

FATE OF GANGLIONIC SYNAPSES AND GANGLION CELL AXONS DURING NORMAL AND INDUCED CELL DEATH

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ABSTRACT

In order to understand the significance of cell death in the formation of neural circuits, it is necessary to determine whether before cell death neurons have (*a*) sent axons to the periphery; (*b*) reached the proper target organs; and (*c*) have established synaptic connections with them. Axon counts demonstrated that, after sending out initial axons, ciliary cells sprouted numerous collaterals at the time of peripheral synapse formation. Subsequently, large numbers of axons were lost from the nerves, slightly later than the onset of ganglion cell death. A secondary loss of collaterals later occurred unaccompanied by cell death. Measurements of conduction velocity and axon diameters indicated that all ganglion cell axons grew down the proper pathways from the start, but it was not possible to determine whether all axons had actually formed proper synapses. This was ascertained, however, in the ganglion itself where preganglionic fibres were shown to synapse selectively with all ganglion cells before cell death. During this period, degenerating preganglionic synapses were observed on normal cells. It can therefore be inferred that at least some preganglionics established proper synapses before dying and that a single synapse is not sufficient to prevent cell death. In this system neither preganglionic nor ganglionic cell death seems designed to remove improper connections but rather to remove cells that have not competed effectively for a sufficient number of synapses, resulting in a quantitative matching up of neuron numbers.

While the existence of extensive neuronal death at precise times during development is well documented, its significance in the formation of neural circuits is not clear. Since similar cell death has been observed in non-nervous tissues (9, 20), it may be unrelated to any special aspect of the nervous system. On the other hand, cell death has been postulated to remove improperly connected neurons (13), or those that have failed to form adequate peripheral connections (4), thus playing a direct role in the shaping of neural circuits.

The previous paper (24) described the ultrastructure of neuron death occurring in normal and

peripherally deprived ciliary ganglia during embryogenesis. Observed differences in the ultrastructural sequence in the two cases implied that normal cells which degenerated had undergone an interaction with their peripheral target organ before cell death, which triggered them into the secretory state.

This suggested that cells which normally die may have formed at least some synapses before degenerating, indicating a possible competition for postsynaptic sites. However, to better understand the significance of the ganglion cell death, it was necessary to determine more directly (*a*) whether

such ganglion cells had actually sent axons into the peripheral nerve before cell death, as appears to be the case for both dorsal root ganglion cells and spinal motoneurons in amphibia (14, 25, 26, 27); and (b) whether these axons had reached the proper peripheral target organ and established synaptic contact. Therefore, the present paper quantitatively compares the loss of axons in the peripheral nerve with the previously described loss of ganglion cells during normal development (18). It further attempts to determine whether the cells which die have actually sent axons to the wrong peripheral target; such evidence would be necessary in deciding whether cell death functions to remove improper connections.

This possibility can also be assessed by considering the cells in the ciliary ganglion as the peripheral targets of the two classes of preganglionic fibers (17, 18, 21) whose cell bodies lie in the accessory oculomotor nucleus in the midbrain (5). It has been shown that each preganglionic class selectively synapses with one of the two classes of ganglion cells (17). Therefore, it should be possible to assess whether any preganglionic fibers degenerate during normal development and whether those that do have actually formed synapses, and it can further be determined if they have synapsed with the proper type of ganglion cell.

Virtually all preganglionic cells die following early ablation of the optic vesicle and the consequent degeneration of the ciliary ganglion (5). However, it was not previously known whether cell death played a role in the normal development of the accessory oculomotor nucleus as well. Therefore, the degeneration of ganglionic synapses and preganglionic fibers was studied in both normal and peripherally deprived ganglia. Since we found (18, 19) that functional synapses formed on ganglion cells that were destined to die in both normal and peripherally deprived ganglia, it also seemed of interest to ascertain the fate of these synapses as ganglion cell death ensued.

This paper does not, however, attempt a detailed ultrastructural study of the development of myelinated axons (8, 24, 31), nor of the degeneration of myelinated or unmyelinated axons (6, 7, 22, 30), both of which have been described in other systems.

MATERIALS AND METHODS

Axon Counts

Methods which have already been described include electrophysiological estimations of the degree of gangli-

onic transmission and conduction velocity (17, 18), electron microscopical procedures (24), and surgical procedures (18). Cross sections of presynaptic and postsynaptic nerves were made in order to quantify the changes in axon numbers during development and to identify the degenerating profiles when present. During embryonic stages, because most of the nerve fibers were unmyelinated, both experimental and control nerves were studied with electron microscopy and, after hatching, with light microscopy. The sections were done for the presynaptic nerves just proximal to the ganglion in order to avoid contamination from the motor nerves innervating the extraocular muscles. The sections of the postsynaptic nerves were done immediately distal to the ganglion in order to keep away from the axons of the sensory ramus, which joins the ciliary nerves before they enter the sclera (21). The ultrathin sections were picked up in a slot grid which had previously been coated with a carbon-stabilized Formvar (Belden Mfg. Co., Chicago, Ill.) film. Pictures of the entire cross sections were photographed, and the measurement of the number of axons was done in a composite montage at a final magnification of 12,000-20,000. When the axons were identified, the diameter was measured, and histograms were plotted. Mean, standard deviation, and standard error were computed with a programmable Hewlett-Packard calculator (Hewlett-Packard Co., Palo Alto, Calif.). This procedure was applied to half of the samples. In the others, a less laborious counting method was used. Semithin plastic sections of the entire cross section were photographed at a magnification of 1,000-2,000, and their areas were measured. Approx. 10% of this area was then photographed with electron microscopy, enlarged, and axons were counted. An estimation of the total axon population was calculated from the total number of axons counted in the sample area. In one case, both procedures were applied to one cross section of the ciliary nerve, and comparable values were obtained, the difference being only 5% of the total number. Samples of cross sections were also taken at higher magnification for more detailed observation. This entire procedure was carried on for ciliary nerves of control ganglia. For the estimation of degenerating profiles and axons, the latter method was used. Montages of the nerve cross sections were divided into squares of $125 \mu\text{m}^2$. Axons and profiles were counted in a number of the squares, and mean and standard deviation of the values were calculated. Squares continued to be counted until the new values did not change the mean and standard deviation. These final numbers were used for the estimations. In experimental ganglia, sections of the oculomotor nerve, N1 (nerve branch between G_1 and G_2) and N_2 (the output of G_2 , see Landmesser and Pilar, 18), were also done.

At early stages (St.) (30-34), it was necessary to develop criteria for distinguishing filament-containing Schwann cell processes from axons. In some cases, it was possible to follow the profile to where it emerged from a clearly identified Schwann cell. In other cases, the presence of ribosomes, cisternae of rough endoplasmic

reticulum (RER), as well as mitochondria twice the size of those found in the axons, allowed us to distinguish the Schwann cell processes. Longitudinal sections of the axons were also done.

Synaptic Profiles

The photographs published in the present paper were selected from approx. 3,000 electron microscope glass plates. Although no serial sections of the ganglia were done with the electron microscope, the investigated ganglia isolated from St. 30–43 were sectioned at different levels, and samples of each level were taken until all ganglia were sectioned. In some cases, large montages of cross sections of the ganglia were taken in order to gain an unbiased estimation of the changes observed in a particular ganglion.

RESULTS AND OBSERVATIONS

Development of Peripheral Nerves

The larger ciliary and smaller choroid nerve cells send their axons out the ciliary and choroid nerves, respectively (17, 21). By St. 30, both sets of nerves are well formed, and at least the ciliary nerves have reached their peripheral target organ. In cross sections, both nerves can be seen to be formed by large numbers of small, naked axons ($0.3 \mu\text{m} \pm 0.001$ in diameter for the ciliary and $0.26 \mu\text{m} \pm 0.002$, for the choroid, mean \pm SE) separated into bundles by Schwann cells, there being roughly 30–70 axons per bundle. Such axon profiles contain neurotubules, neurofilaments, occasional cisterns of smooth endoplasmic reticulum (SER), and mitochondria.

Later, Schwann cell processes interdigitate between the axons, greatly increasing the ratio of the area of Schwann cells to axons, as has been described in other developing nerves (1, 23, 27, 31). By St. 39, Schwann cells begin to wrap around individual axons forming myelin sheaths (8). The cisternae of Schwann cell SER develop a somewhat dilated appearance at this time, and contain a finely floccular material, possibly associated with the formation of myelin. (An example of this can be seen in Fig. 3, and also in Fig. 14 of the previous paper.) 60% of the ciliary cells have been invested by myelin by St. 43, a process which is complete by hatching. The choroid cells are slower to myelinate, the process only beginning around hatching.

Since the long, parallel ciliary nerves were more amenable to study and since the time of peripheral synapse formation by them had already been documented, this population was selected for the quantitative study that will follow. The choroid

nerves were also studied, although not systematically.

Degeneration of Ganglion Cell Axons During Normal Development

In order to determine whether normally degenerating ciliary cells had axons in the peripheral nerve, electron microscope montages of ciliary nerves were surveyed for degenerating axon profiles from St. 30 to hatching. As can be seen in the graph of Fig. 1, degenerating axon profiles are first observed at St. 34 and reach a peak of approx. 500 profiles per nerve by St. 37. The number then declines, and no degenerating profiles are observed after hatching. Thus, at least some of the degenerating ganglion cells are represented by axons in the postganglionic nerve. It further appears that the degeneration of axons lags slightly behind the death of ganglion cells as previously described (18).

