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Developmental neuron death in the rat superior cervical sympathetic ganglion: cell counts and ultrastructure

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Summary

Counts of neurons of the rat superior cervical ganglion (SCG) were made at two days before birth and at several postnatal ages. There is a significant decline in the number of apparently normal neurons over the first postnatal week, with the number falling from 39 500 at 3 days to 26 500 at 7 days. Cell numbers then remained constant up to day 60 when the number of neurons was 27 500. The incidence of degenerating neurons, identified by light and electron microscopy, was correlated temporally with the loss of normal neurons. The early manifestations of the neuron degeneration were chromatin clumping and the presence of free monoribosomes. Later stages were characterized by increased chromatin clumping, dense aggregations of monoribosomes, numerous intracytoplasmic vacuoles, and only short segments of rough endoplasmic reticulum. The ultrastructure of the majority of these dying neurons is similar to the 'nuclear' types of degeneration described by Pilar & Landmesser (1976) and Chu-Wang & Oppenheim (1978). Based on the presence of degenerating neurons coincident with the reduction in neuron numbers, we conclude that neuron death is an important aspect of early postnatal development in the rat SCG.

Introduction

Neuron death during the development of several regions of the nervous system results in a substantial loss of the original cell population (see Oppenheim, 1981, for a review). One explanation of naturally occurring neuron death is that neurons die unless they receive a certain minimum amount of a target trophic factor during development. Nerve Growth Factor (NGF) is thought to be the trophic agent for sensory and sympathetic neurons since it promotes survival of these cells *in vivo* and neurite outgrowth from these cells in culture (Levi-Montalcini & Booker, 1960; Levi-Montalcini, 1966; Hendry & Campbell, 1976; Carr & Simpson, 1978). For example, NGF given to embryonic chicks

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increases neuron numbers in the spinal ganglia, presumably by sustaining cells which would normally die (Hamburger *et al.*, 1981). Similarly, NGF given to rats during the early postnatal period results in an increased number of neurons in the sympathetic superior cervical ganglion (SCG) (Hendry & Campbell, 1976). It is logical to postulate that there is a normal developmental loss of these sympathetic neurons during the postnatal period and the action of NGF in this instance also is to support those cells that normally would have died as a result of insufficient trophic support. The occurrence of normal developmental cell death in the ganglia is a necessary logical link in the trophic support hypothesis.

Normally occurring neuronal death in the lateral motor column occurs at about the time that afferent and efferent contacts begin to form (Chu-Wang & Oppenheim, 1978), (see Oppenheim, 1981). In the rat SCG the development of afferent synapses (Smolen & Raisman, 1980) and target innervation occurs during the first few postnatal weeks; therefore, it is at this age that developmental neuronal death would be expected. However, there is controversy over whether neuron death in the rat SCG occurs. Hendry & Campbell (1976) have reported a 30% decrease in SCG neurons during the first postnatal weeks. However, Davies (1978) shows either no cell loss or an increase in the number of neurons in the SCG during the postnatal period, depending on the choice of factor to correct for over-counting of split cells. Eränkö & Soinila (1981) show an increase in cell numbers between birth and day 8, and then a decrease that continues through the first two postnatal months.

The aim of this study was to determine whether natural cell death occurred in the developing rat SCG by undertaking a correlative light and electron microscopy study in which neuron counts were performed at different ages, and the tissue was examined for evidence of degeneration.

Methods

Superior cervical ganglia were removed under chloral hydrate anaesthesia from 18-day-old foetal Sprague-Dawley rats, and postnatal rats 3, 5, 7, 15 and 60 days old. The ganglia were fixed in cold 1% glutaraldehyde, 1% paraformaldehyde in 0.1 M phosphate buffer (pH 7.2) either by gravity perfusion for 10 min followed by overnight immersion or by immersion alone (for foetal rats). Only perfusion plus immersion-fixed ganglia were used for electron microscopic analysis. This procedure has been recommended to reduce the presence of 'dark' neurons that could be mistaken for degenerating cells (Cammermeyer, 1978). We saw no 'dark' neurons with either type of fixation. After rinsing in buffer and postfixing for 1 h in 1% osmium tetroxide, the ganglia were again rinsed in 0.1 M phosphate buffer, dehydrated in a series of alcohols, and embedded in Epon. Semithin (1.5 μm) transverse sections were taken with a calibrated ultramicrotome at 100 μm intervals and stained with toluidine blue. Thin sections were taken at 500 μm intervals throughout the ganglia. These sections were mounted on 400 mesh grids, stained with uranyl acetate and lead citrate and examined in the electron microscope.

