig. 10).

HRP injections into the ciliary and choroidal targets of the deafferented CG neurons on E8 result in the retrograde labeling of large numbers of ganglion cells (Fig. 11). This supports the evidence obtained from axon counts and shows further that many of the deafferented neurons actually innervate the eye.

In a previous study of cell death in the CG, Pilar and Landmesser (1976) found that naturally-occurring cell death and death brought about by target deletion (eye removal) differed in the ultrastructural characteristics observed during their development and subsequent degeneration. Before the onset of degeneration, neurons in the peripherally deprived ganglia failed to develop the well-organized rough endoplasmic reticulum (RER)

Figure 7. Transverse section of control (*A*) and deafferented (*B*) ciliary ganglion on E14 (stage 40). Bar, 100 μ m for *A* and *B*. Insets, Examples of degenerating CG neurons (*) on E14. Bar, 20 μ m.

characteristic of normal ganglion cells. Degeneration in peripherally deprived ganglia was primarily marked by nuclear changes followed by the breakdown of polysomes to single or free ribosomes. In contrast, naturally-occurring cell death was char-

acterized by dilation of the previously well-organized RER followed by cytoplasmic breakdown and only at terminal stages by nuclear disruption.

Although we have made a careful study only of the ultrastruc-

Figure 8. Histogram of cell (nuclear) size in control and deafferented embryos on E15 (stage 41). (See text for details.)

ture of CG neurons on E12 and E15, with the one possible exception noted below, we failed to detect any differences in either the development or characteristics of degeneration of control and deafferented ganglion cells. In both groups, normal (non-dying) neurons exhibit all of the same ultrastructural features previously described by Pilar and Landmesser (1976) for control CG neurons (Fig. 12). Furthermore, dying cells in both groups appear to undergo the sequence of degenerative changes designated "cytoplasmic" by those authors, in which the most notable changes involve dilation of the cisternae of the RER, mitochondrial swelling and disruption, and terminal nuclear changes (Fig. 13). However, we have noticed an apparent tendency for putative dying neurons in the deafferented ganglia to exhibit an early alteration in the nucleus involving an irregularity (crenulation) in nuclear shape (Fig. 14), a feature previously noted by Pilar and Landmesser (1976) as representing an early sign of degeneration in peripherally deprived ganglia. However, because this nuclear change was by no means found in all dying deafferented neurons and was also occasionally observed in neurons in control ganglia (Fig. 12), it is conceivably a normal variant of CG degeneration.

Although we never observed the typical calyciform synapses on deafferented CG neurons, we have seen an occasional boutonlike synapse in these preparations (Fig. 15). The presence of such synapses, though rare, could indicate the presence of a few residual preganglionic fibers in these preparations. However, because these rare synapses were present in embryos that by all other criteria (i.e., absence of the oculomotor nuclear complex and no detectable preganglionic axons entering the CG) were considered to have been completely deafferented, we are inclined to attribute them to aberrant *intraganglionic* contacts (see Margiotta and Berg, 1984).

Discussion

From previous reports, the CG of the chick is thought to represent a population of neurons in which the efferent targets in the eye exert the major, if not the sole, control over how many CG cells will survive the period of naturally-occurring cell death (e.g., Landmesser and Pilar, 1974a, b). Despite the strength of these data, however, the role of afferents in CG survival had not previously been examined systematically (but see Levi-Montalcini, 1947, 1949). The fact that considerable pregan-

Figure 9. Number of ciliary ganglion cells on E12 (stage 38) in control, deafferented (AON), eye removal, and combined eye removal and deafferented embryos. *, $p < 0.05$ compared to control; **, $p < 0.05$ compared to AON and Eye groups. All data depicted in this figure for control and AON are from different embryos than shown in Figure 6.

