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The development of the neurons of the glossopharyngeal (IX) and vagal (X) sensory ganglia in chick embryos

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Summary. The timetable of cell generation, neuronal death and neuron numbers in the fused proximal glossopharyngeal (IX) and vagal (X) ganglion and distal IX and X ganglia were studied in normal and nerve growth factor (NGF) treated chick embryos.

^{3}H-thymidine was injected between the 3rd and 7th days of incubation and embryos sacrificed on the 11th day. Neurons in the distal IX and X ganglia were generated between the 2nd and 5th days of incubation, the peak mitotic activity occurring on the 4th and 3rd days, respectively. Neurons of the proximal IX and X ganglion were generated between the 4th and 7th days, with maximum neuron generation on the 5th day of incubation. Counts of neurons in the 3 ganglia between the 5th and 18th days of incubation showed a maximum of 22,000 on the 8th day in the proximal IX and X ganglion and this decreased to 12,000 by the 13th day. In the distal IX ganglion, the neuron number decreased by 44% from 4,500 on the 6th day to 2,500 by the 11th day. A similar decrease of 43% was found in the distal X ganglion, the neuron number falling from 11,500 on the 7th day to 6,500 by the 11th day of incubation. Neuronal cell death in these ganglia extended from the 5th to the 12th day of incubation, maximum cell death occurring at or after the cessation of mitotic activity. NGF administration from the 5th to the 11th day of incubation did not have a measurable effect on the neurons of proximal IX and X and distal IX ganglia, but increased neuronal survival by 30% in the distal X ganglion.

The temporal patterns of cell generation and neuron death are discussed in relation to embryonic origin of neurons and factors controlling the survival of cranial sensory neurons.

Key words: Sensory neurons - Cell generation - Neuron death - Autoradiography - NGF - Chick embryo

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Introduction

Neurons of the sensory ganglia associated with glossopharyngeal (IX) and vagal (X) nerves derive either from the medullary neural crest or the epidermal placode material. The proximal sensory ganglia of IX (superior) and X (jugular) are fused in birds -and referred to as the proximal IX and X complex- consisting of small neurons of neural crest origin (Ayer-LeLievre and L'Douarin, 1982; D'Amico-Martel and Noden, 1983). The generation time in this neuron pool extends from the 4th to 7th day of incubation (D'Amico-Martel, 1982). The distal sensory ganglia of IX (petrosal) and X (nodose) contain only large neurons of placodal origin, generated between the 2nd and 5th day of incubation. Thus, unlike the trigeminal ganglion, where the small and large neurons of neural crest and placodal origins are juxtaposed (Hamburger, 1961; Narayanan and Narayanan, 1980), neurons from the two sources form separate ganglia of the IX and X nerves.

To date, quantitative studies on the generation, differentiation and cell death of sensory neurons have been mostly confined to dorsal root ganglia (Hamburger and Levi-Montalcini, 1949; Carr and Simpson, 1978). Despite the homogenous neural crest origin of dorsal root ganglion cells, two distinct waves of neuron generation and death have been reported. The large ventrolateral neurons are generated from days 2 to 5 and the small dorsomedial

neurons from days 4 to 7% of incubation. Neuronal degeneration occurs among large neurons between days 4% and 7% and among small neurons between 7½ and 12 days of incubation. Thus generation and cell death overlap to a considerable extent in the dorsal root ganglia.

The cause of naturally occurring neuron death is not yet fully understood. It is generally assumed that neurons die because of competition for their synaptic target and/or for the limited supply of target derived factor/s (Oppenheim, 1981). Nerve growth factor (NGF) has been found to affect the survival of neural crest derived sensory neurons in vivo both in the dorsal root ganglia (Hamburger et al., 1981) and in the Gasserian ganglion of the trigeminal nerve (Davies and Lumsden, 1983; Straznicky and Rush, 1985a). Tissue culture studies on the placodal derived chick nodose ganglion cells have shown increased survival and neurite outgrowth in response to NGF (Hedlund and Ebendal, 1980).