A quantitative comparison was not made of degenerating profiles, for, as seen in Fig. 2 (arrow), at early stages (36–37) degeneration occurred in clusters, with groups of adjacent axons enclosed by a common Schwann cell degenerating simultaneously (6). As degeneration ensued, the dark axonal profiles became clumped together, preventing a quantitative estimate of the number of axons involved. Moreover, since the time taken by degenerating profiles to disappear is not known, a

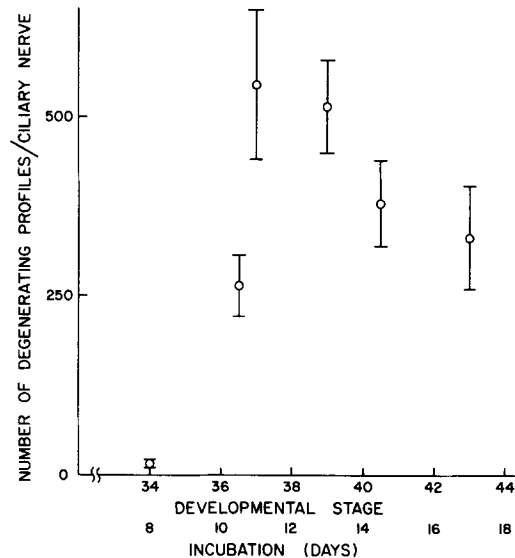


FIGURE 1 Number of degenerating axonal profiles in the ciliary nerves during the period of cell death. (mean \pm SE).

quantitative analysis is further complicated. Not all the axons enclosed by a single Schwann cell degenerated; however, some of them appeared completely normal (Fig. 2).

At later stages, degenerating axons did not

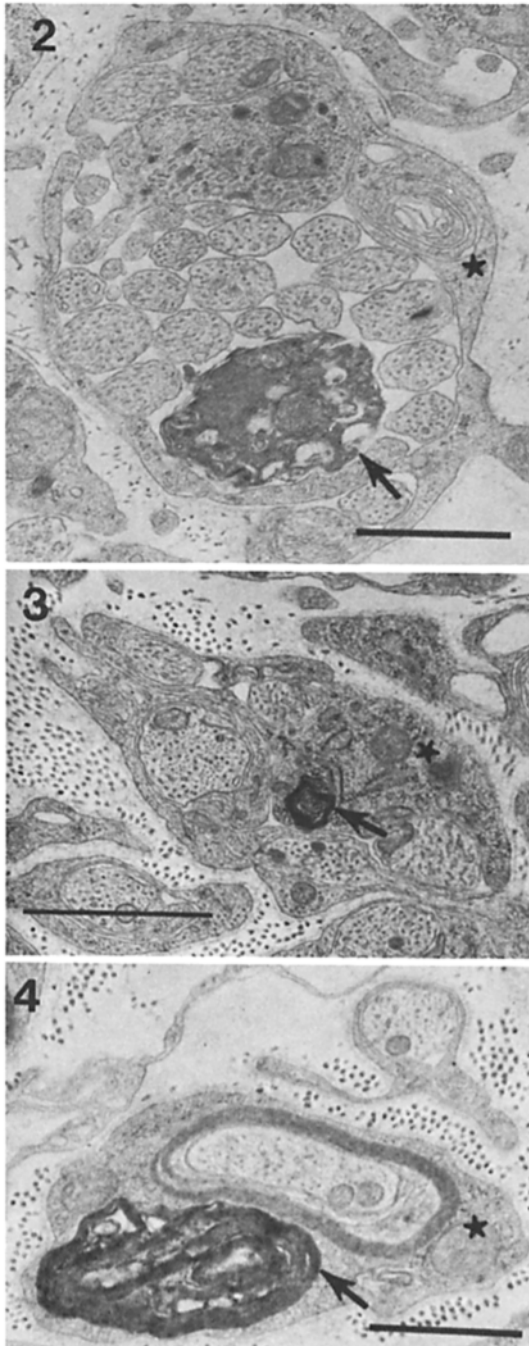
occur in clusters, but rather singly as shown in Fig. 3 for St. 39 and Fig. 4 for St. 42. In the latter case, both the degenerating and adjacent normal profile contain myelin sheaths and share a common Schwann cell. Thus, some of the axons which degenerate have already been myelinated.

A Comparison of Ganglion Cell and Axon Number in Normal Ganglia

If the total number of axon profiles in the ciliary nerves at different developmental stages (Fig. 5, middle graph) is compared with ganglion cell number (upper graph) taken from an earlier study (19), it is apparent that there is a large loss of axons between St. 36 and 40 which parallels but lags slightly behind the loss of cells. This corresponds with the results obtained by observing degenerating axonal profiles (Fig. 1), and indicates that many cells which die have axons in the postganglionic nerve. Further, degeneration appears to proceed in a direction from soma to axon.

However, another phenomenon which cannot be explained solely by the degeneration of existing profiles is apparent in the data of Fig. 5. The total number of axons in the ciliary nerves actually increases from approx. 7,000 axons at St. 30 to 13,000 at St. 36½. Since cell bodies have already begun to be lost during this period, the net increase must be even greater. If one expresses the data in terms of axons per ciliary cell, assuming that all cells have sent out axons (Fig. 5, lower graph), then each ciliary cell has approximately two axon branches at St. 30, a value which increases to five by St. 36. (Ciliary cells account for approximately half the total ganglion cell population.)

If all ganglion cells have not sent axons into the nerve by St. 30, then those that have must possess more than two axonal branches. While the increase in axon number between St. 33 and 36 could be explained by a late outgrowth of axons from some ganglion cells, it is also consistent with an increase



FIGURES 2, 3, and 4 Appearance of degenerating axonal profiles (arrows) in ciliary nerves at different developmental stages. At St. 36 (Fig. 2), axons are naked and degenerate in clumps, whereas at St. 39 (Fig. 3) and St. 42 (Fig. 4) they degenerate singly. Schwann cells (asterisks) have wrapped around the axons in Fig. 3, and both the degenerating and normal profile seen in Fig. 4 are myelinated. Calibration = 1 μ m; \times 19,000; \times 20,000; \times 20,000.

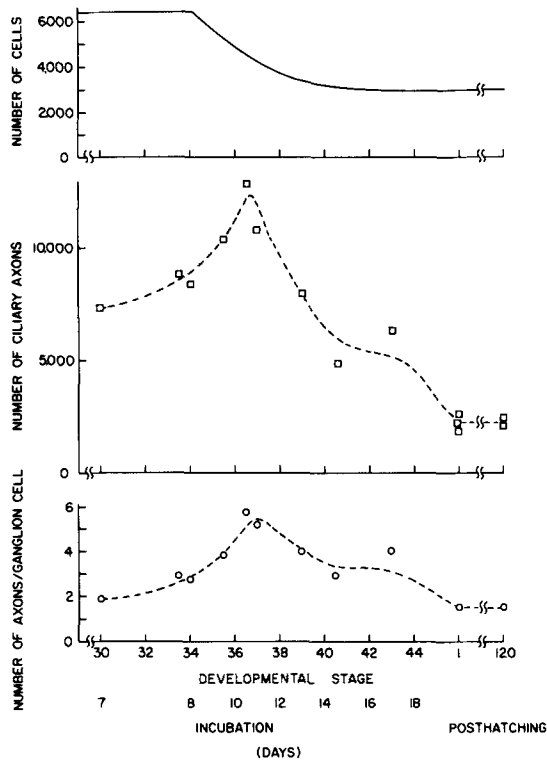


FIGURE 5 The decline in ganglion cell number during normal development (upper graph) compared with the number of axons in the ciliary nerves over the same developmental stages. This is expressed as total number of axons per ciliary nerve (middle graph) and axons per ciliary cell (lower graph). Curves fitted by eye.

in collateral sprouting of axons already in the ciliary nerves.

Ultrastructural evidence in support of this proposition was obtained. When histograms of axon diameters were made of ciliary nerves between St. 34 and 36, two populations of profiles with mean fiber diameters of $0.60 \mu\text{m}$ and $0.3 \mu\text{m}$ were observed. The smaller, more numerous profiles (85% of the total) contained mostly neurotubules oriented parallel to the long axis and occasional mitochondria (a, Figs. 6 and 7). Microtubules were more irregularly distributed in the larger profiles which contained more filamentous membranous material and vesicles (v, Fig. 6). Longitudinal sections (Fig. 7) demonstrated that the larger profiles were not a separate population but rather varicose swellings along axons in which the ordered array of microtubules was disrupted. Since, in fortuitous sections, multiple varicosities were

observed along single axons, they do not appear to be terminal growth cones.

