ULTRASTRUCTURAL DIFFERENCES DURING EMBRYONIC CELL DEATH IN NORMAL AND PERIPHERALLY DEPRIVED CILIARY GANGLIA

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ABSTRACT

Normally occurring neuron death and that brought about by prior removal of the peripheral target organ was studied ultrastructurally in embryonic chick ciliary ganglion in order to better understand the mechanism of cell death in this system. Before the period of cell death, all neurons in the normal ganglion developed a well-organized rough endoplasmic reticulum (RER) which coincided with peripheral synapse formation. None of the peripherally deprived neurons underwent this change, suggesting that some interaction with the periphery, possibly synapse formation, triggered them into the secretory state. Cell death in peripherally deprived neurons was signalled by nuclear changes followed by freeing of ribosomes from polysomes and RER and presumably cessation of protein synthesis. In contrast, normal cell death was brought about by dilation of the RER with eventual cytoplasmic disruption, nuclear changes appearing only secondarily. It is suggested that failure to form or maintain peripheral synapses could result in the accumulation of transmission-related proteins with consequent cisternal dilation, and eventual cell death.

In vertebrates, histogenetic cell death (13) appears to be a common if not ubiquitous occurrence during normal neural development (17, 19, 42, 43; see reference 7 for review), with rather synchronous waves of neuron death having been described in different parts of the nervous system. In some cases it has been found that the normally occurring cell death can be greatly increased by prior removal of the peripheral target organ (8, 14, 15, 42, 43), and a role for the periphery in sustaining neurons past critical developmental stages has therefore been proposed.

Most cases of neuronal cell death have been studied only anatomically at the light microscope level, presenting a picture of cell number vs. time. Thus, the physiological state of various neurons at the time of cell death is not known, nor is information on whether these cells have already synapsed with the periphery or been synapsed upon. This has hampered our understanding of the significance of neuronal cell death.

Furthermore, ultrastructural studies of neuron cell death are scant (5, 33), and none have compared normally occurring cell death with that brought about by peripheral ablation in the same neuronal population. If normal cell death results simply because neurons have failed to form peripheral connections, one would expect the appearance of cell death at the ultrastructural level to be similar in the two cases. In addition, an ultrastruc-

tural analysis of cell death might give some clues as to its mechanism (33).

Previous work on the avian ciliary ganglion has anatomically and functionally defined the sequence of ganglion cell differentiation, synapse formation onto ganglion cells, and synapse formation by ganglion cells with their peripheral targets, the intrinsic, and vascular smooth muscle of the eye (21). It was found that early removal of the periphery did not affect differentiation of ganglion cells, nor synapse formation onto them (Stages [St.] 25–33). However, between St. 34 and 38 all but a few peripherally deprived neurons degenerated (22). During this same period half of the neurons in the control ganglion also degenerated, the time of cell death coinciding with the formation of peripheral synapses (23).

The present paper presents the ultrastructural correlates of these developmental events, specifically comparing the normally occurring ganglion cell death with that brought about by peripheral removal. It notes differences in the sequence of cell death in the two cases, and speculates as to the possible mechanism. A brief summary appears elsewhere (41).

MATERIALS AND METHODS

Electron Microscopy

The results described in this paper are based on examination of 50 embryonic chick ciliary ganglia staged according to Hamilton and Hamburger (16) which were part of a larger study of cell death in normal and peripherally deprived ganglia (22, 23). Several combinations of dissection and fixation procedure were used in order to obtain the most satisfactory result. Since the intent of this investigation was to compare the ultrastructure of cell death in normal and peripherally deprived ganglia and to relate these observations to previous physiological results in the same tissue, the following procedure was followed.

Both control and peripherally deprived ganglia were routinely dissected from heads of freshly decapitated white Leghorn chick embryos which were placed immediately into oxygenated Tyrode's at room temperature (20–22°C), the same conditions under which previous electrophysiological experiments had been carried out. After dissection, which took 10–45 min, the ganglia with pre- and postganglionic nerves were fixed by small pins to pieces of cardboard (in proper orientation so that the pre- and postsynaptic nerves could be transversely sectioned) and placed in 2% glutaraldehyde in phosphate buffer (pH 7.3) for 2–3 hr. They were then postfixed overnight in 1% osmium tetroxide at 4°C, embedded in Epon (Shell Chemical Co., New York, N. Y.), sectioned on a Huxley (Cambridge Instrument Co., Inc., Ossining, N. Y.), or LKB (LKB Instruments, Inc., Rockville, Md.), ultratome microtome, stained with 2% uranyl acetate and 1.5% lead citrate, and viewed on a Philips EM 300 electron microscope at 80 kV.

Control ganglia from St. 40 embryos treated in this manner did not differ ultrastructurally from ganglia that were removed from the animals within 2–5 min after decapitation and placed directly into cold fixative.

The dissection of ganglia at 20–22°C can present some problems since Morimoto et al. (31, 32) have shown that the ribosomal complement derived from whole chick embryos dissociates from polysomes into free monosomes with slow cooling for 30 min at 4°C. These free ribosomes may then crystallize to form tetramers with an additional cooling period in excess of 2 h (see Discussion for further details). Since ganglion isolation generally took 10–30 min, it was possible that the organization of ribosomes could be affected in dissections taking 30 min or longer. To test for this, two pairs of normal and peripherally deprived ganglia, at St. 37–38, were dissected out in oxygenated Tyrode's at 37°C. Normal ganglia showed the characteristic predominance of polysomes and abundant rough endoplasmic reticulum (RER), while peripherally deprived ganglia had only sparse RER, few polysomes, and relatively more free ribosomes. These ganglia were indistinguishable from similar ganglia dissected out at room temperature for 30 min and indicate that the observed difference in ribosome complement at St. 37–38 between normal and peripherally deprived cells (see Results) is not an artifact of the dissection procedure.

Similar control experiments at St. 34 showed that there was a slight increase in the proportion of ribosomes as polysomes in ganglia dissected at 37°C instead of room temperature, and that this was true for both normal and peripherally deprived ganglia. However, the basic state of RER and ribosomal content was not greatly affected. Thus, the striking difference seen in ribosome organization between St. 34 and St. 37–38 ganglia cannot be ascribed to the preparative procedure.

In peripherally deprived animals two separate ganglia were formed (22). Each contained both ciliary and choroid cells and did not differ from each other ultrastructurally. The observations in this paper are based on both ganglia and cell types, although ciliary cells, being more obvious and containing calyces, were investigated to a greater extent.

Light Microscopy

Thick plastic sections of ganglia stained with toluidine blue were observed in the light microscope, but usually for ganglion cell counts or observations of the anatomical arrangement of whole ganglia, the tissue was fixed in Bouin's, paraffin embedded and serially sectioned at 7–8 μm. It was then stained with haemotoxylin & eosin and orange G. Other techniques, including embryonic abla-

tion of the optic vesicle and electrophysiological methods of estimating transmission through the ganglion at different developmental stages, have been described (22, 23).

OBSERVATIONS

Effect of the Periphery on Ganglion Cell Differentiation

In the peripherally deprived ciliary ganglion, both ciliary and choroid cell populations differentiate to a degree that makes it possible to distinguish one from the other in the light microscope (22). The main ultrastructural features appear normal and have been briefly summarized (see Fig. 9 in reference 22). However, in order to compare in more detail the normal and peripherally deprived situation, a description of ganglion cell fine structure at St. 34 will follow and will apply for either case since no differences between the two were detected up to this stage. Further, it will serve as a basis with which to compare the changes occurring later during cell death.

A typical ciliary ganglion cell soma, part of which is shown in Fig. 1, is ovoid in appearance and contains abundant cytoplasm. The eccentric, spherical nucleus contains finely dispersed chromatin with several nucleoli usually present. Small fingerlike indentations of the cytoplasm into the nucleus are common. The Golgi apparatus is well developed, and the cytoplasm contains a sparse network of smooth endoplasmic reticulum (SER), abundant mitochondria, and scattered neurotubules, especially at the perimeter of the cell. Occasional dense bodies, autophagic vacuoles, and multivesicular bodies are also seen. The somata of all ganglion cells at this stage, both normal and peripherally deprived, possess only scant RER, an appearance consistent with light microscope observations showing no clearly defined Nissl substance (see *inset* on Fig. 1). Some of the abundant ribosomes are organized into polysomes, but many single ribosomes are scattered randomly through the cytoplasm. This appearance is similar to other developing neurons at early to intermediate stages of development (28, 33, 36), although free ribosomes were somewhat more abundant in our tissue.

