Histol Histopath (1986) 1: 315-321

Histology and Histopathology

The thoracic sympathetic neurons of the chick: normal development and the effects of nerve growth factor

Peter Smet[2], Robert A. Rush2 and Charles Straznicky[1]

Departments of Anatomy & Histology[1] and Physiology[2], School of Medicine, The Flinders University of South Australia, Bedford Park 5042, Australia

Summary. The generation and degeneration of sympathetic neurons in the third thoracic ganglion (segment 19) of the chick were studied between embryonic days (E) 7-18 using 3H-Thymidine autoradiography and routine cell counts. Cumulative radiolabelling experiments indicated that few sympathetic neurons were generated on E6-7. 10% of the sympathetic neurons were generated on E8 and a further 20% on E9. The final 70% of neurons completed the mitotic cycle between E10-12. Cell counts demonstrated that the neuronal population increased from 10,166 ± 423 (mean ± SEM) to 22,291 ± 767 between E8-10 and remained stable up to E14. The population subsequently declined by 37%, to 14,157 ± 831, by E18. Pyknotic neurons were found at all stages of development, but were most apparent between E7-15. The effects of Nerve Growth Factor (NGF) on the number of both surviving and pyknotic neurons in the ganglion were also examined. E9 embryos treated with NGF from E5-8 showed a 57% increase in the number of sympathetic neurons. This increase therefore occurred prior to the decline in neuronal number and was not accompanied by a decrease in the number of visibly pyknotic neurons. It is therefore possible that early NGF treatment increases the number of sympathetic neurons through a mechanism other than the attenuation of cell death.

Key words: Sympathetic neurons - Neuron generation - Neuron death- Nerve Growth Factor - Chick embryos

Offprint requests to: A/Prof. C. Straznicky, School of Medicine, The Flinders University of South Australia, Bedford Park 5042, Australia

Introduction

The chick embryo is extensively used as an experimental model for developmental studies and, as a result, much is known regarding the development of particular neuronal pools. For example, the generation, formation of connections and period of cell death have been determined for both motoneurons of the lateral motor column (Hollyday and Hamburger, 1977; Oppenheim and Chu-Wang, 1977) and the sensory neurons of the dorsal root ganglia (DRG) (Carr and Simpson, 1978; Hamburger et al., 1981; Hamburger and Oppenheim, 1982). However, little is known regarding the development of neurons of the sympathetic ganglia in the chick, despite a detailed description of the initial derivation of sympathetic precursors from the neural crest (Le Douarin, 1984).

A preliminary study of the later development of chick sympathetic ganglia of the thoracic region has been reported (Oppenheim et al., 1982). This study, however, only examined 3 developmental stages and did not establish at which stage the neuronal population declines. A detailed study of both the generation and loss of sympathetic neurons is therefore necessary, particularly since such information is essential to studies of the mechanism of action of neuronal growth factors. These factors are thought to be important in regulating neuronal survival (Thoenen and Edgar, 1985); and it is, therefore important to determine when neurons are normally lost during development.

Sympathetic neurons require Nerve Growth Factor (NGF) for their normal development (Levi-Montalcini and Booker, 1960b; Goedert et al., 1978). Previous studies have shown that the number of neurons within the sympathetic ganglia of rodent and chick embryos can be dramatically elevated by NGF treatment (Levi-Montalcini and Hamburger, 1953; Levi-Montalcini and Booker, 1960a;

Kessler and Black, 1980; Oppenheim et al., 1982). In the rat superior cervical ganglion (SCG), such an increase approximates the number of neurons which are normally lost during development and coincides temporally with the period of naturally occurring death (Hendry, 1977a). Other studies have demonstrated that NGF treatment decreases the number of pyknotic neurons both in chick DRG (Hamburger et al., 1981; Hamburger and Yip, 1984) and sympathetic ganglia (Oppenheim et al 1982). Taken together, these results indicate that exogenous NGF attenuates the naturally occurring loss of neurons.

Most of these studies, however, involve the administration of NGF over long time courses from relatively early stages. It is therefore not possible to determine at exactly which time point the neuronal population is increased. The present experiments were undertaken in order to determine the time and extent of the generation and loss of sympathetic neurons. It was further aimed to establish whether NGF could induce an increase in the neuronal population early in development, prior to the onset of cell death.