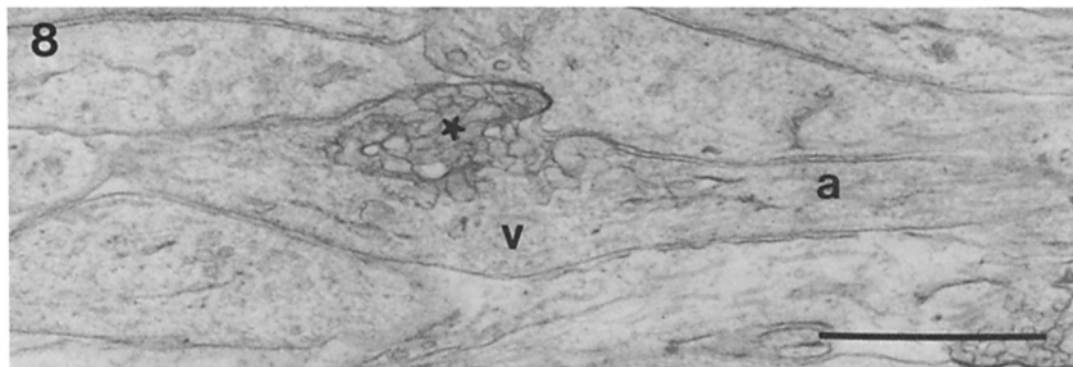
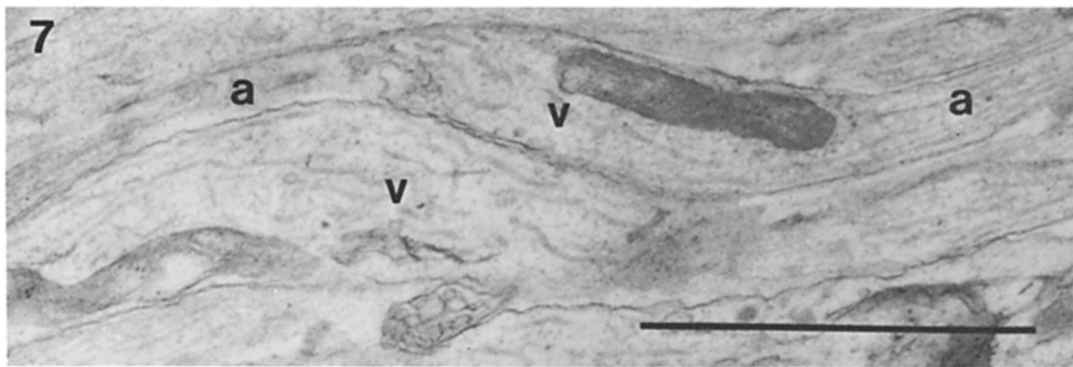
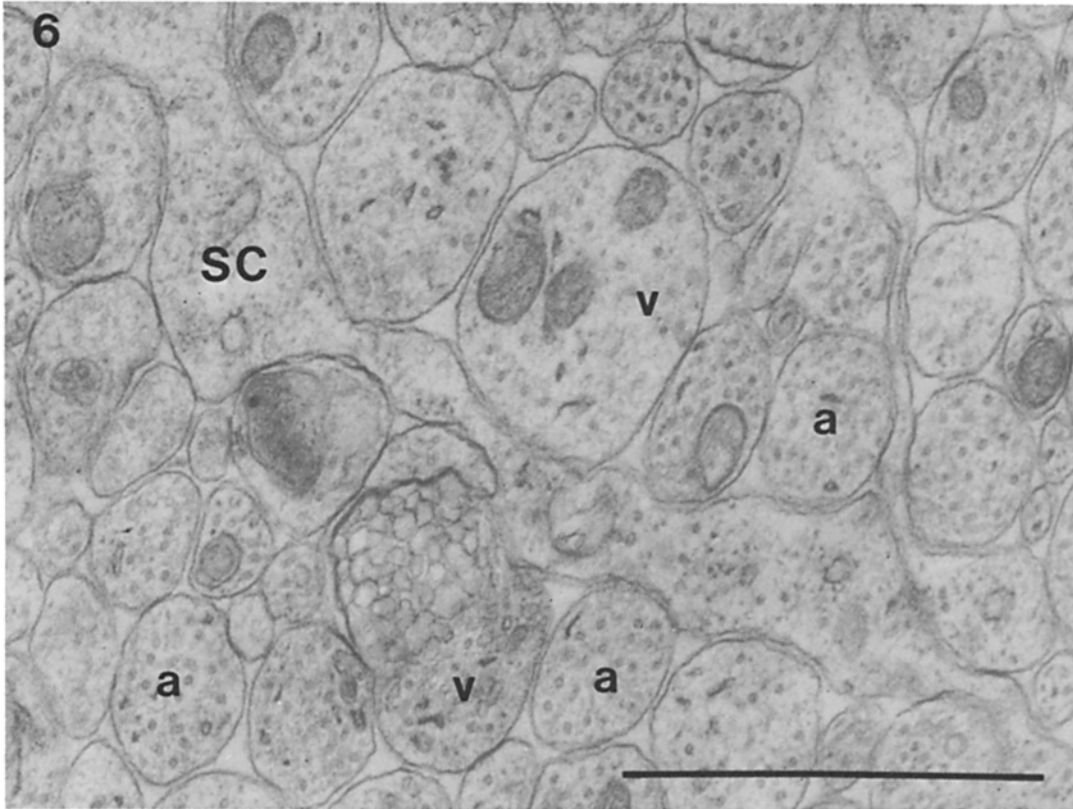
However, at St. 34 it was common for a proportion of such varicosities to contain a mass of vesicular material (Fig. 6, asterisk). Such profiles declined from a frequency of 2-3 per $125 \mu\text{m}^2$ at St. 34 to less than 1 by St. 36. In longitudinal sections (Fig. 8, asterisk) these vesicle-filled structures appeared to bud off the axonal varicosity, were reminiscent of growth cones, and may represent the initiation sites of axonal sprouts. Since they were observed to arise from axons which could be followed for some distance distally, they do not seem to be terminal growth cones but rather the start of collateral branches. Such sprouting beginning at St. 34 could result in the increased number of axons seen at St. 36.

What triggers this apparent increase in axonal sprouting? Since this occurs shortly before synapses are formed with the peripheral target organ, some interaction with the periphery, not necessarily synaptic, may be involved. In fact, in the postganglionic nerves of peripherally deprived ganglia, these profiles are not observed at St. 34 when they are very common in control nerves.

In Fig. 5 a secondary loss of axons is seen to occur between St. 40 and hatching. Since cell number is constant over this period, the axon loss cannot be explained by loss of ganglion cells. As pointed out earlier, axons during this period do not degenerate in clusters, and the degeneration observed here may represent a phenomenon different from that seen earlier when clumps of axons degenerate at the time cell bodies are dying. Since degenerating myelin profiles were observed (Fig. 4) and since myelination does not begin until St. 40, it is likely that such profiles represent axons that have died after St. 40 and are not merely degenerating profiles persisting from earlier stages. Therefore, it seems that there is a secondary loss of axon collaterals not associated with cell death, and it can further be inferred that not all axons which become myelinated ultimately survive.

Axons from Peripherally Deprived Ganglion Cells

A systematic quantitative study of ganglion cell axons was not made for peripherally deprived ganglia, for in such cases the postganglionic nerves varied considerably in anatomical arrangement and contained a mixture of ciliary and choroid axons (see Materials and Methods and 18). How-



FIGURES 6, 7, and 8 Evidence of collateral sprouting in the ciliary nerves. A cross section of a St. 34 ciliary nerve shows numerous axonal profiles (*a*) which contain mostly an ordered array of neurotubules. Larger profiles (*v*) can be seen, in Fig. 7, to be varicose swellings along the axon. Growth conelike structures were seen to bud off the varicosities in both longitudinal (Fig. 8, asterisk) and cross sections (Fig. 6, above lower *v*). Schwann cell (*SC*). Calibration = 1 μm ; $\times 56,000$; $\times 52,000$; $\times 30,000$.

ever, such axons went through a sequence of events comparable to that described for normal development, with the exception that axon death was much more extensive and occurred mostly before myelination with few profiles persisting past St. 40. An additional difference was that they failed to show ultrastructural evidence of collateral sprouting seen in control ciliary nerves between St. 34 and 36. In a previous study, axon counts were performed on one peripherally deprived ganglion at St. 30 (18). The number of axons was comparable to the control number, indicating that most of the peripherally deprived cells had sent out axons. In conclusion, the periphery is not required for initial axon outgrowth, but appears necessary for the secondary collateral sprouting that occurs during synapse formation.

Fate of Synapses on Peripherally Deprived Ganglion Cells

It is now possible to turn from the fate of ganglion cells themselves to a consideration of the preganglionic fibers which synapse upon them. Previous electrophysiological evidence has shown that by St. 34 all ganglion cells, both normal and peripherally deprived, have been synapsed upon (18). Similar types of synapses were observed in both the peripherally deprived (Reference 18, plate 2 & 3) and control ganglia as shown in Figs. 9, 10, and 11.

By St. 34, some of the calyciform endings have already formed and, as shown in Fig. 9, contain a relatively large amount of SER as well as scattered mitochondria, and some neurotubules and neurofilaments. Synaptic vesicles are scarce and occur only in small groups close to postsynaptic densities (arrow) which are spaced intermittently around the calyx-ganglion cell boundary. However, most of synapses still persist in the form of the initial contacts as small boutons (1-2 μm) on either cell processes or soma (Figs. 10-11). These boutons contain an array of organelles similar to that described for the calyx.