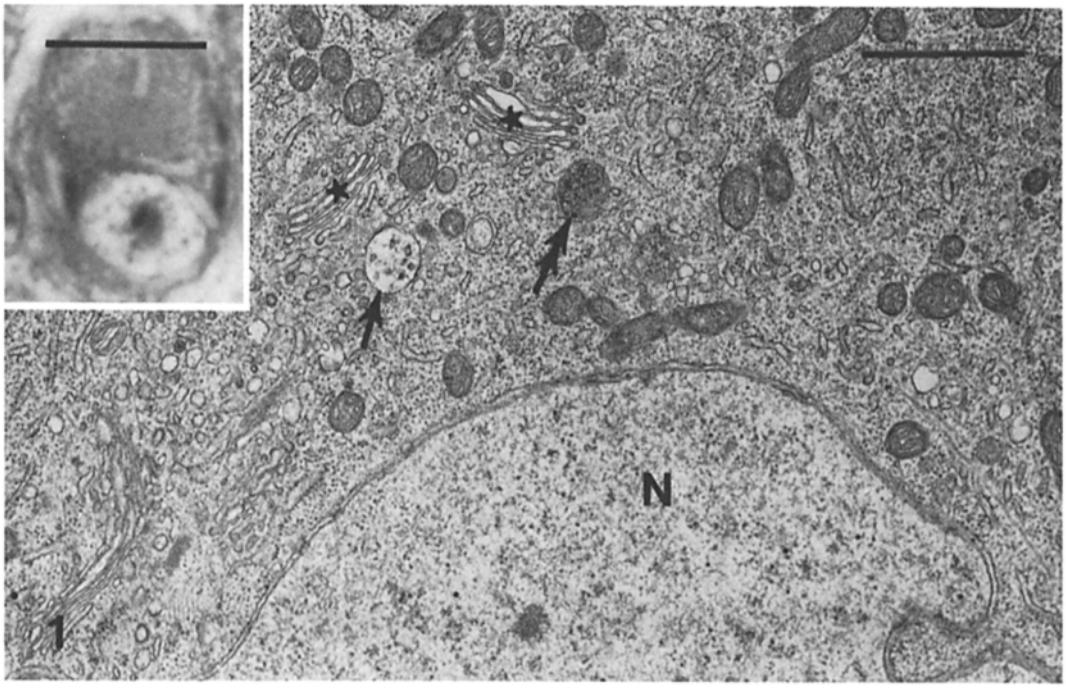


FIGURE 1 Ganglion cell cytoplasm and nucleus at St. 33 ½. The nucleus (*N*) contains dispersed chromatin. In the cytoplasm are seen the Golgi apparatus (asterisks), a multivesicular body (arrow) and dense body (double arrow). Calibration = 1 μm. *Inset*. Light micrograph of a ciliary cell from same stage. *Inset:* Calibration = 10 μm; × 2,000.

The ganglion cells at St. 34 possess numerous processes that are interdigitated with preganglionic axons and terminals to form a complex neuropile. Some are long and irregular (1–2 cell diameters in length) transient structures which appear to be retracted as the initial, multiple, bouton-type preganglionic terminals give way to single calyciform endings on the ciliary cells (21). Other somatic processes, seen at all stages of development, are small fingers which project in clusters a short distance (1–3 μm) from the soma, sometimes into the preganglionic ending. Both types of processes appeared as abundant in peripherally deprived ganglia as in the control, so that this aspect of ganglion cell differentiation was not affected by peripheral removal.

Evidence for the dynamic state of ganglion cell processes at this time was the existence of numerous growth cones from the soma or somatic processes. As seen in Fig. 2 (asterisks), some of these growth cones contain a mixture of clear vesicles of various sizes, large dense-core vesicles, SER, neurotubules, and neurofilaments similar to previously described axonal or dendritic growth cones (3, 48, 52). Much more common were somatic protrusions containing mostly membranous vesicles of various diameters, similar to growth cones described by Bodian (2), and also to the domelike structures seen to occur near growth cones in more recent studies (37).

Other structures, common at this time, were lamellated bodies (not shown), composed of numerous layers of membrane or membrane whorls, which appeared to be undergoing digestion in autophagic vacuoles, perhaps indicating large-scale destruction of membranes.

The glial cells exist as Schwann and satellite cells. While their appearance varies considerably, at St. 34 the satellite cells have a generally astrocyte-like appearance. Their nuclei are smaller and more irregular than those of the neurons, with a distinct rim of chromatin beneath the nuclear membrane. The cytoplasm is considerably less dense in appearance than that of the neurons, with characteristic sparse, wide-diameter cisternae of RER, and scattered polysomes. Neurotubules and filaments may also be present, and glial cell mitochondria are generally twice as large as those in either preganglionic endings or ganglion cells. An occasional Golgi apparatus is often visible near the nucleus. (Examples of glial cells can be seen in Figs. 9, 14, 15, 16, and 17.)

Effect of the Periphery on Ribosome Organization

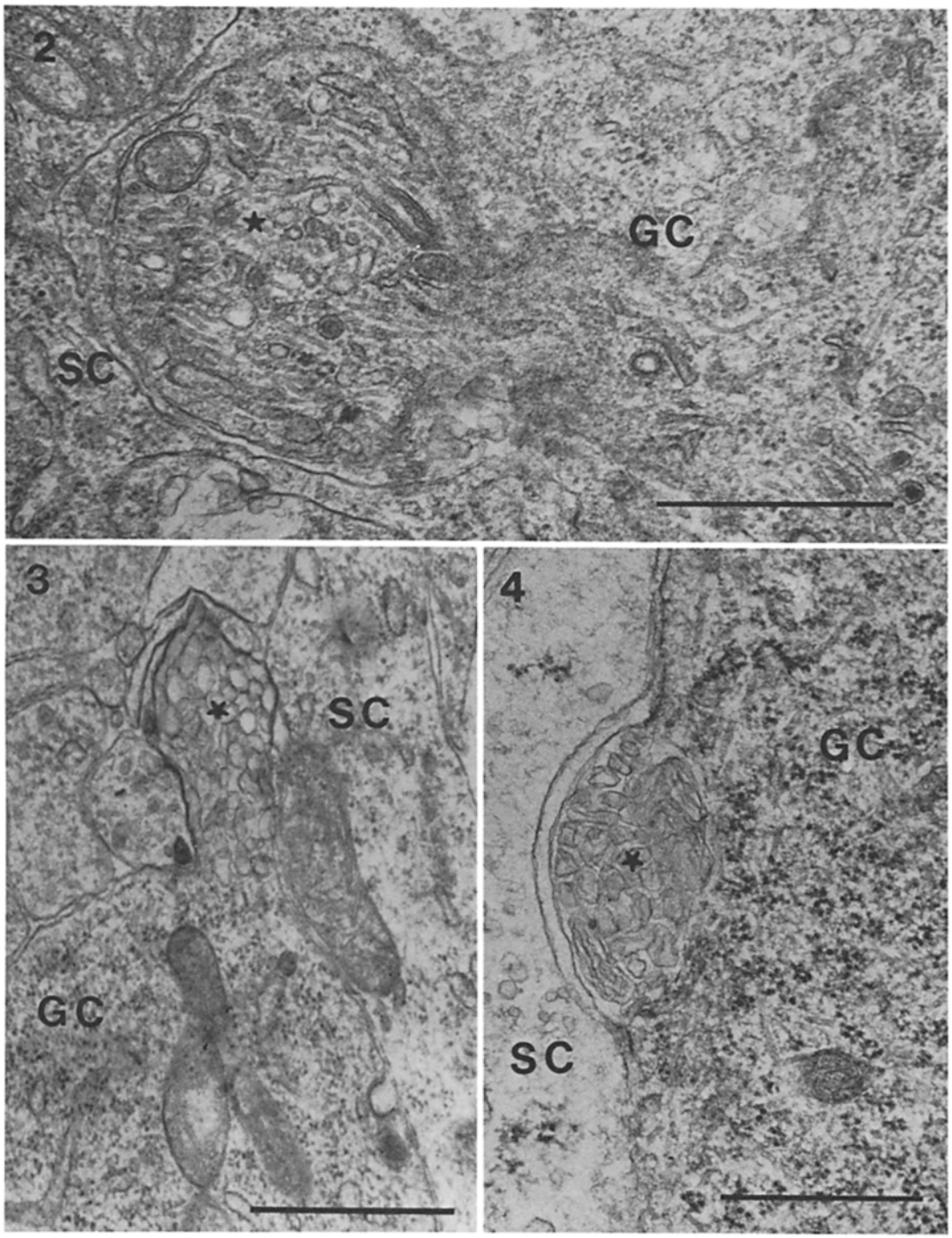
From St. 36–40, all ganglion cells from control ganglia, even those that will eventually degenerate, undergo a striking alteration in appearance. The nucleus loses its fingerlike indentations, and there is a rapid grouping of ribosomes into polysome clusters, so that by St. 39–40 there are essentially no free ribosomes. Concomitantly there is a large increase in the amount of RER with organized layers developing primarily around the circumference of the cell (see Fig. 5). It is at this time that the cells have been shown to form synapses with the periphery (23) and to increase their production of enzymes involved in transmitter synthesis (39).