Materials and methods

Fertile eggs from White Leghorn/Austral Leghorn crossbred chickens were obtained from a local poultry supplier (Parafield Poultry, Adelaide) and incubated at 37.7°C in a forced draught incubator at 70% relative humidity.

NGF purification

B-NGF was purified from adult male mouse saliva according to Burton et al. (1978) and Mobley et al. (1976). The purified NGF was detectable as a single band on a sodium dodecyl sulphate (SDS) polyacrylamide electrophoretic gel. Biological activity was assayed on E8 chick DRG explants *in vitro* (Levi-Montalcini et al., 1954), and demonstrated to be optimal at concentrations of 1-10 ng/ml.

Injection procedure

Embryos received 20μCi of methyl 3H-Thymidine (3H-T; Amersham, 25 Ci/mmol) in a volume of 20μl, which was injected through a hole in the shell onto the chorioallantoic membrane. The injection site was subsequently sealed with sterile tape. Embryos were injected either on embryonic days (E) E5-7, 6-8, 7-9, or 10-12, and sacrificed on E12. At later stages, 3H-T was administered on E13-14 or E14 only, and embryos were sacrificed on E15. NGF-treated animals were injected in an identical manner, from E5-8, and sacrificed on E9. The dose consisted of 20μg NGF in a volume of 100μl physiological saline. Control embryos received an equal volume of physiological saline.

Histology

Chick embryos were sacrificed by decapitation and staged according to Hamburger and Hamilton (1951). The spinal cord and surrounding tissue were dissected out, fixed in Carnoy's solution for 4 hours, dehydrated, and embedded in paraffin. E12 or younger specimens were serially sectioned at 7pm in the transverse plane. Older specimens were sectioned at 10 pm. Tissues were prepared for either thionin stain or autoradiography. 3H-T treated animals were processed for autoradiography according to Rogers (1979). Briefly, slides were dipped in llford K2 emulsion, dried, exposed at 4°C for 21 days, and developed in Kodak D19 developer. Autoradiographic material was then stained with Harris's haematoxylin and mounted in DePeX (Gurr).

Cell counts and morphometric measurements

Both radiolabelled and normal cell counts were performed on every fifth section of sympathetic ganglion 19 at a magnification of 630X and the obtained figures multiplied by 5. The number of labelled neurons was expressed as a percentage of the total number of neurons present in the ganglion at the particular stage examined. 3H-T labelled neurons could be clearly identified by the accumulation of silver grains over the nucleus. On thionin stained sections, only neurons with a clear nucleus and at least one large nucleolus and substantial cytoplasm were counted. No corrections were made for split cell counts (See Oppenheim et al., 1982). Pyknotic neurons were identified according to previously established criteria (Pannese et al., 1976; Chu-Wang and Oppenheim, 1978) as having fragmented nuclei and the appearance of darkly stained homogeneous spheres.

The average area of the ganglion was determined by tracing the ganglion circumference in every fifth section at 125X with a HIPAD digitizer pad connected to a North Star Z80 computer. This measurement was multiplied by the total number of sections in which the ganglion appeared and the section thickness to obtain the ganglion volume. Nuclear area measurements were obtained by tracing the outline of 300 nuclei at 630X at the maximum extent of the ganglion of selected specimens.

Results

The generation of neurons

To determine the number of neurons generated at various developmental stages, a total of 23 embryos were injected cumulatively with 3H-T. From E7 onwards, increasing numbers of unlabelled neurons were apparent. Essentially all of the sympathetic neurons of embryos sacrificed on E12 and injected with isotope on E5, 6 and 7 were labelled. Approximately 10% of the neurons were unlabelled in embryos injected on E6, 7 and 8, 60% in embryos injected on E7, 8 and 9, and 90% in embryos receiving isotope on E10, 11 and 12. Some scattered labelling was seen in specimens injected on E13 and sacrificed on E14, no neurons were labelled in embryos injected on or after day 14. The generation of sympathetic neurons on a day by day basis is shown in Figs. 1 and 2 (A). Since injected isotope is not decomposed in the egg, it was assumed that 3H-T remained available for uptake to all dividing cells during the course of the experiment.