Neuron counts

All neuron nuclei in each semithin section were counted at a final magnification of × 555. To ensure that the cells that were counted were neurons, semithin sections were compared with

electron micrographs of thin sections from the same ganglia, in which neuronal characteristics were evident. Cells counted as neurons at the light microscopic level corresponded with neuroblasts in the primitive neuroblast stage (Papka, 1972) or more mature stages of development. In the foetus, these neurons could be distinguished from satellite cells by their more abundant cytoplasm and their longer, narrower rough endoplasmic reticulum, and from indifferent cells by their electron-lucent nuclei. Small, intensely fluorescent (SIF) cells could not be distinguished from principal ganglion neurons at the light microscopic level, so these cells were undoubtedly included in the neuron counts.

Nuclei, rather than nucleoli were the particles counted, because the SCG neurons contain a variable number of nucleoli (see Fig. 6). Ten nuclear profiles from each section counted (for a total of 150–200 nuclei per ganglion) were measured with the aid of a drawing tube attached to the microscope and a digitizing planimeter. Neighbouring nuclear profiles were selected to minimize the bias toward selection for larger nuclei. The modification of Hendry's (1976) correction described in the accompanying paper (Smolen *et al.*, 1983) was used to calculate the mean nuclear diameter. The correction factor was calculated separately for each ganglion to ensure that differences in nuclear size due to age or fixation differences would not systematically bias the calculation of neuron number.

Degenerating neurons

Debris profiles consisting of a dense clump surrounded by a cytoplasmic rim (Fig. 4 inset; Fig. 6) were frequently encountered in this material. We identified these profiles in the SCG with the light microscope, and counted them from each of the sections used for neuron counts. Debris profiles appearing smaller than nearby satellite nuclei were not counted. Degenerating neurons were also examined with the electron microscope.

Results

Counts of normal and degenerating neurons

The results of the neuron counts are shown in Fig. 1A. Neuron numbers remained at a constant level of about 39 000 cells between two days before birth (E19) and three days after birth (P3). Thirty-seven per cent of these neurons were lost by day 15, with a significant (33%) drop occurring between day 3 (39 000 ± 3300) and day 7 (26 500 ± 3900). The mean number of neurons on day 15 was 25 000 ± 3400. The mean nuclear diameter increased from 10 μm at E18 to 14 μm at P60.

The results of the counts of dying neurons (Fig. 1B) indicate that there was a significant increase in numbers of degenerating neurons between days 3 and 5. A significant decrease in profiles occurred between days 15 and 60. The mean diameter of the degenerating profiles was 11 μm.

Ultrastructure

Comparison of light and electron microscopic evidence suggests that most of the degenerating neuron profiles counted at the light microscopic level correspond to the later and terminal phases of neuron degeneration. With the electron microscope we were able to identify earlier phases of neuron degeneration which were not recognizable in the light microscope. These early phases were most numerous in ganglia of 5-day-old rats, but were also seen at all ages examined except in day 60 ganglia. Early phases of