This alteration does not occur in any of the peripherally deprived ganglion cells. This is true even of peripherally deprived cells which do not degenerate until later stages (37, 39–41) and which, although showing an increase in size, possess only sparse polysomes and RER with many free ribosomes dispersed in the cytoplasm (Fig. 6). Thus, it can be inferred that some form of interaction between the periphery and ganglion cells occurs even before the period of cell death and that it results in the development of an organized RER.

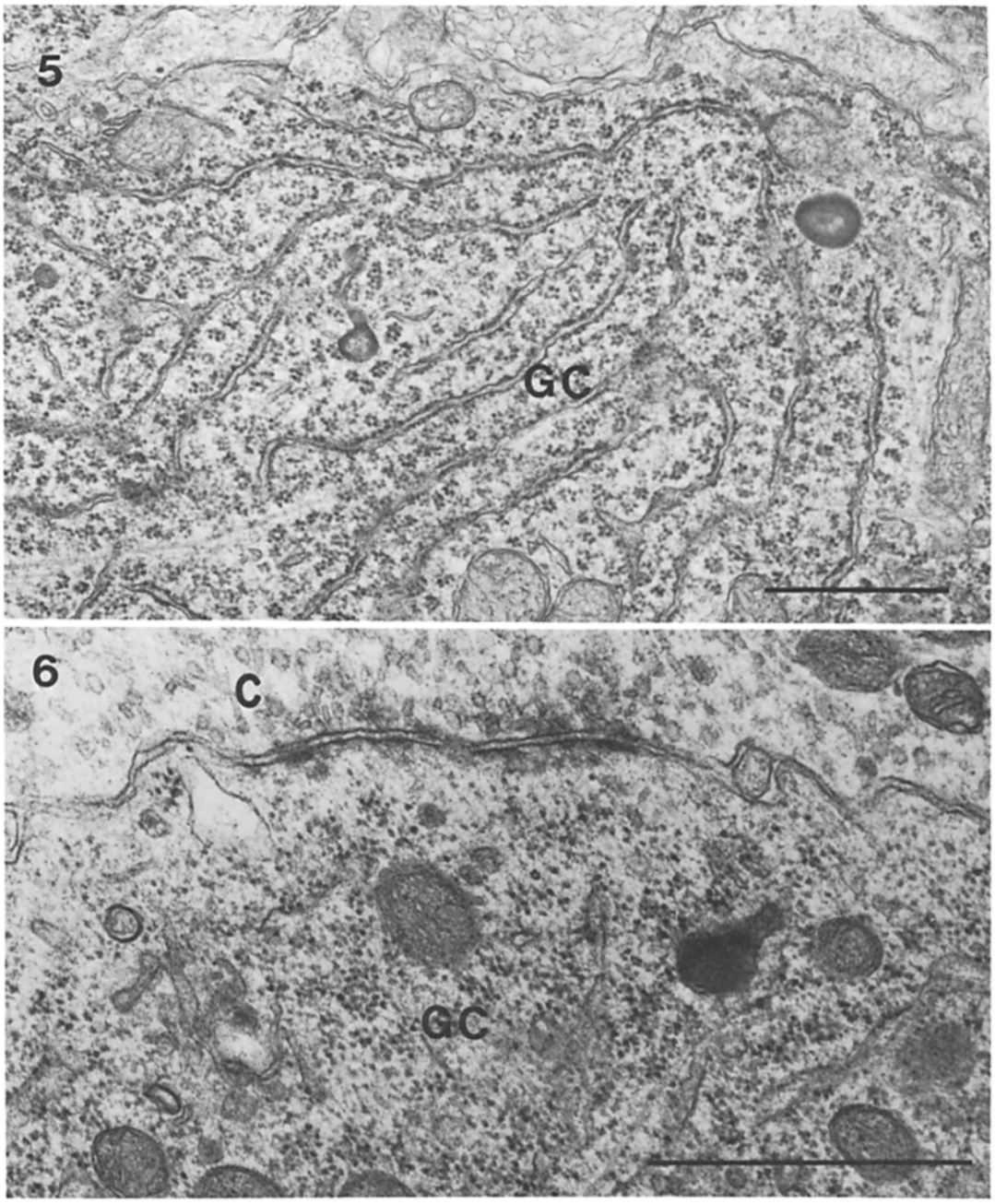
Neuron Death in Peripherally Deprived Ganglia

The first indications of cell death following removal of the target organ are seen at St. 35–36, in the form of increased density of nuclear chromatin. The first changes observed by others were also found to be nuclear, but we did not find the extreme clumping of chromatin beneath the nuclear membrane as was observed by O'Connor and Wyttenbach (33). Initially, the cytoplasm of such cells does not differ from normal. The nucleus gradually becomes more irregular and the nucleoplasm more dense, although the nuclear membrane remains intact. At this stage (Fig. 7), cytoplasmic changes can be detected as a large increase in the number of free ribosomes, any previous tendency toward polysomes disappearing. Ribosomes are also set free from whatever RER exists. This is the first stage that can be detected with the light microscope due to an increase in nuclear density and cytoplasmic staining (*inset*, Fig. 7).

Cells rapidly pass into the next stage in which many cells are found between St. 36 and 38. As shown in Fig. 8, the crenulated, electron-dense