The aim of the present study is to document the timing and extent of naturally occurring neuron death as well as the time scale and magnitude of cell generation in IX and X sensory ganglia in chick embryos. An account of the effect of NGF treatment on these ganglia during development is also given. An abstract of the main observations of the present study has already been published (Hiscock and Straznicky, 1985).

Materials and methods

Experiments were carried out using white leghorn embryos. Eggs, obtained from a local supplier (Parafield Poultry, Adelaide), were incubated at 38.2°C in a forced draft incubator at 70-75% relative humidity and rotated every 4 hours throughout the period of incubation.

Autoradiography

A daily dose of 20 µCi Methyl-^{3}H-thymidine (Amersham, 25 Ci/mmol) was injected cumulatively into chick embryos from day 3 to 7 of incubation. Isotope was introduced onto the yolk sac near the embryo through a hole in the shell which was sealed with sterile tape and the embryo returned to the incubator until the time of sacrifice on the 11th day of incubation.

NGF adminktration

NGF was purified from male mouse salivary gland by the method of Mobley et al. (1976). Twenty micrograms of sterile NGF dissolved in 20 pl of 5 mM Tris-HCl buffer, pH 7.4 containing 0.14 M NaCl was administered daily from the 5th to 11th day of incubation. Injections were made with a 100 µl Hamilton microsyringe onto the chorio-allantoic sac of the embryo. Embryos were sacrificed between the 9th and 12th day of incubation.

Histological processing

^{3}H-thymidine, NGF injected embryos and normal chick embryos from the 5th to 18th day of incubation and hatched chicks were sacrificed and prepared for histology. The ages of the embryos were determined according to the Hamburger and Hamilton (1951) developmental series. The heads and brachial segments of the trunk of the animals were fixed in Carnoy's solution for 3-6 hours according to the size of the tissue. Tissues from embryos after the 12th day of incubation and hatched chicks were decalcified for ½-1 hour in HCl/EDTA solution. Tissues were then processed for paraffin embedding and serial sections of 7 or 10 pm were cut in the transverse plane at the level of proximal IX and X, distal IX and distal X ganglia. The closely spaced serial sections were stained with Thionin. Serially sectioned autoradiographic material was dipped in llford K2 emulsion, exposed at 4°C for 3 weeks, developed and counterstained with Harris' haematoxylin.