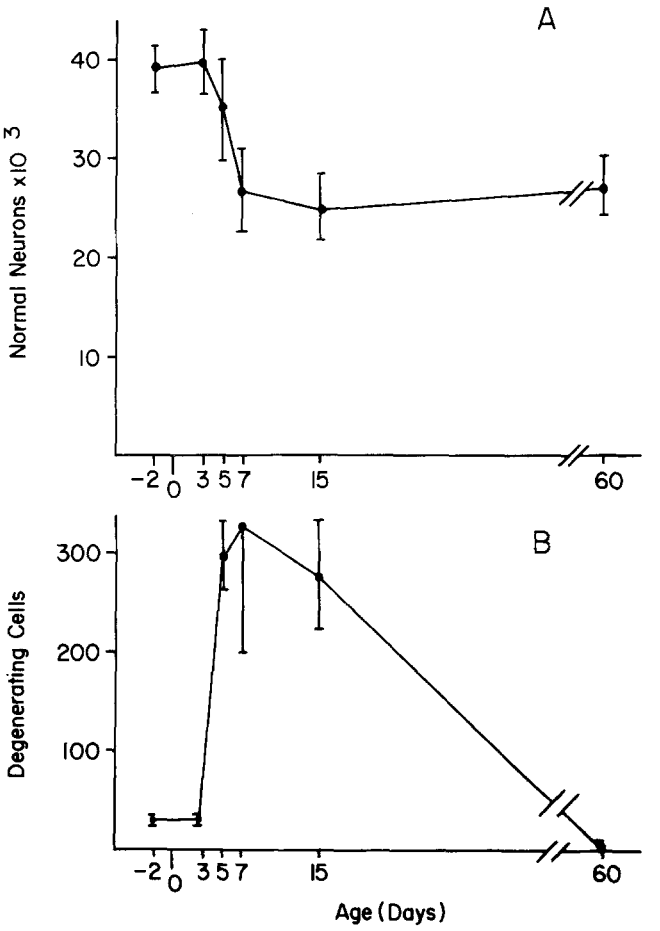
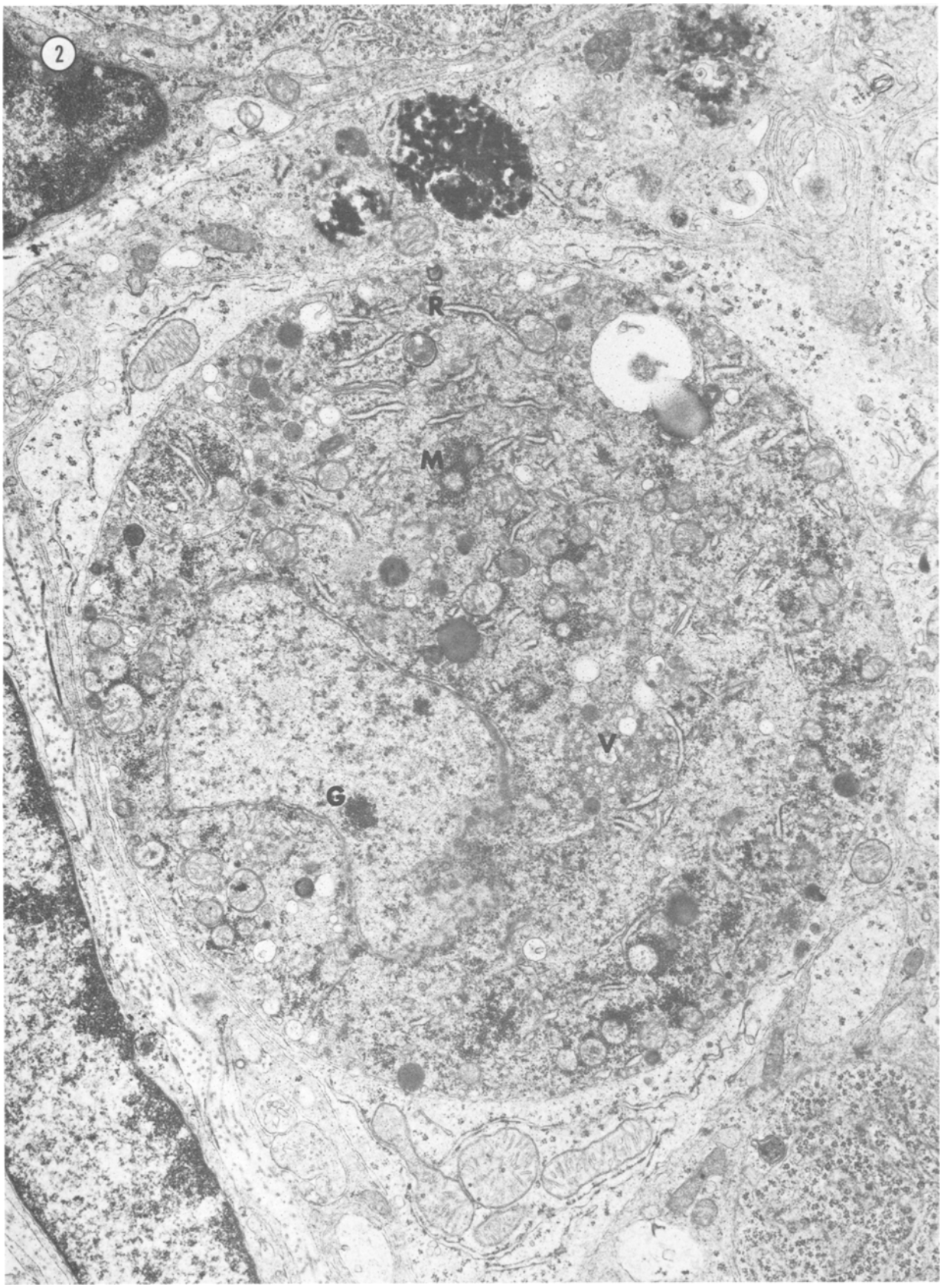