Changes in the total neuronal population over t h e

A total of 62 embryos were sacrificed in order to establish the neuronal population of sympathetic ganglion 19 at various developmental stages, ranging from E7-18 (Fig. 3). Although the counts were variable, there was a dramatic increase in the population, from 10,166 ± 423 (mean ± SEM) at E8, to 22,291 ± 767 at E10. The population then remained stable, within the range of experimental error, up until E14, and subsequently declined by 37%, to 14,157 ± 831, at E18. The neuronal counts were consistent with the autoradiographic studies, as most neurons were still in the mitotic cycle between E6-7, just prior to the large increase in the population. Between E10-12, when the population levelled off, the remaining 30% of neurons completed the mitotic cycle.

Pyknotic cell numbers

Pyknotic neurons were counted on the same sections used for normal neuron counts. The percentage of visibly degenerating neurons was extremely low at all stages examined, never exceeding 0.7% of the total neuronal population (Fig. 3). This indicates that only a very small proportion of neurons which undergo pyknosis during development can actually be observed at any particular time. The duration of the pyknotic cycle is therefore considerably shorter than the sampling interval of 24 hours. However, there were many more pyknotic neurons during the early stages of development (E7-12) than at the later stages (E14-18). Although some pyknosis therefore occurs throughout development, the peak does not coincide with the decrease in neuron numbers observed between E14-18.

Effects of NGF treatment

Seven embryos were treated with NGF from E5-8 and compared to 9 control animals to determine the increase in the neuronal population induced by NGF. The average number of sympathetic neurons was elevated by 57% in the NGF treated (25,034 ± 3686) as compared to the control group (15,899 ± 1288) (Fig. 4). It is important to note that this increase occurred prior to the decline in the neuronal population, i.e. prior to the apparent onset of cell death. The ganglion volume of E9 embryos treated with NGF daily from E5-8 was increased to a greater degree, by 93% (Fig. 4). To determine if a reduction in neuronal death could be a factor contributing to the detnonstrated population increase, the degeneration indices of NGF-treated and control embryos were compared. The 2 groups did not significantly differ (Fig. 5), indicating that early NGF treatment does not reduce cell death.

The neuronal nuclear area frequency distributions of 3 NGF-treated and 3 control embryos were compared to determine whether this parameter was influenced by early NGF administration. There was a minor but significant increase in the nuclear area of the sympathetic neurons of NGF-treated embryos (Fig. 6). The present and previous studies therefore indicate that NGF increases the nuclear area of developing sympathetic (Hendry and Campbell, 1976; Hendry, 1977b) and DRG sensory neurons (Straznicky and Rush, 1985).

Fig. 1. Histogram showing the percentage of segment 19 sympathetic neurons generated at various developmental stages (hatched) and the cumulative generation of neurons (open). Unless otherwise stated, the numbers in parentheses represent the number of embryos examined per data point. Injection schedules are outlined in the text.

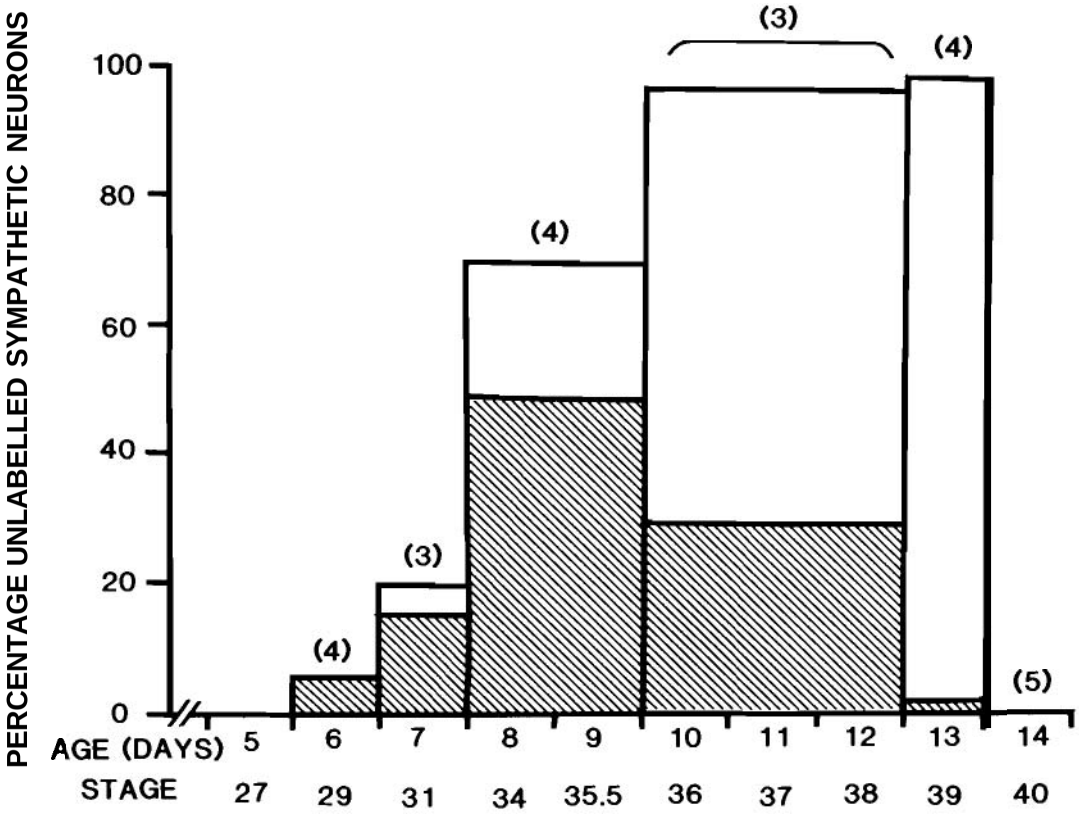
Fig. 2. A. Microphotograph of radiolabelled sympathetic neurons within the thoracic ganglion of an E12 chick embryo injected with 3H-T on E9, 10, and 11. B. Fragmented pyknotic nucleus (arrow) in a thionin stained section of the sympathetic ganglion of an E9 embryo. C and D. Low-power magnification of the spinal cord and the sensory and sympathetic ganglia (arrowed) of a normal (C) and NGF-treated embryo (D). The hypertrophy of the ganglia following NGF treatment is apparent. Bars in A = 75 pm, B = 30 μm, C and D = 500 pm.