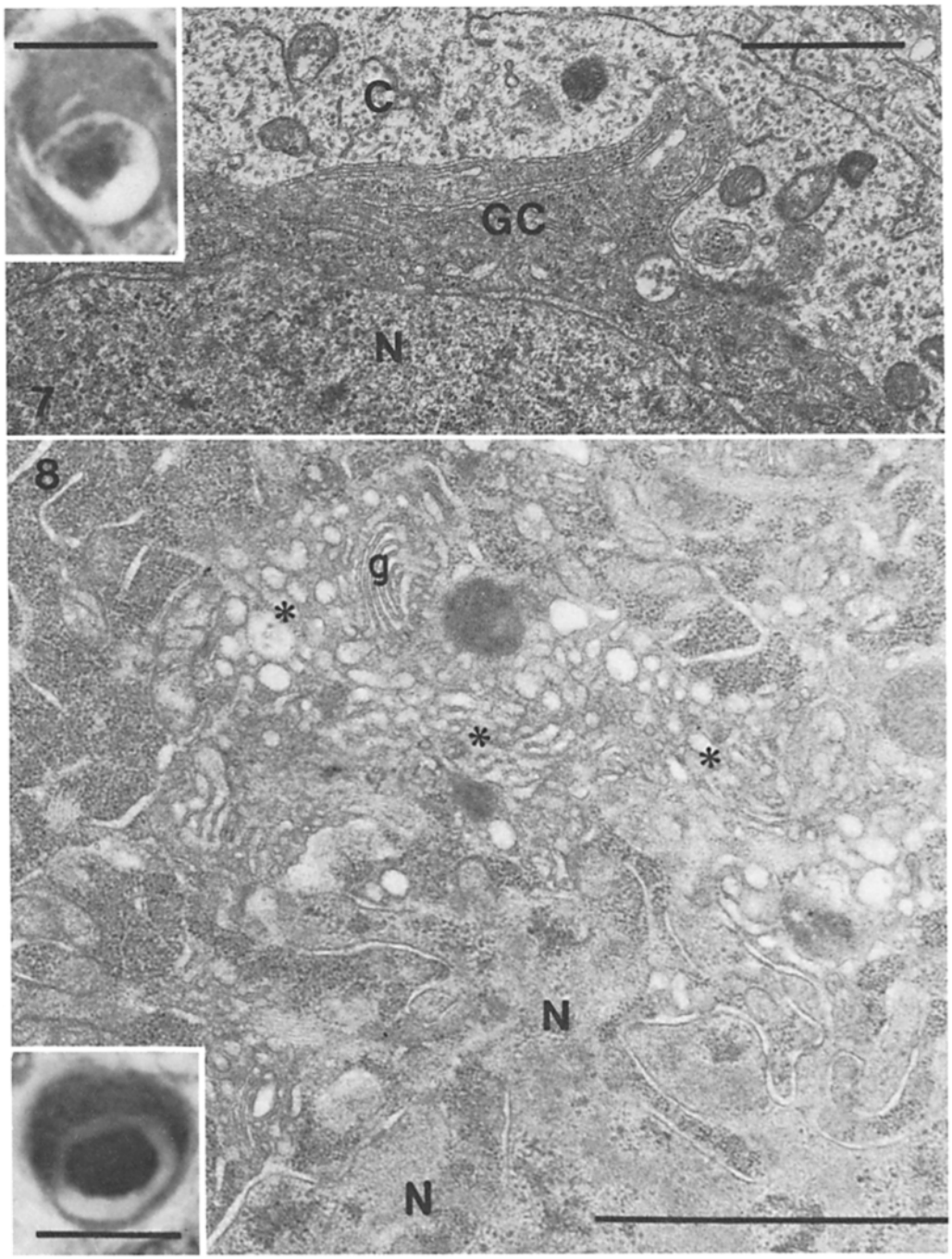


FIGURES 2, 3, and 4 Growth cones (asterisks) were commonly observed on ganglion cell somas (*GC*) at St. 34. *SC*, satellite cell. Calibration = 1 μm; × 35,200; × 30,000; × 30,400.



FIGURES 5 and 6 A comparison of RER in a St. 39 normal and peripherally deprived ganglion cell. Normal ganglion cell (*GC*, Fig. 5) contains abundant RER, and polysome clusters. Peripherally deprived cell (Fig. 6) which is synapsed with a calyx (*C*) contains sparse RER and mostly free ribosomes. Calibration = 1 μm; × 26,300; × 46,100.

nucleus has chromatin clumped in irregular masses, and the nuclear membrane is indistinct in places. The cytoplasm, of much increased opacity, consists of areas of clumped free ribosomes separated by clear spaces representing the cisternae of RER, which, it should be pointed out, are not unusually dilated. Other areas contain fibrous or filamentous material. The Golgi apparatus is en-



FIGURES 7 and 8 Appearance of degenerating ganglion cells from a peripherally deprived ganglion. In Fig. 7, the nucleus (*N*) of a St. 36½ ganglion cell shows an increase in granularity with some clumping of chromatin. A calyx (*C*) is in contact with soma (*GC*) which shows an increased electron density. Fig. 8, a later stage of degeneration in which nucleus (*N*) is very dense and cytoplasm contains clumped free ribosomes. Golgi apparatus (*g*) is hypertrophied with large increase in Golgi-associated ER and vacuoles (asterisks). Calibration = 1 μm; × 23,600; × 51,000. *Insets* show corresponding stages of degeneration seen with light microscope. Calibration = 10 μm × 2,000.

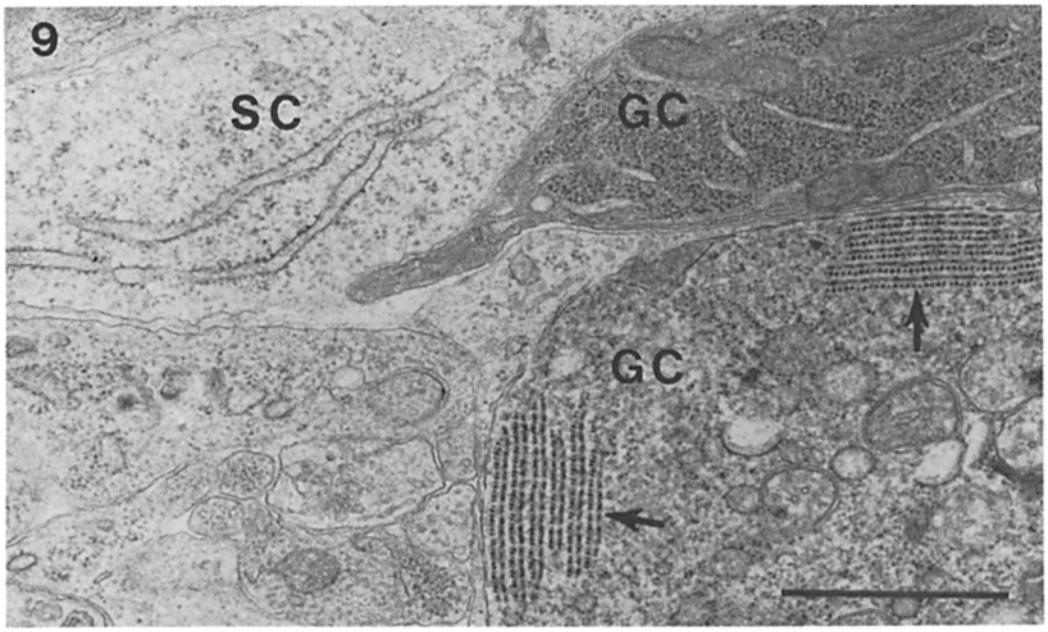


FIGURE 9 Ribosome crystals (arrows) were occasionally observed in peripherally deprived degenerating ganglion cells, although clumped ribosomes seen in upper degenerating ganglion cell were more common. St. 36½ *GC* = ganglion cell. *SC* = satellite cell. Calibration = 1 μm; × 30,000.

larged and associated with increased SER and large clear vesicles and vacuoles. Mitochondria are still recognizable and, although more dense, are not greatly swollen. Multivesicular bodies, dense bodies (possibly lysosomes), and autophagic vacuoles are present but not unusually frequent. These processes continue, with the cell becoming progressively darker and more rounded.

Ribosome crystals previously described in both normally occurring and induced cell death during embryonic development (10, 33) were only occasionally seen. As shown in Fig. 9 (arrows), they conformed in appearance to the ribosome crystals seen in degenerating chick spinal motoneurons (33) and to those brought about by hypothermic treatment of chick embryos (4, 29) (see Discussion). The upper ganglion cell process seen in Fig. 9 was, however, the more common form that degeneration took.

Since cell death is not completely synchronous, between St. 36 and 38, cells in the last stages of degeneration are intermixed with cells that are as yet showing only nuclear changes. However, all peripherally deprived cells appear to go through the sequence described above, and by St. 37–38 many of the cells have disappeared, and most of the remainder are in late stages of degeneration.

An occasional degenerating neuron with an electron-dense nucleus was observed between St. 30 and 34, but since it occurred before the major cell death period it was not further investigated.

Normally Occurring Ganglion Cell Death

While the appearance of cell death in peripherally deprived and control ganglia seems to be synchronous, not enough cell counts of this critical stage were done in control ganglia to make definite conclusions with respect to the time of its onset (22). However, the present electron microscope observations lead us to suspect that the initial occurrence of cell death in the normal ganglion is one or two stages later than that observed in the peripherally deprived ganglion. This difference has also been noticed in the study of cell death in normal and peripherally deprived motoneurons in amphibia (44).

The first sign of degeneration (St. 36) in the normally occurring cell death was observed in the cytoplasm, rather than in the nucleus; there was an increase in the diameter of the cisternae of RER (Fig. 10). In Fig. 11, the cisternae, which show a moderate degree of dilation, can be observed to contain a finely granular material. While it is

difficult to construct a temporal sequence from still images, intermediate stages of dilation were observed and seemed to indicate a progression to the extremely dilated reticulum seen in Figs. 10 and 12. The occurrence of such cells with dilated endoplasmic reticulum (ER) is probably not a fixation artifact, since these changes were seen only during the normal period of cell death (St. 36-38). Further, cells with dilated ER were observed bordering cells of completely normal cytologic appearance.

Ribosomes are still attached to the greatly distended cisternae shown in both Figs. 11 and 12 (arrows). There is, in fact, no loss of ribosomes from the RER until so late that mechanical distortion may have occurred. There is also no dispersion of polysomes into free ribosomes, both observations contrasting with the peripherally deprived situation. No distinct increase in nuclear opacity or clumping of chromatin was observed when cisternal dilation was already underway (as shown in Fig. 10). At late stages of dilation an increase in the "granularity" of the chromatin was apparent, but by this stage disruption of the cytoplasm was extensive enough to possibly result in direct damage to the nucleus.