Fig. 1. (A) Numbers of neurons in the rat SCG at various ages. The day of birth is 0. Each point represents the mean count from three animals. The bars are standard errors. (B) Numbers of degenerating neurons in the rat SCG at various ages. The day of birth is 0. Each point represents the mean count from three animals. The bars are standard errors.

neuronal degeneration were characterized by the presence of free monoribosomes and some chromatin clumping (Fig. 2). The Golgi apparatus was broken down into aggregations of vesicles. In these early phases, the nuclear membrane was intact, and most of the mitochondria appeared normal. Segments of apparently normal rough endoplasmic reticulum were seen in these neurons, with only occasional lengths being

Fig. 2. SCG neuron of a 5-day-old rat in the early phase of degeneration. Mitochondria are generally intact, and strands of rough endoplasmic reticulum appear normal (R). The first signs of degeneration are aggregations of free monoribosomes (M), dissociation of the nucleolus, as indicated by the presence of granular clumps (G), and the breakdown of the Golgi apparatus into vesicles (V). × 15 000.


2
R
M
V
G

free of ribosomes. Degenerating processes were seen frequently near these dying cell bodies.

In later phases of degeneration the nucleus was irregular in shape, showed more chromatin clumping, more frequent occurrences of granular material in the nucleus, and more nuclear membrane disruption (Fig. 3). Short segments of rough endoplasmic reticulum (RER) were scattered throughout the cytoplasm but did not appear dilated. Most mitochondrial cisternae had degenerated. Still later phases of neuronal degeneration (Figs. 4,5) showed more dense aggregations of free monoribosomes and numerous vacuoles.

In the latest phases of degeneration, cells displayed little structural detail, and frequently consisted of a dense chromatin clump engulfed in glial cytoplasm (Figs. 5,6). This phase of degeneration was the most frequently encountered phase at all ages.

Ganglia fixed by immersion contained occasional neurons with dilated cisternae of endoplasmic reticulum. These dilated cells appeared in ganglia at all ages studied, including 15 and 60 days, when much of the reduction in neuron numbers is complete. This dilation of cisternae was thought therefore to be an artifact of immersion fixation. Only two cells with dilated cisternae were seen in perfused ganglia. In these instances the cytoplasm was slightly hyperchromatic, and free monoribosomes were present along with polysomes.

Discussion

In this study we have correlated a reduction in the number of apparently normal neurons in rat SCG with the presence of degenerating neurons seen with the light and electron microscopes. We conclude that neuron death does occur in the postnatal rat SCG. The number of neurons declines by one-third between two days before birth and one week after birth. The number of degenerating cells seen on days 5, 7 and 15 corresponds with the significant reduction in the number of neurons seen during the first weeks. Approximately 90% of the neuron loss occurs between 3 and 7 days of age.