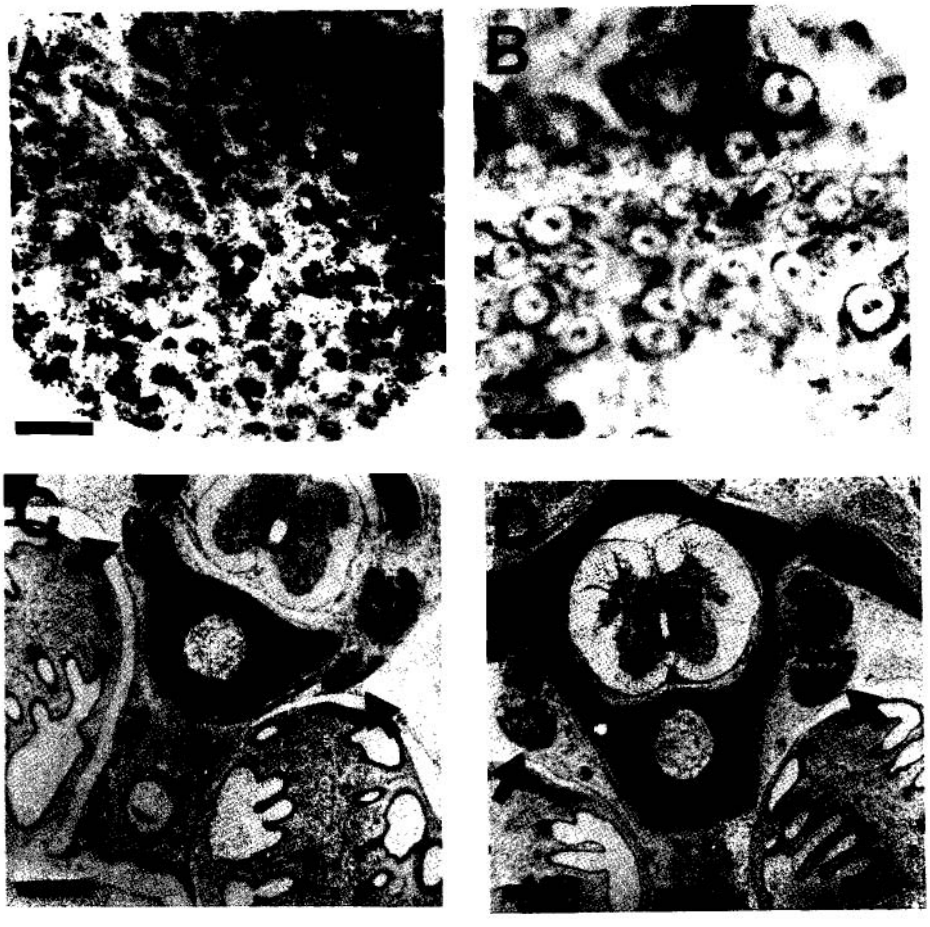
Fig. 3. Graph showing the neuronal population and degeneration index of sympathetic ganglion 19 at various stages of development. The degeneration index was obtained by expressing the number of pyknotic neurons as a percentage of the total number of neurons within the ganglion. The bars represent the SEM.