This form of degeneration, with cisternal dilation as the primary sign, resulted in eventual rounding up of the cells and loss of the integrity of intracellular organelles, although the possibility exists that some of the cells showing moderate dilation could revert to normal. Another possible form of normal degeneration resulted in primarily mitochondrial swelling, which was not necessarily accompanied by cisternal dilation. Cells with moderately swollen mitochondria (Fig. 13) were com-

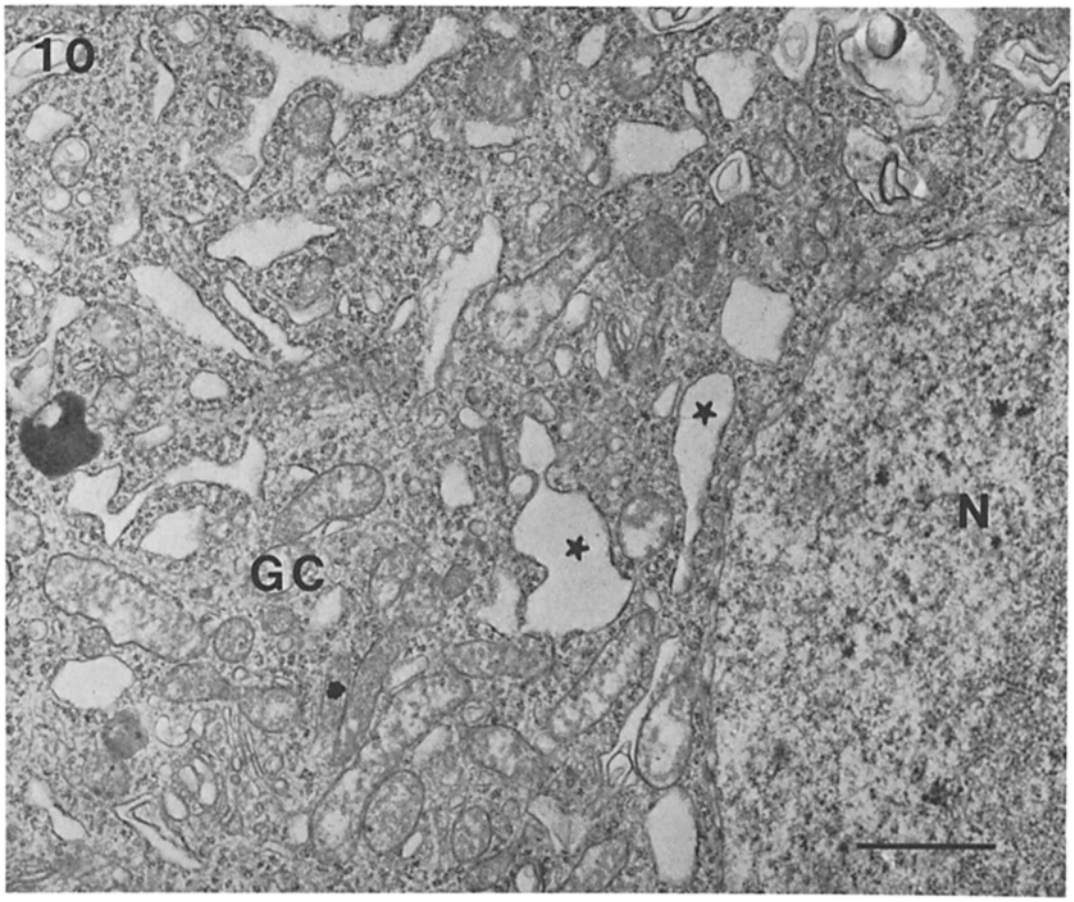
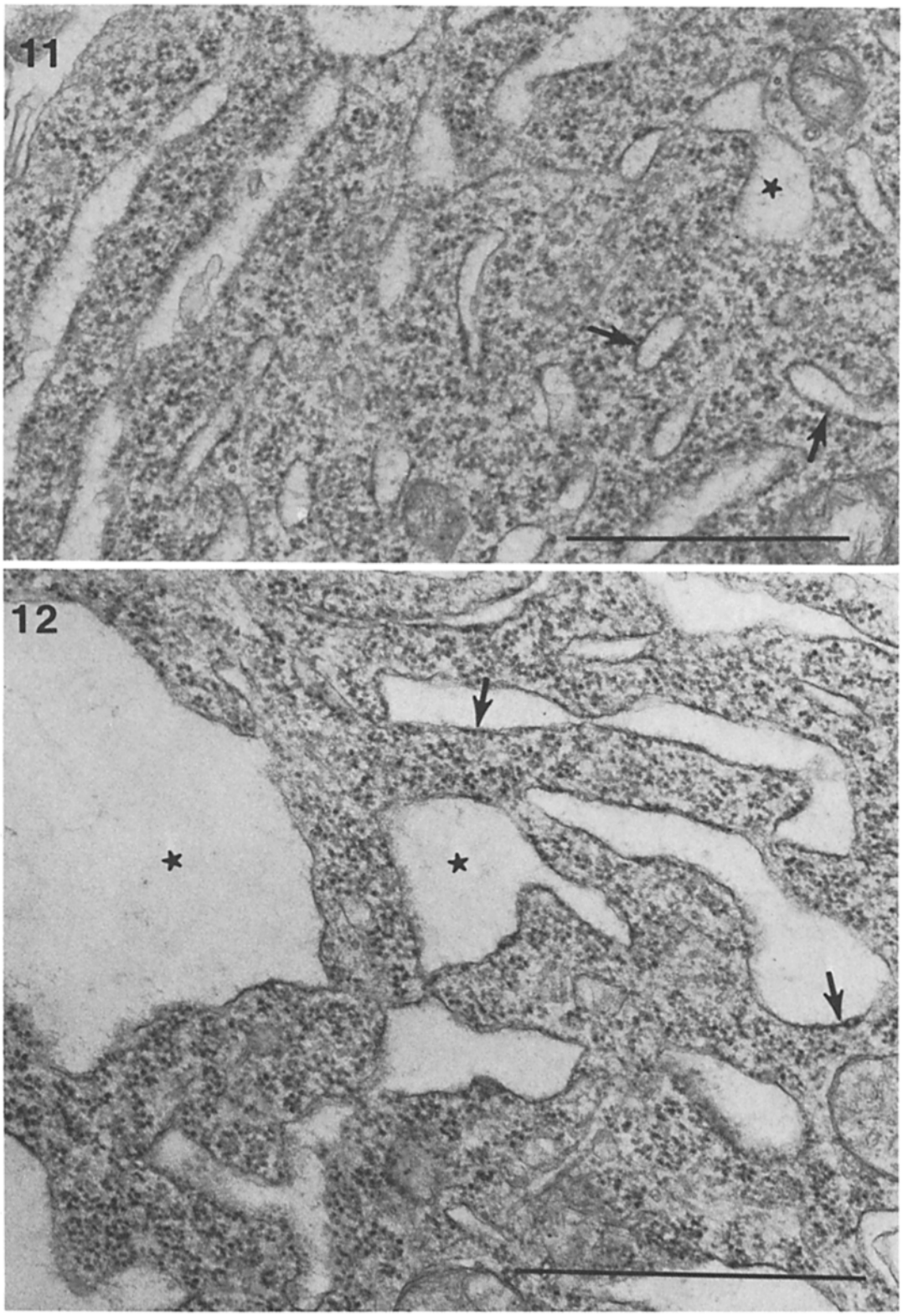


FIGURE 10 The nucleus (*N*) retains a normal appearance in this ganglion cell (*GC*) undergoing degeneration during normal development, although the RER is extremely dilated (asterisks). St. 37. Calibration = 1 μm; × 17,600.



FIGURES 11 and 12 Dilation of ER (asterisks) appeared to progress from moderate (Fig. 11) to extreme (Fig. 12) levels although ribosomes can still be seen to be attached in both cases (arrows). Also note clumping of ribosomes into polysome clusters. St. 37. Calibration = 1 μm; × 45,300; × 55,200.

mon at St. 36, and it is possible that this condition is reversible with some of the cells returning to normal. In other cases it appeared to progress to the degree seen in Fig. 14 where the degenerating neuron profile consists mostly of greatly ballooned mitochondria with disrupted cristae. Satellite cells at this time had dilated RER that contained a floccular material (Fig. 14, arrow), and was presumably associated with myelin formation, which began at this time. Although we did not observe the type of cell death involving a primary nuclear change in control ganglia, it is possible that it could occur to some extent.