The study of Cowan and Wenger (5) showed that, following degeneration of peripherally deprived ganglion cells, a retrograde transneuronal degeneration of the preganglionic cell bodies began at St. 35 and was virtually complete by St. 40. If only one preganglionic cell synapsed on a single ganglion cell, one might expect that with the onset of ganglion cell degeneration the preganglionic ending would be affected in some manner that

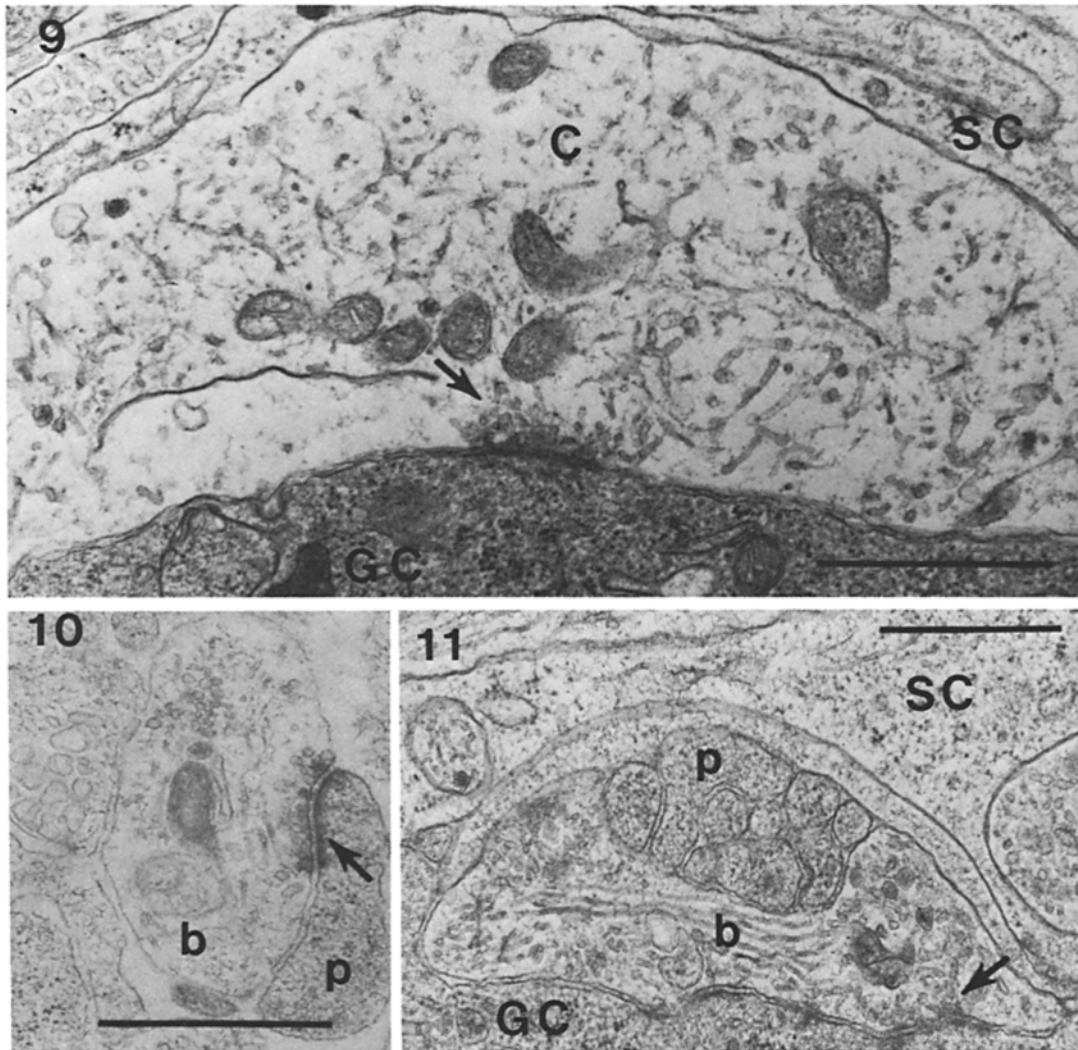
would ultimately cause the parent cell body to degenerate. In fact, by St. 36 $\frac{1}{2}$ calyces ending on very degenerate cells can be seen to contain numerous fingers of degenerating postsynaptic cytoplasm (Fig. 12, asterisks). It appears that some of the degenerating fingers are phagocytosed by the ending and that this phagocytosis together with other alterations of the preganglionic structure might result in death of the parent cell body.

However, it is known from cell counts of the ciliary ganglion (18) and the accessory oculomotor nucleus (5) that a one-to-one relationship does not exist, there being an excess of ganglion cells. Further, single preganglionic fibers have been observed to synapse with more than one ganglion cell (our unpublished observations). This, as well as the fact that ganglion cell deaths occur over a period of 4 days (18), complicates the analysis of the ultrastructural observations.

These factors, though, make it possible for a single preganglionic cell to simultaneously be in synaptic relationship both with degenerating and with as yet normal ganglion cells. This would explain the observation of normal synapses on degenerating postsynaptic cells (Figs. 13 and 14), since the parent cell body in these cases might be sustained by synapses with other normal cells. However, extremely degenerated synapses were also observed on relatively normal ganglion cells. In order to explain this phenomenon, it is necessary to postulate that a single synapse is not sufficient to prevent the death of the preganglionic cell.

Degeneration of preganglionic elements was first detected as a loss of most intracellular organelles, resulting in a rather watery appearance. There was also an increase in fine filamentous material, as seen in the calyciform ending shown in Fig. 15. Later, typical dense, fibrous, degenerating profiles were common.

Since synaptic vesicles were sparse in all calyces up to St. 40, presumed degenerating endings did not contain clumped synaptic vesicles. This made it difficult to be sure that such profiles represented endings and not just adjacent axons, as similar dense profiles are common in the oculomotor nerve during the period of cell death. However, the clear calyciform nature of some of these processes, with parts of the calyx interdigitating with the finger like somal processes (Fig. 16), made identification possible. In this case, the extremely degenerated ending occurred on a relatively normal cell. Fingers of glial cytoplasm were seen to interpose



FIGURES 9, 10, and 11 Normal synapses on ganglion cells (GC) at St. 34 were in the form of calyces (Fig. 9, C) or boutons (Fig. 10 and 11, b) and could occur either on the cell soma or on somal processes. Synaptic areas indicated by arrows. SC = satellite cell. Calibration = 1 μ m; \times 31,000; \times 30,000; \times 24,000.

between such degenerating endings and the postsynaptic cell. As seen in Fig. 17, such glial cytoplasm was watery in appearance with few organelles, and desmosome-like contacts (arrows) were seen to join adjacent glial processes. Degenerating endings eventually were phagocytosed by the surrounding satellite cells.

The above observations together with those of Cowan and Wenger (5) show that preganglionic cells, many of which have formed synapses with ganglion cells, undergo a form of transynaptic degeneration and die when their peripheral target

organ degenerates. However, the occurrence of degenerating synapses on normal cells suggested that preganglionic cell death might be a normal occurrence which is only intensified by degeneration of the ciliary ganglion.