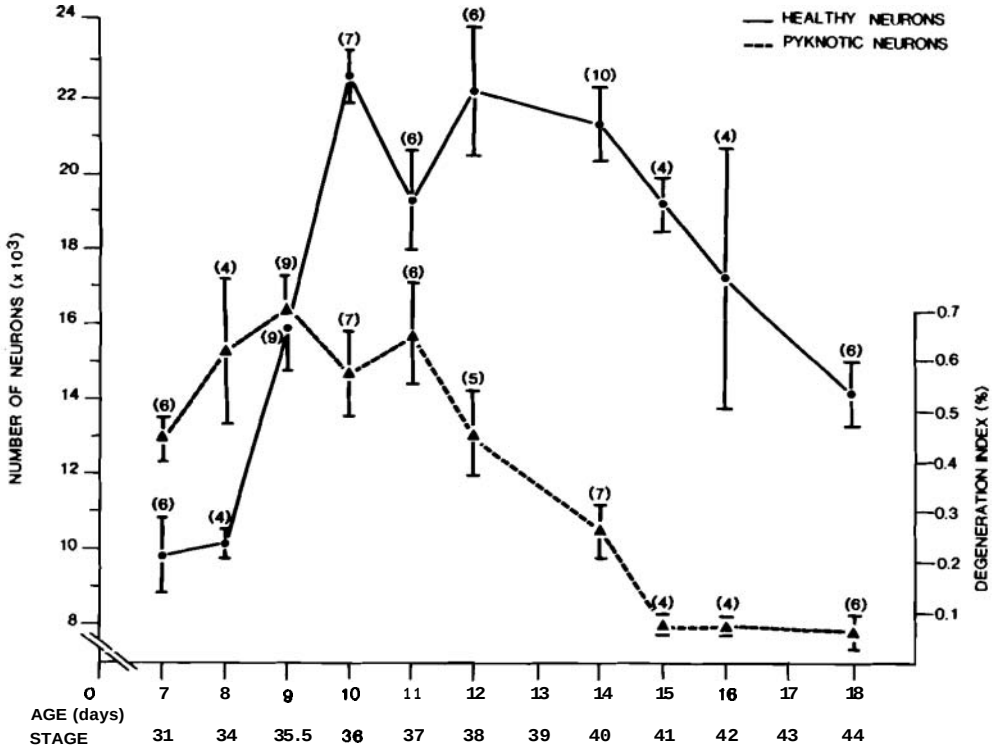
Fig. 4. Histogram showing increases in both the volume (right) and the neuronal population (left) of sympathetic ganglion 19 of E9 embryos treated with 20μg NGF over E5-8. The volume is significantly increased by an average of 93% (t-test $p < 0.05$) and the neuronal population by 57% (t-test $p < 0.05$).

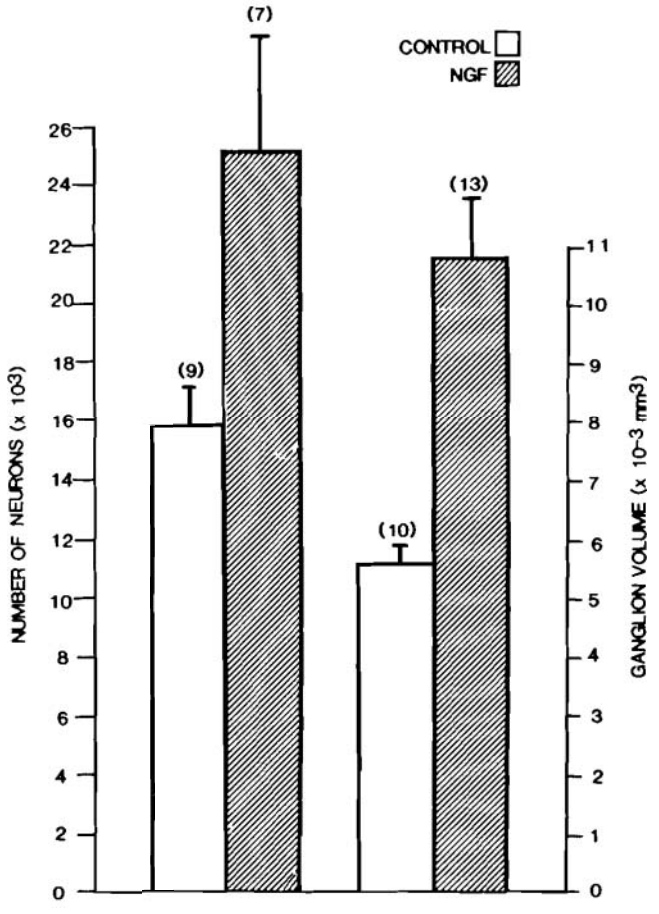
Fig. 5. Histogram indicating the degeneration indices of E9 NGF-treated and control embryos. The groups did not significantly differ (t-test, $p < 0.05$).