glionic input to the CG exists prior to the onset of cell death on E8 (Landmesser and Pilar, 1974a, b) is consistent with the possibility that afferents may play a role in normal cell death in this system. As noted in the introduction, the fact that the avian CG receives all of its afferent input from a single source, the preganglionic AON, makes it a particularly appealing system in which to examine this question experimentally. In the present paper we have surgically destroyed the AON bilaterally on E4, several days prior to the onset of cell death in the CG. Following deafferentation, the CG develops normally up to E9, after which the amount of cell death is enhanced such that the number of surviving cells on E15–E18 is reduced to less than 10–15% of the peak number present prior to cell death. During this same period, control embryos with intact afferents lose only about 50% of the pre-cell death population. Although it is possible that at later stages (e.g., posthatching) all deafferented CG neurons may degenerate, this seems unlikely. Cell number in the deafferented CG appears to stabilize by E14–E15 and shows no further significant decrease up to E18, the oldest age examined by us. It is interesting that the amount of enhanced cell loss in the CG following complete *target* removal (also see Landmesser and Pilar, 1974b) is quantitatively similar to that found following total deafferentation. (In both cases, 15–20% of the deprived neurons continue to survive.) By contrast, when *both* the targets and afferents are removed, virtually all of the CG neurons are lost by E15. Thus, neither targets nor afferents alone are able to sustain more than a small number of CG neurons (<15%). Although it is conceivable that some of the surviving neurons in both cases are sustained by aberrant intraganglionic connections, the virtually complete degeneration of the CG following combined target and afferent removal indicates that this putative source of support is probably insignificant.

The enhanced neuronal loss following deafferentation could be the result not of deafferentation per se but, rather, of a secondary deafferentation-induced abnormal development of target innervation. If the deafferented neurons were impaired in their ability to project an axon and make target contacts, then the enhanced cell death could be due to the same or similar factors that lead to increased cell death following target removal (e.g., loss of trophic support from the target). The quantitative similarity in the extent of cell loss following either operation

Figure 10. Transverse sections of postganglionic ciliary nerve from control (*A*) and deafferented (*B*) embryos on E15 (stage 41). Except for the presence of more axons and less extra-axonal space in *A*, there are no obvious differences in the appearance of the nerve in the 2 groups. *, degenerating axon. $\times 3300$ (see text for details).

Figure 11. HRP-labeled ciliary ganglion in control (*C*) and deafferented (*A*, *B*, *D*) embryos on E8 (stage 34). *A*, Ciliary ganglion enclosed by dots with HRP-labeled postganglionic nerve (arrow) projecting towards eye (*e*). Unintensified DAB procedure. Bar, 200 μ m. *B*, High magnification of labeled ganglion cells from same embryo as shown in *A*. Bar, 17 μ m; same for inset in *C*. *C* and *D*, Labeled ganglion cells from control (*C*) and deafferented (*D*) embryo. Intensified DAB procedure. Note the presence of many labeled ganglion cells with dendritic processes (arrow and inset). White arrows indicate postganglionic nerve. Bar, 45 μ m (*C* and *D*).

(i.e., an 80–90% cell loss) could even be used as evidence in support of this argument. Nonetheless, several lines of evidence appear to refute this notion. Silver-stained preparations, axon counts, and retrograde labeling of the CG following HRP injection into the eye indicate that the deafferented ganglion cells do, in fact, project postganglionic axons to and innervate their targets in the eye. Moreover, if the cell loss following either target removal or deafferentation is actually due to a failure of CG neurons in both situations to innervate their targets, then the combined removal of both should not result in any greater amount of cell loss than following either operation alone. But as shown here, there is, in fact, a significantly greater cell loss in this situation. Based on these 2 arguments, we conclude that it is unlikely that the enhanced cell death following deafferentation results from failure to initiate target contact. It is also conceivable that deafferentation leads to a transganglionic alteration of target cell development that results in the increased death of neurons. Although examination of the muscular targets of the CG in the light microscope following deafferentation has not revealed any obvious changes in their development, an ultrastructural analysis is needed in order to rule out this possibility conclusively.

The ultrastructural characteristics of cell death in the CG have been previously described by Pilar and Landmesser (1976). In a comparison of naturally-occurring and induced cell death fol-

lowing target deletion they observed that the ultrastructural features of degeneration differed in these 2 situations. Whereas normal cell death was initiated by dilation of the RER and cytoplasmic disruption, and only later by nuclear changes, cell death in the deprived CG neurons was heralded by nuclear changes and increased free ribosomes and only at later stages by cytoplasmic disruption. Pilar and Landmesser (1976) designated these 2 kinds of cell death *cytoplasmic* and *nuclear*. Furthermore, they also observed that even prior to degeneration the peripherally deprived neurons failed to differentiate normally. Whereas normal CG neurons develop extensive RER and polyribosomes at the time that the postganglionic axons form recognizable synapses with their targets in the eye (E10–E14, stages 36–40), the deprived neurons never undergo this developmental transition.