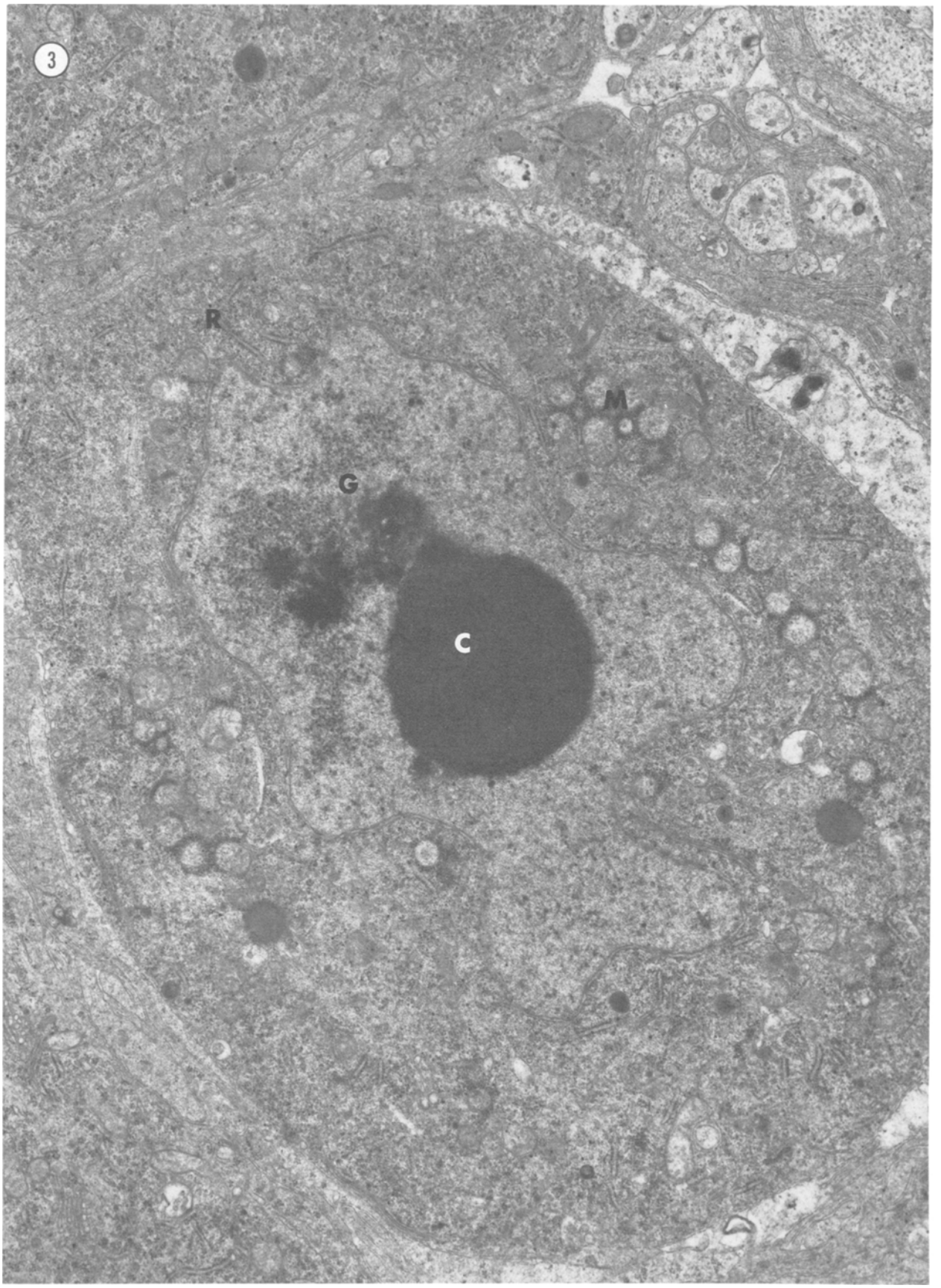
On embryonic day 19 some of the SCG neurons are still proliferating (Hendry, 1977).

Fig. 3. SCG neuron of a 5-day-old rat in the early phase of degeneration showing a large chromatin clump (C) and granules (G). × 15 000.

Fig. 4. SCG neuron of a 5-day-old rat in the late phase of degeneration. Aggregates of free monoribosomes are now more dense (M), although occasional rosettes can be seen. Both granular (G) and fibrillar (F) components can be seen in the nucleus. Strands of rough endoplasmic reticulum remain undilated (R). × 15 000. Inset: Neuron at a comparable stage of degeneration seen with the light microscope. × 1875.

Fig. 5. SCG neuron of a 5-day-old rat in the terminal phase of degeneration. The central clump is surrounded by cytoplasmic remnants lacking in ultrastructural detail. × 12 000.