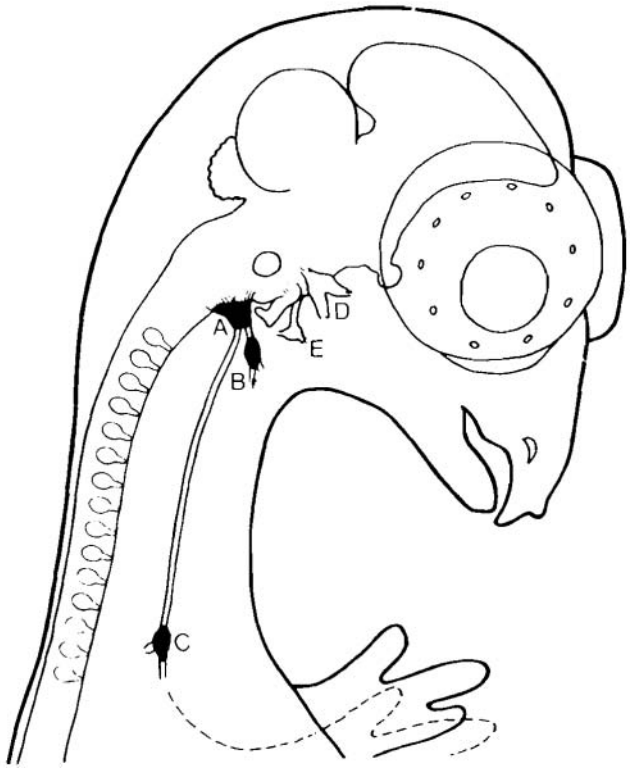
Morphometry

The absolute number of neurons in each of the three ganglia was estimated by counting nuclei in every 5th section at x 600 magnification. Only nuclei with clearly discernible nucleoli were counted. Total counts estimates were obtained by multiplying the sum of individual section by 5. No correction was made for split nuclear error or double counting. All counts were carried out by one person to minimise counting errors. Pyknotic nuclei were counted at x 1,000 under oil immersion magnification on every 3rd section and the counts multiplied by 3. Criteria used for the identification of pyknotic nuclei were taken from the descriptions given by Pannese (1976). Nuclear area measurements on the nuclei of the 3 ganglia were carried out at a magnifiction of ×600 with a HIPAD digitizer pad linked to a North Star Z80 computer. In each case 400 cell nuclei were measured at the maximum extent of the ganglia on about 3-4 consecutive sections. The cross-sectional area of the ganglia was measured on every 5th section and the average multipled by the antero-posterior extent (number of sections × section thickness) to obtain the ganglion volume expressed in $mm^3 \times 10^{-2}$.

Results

The location of the sensory ganglia of the glossopharyngeal (IX) and vagus (X) nerves is shown in Fig. 1. The superior ganglia of IX and X are fused in birds and the complex is located intracranially adjacent to the IX and X root fibres. The distal (petrosal) ganglion of IX is extracranial and is positioned ventral to the petrosal fossa. The distal (nodose) ganglion of X is at the level of the 14th and 15th spinal cord segments, behind the developing thyroid and parathyroid glands. The results of the present study were obtained on seventy five 5 to 18 day old normal embryos and hatched chicks, fourteen 9-12 day old NGF treated embryos and thirty 11-12 day old chick embryos with ^{3}H-thymidine injection.

Fig. 1. Schematic illustration of the position of the proximal IX and X (A), distal IX (B) and distal X (C) ganglia. V (DI and VII (E) sensory ganglia are also indicated.



Ganglion size

The developing ganglia are clearly separated from the surrounding tissue from the 5th day of incubation. Volume measurements of the 3 ganglia were undertaken on serial sections of embryos between the 7th and 12th day of incubation. Over this period there was a marked increase in volume of about 100% in the proximal **IX** and **X** ganglia, 45% in the distal **X** and only a modest increase of 30% in the distal IX (Fig. 2).

Cell generation

Cumulative ^{3}H-thymidine injection schedules on the 3rd, 4th and 5th days; 4th, 5th, and 6th days; 5th and 6th and 7th days were carried out in at least 5 embryos in each group. The embryos were sacrificed on the 11th day of incubation and the number of labelled and unlabelled neurons for each of the 4 injection schedules was determined on the autoradiographic material. The results are shown in Fig. 3, expressed as the percentage of surviving neurons on each day during the generation time of the neuron pools.

In the proximal **IX** and X ganglion peak cell generation was found on the 5th day of incubation (55%) with 15% and 27% generated on the 4th and 6th day of incubation, respectively. Few labelled cells appeared on the 3rd and 7th days. **In** the distal **IX** ganglion 49% of neurons were generated on the 4th day and 35% and 10% on the 3rd and 5th days, respectively. Cell generation in the distal X ganglion occurred between the 2nd and 5th days similar to the time course of the distal **IX** ganglion; however, most of the neurons were generated on the 3rd day (70%) and 12% on each of the 2nd and 4th days of incubation. Few cells were added on the 5th day of incubation in either the distal **IX** and **X** ganglia. Examples of the patterns of autoradiographic labelling are given in Fig. 4. These observations show that the period of neuron generation extends from the 2nd to the 5th day in the distal ganglia and from the end of the 3rd to the 7th day of incubation in the proximal **IX** and **X** ganglion.