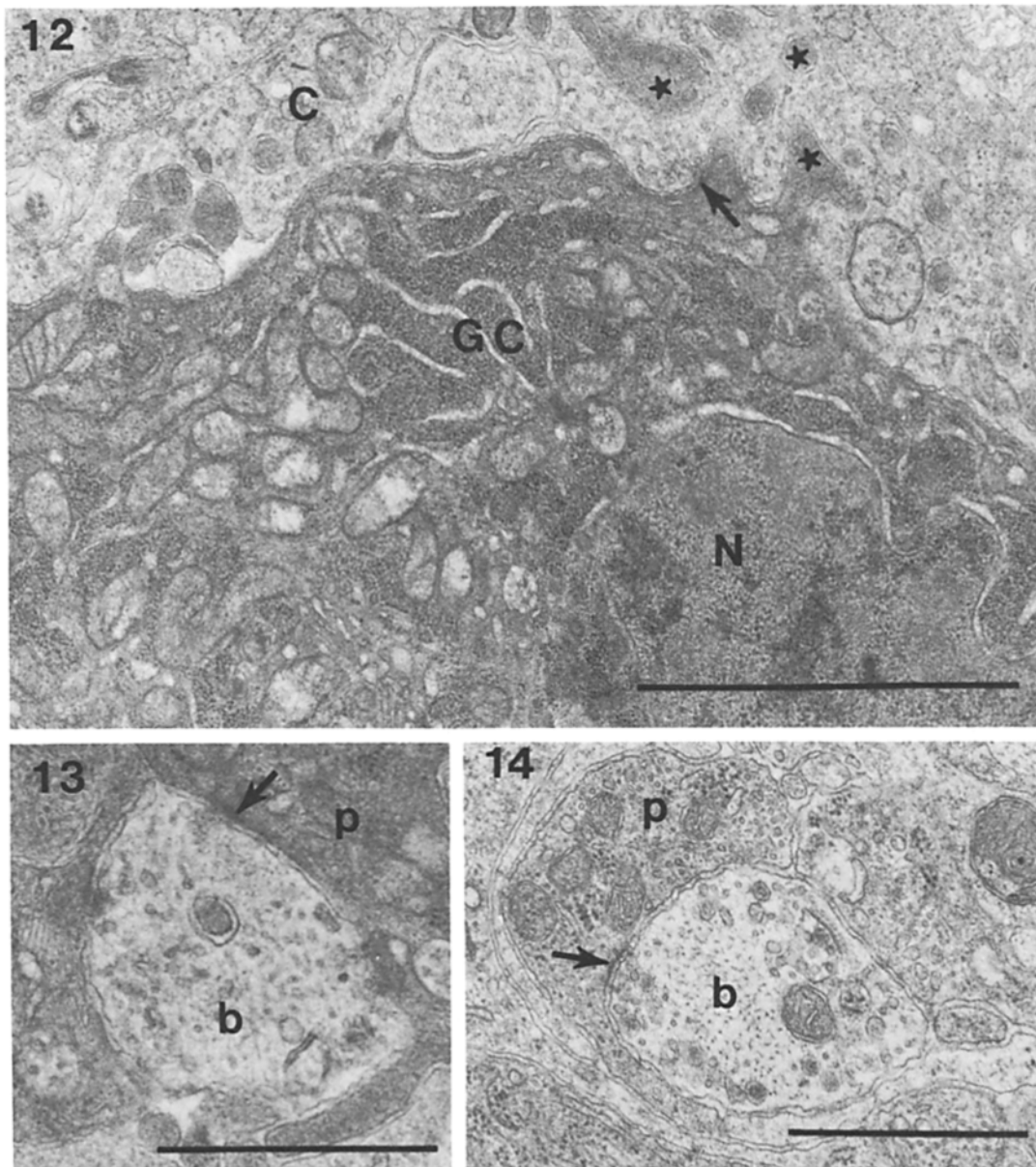
Degeneration of Preganglionic Fibers in the Oculomotor Nerve

No quantitative counts were made of the total number of preganglionic axons in the oculomotor nerve, but photographic montages of this nerve

were surveyed at various stages of development in both normal and peripherally deprived embryos. As can be seen in Figs. 18 and 19, by St. 37 the peripherally deprived oculomotor nerve at its entry into the ganglion contains many axons in various stages of degeneration, these representing 56% of the total number of axons. This is consistent with

the observation that degeneration of preganglionic cell bodies is, by this time, extensive (5).

While less severe, degeneration is also a marked phenomenon in the normal oculomotor nerve. At this stage, approx. 32% of the preganglionic fibers are showing definite signs of degeneration. Therefore, it can be concluded that death of pregangli-



FIGURES 12, 13, and 14 Synapses on degenerating peripherally deprived ganglion cells (GC) could contain profiles of degenerating postsynaptic cytoplasm (Fig. 12, asterisks) or could be relatively intact (Figs. 13 and 14). C = calyx; b, bouton; p, ganglion cell process; N, nucleus. Synaptic sites indicated by arrows. Calibration = 1 μ m; \times 52,000; \times 37,000; \times 32,000.

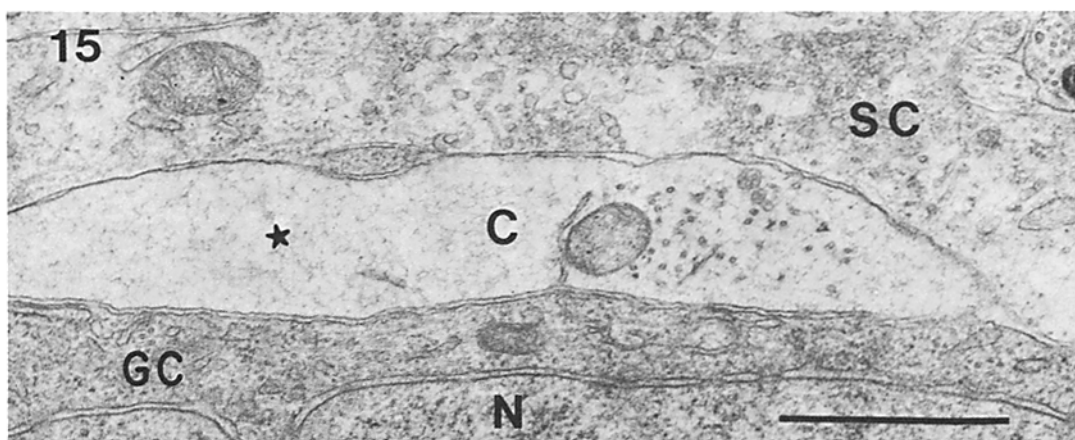


FIGURE 15 Early stage of synaptic degeneration showing a calyx (C) containing fibrous material but few organelles (asterisk) synapsing on a peripherally deprived ganglion cell (GC). SC, satellite cell; N, nucleus. Calibration = 1 μ m; \times 31,200.

onic fibers is a part of normal ganglionic development. The cells which die have managed to send axons as far as their peripheral target organ. The next question is whether these degenerating fibers have actually formed ganglionic synapses.

Synapses on Normal Ganglion Cells During the Period of Cell Death

It is not possible to determine in a quantitative manner whether all of the degenerating fibers in the oculomotor nerve have formed ganglionic synapses. However, it was possible to demonstrate qualitatively that degenerating synapses were relatively common during the period of preganglionic fiber degeneration, indicating that some preganglionic cells had formed synapses before dying.

The actual form of degeneration was similar to that already described for the peripherally deprived ganglion. By St. 36 $\frac{1}{2}$ -37, degenerating profiles were observed on both normal (Fig. 20) and degenerating (Fig. 21) ganglion cells. These figures represent a slightly earlier stage of degeneration than that shown in Figs. 16 and 17 for the peripherally deprived ganglion.

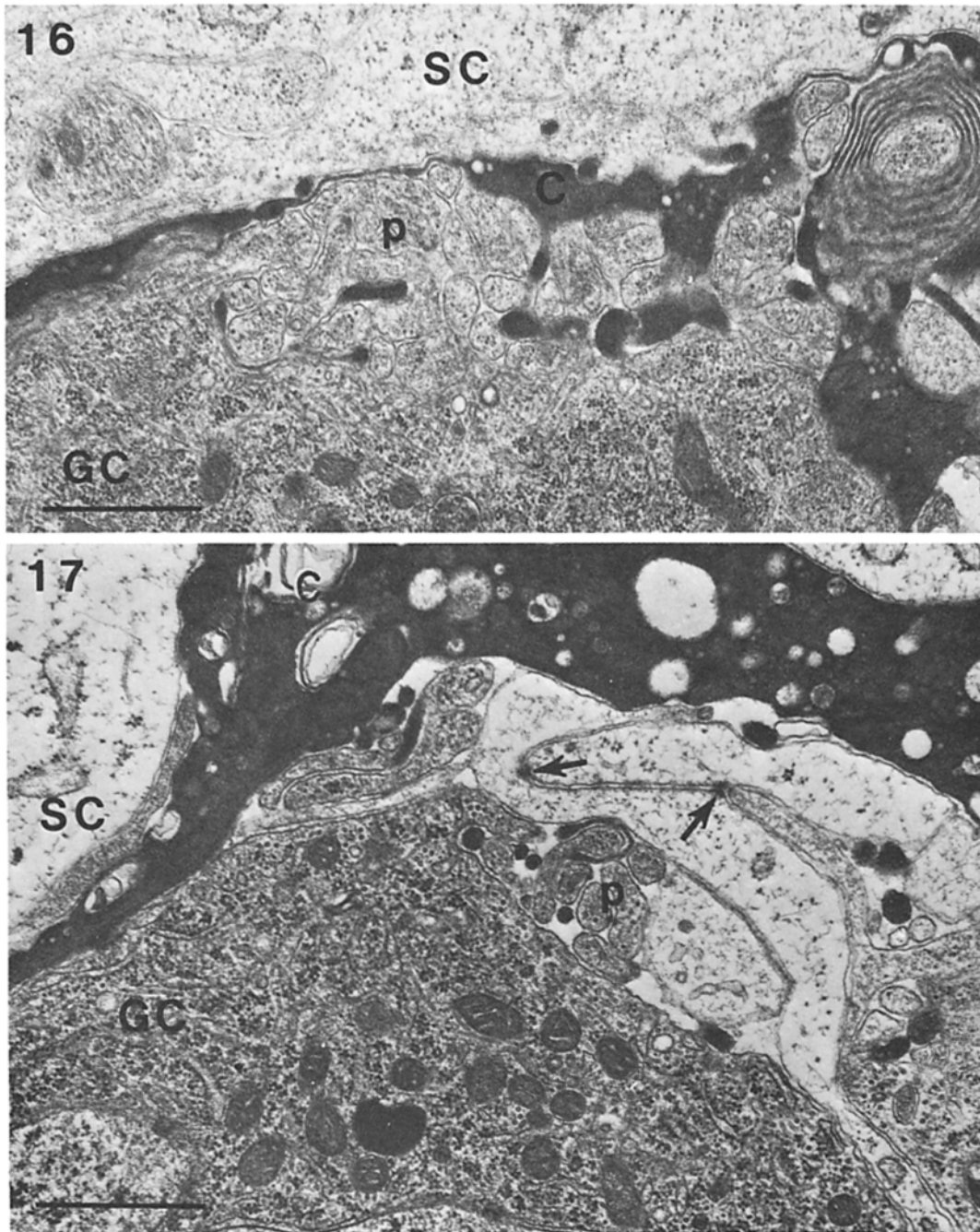
Both degenerating boutons and calyces were seen. As in the peripherally deprived situation, it appeared that glial processes (arrow, Fig. 22) interposed between the degenerating ending and the ganglion cell soma.