In both normal and peripherally deprived ganglia most of the degenerating debris seems to be phagocytosed by glial cells. In normal ganglia, as already shown in Fig. 14, satellite cells surrounded whole degenerating cells. Later, large clumps of debris, probably representing cells in the final stages of disintegration, were observed enclosed within glial cytoplasm (Figs. 15 and 16). In the peripherally deprived ganglion (St. 36–40), glial cells appeared to pinch off small pieces of the dark cytoplasm from degenerating neurons (Fig. 17). Similar degenerating structures, as well as myeloid and dense bodies, were seen within glial profiles at this stage (Fig. 15). In the normal ganglion, glial cells were not observed to pinch off pieces of ganglion cell soma, but rather entire rounded-up ganglion cells appeared to be surrounded by glial processes as shown in Fig. 16. Large clumps of debris, probably representing cells in late stages of disintegration, were observed in glial cells from St. 37 to 38. While an occasional macrophage was observed at the peak of degeneration in both normal and peripherally deprived ganglia, most of the cellular debris appeared to be removed by the normal glial population. Glial cells associated with degenerating neurons showed a definite increase in microtubules and microfilaments. Their processes that enveloped degenerating cells contained few organelles, giving them a watery appearance, and they often lacked ribosomes, making them difficult to distinguish from calyces at the same stage.

The entire sequence of differentiation and cell death described for both normal and peripherally deprived ganglion cells is summarized in Fig. 18.

DISCUSSION

Many studies of cell death in the developing nervous system have implied that neurons are not genetically programmed to die, but rather that their survival is contingent on peripheral factors, possibly the establishment of synapses with the peripheral target organ (4, 15, 19, 22, 23, 25, 38, 42, 43, 47). These ideas arise because in many systems the normally occurring cell death and that brought about by removal of the peripheral target organ were found to occur at approximately the same time (7, 14, 15, 22, 23, 42, 43), and coincide with the time of peripheral synapse formation (19, 22, 23). Since normally occurring cell death would then result because some of the neurons had failed to form peripheral synapses, we expected both normally occurring and induced cell death to be caused by the same mechanism, and to present a similar ultrastructural sequence. These expectations, however, were not fully supported by the present studies.

In order to facilitate understanding of the following discussion, the reader is referred to Fig. 18, which summarizes the more important findings of this paper. Differences between normal and peripherally deprived neurons were detected even before cell death. Until St. 34, both types of ganglion cells are similar in appearance, containing only sparse polysomes, and RER and many single ribosomes scattered throughout the cytoplasm. Since free ribosomes are not involved in protein synthesis (18), the appearance of such neurons is consistent with a moderate amount of protein synthesis, probably sufficient for axon growth and self maintenance (Fig. 18 A).

However, beginning at St. 36 and continuing through St. 40, all neurons from control ganglia, even those that will eventually degenerate, show a characteristic organization of ribosomes into polysome clusters, so that by St. 40 few free ribosomes remain. In addition, the amount of RER is dramatically increased (Fig. 18 B'). The former observation probably reflects a general increase in protein synthesis, the latter an increase in synthesis of proteins destined for export to the axon terminal (11, 12).

The last hypothesis is supported by two lines of evidence: (*a*) electrophysiological and ultrastructural evidence (22, 23, 26) that ciliary cells establish functional synapses with the iris and ciliary muscles between St. 35 and 40; (*b*) biochemical observations that ganglion cells show a large increase in choline-acetyltransferase and acetylcholinesterase between St. 36 and 40 (39). Increased protein synthesis may also be required for the collateral sprouting that occurs around St. 34–36 (see subsequent paper).

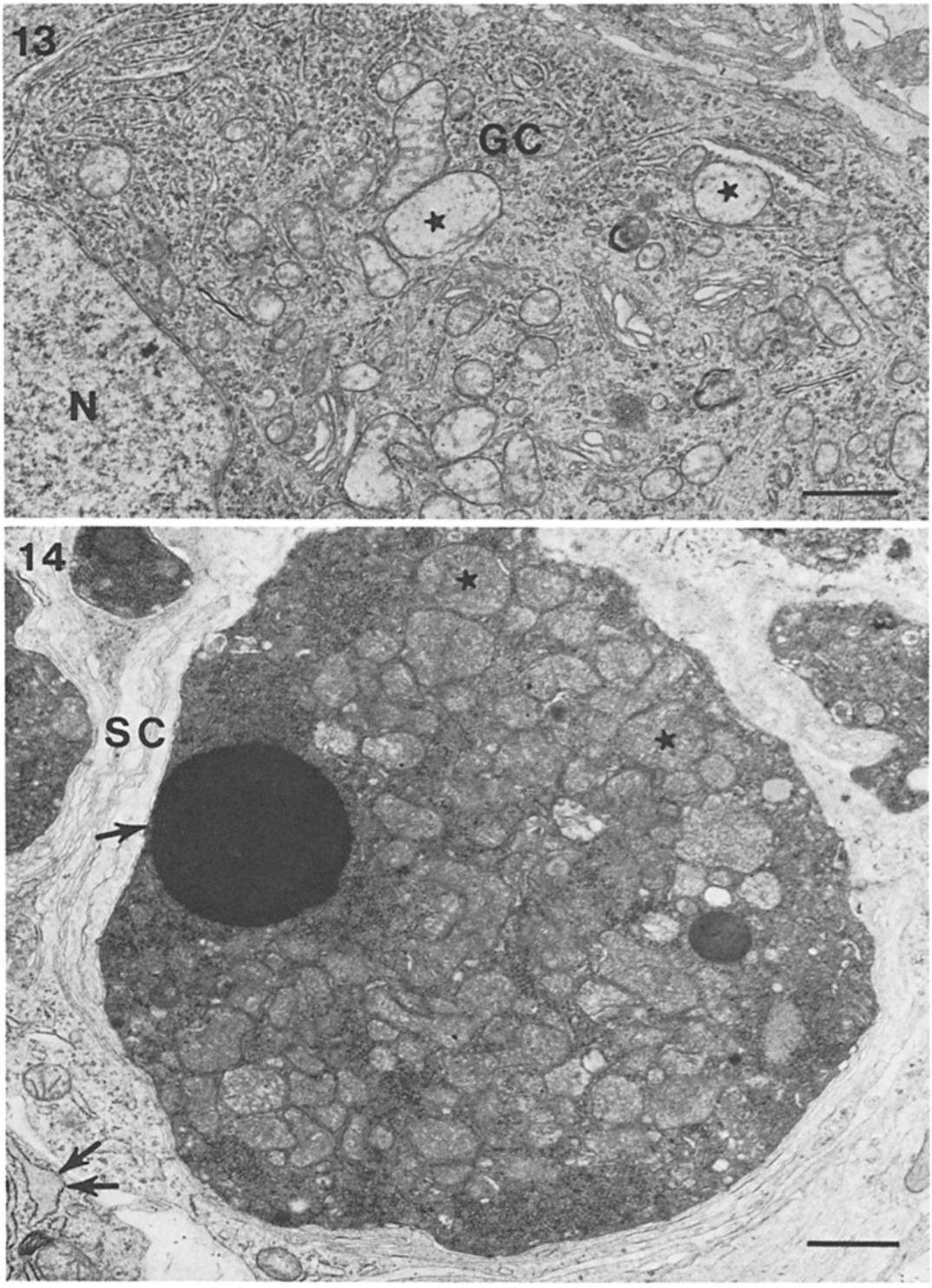
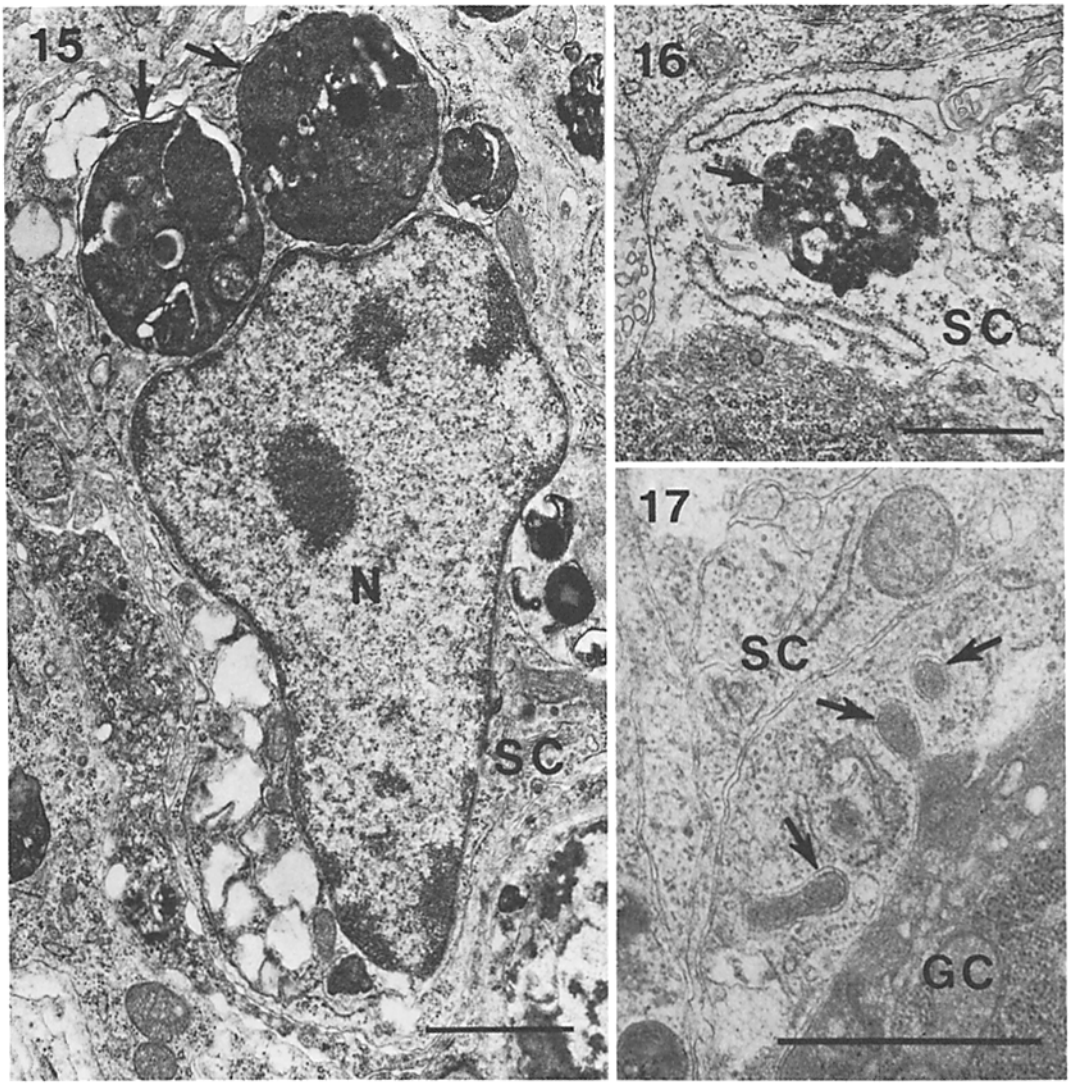