In the present study we have found that the most prevalent type of degenerative changes observed in both the deafferented and normal CG are quite similar to the *cytoplasmic* form described by Pilar and Landmesser (1976) for control ganglia. Moreover, prior to their degeneration the deafferented ganglion cells undergo the same developmental transitions in RER and polyribosomes as seen in control ganglia. Thus, although it has been argued that deafferentation of some neuronal populations alters their differentiation in a manner similar to target deletion and also transforms the type of degeneration observed from

Figure 12. Comparison of the ultrastructure of normal-appearing control (A) and deafferented (B) ciliary ganglion neurons on E15 (stage 41). $\times 5500$.

primarily *cytoplasmic* to primarily *nuclear* (Cunningham, 1982), this is clearly not the case for the chick CG. Consequently, target influences must play some special role in *initial* CG development not shared by afferents (see Chiappinelli et al., 1978).

However, this difference is not critical for the *maintenance* and *survival* of neurons since, as we have shown, following deafferentation target contacts alone are not sufficient to prevent the increased loss of cells. These observations raise some intriguing

Figure 13. Ultrastructural features of different phases of degeneration in control (*A, D*) and deafferented (*B, C*) ciliary ganglion neurons on E14 (stage 40). Note the progressive dilation of RER (*arrowheads*), swelling and disruption of mitochondria (*asterisks*), and gradual breakdown and pyknosis of nuclear material in *C*. Fragments of degenerated cells being phagocytosed can be seen in *D* (*asterisks*). $\times 4300$ (*A, B*), $\times 7000$ (*C, D*).

questions but regrettably ones for which we have no final answers; namely, what is different about those cells that survive deafferentation and what is responsible for their maintenance? Except for the virtual absence of afferent input, up to E18 the

surviving deprived CG neurons exhibit no obvious ultrastructural differences from controls. Of course it is conceivable that these cells exhibit important biochemical or other differences that remain to be discovered. By contrast, *target-deprived* CG

Figure 14. Irregular (crenulated) nuclear outlines in control (*A*) and deafferented (*B*) ciliary ganglion neurons on E12 (stage 38). Compare with Figure 12*A*. $\times 7000$ (*A*), $\times 5500$ (*B*).

Figure 15. Deafferented ciliary ganglion neuron with a distinct synaptic contact (arrow). E14 (stage 40). $\times 4300$.

neurons that survive to E15 continue to exhibit obvious abnormalities in ribosomes and RER (Pilar and Landmesser, 1976).

Although the literature on the role of afferents in the maintenance and survival of neurons is limited, there are an increasing number of reports in support of their importance (see LoPresti et al., 1973; Sohal, 1976; Mouze, 1978; Parks, 1979; Cunningham, 1982; Okado and Oppenheim, 1984; Clarke, 1985; Kwei and Fite, 1985; Friedman and Price, 1986). Consequently, the major challenges now are to understand the mechanisms by which afferents exert this influence and to determine how afferent input and target contact interact in determining whether a given neuron will survive the period of cell death. With regard to the first question, there is evidence that in the case of the CG, chronic blockade of ganglionic transmission (activity) during the cell death period mimics the effects of surgical deaffer-

entation (Wright, 1981; Maderdrut et al., 1984; see also Chiappinelli et al., 1978). Blockade of activity by TTX in cocultures of spinal cord and dorsal root ganglia (DRG) results in the death of neurons within the spinal cord cultures (Nelson and Brenne- man, 1982). One explanation for this result is that synaptic transmission between the DRG and spinal cord also mediates an orthograde or afferent influence on neuronal survival. Afferent influences on neuronal survival in the chick auditory system are also mediated by synaptic transmission and/or postsynaptic activity (Born and Rubel, 1986). If activity is the critical factor mediating afferent influences, then one might expect that the onset of this effect would coincide with the initiation of afferent synaptic transmission. Whereas this appears to be the case in the chick auditory system (Parks, 1979), in the CG, ganglionic input is functional 4–5 d before the first effects of deafferentation

are observed. However, Landmesser and Pilar (1974a, b) have observed that in normal control embryos there is a transient naturally-occurring failure of synaptic transmission through the CG that reaches a peak reduction of 70% at E11–E13 (stages 37–39), which is when we observe the onset and major occurrence of deafferentation-induced cell loss. Thus, both denervation and ganglionic blockade could induce more cell death by augmenting the normal transient loss of a functional afferent input that the CG neurons may require for their survival. Alternatively, the increased cell loss following ganglionic blockade may have nothing to do with activity during normal development. Neurotransmitter receptors may be the sites at which a putative afferent-derived neurotrophic factor is bound and internalized. Ganglionic blockade would then be acting to alter the uptake of this *anterograde* factor by occupying receptors, with the resulting inactivity only being incidentally involved.