Cell numbers

Cell counts were begun approximately at the end of the time of cell generation. For the neural crest derived

proximal IX and X ganglion, the 6th day of incubation was chosen as the starting point and the 5th day of incubation for the placodal derived distal IX and X ganglia. The changing size of the ganglion cell population can be seen in Fig. 5. In the proximal IX and X ganglion a maximum cell number of 22,000 was found on the 8th day, followed by a steady decrease to just under 12,000 by the 13th day of incubation. Thereafter, the neuron number remained unchanged. Counts in the distal IX and X ganglia showed that maximum neuron number occurred around the 6th day of incubation at 4,500 and 11,500, respectively. In the distal IX ganglion, the cell number decreased in a linear manner until the 11th day to 2,500, which corresponds to the numbers in post-hatched animals. In the distal X ganglion, the cell numbers decreased to 6,500 by the 12th day and thereafter the neuron population remained steady.

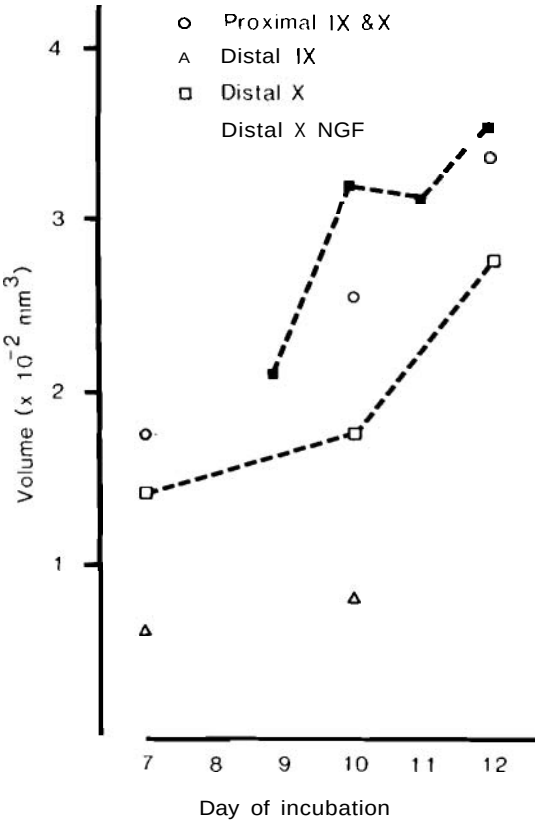


Fig. 2. Histogram giving the mean volume (in mm^3 x 10^2) of the IX and X ganglia in normal and NGF treated animals. Note the considerable increase of the volume (broken lines) of distal X ganglion following NGF administration, in contrast to the proximal IX and X and distal X ganglia where no response was obtained.

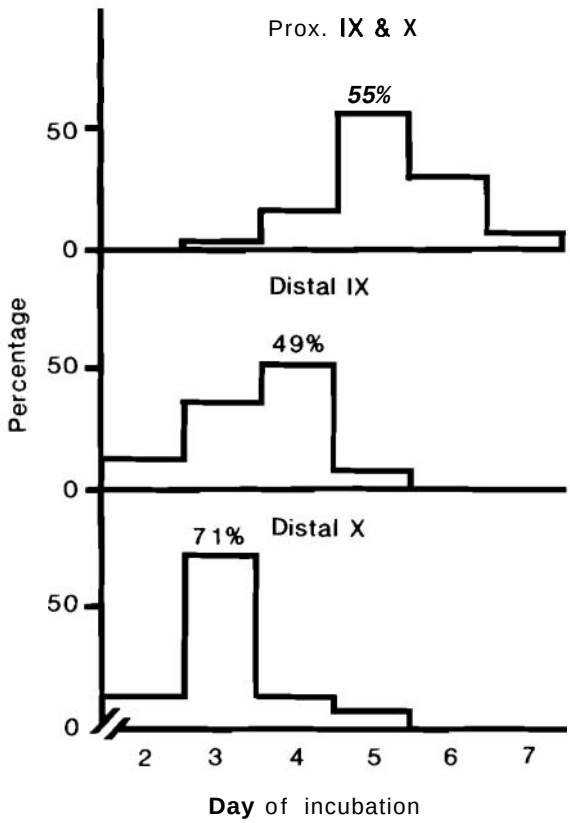


Fig. 3. Histogram showing the percentage of surviving neurons generated day by day from the 2nd to 7th day of incubation in the IX and X ganglia.