FIGURE 13 Early stages of normally occurring cell death involving primarily swelling of mitochondria (asterisks). St. 37. *GC*, ganglion cell; *N*, nucleus.

FIGURE 14 Degenerating profile containing mostly greatly dilated mitochondria (asterisks) with a clump of chromatin (arrow). *SC*, satellite cell. Double arrow points to dilated cisternae of satellite cell RER containing a floccular material. St. 37½. Calibration = 1 μm; × 13,700, × 14,000.



FIGURES 15, 16, and 17 Commonly found cellular debris (arrows) in glial cells between St. 37 and 39 can be seen in Fig. 15. In normal ganglion, satellite cell (*SC*) showing nucleus (*N*) contains two large degenerating profiles. Watery appearance of satellite cell processes which surround degenerating profiles can be seen (arrow) in Fig. 16. Pieces of degenerating cytoplasm (arrows) from a peripherally deprived ganglion cell (*GC*) appear to be engulfed by satellite cell (*SC*) in Fig. 17. Calibration = 1 μm, Figs. 15 and 16; 0.5 μm, Fig. 17; × 19,000; × 17,800; × 70,000.

Interestingly, none of the peripherally deprived cells, some of which may survive until St. 40, show this change in ribosome complement. These cells continue to grow in size, yet possess the same abundance of free ribosomes and sparse RER as earlier stages of both normal and peripherally deprived cells (Fig. 18 B). Thus, the organization of polysomes and RER does not ensue automatically at a certain time in development, but appears to be dependent on peripheral factors. Since peripherally deprived ganglion cells possess normal preganglionic synapses (22, 23), the target organ would seem to play the primary role in triggering the cell into its secretory state.

How is this interaction mediated? Axons from ganglion cells are in contact with the presumptive

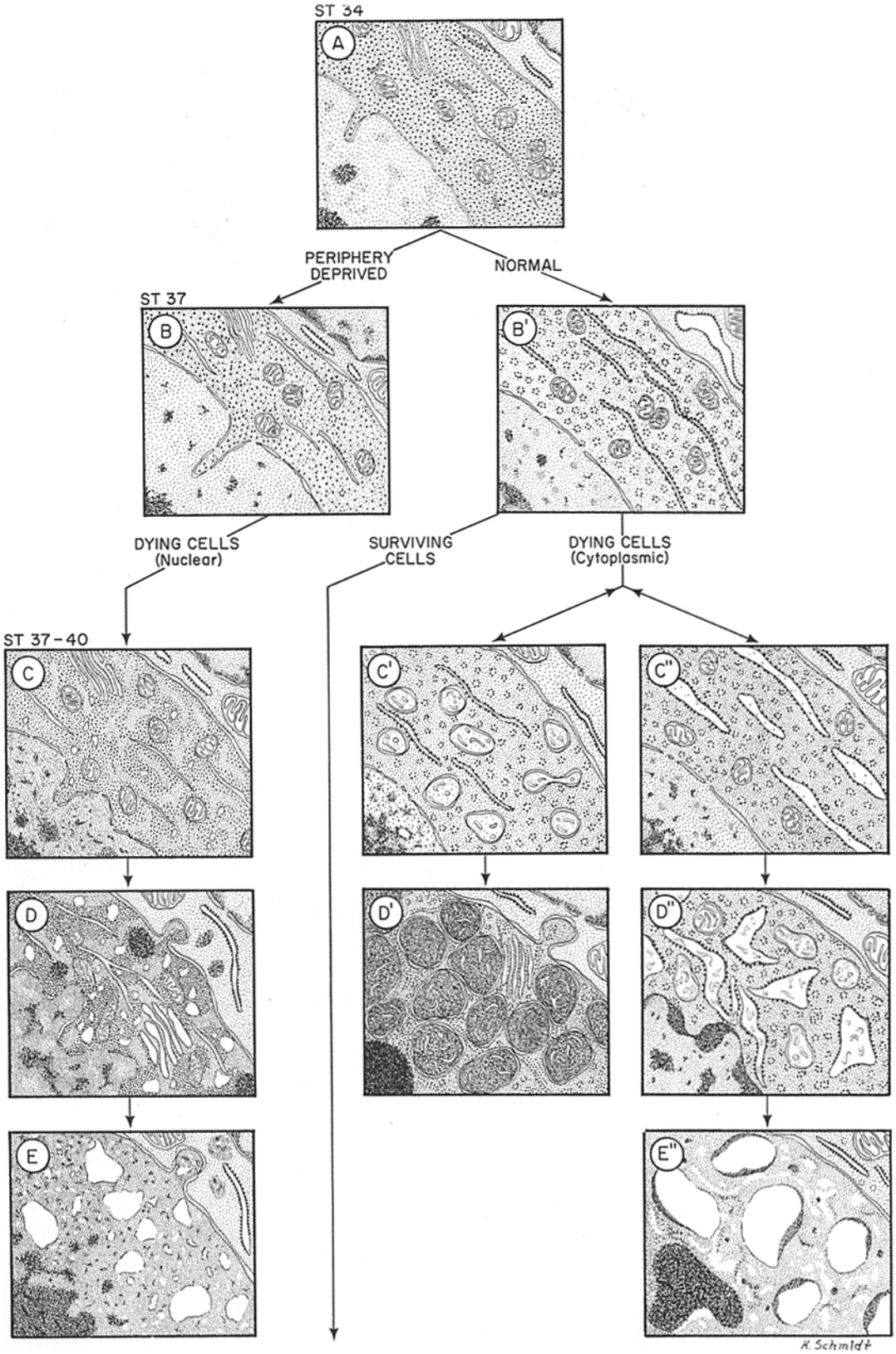


FIGURE 18 Summary of the cytologic changes occurring in peripherally deprived (B, C, D, E) and normal (B′, C′, C″, D′, D″, E″) ciliary ganglion during both ganglion cell differentiation (B, B′) and death (C, C′, C″, D, D′, D″, E, E″). Upper right corner of all diagrams: Schwann cell. Arrows indicate probable sequence of ultrastructural changes. Note that the changes in RER and mitochondria may be reversible at early stages.