Fig. 6. SCG neuron of a 5-day-old rat in the terminal phase of degeneration, indicated by the arrow. × 1875.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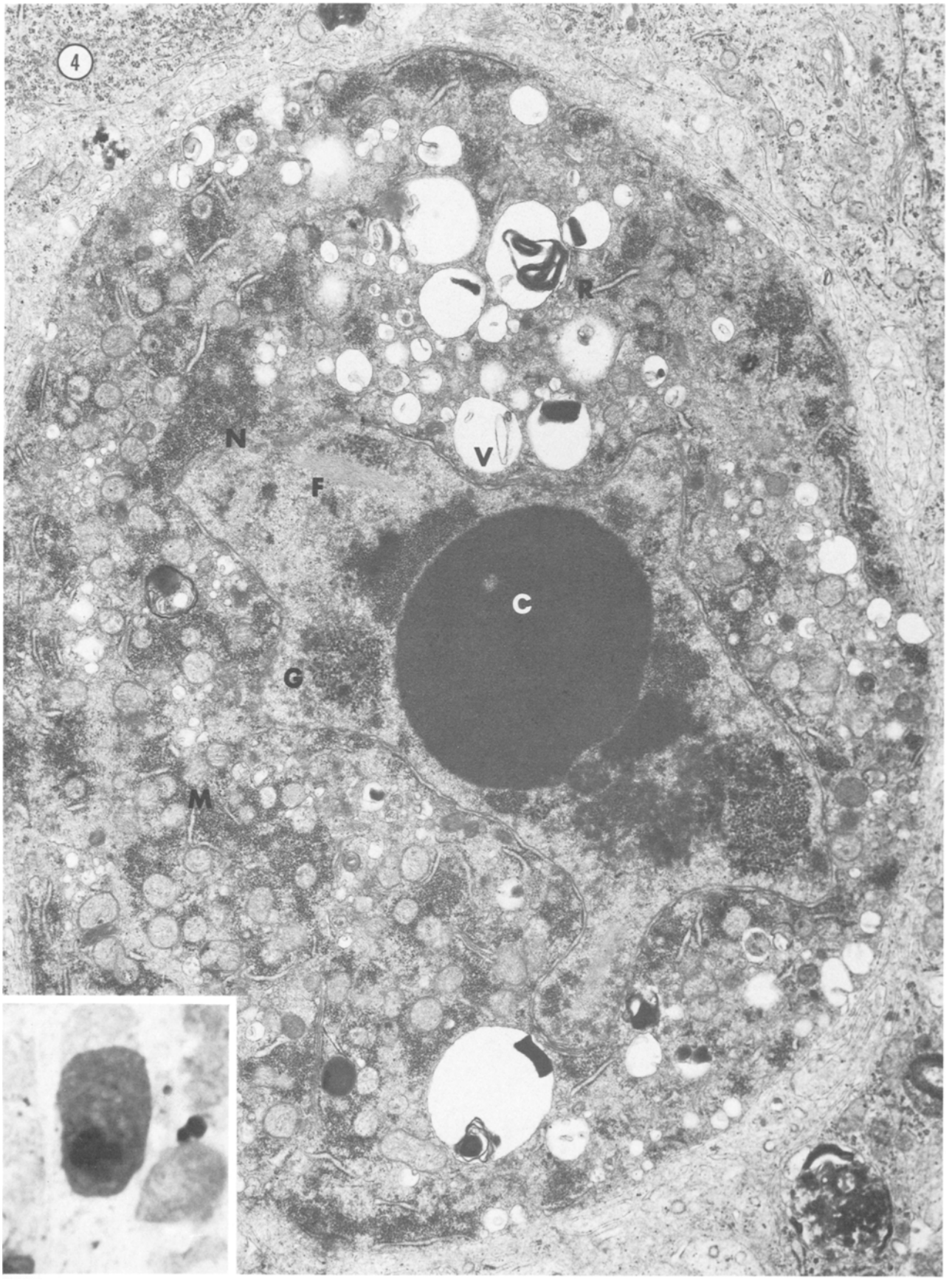
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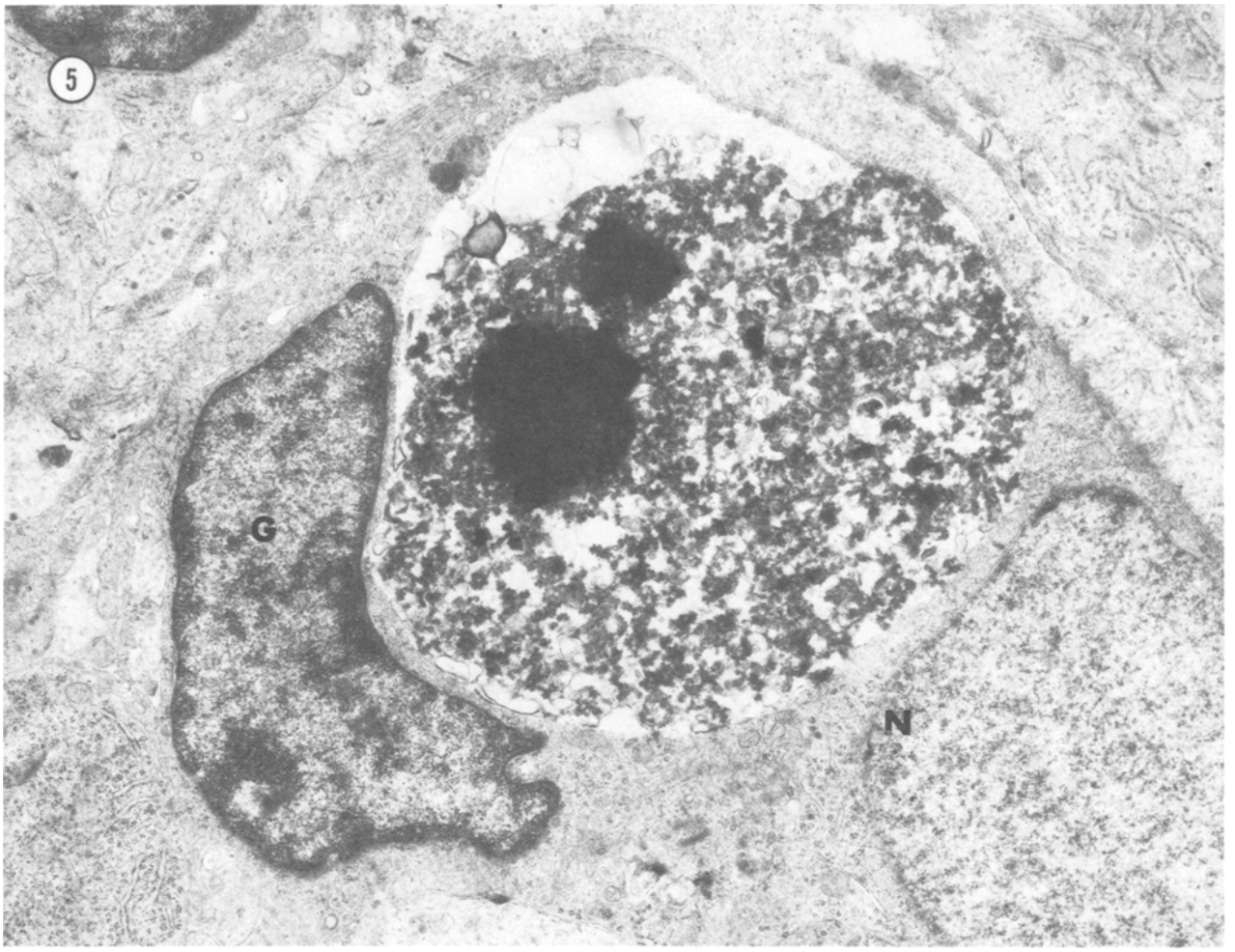
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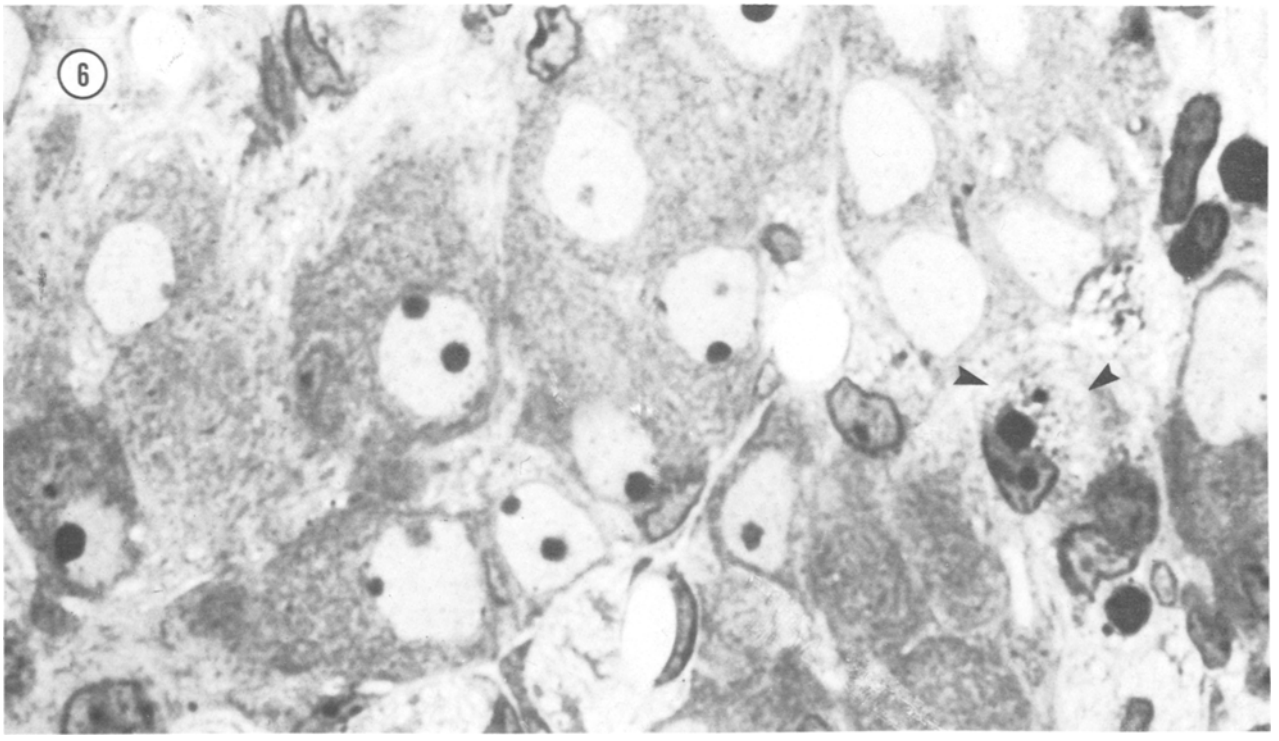
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Our results show that neuron degeneration has already begun in these foetal ganglia. Because the population is in flux, our neuron counts at this age do not represent the total number of neurons produced. Our estimate of the percentage of the original neurons that degenerate during normal cell death is therefore an underestimate. Our data suggest that most of the neuron death in SCG occurs after day 3, since the number of neurons is stable between days E18 and P3, and few degenerating cells are seen before day 5.