If for the moment, however, we assume that the afferent-driven activity of ganglionic neurons is directly involved in survival, we may speculate that inactive cells are (for unknown reasons) at a competitive disadvantage in gaining access to or utilizing a putative neurotrophic factor supplied by the afferent terminals. If correct, this would mean that neuronal survival in the CG is regulated by competition for at least 2 different neurotrophic factors, a retrograde factor supplied by targets and an anterograde factor supplied by afferents. Recent evidence supports this assumption in showing that at least certain classes of neurons (e.g., proprioceptive neurons in the chick trigeminal mesencephalic nucleus) require at least 2 different factors for survival, one supplied by peripheral targets and one by central targets (Davies et al., 1986). Alternatively, neurons made inactive by loss of afferent input may be at a disadvantage in competing for target-derived survival factors.

With regard to the question of the relative roles of target and afferent influences in neuronal survival, Cunningham (1982), who was the first to focus attention on this issue, has formulated a model—the “balance”-hypothesis—that attempts to explain how target contacts and afferent inputs together regulate neuronal survival. According to this hypothesis, neurons are thought to have an intrinsic requirement for a quantitative, but not necessarily one-to-one, balance or matching of afferent and target contacts. If in normal development target structures are contacted before substantial contact with afferents occurs, then the subsequent afferent contacts should be regulated so as to balance or match quantitatively with the amount of previously established target contact. And, of course, the converse would be true if afferent contacts occur first during development. Although no critical tests of this hypothesis have been made, there is some circumstantial evidence in support of certain aspects of the proposal (Cunningham, 1982). On the other hand, one prediction of the balance-hypothesis that has *not* been supported in the present study is the observation that deletion of afferent contacts does not impair the initial differentiation of CG cells in the same way that target removal does (Pilar and Landmesser, 1976). Deafferented CG cells show the same degree of initial ultrastructural differentiation and the same type of subsequent degeneration (cytoplasmic) as controls. Somewhat stronger evidence against the balance-hypothesis comes from the situation in which spinal cord motoneurons are rescued from dying by chronic neuromuscular blockade (Pittman and Oppenheim, 1979). In this case, target size is *reduced* (muscle atrophy), motoneuron numbers and efferent (neuromuscular) contacts are both *increased* (Oppenheim and Chu-Wang, 1983; Ding et al.,

1983), yet the packing density of axodendritic and axosomatic *afferent* synapses on these motoneurons remains unchanged (Okada et al., 1985). Furthermore, the increased supporting capacity of the targets that is assumed to occur in these curare-treated preparations is not effective in rescuing motoneurons from deafferentation-induced cell loss (Okado and Oppenheim, 1984). That is, an increased maintenance from the target cannot compensate for a depletion of afferents (cf. Clarke, 1985).

In conclusion, the present results would appear to be incompatible with a model of naturally-occurring neuron death in which the target is thought to be the major source of information for regulating neuronal survival. Although experimental situations exist *in vitro* in which virtually all CG neurons can be rescued by target-derived factors (e.g., Nishi and Berg, 1979), the regulation of normal development *in vivo* may be considerably more complex than is implied by these data. Alternatively, it must also be considered whether or to what extent deafferentation studies of the sort reported here provide evidence that is pertinent to the normal biological role of afferents *in vivo*. Perhaps normally, populations of neurons are buffered against small to moderate fluctuations in afferent input (Finlay et al., 1986), but that following more drastic experimental perturbations they respond in a manner (increased cell death) that is normally never operative. Although this seems unlikely, the possibility cannot be excluded until it can be conclusively demonstrated that *increased* afferent input can rescue neurons normally destined to die. The CG neurons may be an advantageous population in which to examine this.

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