peripheral target from as early as St. 28–30, yet it is only between St. 36 and 40, when peripheral synapses actually form, that the full organization of polysomes and RER occurs. Synapse formation *per se*, with consequent alteration of some substance carried via axonal flow to or from the soma, may be a direct trigger. A similar progression from polysomes dispersed in the cytoplasm to organized arrays of RER has been described elsewhere during normal development (12, 28, 36). Yet, in most cases the time of peripheral synapse formation is unknown and, without comparison with a peripherally deprived tissue, it is not possible to rule out the coincidental nature of these events.

Following axotomy of adult neurons, there is a dispersion of ribosomes and loss of RER (35, 53). This is accompanied by a fall in some enzymes involved in neurotransmission (6, 45). After regeneration of the axon, the neuron regains its complement of RER (53), although this has not yet been clearly associated with peripheral synapse formation. As during development, these changes seem to be brought about by some interaction with the peripheral target.

The nature of the signal for chromatolysis is still uncertain (see reference 9 for review), but several possibilities have been excluded in the ciliary ganglion system (40). The most likely remaining one is that, following axotomy, some substance normally utilized at the nerve ending builds up in the soma and triggers chromatolysis. It is, therefore, possible that the organization of ribosomes may be brought about by a similar signal during both development and axon injury. Alternatively, the signal may differ, and, during development, some nonsynaptic inductive interaction with the periphery may occur. If synapse formation is involved, it follows that the cells which die in control ganglia have formed at least some synapses, since these degenerating cells possess abundant RER.

The observed sequence of ultrastructural changes during cell death in peripherally deprived ganglia is quite like that described by O'Connor and Wyttenbach (33) in the chick spinal cord and may be similarly interpreted. The first detectable change was nuclear clumping of chromatin followed by a shedding of ribosomes from the RER and the breaking up of polysomes into single ribosomes. Since dense chromatin is inactive in RNA synthesis (27), one of the first signs of cell death, that of chromatin clumping and nuclear opacity, can be tentatively interpreted as nuclear inactivation with a cessation of transcription. With normal turnover of messenger ribonucleic acid (mRNA), single ribosomes would eventually accumulate following completion of translation, and protein synthesis would then cease (Fig. 18 C, D, and E).

However, the formation of ribosome crystals from the resultant pool of free ribosomes, which was part of the sequence of degeneration in spinal cord motoneurons (33), was observed only occasionally in the present study. Ribosome crystals, common in various types of degenerating cells (29, 33), have been shown to be formed from single ribosomes not programmed for protein synthesis and thus not associated with mRNA or nascent polypeptides (31, 32). Ribosome crystals also form in embryonic chick tissue subjected to slow cooling (4, 29), and are composed of a basic tetrameric aggregate. Morimoto et al. (31, 32) have demonstrated that such tetramers form from the pool of free ribosomes which accumulate during slow cooling. Various treatments that prevent the initiation of protein synthesis but allow polypeptide chain elongation and termination to continue (as does slow cooling) result in a pool of free ribosomes which can then crystallize.

Our failure to consistently observe ribosome crystals need not detract from the proposed sequence whereby cessation of transcription results in a pool of free ribosomes, for the actual process of tetramerization and the formation of larger crystalline aggregates may be affected by the intracellular environment and has in fact been shown to vary with tissue and developmental stage (29, 31, 32). Large numbers of free ribosomes may also exist in apparently normal neurons without crystallizing (34).

Following the initial stages of degeneration, the ganglion cells round up and become more dense (Fig. 18 D). In contrast to the chick spinal cord study (33), the Golgi apparatus was seen to persist in all cells, even late in degeneration, and appeared to be somewhat hypertrophied. While typical autophagic vacuoles and lysosomes were not more common than normal, the Golgi-associated SER and vacuoles were greatly increased in number and may possibly be a source of lytic enzymes, resulting in initial intracellular digestion.

As the degenerating neurons become smaller and denser, with loss of identity of intracellular organelles, small pieces of cytoplasm were pinched off by adjacent glial processes (Fig. 18 D and E). Thus, as in the EM study of the chick spinal cord

(33) and in contrast to earlier light microscope studies (14, 25), the normal glial population appeared to be the primary cell type responsible for removal of degenerating cells and cellular debris.

Surprisingly, the normally occurring cell death in the ciliary ganglion, which results in destruction of 50% of the neuron population, did not follow the above ultrastructural sequence, and suggested perhaps another mechanism of cell death. The primary change, at least detectable with the electron microscope, was not nuclear but cytoplasmic, taking the form of a gradual dilation of the RER (Fig. 18 C″). As the dilation became severe, the cytoplasm was greatly disrupted and the mitochondria became swollen (Fig. 18 D″ and E″). Nuclear changes were only seen when disruption of the cytoplasm was extreme and when direct mechanical damage to the nuclear membrane may have occurred. That nuclear inactivation, with the cessation of mRNA synthesis was not the primary cause of death was supported by the presence of polysomes in the cytoplasm until late in the degenerative sequence, which contrasts markedly with the events observed in peripherally deprived cells. Of course, differences in the half-life of mRNA's have not been ruled out.

However, the sequence of events described above suggests an alternate explanation. All normal cells were shown to undergo an interaction with the peripheral target organ and to begin to synthesize proteins for export to the axon terminal at a high rate. Cells which fail to form synapses, or at least a sufficient number of synapses, would not utilize these proteins. The accumulation of these proteins might interfere with normal axonal transport and lead to their accumulation in the cisternae of the RER. Osmotic effects might then ensue and bring about increased swelling of the cisternae and disruption of the cytoplasm. Polypeptides known to cause swelling of mitochondria (24), could also be released in this process.

While dilation of the RER is seen in some neurons in various pathological states associated with an inhibition of protein synthesis (50, 51), moderate dilation also occurs in cells undergoing increased protein synthesis (46, 49) and has been observed in neurons undergoing chromatolysis, especially those which ultimately degenerate (1, 20, 35). Such initial dilation in neurons following axotomy might occur for a similar reason, since these neurons, mature and presumably synthesizing protein for export, are suddenly deprived of their peripheral target. One might note in this respect that a normal part of the regenerative response of some peripheral neurons subjected to axotomy is first a degradation of the RER by lysosomes and autophagic vacuoles (30), similar to involution of secretory cells in the anterior pituitary (49). Consistent with this proposal is the lack of ER dilation in peripherally deprived ganglion cells which are never switched into the secretory state. These ideas as yet are only speculative and require further testing.

Unfortunately, the generality of the two types of cell death described in this paper cannot be assessed, for there are as yet no other ultrastructural studies which compare normal cell death and that induced by peripheral deprivation in the same neuronal population. These observations, at first glance, appear to contradict those of O'Connor and Wyttenback (33), who found that normally occurring cell death in the cervical spinal cord followed the same sequence as cell death in the lumbosacral cord induced by limb removal. However, the cells that die in the cervical cord are preganglionic neurons, and they die because there is no peripheral target organ, i.e., the sympathetic chain, at that level (25, 47). Thus, these cells have probably never interacted with the peripheral target and may be thought of as peripherally deprived by nature.

The second type of cell death, described in the normal ciliary ganglion, affects cells that have already interacted with the periphery. This type of death possibly indicative of competition for synaptic sites, may be widespread in the developing nervous system. In any case, the unexpected differences between normally occurring and induced cell death indicate that caution should be used in drawing conclusions about the nature of cell death from light microscope and cell count studies alone. The actual ultrastructural sequence of degeneration can provide clues to the mechanism of cell death and possibly to the past history of the degenerating neuron, both of which may be important in interpreting the significance of cell death in different parts of the developing nervous system.

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