This degeneration of synapses on normal cells may partially explain the electrophysiological observation that transmission failed on approximately half the cells between St. 36 and 37, but

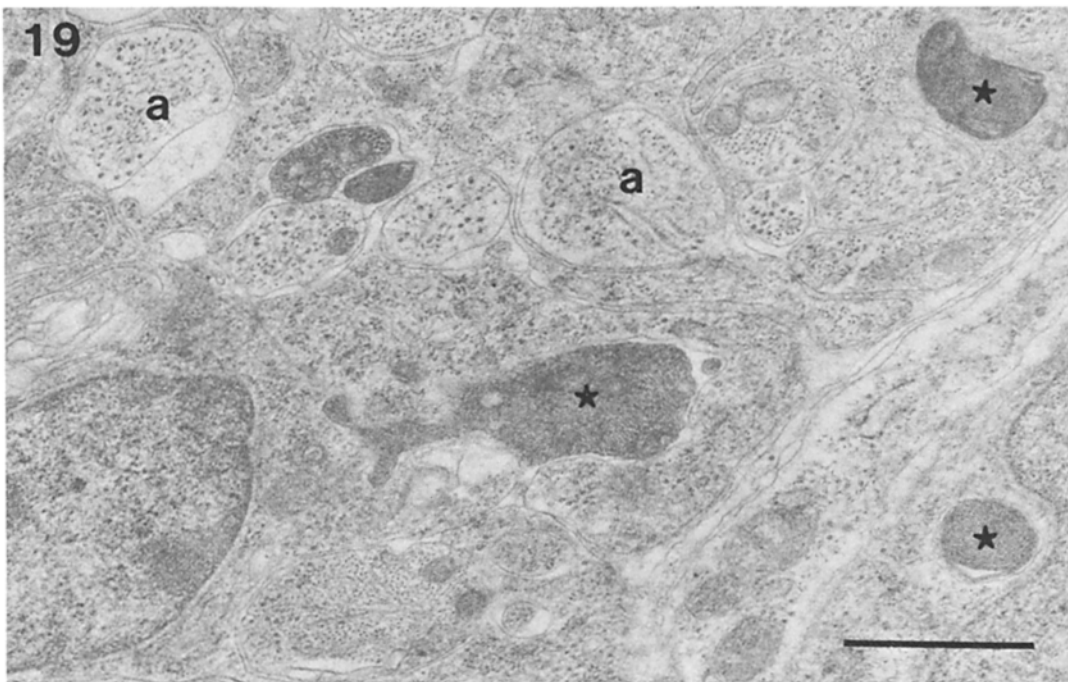
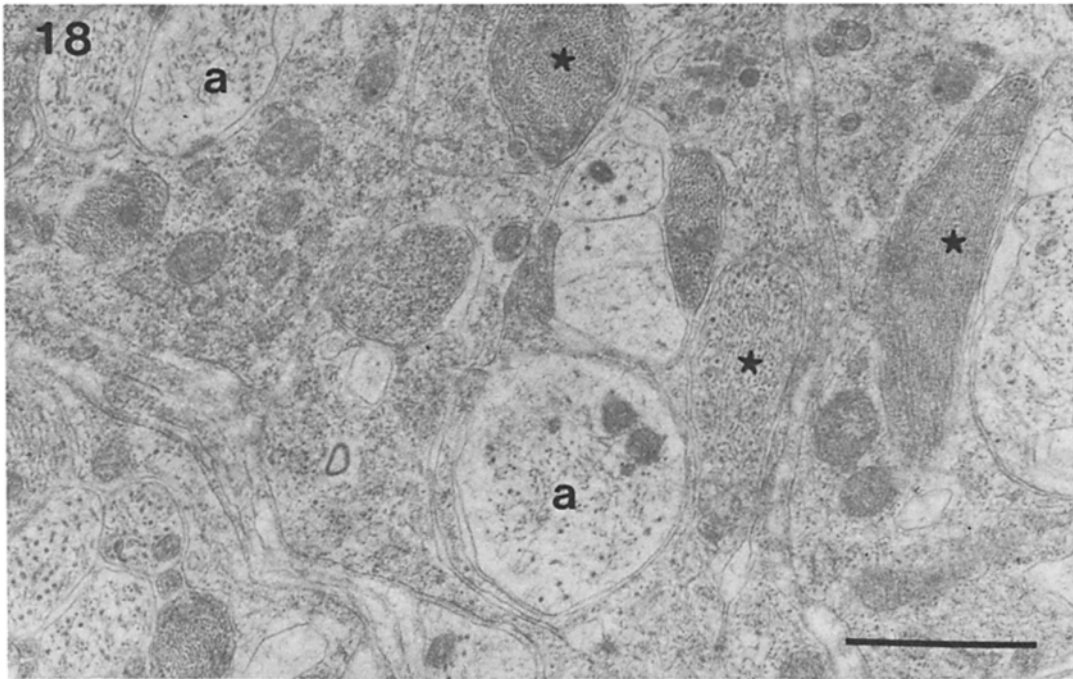
returned to normal (100%) by St. 40. By combining this information with cell counts, we calculated that approximately one third of the cells destined to survive underwent a transient failure of transmission between St. 37 and 38 (19). Although subject to a number of assumptions, this observation received ultrastructural confirmation in the present study. At St. 36, calyces were observed on almost all ciliary cell profiles which passed through the nucleus, but by St. 37 many normal ciliary cells appeared devoid of synapses and were surrounded by glia, which had apparently displaced degenerated synapses. Again, by St. 40 it was rare to find a ciliary cell profile through the nucleus without a calyx. Surviving preganglionic cells presumably form new synapses with these ganglion cells. In fact, between St. 36 $\frac{1}{2}$ and 40, many calyces had an altered appearance and contained many more neurofilaments, SER, large dense-core vesicles, autophagic vacuoles, etc. as seen in Fig. 23. It is suggested that these may be growing calyces, the surviving preganglionic cells now innervating any cells left transiently denervated after the period of cell death.

Connectivity of Surviving and Degenerating Neurons During Normal Development

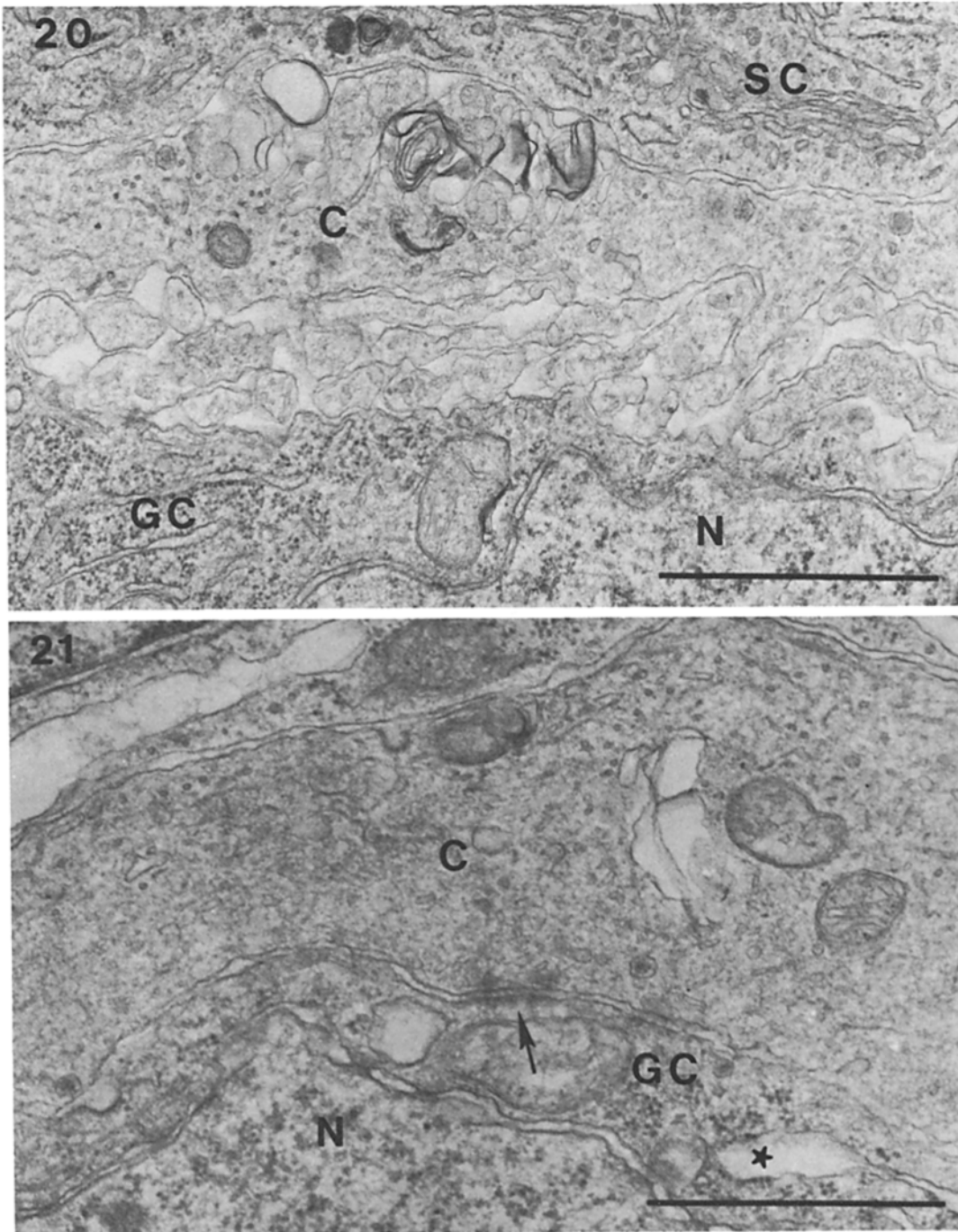
In order to determine whether degenerating neurons were those that had grown down wrong pathways or formed improper connections, conduction velocities of pre- and postganglionic axons were measured. It has been shown that ciliary cells have faster conducting axons than choroid cells,