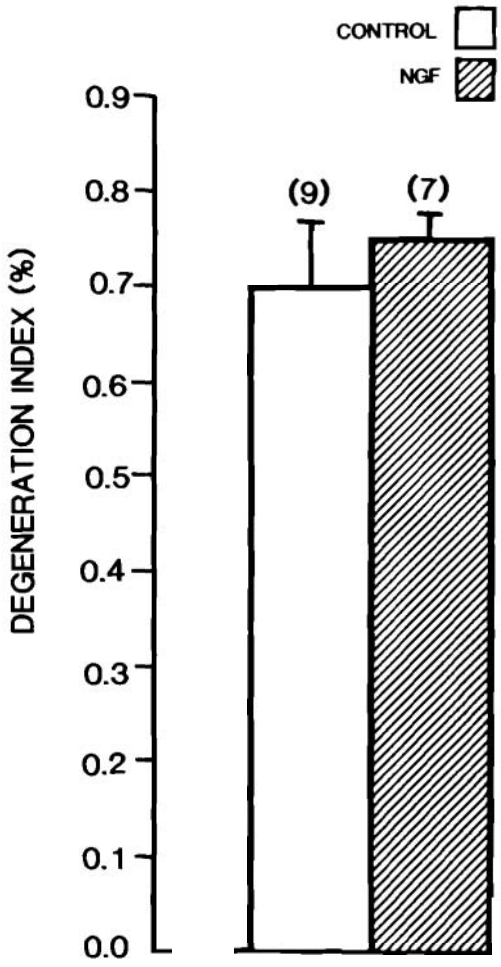
Fig. 6. Frequency histogram of the neuronal nuclear areas of 300 neurons from each of 3 NGF-treated and 3 control embryos. A 2 x 4x2 test indicated that there was a highly-significant shift in the frequency distribution of the NGF-treated animals ($p < 0.001$). The following classes of nuclear area were used to construct the contingency table: $\leqslant 15 pm^2$, $> 15 pm^2 \leqslant 20 pm^2$, $> 20 \mu m^2 \leqslant 25 pm^2$, and $> 25 pm^2$.