Nuclear size changes

Table 1 summarizes the results of nuclear cross-sectional area (size) measurements of the neuron populations in the 3 ganglia between the 7th and 12th day of incubation. Concomitant to volume increase of the ganglia over the same period, nuclear sizes increased by 100% in the proximal IX and **X** ganglion and by 60% in the distal IX ganglion. The least increase of nuclear size was obtained among the neurons of the distal X ganglion of about 30%. Characteristic nuclear size distribution in the 3 ganglia of 12 day old embryos is given in Fig. 6.

Cell loss

Pyknotic nuclei can be distinguished clearly from normal resting and mitotic neurons in these ganglia (Fig.7). Degenerating cells appear with fragmented, shrunken and vacuolated nuclei or disintegrated nuclei with basophilic spheres. Pyknotic counts were made separately for the proximal **IX** and **X,** distal **IX** and **X** ganglia between the 5th and 12th day of incubation (Fig. 8). In all three ganglia, the peak of neuron death occurred around the 7th day of incubation. The number of dying neurons then steadily decreased until the 12th day of incubation. The aggregate cell loss over the time-period of investigation, adding up the numbers of daily neuron death, amounted approximately to 1,950 in the proximal **IX** and **X,** to 650 in the distal IX and to 1,550 in the distal **X** ganglia, falling short of the actual decrease of neuron population (compare Figs. 5 and 8).