FIGURES 16 and 17 Degenerating synapses on peripherally deprived ganglion cells. In Fig. 16, a dark degenerating calyx (C) can be seen in contact with ganglion cell soma (GC) and processes (p). In Fig. 17, a similar degenerating calyx (C) seems to be in the process of being replaced by satellite cell (SC) processes, which are joined by desmosome-like contacts (arrows). Degenerating fingers of calyx cytoplasm are interdigitated with ganglion cell processes (p) in both figures. Calibration = 1 μ m; \times 22,000; \times 25,000.



FIGURES 18 and 19 Both the peripherally deprived (Fig. 18) and the control (Fig. 19) oculomotor nerves at St. 37 contain numerous degenerating axons (asterisks) intermixed with normal axons (a). Calibration = 1 μ m; $\times 25,000$; $\times 25,000$.



FIGURES 20 and 21 Degenerating calyciform synapses observed in control ganglia at St. 37 both on normal (Fig. 20, note normal RER) and on degenerating (Fig. 21, dilated cisternae of RER marked with asterisk) ganglion cells were more electron dense and contained more filamentous material than control calyces (compare with Fig. 9). *GC*, ganglion cell; *SC*, satellite cell; *C*, calyx; *N*, nucleus; arrow, synaptic site. Calibration = 1 μ m; \times 42,000; \times 40,000.

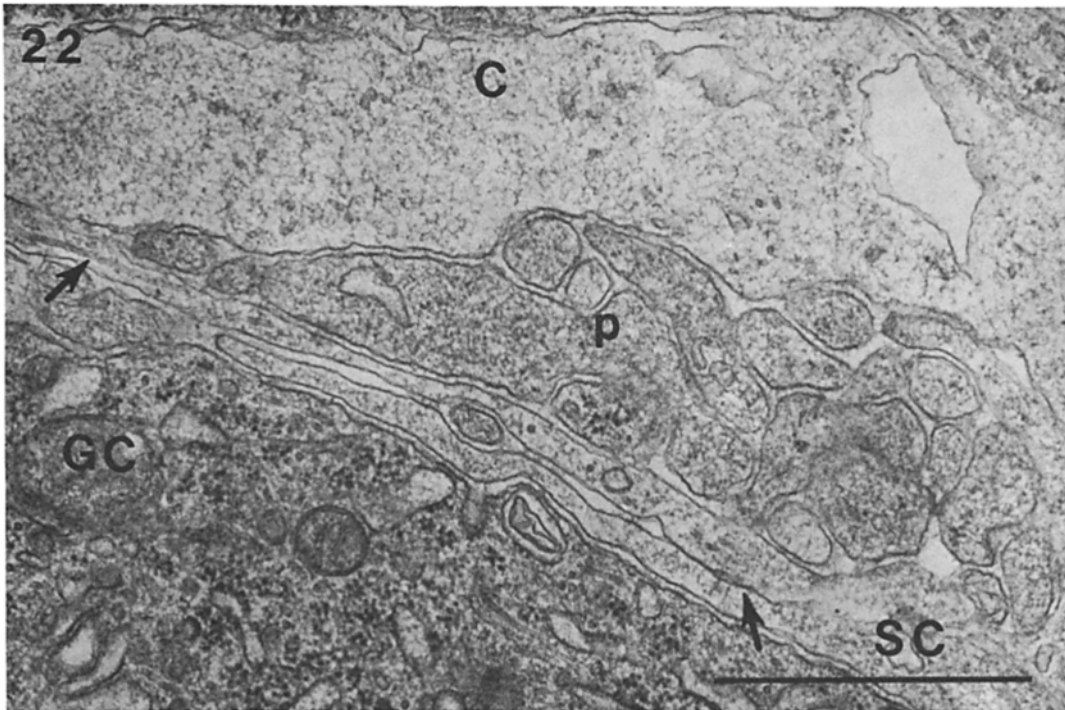


FIGURE 22 Satellite cell (SC) processes (arrows) interpose between degenerating calyx (C) and ganglion cell (GC) from a St. 37 control ganglion. *p*, ganglion cell process. Calibration = 1 μ m; \times 41,600.

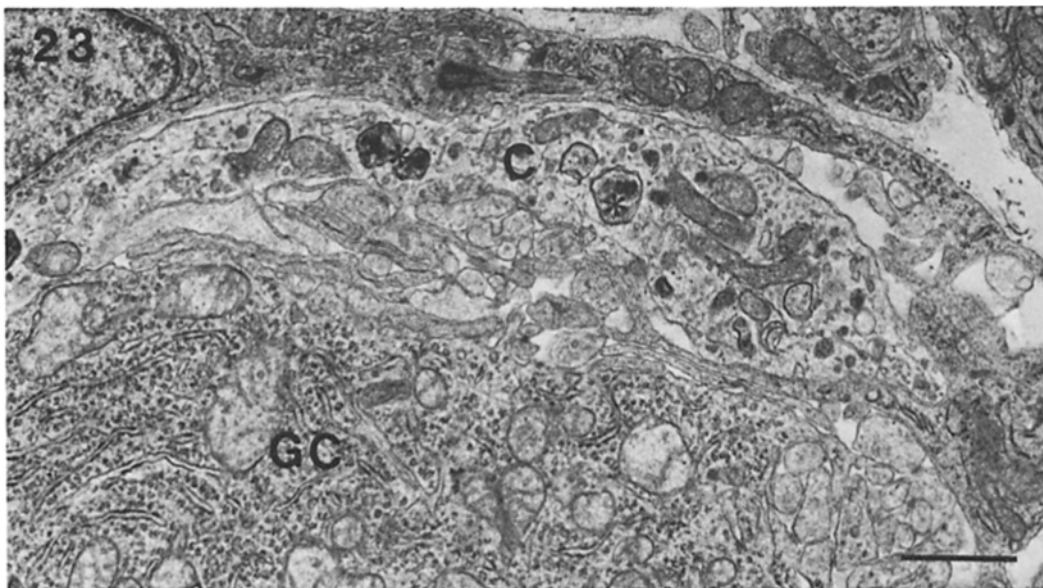


FIGURE 23 A reactive, possibly growing, calyx (C) from a normal St. 37 ganglion contains dense-core vesicles, autophagic vacuoles (asterisk), and increased amount of tubular and vesicular material. GC, ganglion cell. Calibration = 1 μ m; \times 15,000.

and that they are synapsed upon by a faster conducting class of preganglionic fibers, even during development (17).

Since we have demonstrated that many ganglion cells which die have axons in the postganglionic nerve, improper growth of axons down the wrong nerve should be electrophysiologically detectable. However, throughout development, even before the period of cell death, axons in the ciliary nerve conducted at a significantly greater velocity than axons in the choroid nerve. Illustrated in Fig. 24 are combined values for conduction velocity at St. 30-35 (mean \pm SD). This is supported by anatomical measurements of mean axon diameter which were consistently larger for the ciliary population. For St. 30-34, the mean diameter of ciliary axons was 0.30 ± 0.001 (mean \pm SE) and of choroid axons 0.24 ± 0.002 (mean \pm SE). There is therefore no evidence that ganglion cells grow down the wrong nerve, and most of the ganglion cells which die have probably sent axons down the proper nerve. This would presumably guide them to the correct target, since the choice

of growing down the ciliary or choroid nerves is made close to the ganglion.