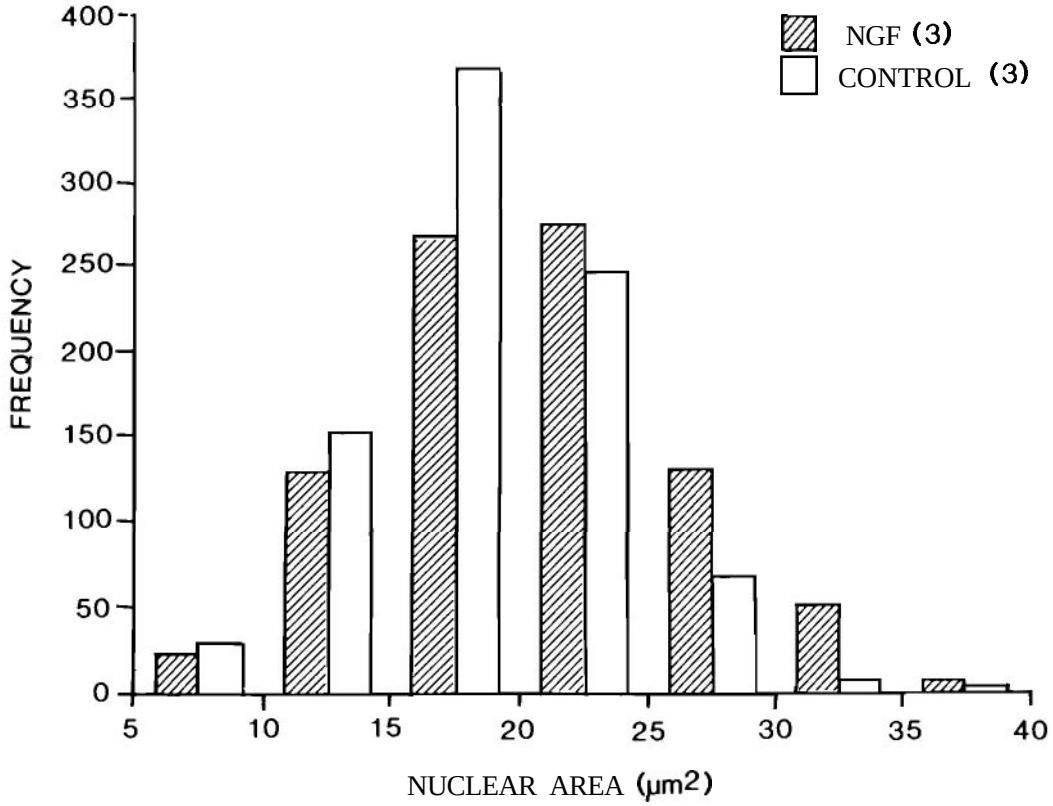

PERCENTAGE UNLABELLED SYMPATHETIC NEURONS
100
80
60
40
20
0
(4)
(3)
(4)
(3)
(4)
(5)
AGE (DAYS) 5 6 7 8 9 10 11 12 13 14
STAGE 27 29 31 34 35.5 36 37 38 39 40


A
B


HEALTHY NEURONS
PYKNOTIC NEURONS
NUMBER OF NEURONS (x 10³)
DEGENERATION INDEX (%)
AGE (days)
STAGE
0 7 8 9 10 11 12 13 14 15 16 17 18
31 34 35.5 36 37 38 39 40 41 42 43 44


CONTROL
NGF
NUMBER OF NEURONS (x 10³)
GANGLION VOLUME (x 10⁻³ mm³)


CONTROL
NGF
DEGENERATION INDEX (%)



Discussion

The present study indicates that the sympathetic neurons of the chick thoracic sympathetic ganglion are generated over a considerable time span. This is in contrast to other neuronal populations, such as chick DRG neurons (Carr and Simpson, 1978) and mesencephalic trigeminal neurons and motor trigeminal neurons (Rogers and Cowan, 1974; Arens and Straznicky, 1986), which are produced over a period of 3 days. Although the sympathetic neurons are generated over an extended period, between E6-13, the maximum cell production is between E8-9.

In sympathetic ganglion 19, the neuronal population decreases by 37% between E14-18. This decrease is consistent with the 30% decrease found in the neuronal population of the rat SCG (Hendry, 1977a; Wright and Smolen, 1983). In the latter case, however, most neurons are lost over a limited period, on postnatal days 5 and 6 (Wright et al., 1983). The time of generation and degeneration within chick thoracic sympathetic ganglia overlap considerably. This suggests that these neurons may comprise a heterogeneous population, made up of subpopulations with distinct periods of generation and degeneration.

The presence of visibly pyknotic neurons indicates that some degeneration occurs at all stages of development. Oppenheim et al. (1982) have also noticed pyknotic sympathetic neurons very early in the developing chick embryo. It is likely that a proportion of the sympathetic neurons degenerate prior to making contact with the target. Carr and Simpson (1982) have indeed drawn a similar conclusion from studies of chick DRG sensory neurons.

Paradoxically, few pyknotic neurons were obvious during the period when neuronal numbers declined and in fact pyknosis occurred more frequently earlier in development. Oppenheim et al. (1982) have also reported that more pyknotic neurons are visible at E8-10 than at E15 in the chick thoracic sympathetic ganglia. At later stages, the number of visible pyknoses may be reduced due to a shortened length of the pyknotic cycle, or a more rapid removal of debris by macrophages. Due to these uncertainties, the degree of neuronal pyknosis in sympathetic ganglia does not appear to be an accurate quantitative measure of the loss of neurons.