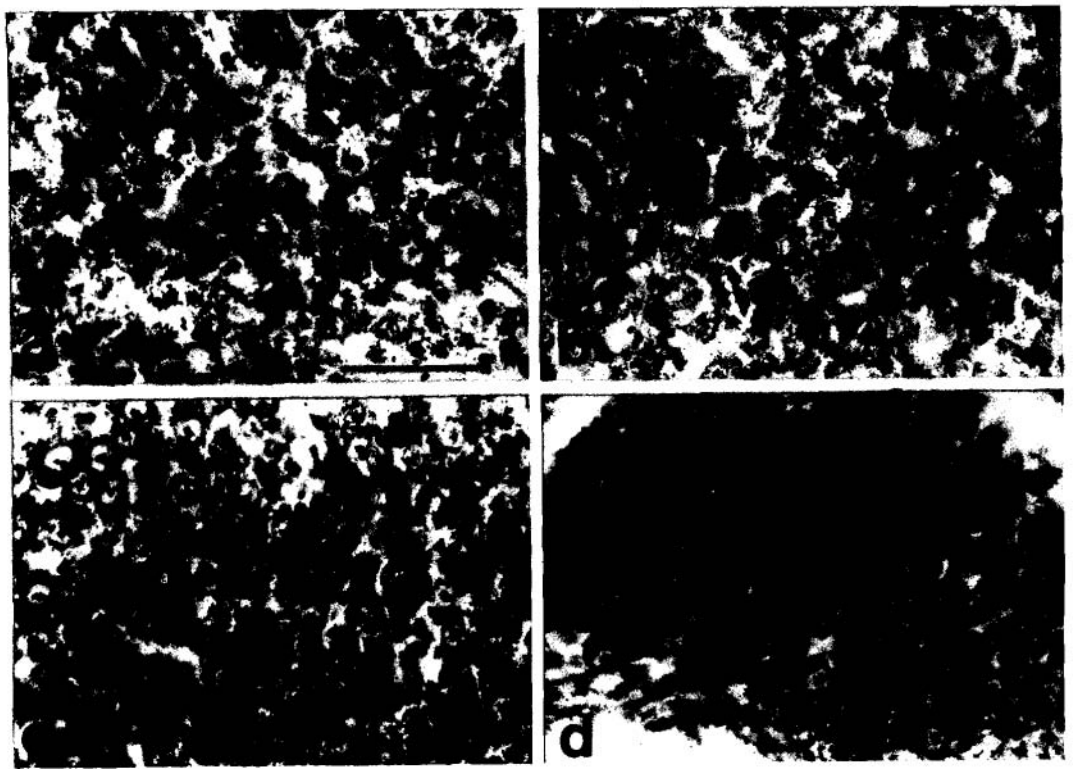


Fig. 4. Microphotographs of ^{3}H-thymidine autoradiographic material of the IX and X ganglia in 11 day old chick embryos. (a) proximal IX and X and (b) distal X ganglia in an animal where isotope was administered on the 3rd, 4th and 5th day of incubation. Note the very high percentage of labelled neurons in both ganglia. A few unlabelled cells which became postmitotic before the 3rd day of incubation, are present in the distal X ganglion (arrowhead). Proximal IX and X (c) and distal X (d) ganglia following isotope administration on the 5th and 6th day of incubation. Unlabelled neurons in (c) (arrowhead) became postmitotic before the beginning of isotope administration. Labelled neurons in (d) were generated on the 5th day of incubation. Bar in (d) is 100 μm, applies to all microphotographs.

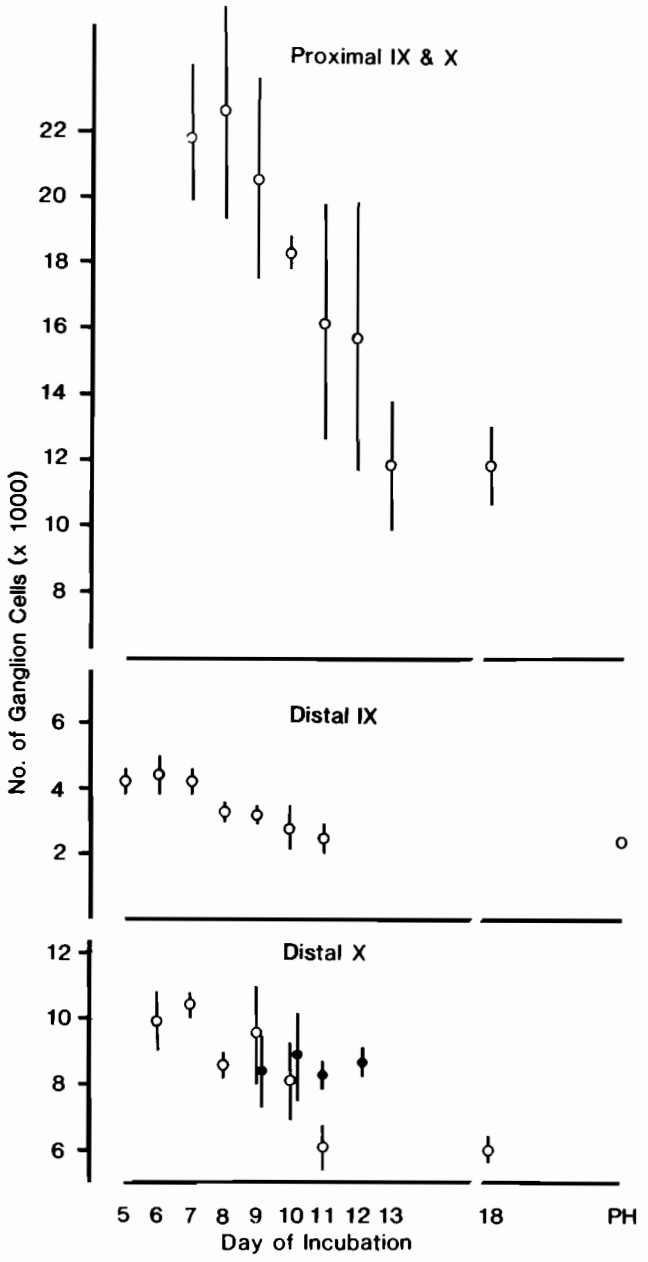


Fig. 5. Developmental changes in the numbers of neurons (means and ± SD) from the 5th day of incubation to hatching (PH) in the IX and X ganglia. Note the substantial increase of surviving neurons (filled circles) in the distal X ganglion following NGF administration.