Unfortunately, it was not possible to determine whether such cells had made synapses with the iris muscle. Degenerating profiles, possibly synaptic, were observed in the iris but, due to their immature state, i.e., sparsity of vesicles, could not be distinguished from axons. However, as already described, many preganglionic fibers had actually formed synapses before degeneration. In order to tell whether they had synapsed with the proper type of ganglion cell, the conduction velocities of preganglionic fibers synapsing with ciliary and choroid cells were determined. As shown in Fig. 24, at St. 34, just before the period of cell death, those fibers synapsing with ciliary cells conducted at 0.54 ± 0.19 m/s and those with choroid cells at 0.18 ± 0.08 m/s (mean \pm SD). Thus, it can be inferred that some of the preganglionic cells that die have actually formed synapses with the proper target organ.

Further, since it has been shown that all ganglion cells, even those that degenerate, are syn-

STAGE 34

PRESYNAPTIC

POSTSYNAPTIC

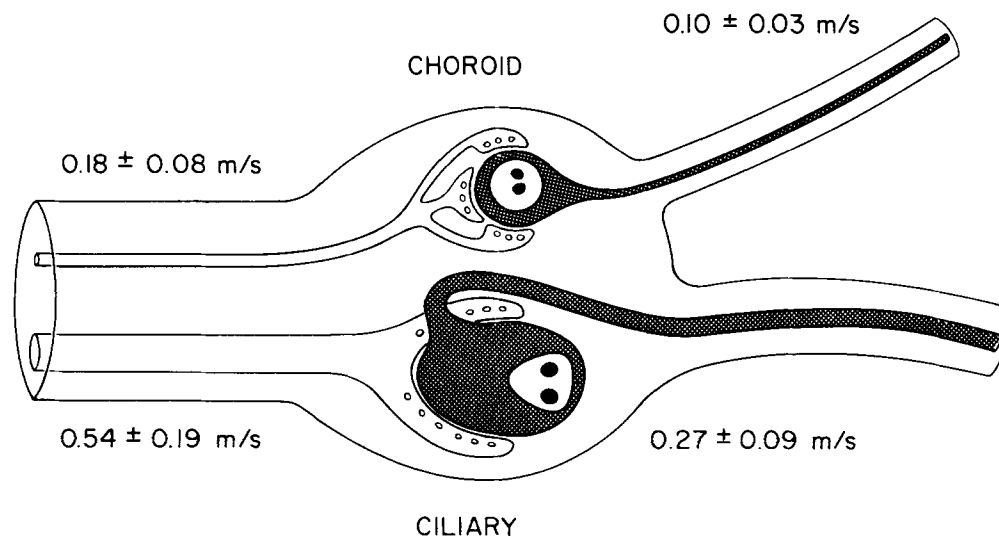


FIGURE 24 A schematic diagram of a St. 34 ganglion containing one ciliary cell and one choroid cell. The faster conducting preganglionic fiber synapses with the ciliary cell, the slower conducting one with the choroid cell. The ciliary cell axon also conducts more rapidly than the choroid axon.

apsed upon (18, 19), the above results would indicate that even degenerating ganglion cells have been synapsed upon by the proper type of preganglionic fiber.

DISCUSSION

This paper attempts to define the role of cell death during normal development, and to further characterize the principles governing the formation of synaptic connections in a simple neuronal system. In summary, the two classes of neurons in the ciliary ganglion appear to send axons toward their proper target organs from an early stage. Later, after an interaction with the peripheral target organ and just before peripheral synapse formation, they send out additional axon collaterals. After a presumed period of competition for peripheral synaptic sites, a large number of cells die, probably because they have formed an insufficient number of synapses. Later, after the period of ganglion cell death and the start of myelination, other, presumably excess, axon collaterals are lost.

Normally occurring death of neurons which have already sent out axons is well documented in other systems (14, 27). Yet, neither the increase in axon collaterals at the time of synapse formation nor the subsequent degeneration of axon collaterals independent of cell death has been observed. However, a normal part of neuromuscular development in both focally and multiply innervated muscles seems to be an early hyperinnervation of the individual muscle fibers followed by subsequent loss of a number of inputs (3, 29). It has been speculated that this reflects motoneuron death (3), which may well be the case in some systems.

Yet, the decrease in multiple innervation observed by Bennett and Pettigrew (3) in developing chick limb muscles appears to be too late to be associated with death of motoneurons (11). Such loss of connections may rather represent atrophy of individual collaterals which have not competed effectively for synaptic sites, as seems to occur in the present study. Since most target organs increase greatly in size after the death of the neurons that innervate them, additional synapses may be required and some further competition perhaps during the development of motor units may occur.

Since quantitative studies were not carried out on the preganglionic fibers, it is not possible to make a complete comparison with the ganglion cell situation. Yet, in many respects, the sequence of events as outlined in Fig. 25 appears similar. If a preganglionic fiber (Fig. 25 A) by chance synapsed

exclusively with ganglion cells which were destined to die by virtue of their failure to form peripheral synapses, it too would eventually degenerate. This would occur extensively in the peripherally deprived ganglion and possibly to some extent in the normal as well. The cell shown in Fig. 25 B would also die since a single ganglionic synapse would not be able to prevent cell death. That multiple synapses are required is apparent from the observation of degenerating preganglionic synapses on normal cells as well as from observations of the period after cell death when many ganglion cells are denuded of synapses. This preganglionic cell death might be intensified by the transition from multiple bouton contacts to single calyces on each ciliary cell, which occurs just before the period of ganglion cell death and during which time some preganglionic fibers may be outcompeted for synaptic sites. It seems that this process can account for the previously described transient failure of ganglionic transmission, and the fact that transmission failure seems to precede cell death, at least for some cells (19).

The cell shown in Fig. 25 C survives because it has formed synapses with a sufficient number of ganglion cells, and, after the period of cell death,

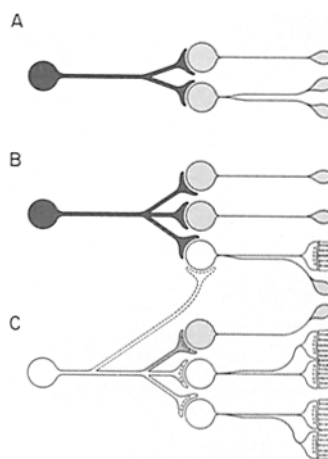


FIGURE 25 Summary of anatomical alterations that occur in the ciliary ganglion during and after the period of cell death. Ganglion cells (center) either contact or fail to contact the iris muscle (right). Light stippling indicates ganglion cells which degenerate; dark stippling, preganglionic cells (left) which subsequently degenerate (A, B). The preganglionic cell in C survives and sprouts a collateral branch (dashed line) to innervate the denervated ganglion cell. Another collateral (diagonal lines) from the same cell degenerates after death of the ganglion cell with which it was synapsing.

sprouts a collateral to innervate the recently denervated ganglion cell in Fig. 25 B. That such events occur is supported by the large number of reactive, apparently growing, calyces observed after the period of cell death, as well as by a transient period when many ganglion cells seem devoid of functioning synapses (19). It has been demonstrated in studies of mature nervous systems that partial denervation of a tissue results in innervation of the denervated cells by collaterals from the surviving neurons (10, 28).

The preganglionic cell shown in Fig. 25 C has one collateral innervating a cell destined to die. After death of the postsynaptic ganglion cell, it is postulated that this collateral is lost, similar to the loss of ganglion cell axon collaterals demonstrated after St. 40. Presumably, loss of such collaterals could not occur by simple retraction as happens over a relatively short distance in growing neurites (32), since many degenerating collaterals were observed after they had been myelinated and when the distance from ganglion cell to target organ was in excess of 5 mm. It is possible that such collaterals are cut off from the cell body and die, although we do not know of any descriptions of this happening elsewhere, nor can we speculate as to the cellular mechanism involved.

Neither preganglionic nor ganglion cell death in this system seems designed to remove improper connections. The evidence shows that ganglion cells do not grow to the wrong targets and do not have improper synaptic connections made upon them even before the period of cell death. Thus, in the ciliary ganglion system a highly selective neuron outgrowth and synapse formation is followed by a period of cell death which presumably removes cells that have not competed successfully for synaptic sites. Unfortunately, in many systems where neuron death has been described, the specificity of connections before cell death has not been determined. In the amphibian, motor neuron death during normal development appears to make neuromuscular connections more precise (12, 15); in the chick, neuromuscular connections are very selective (2, 16), even before cell death (11).

The apparent quantitative nature of the synapse requirement needed to prevent cell death makes a more direct testing of the hypothesis or a demonstration of the mechanism more difficult, but it is hardly surprising since almost all nerve cells synapse with more than one postsynaptic cell.

One is struck by the apparent inefficiency of such an overproduction of neurons and of synapse

formation onto cells destined to die. However, this mechanism for the quantitative matching up of groups of synaptically related cells requires little genetic information. It may well be the most efficient way to bring about precise and adequate numbers of cells, and to remove cells that have not formed adequate connections. Of course, it should be stressed that this type of mechanism only accounts for quantitative matching up and selection of the fittest neurons. Additional information is necessary to ensure proper connectivity. For the ganglion cells, directed growth to the proper target organ may suffice. An additional recognition mechanism would be required in the ganglion where preganglionic fibres must choose between two types of anatomically adjacent ganglion cells.

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