Neuron death has been shown to coincide with the time when target innervation occurs (Oppenheim, 1981; Cowan et al., 1984), although some neurons may die prior to target contact (Carr and Simpson, 1982; Hiscock and Straznicky, 1986). The first sympathetic fibres reach the wing tissue on E10 (Saltis et al., 1986) and the expansor secondariorum, a smooth muscle of the chick wing, becomes innervated between E14-15 (Rush et al., 1986). Since outgrowth from

thoracic ganglia may have a similar time course as that of the brachial ganglia, and since the sympathetic neuron population declines after E14, it is likely that segment 19 sympathetic neurons innervate their targets prior to E15.

There is considerable inconsistency in the literature regarding the size of the sympathetic neuron population. In the rat SCG for example, counts range from 13,000 to 45,000 (reviewed by Smolen et al., 1983). Oppenheim et al. (1982) have reported the neuronal population of chick sympathetic ganglion segments 21-22 at E10 to be 5,296. This is substantially lower than our estimate, using identical criteria, of 22,291. The latter figure, however, was partially confirmed by excising and dissociating the sympathetic chain from E10 embryos and counting the cells under a haemocytometer (Varon and Raiborn, 1972). Although less accurate, this procedure yielded an average of 15,000 neurons per ganglion after adjusting for the presence of non-neuronal cells (Smet, unpublished). Comparable cell counts from dissociated chick sympathetic ganglia have also been obtained by Leah and Kidson (1983).

There is further controversy regarding the extent to which NGF treatment increases the number of sympathetic neurons. In the rat SCG, estimates range from 35% (Hendry, 1977a) to 250% (Banks et al., 1975a,b). In the present study, NGF increased the neuronal population by 57%. Oppenheim et al. (1982) have found that NGF administered from E3-7 induced an 85% increase in the number of sympathetic neurons. NGF administered from E10-14, however, only produced a 20% increase. Recent observations from our laboratory also suggest that NGF induces a smaller increase if administered at later stages (E12-17) (Murdoch, Straznicky and Rush, unpublished). These results suggest that NGF may act to decrease cell death at later developmental stages, while acting in a different manner earlier in development. It has recently been shown that NGF acts as a mitogen to immature chromaffin cells *in vitro* (Lillien and Claude, 1985). The chromaffin cells are, like the sympathetic neurons, derivatives of the neural crest and are closely related to the sympathetic neurons (Doupe et al., 1985). More mature chromaffin cells respond to NGF by leaving the mitotic cycle and assuming a phenotype indistinguishable from a sympathetic neuron (Lillien and Claude, 1985).

Although NGF has been reported to lack mitogenic activity for sympathetic neurons (Hendry, 1977a), only postmitotic neurons were examined; hence, no definite conclusions can be drawn regarding the action of NGF on immature neurons. The population increase induced by NGF in the present study and the increase reported by Oppenheim et al. (1982) are consistent with a mitotic effect, although no direct evidence is available. In both cases the increase induced by NGF appears to be greater than the normally occurring loss of neurons. Furthermore, in the present study, NGF treatment did not reduce the number of pyknotic neurons. This is in direct contrast to both Oppenheim et al. (1982) and Hamburger and Yip (1984). Since pyknotic neurons, as indicated in the present study, are not always a valid measure of cell death, it is still possible that NGF reduces the loss of neurons without producing a detectable decrease in the number of pyknotic neurons.

The possibility still remains that NGF increases the neuronal population in alternative ways, for example, by enhancing the rate at which the sympathoblasts differentiate. There is strong evidence that NGF increases the rate of both biochemical (Harper and Thoenen, 1981) and morphological maturation of the sympathetic neurons (Papadaki, 1972). Undifferentiated neurons, which are excluded from counts, would therefore be more prevalent in control relative to experimental embryos. We consider this unlikely as the number of both undifferentiated and non-neuronal cells excluded from counts was only 15-25%. Although an acceleration of differentiation may therefore partly contribute to the increase induced by NGF, it cannot be entirely responsible. The characterization of the actions of NGF during early neuronal development *in vivo,* in particular with regard to mitogenic activity, therefore warrants further investigation.

Acknowledgements. The authors acknowledge the assistance of Susie Murdoch, Phillipa Wilson, and Jennifer Hiscock. This work was partially supported by a grant from the Australian National Heart Foundation.

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Accepted June **20, 1986**