The ultrastructural characteristics of the vast majority of the neurons that die in the SCG are similar to type I degeneration described in the lateral motor column during normal cell death (Chu-Wang & Oppenheim, 1978). This type of degeneration is also observed during normal ganglion cell death in the retina (Hughes & McLoon, 1979; Cunningham *et al.*, 1981), and in the spinal ganglia (Pannese, 1976), after target removal in the ciliary ganglion (Pilar & Landmesser, 1976), and after deafferentation of the superior colliculus (Giordano & Cunningham, 1982). Pilar & Landmesser (1976) refer to this type of degeneration as 'nuclear' because of the early appearance of clumped chromatin.

A second type of degeneration, termed 'cytoplasmic' by Pilar & Landmesser (1976), is characterized by early dilation of the cisternae of the endoplasmic reticulum with only later changes in the nucleus. It has been reported during normal cell death in the trochlear nucleus of the duck (Sohal & Weidman, 1978), lateral motor column (Chu-Wang & Oppenheim, 1978), ciliary ganglion (Pilar & Landmesser, 1976), and superior colliculus (Giordano *et al.*, 1980). In the perfused rat SCG, we found only two instances of neurons with dilated cisternae. Thus, a small population of SCG neurons may undergo cytoplasmic cell death. The presence of mixed types of neuron death has also been reported in the lateral motor column of the chick (Chu-Wang & Oppenheim, 1978).

The nuclear form of degeneration is therefore, the predominant type seen in the SCG. Pilar & Landmesser (1976) suggested that the ultrastructural type of degeneration seen may reflect the maturity of the neuron at the time of degeneration. Normal cell death in the ciliary ganglion is of the cytoplasmic type but after removal of the target, the eye, the cells die with the nuclear pattern of degeneration. These neurons apparently require the presence of their target region to mature: target-deprived neurons contained less rough endoplasmic reticulum than non-deprived controls. Likewise, deafferentation of the superior colliculus by eye removal changes the predominant form of degeneration from cytoplasmic to nuclear (Giordano & Cunningham, 1982). Thus, it appears that contact with afferents or efferents may initiate maturation of the neurons, and thereby regulate the ultrastructural characteristics of neuronal degeneration (see Cunningham, 1982).

At the time that SCG neurons die, they are undergoing rapid development. Synapse formation in the SCG by preganglionic axons occurs largely during the first postnatal week, and is 85–90% complete by 15 days (Smolen & Raisman, 1980). The onset of functional ganglionic transmission has been shown to occur between 5 and 10 days after birth (Smith *et al.*, 1982). Similarly, innervation of its peripheral targets occurs during the

first three weeks after birth (deChamplain *et al.*, 1970; Owman *et al.*, 1971). Biochemical indices of maturation of the SCG (Black *et al.*, 1971, 1972, 1974; Black & Geen, 1974; Thoenen *et al.*, 1972), rise during the early postnatal period, suggesting that SCG neurons have not yet reached maturity by the time neuron death occurs. Thus, the nuclear type of degeneration seen in these young neurons is consistent with the suggestion of Pilar & Landmesser (1976) that the ultrastructure of degeneration is correlated with maturity at the time of death.

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