The effect of NGF

Counts of neurons in the proximal IX and X and distal IX ganglia in NGF treated embryos on the 11th day of incubation (mean 15,920 ± S.D. 1,850 and 2,310 ± 260) did not show any change from values obtained from normal embryos. In the distal X ganglion, however, NGF treatment resulted in the elevation of cell numbers and ganglion sizes of 10 to 12 day old embryos when compared with controls (Figs. 2, 5). Surviving neuron numbers increased from 8,022 (± 490) to 8,520 (± 335) in 10 day old embryos; from 6,212 (± 639) to 8,210 (± 723) in 11 day old embryos and to 8,440 (± 446) in 12 day old embryos. Thus, approximately 30% more neurons survived in the NGF treated distal X ganglia than in the normals by the 12th day of incubation. The number of pyknotic neurons on the 10th (365 ± 136), 11th (71 ± 55)

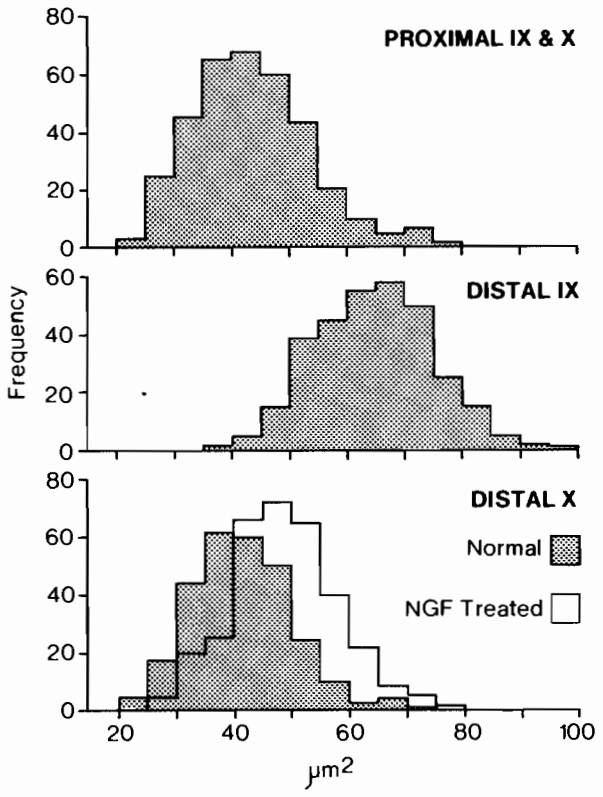


Fig. 6. Bar chart showing nuclear size distribution among neurons of the IX and X ganglia in 12 day old embryos. Note the considerable increase of nuclear size in the distal X ganglion following NGF treatment.

Table 1. Nuclear cross-sectional area measurements (means + SD in μm^2) in the proximal IX and X, distal IX and X ganglia in normal and nerve growth factor treated embryos

Animal	Proximal IX and X	Distal IX	Distal X
7 N1	24.7 ± 5.6	33.7 ± 6.5	38.6 ± 7.7
N2	22.5 ± 4.4	32.0 ± 6.9	37.7 + 7.7
N3	22.9 ± 4.9	32.2 ± 8.1	36.1 + 7.5
10 N1	41.2 + 6.1	49.5 ± 8.6	47.4 ± 8.0
N2	39.7 ± 7.7	50.9 ± 8.9	51.6 ± 9.1
N3	42.8 + 7.2	52.2 + 9.2	52.7 ± 7.8
11 N1	—*	—	50.4 ± 4.6
N2	—	—	51.0 ± 8.8
12 N1	51.8 ± 10.2	55.2 ± 10.9	52.7 ± 6.9
N2	48.0 ± 8.3	54.4 + 9.1	48.3 ± 8.7
N3	50.4 ± 7.8	53.8 ± 9.2	51.9 + 5.7
9 NGF1	—	—	42.6 ± 7.9
NGF2	—	—	42.5 ± 7.5
10 NGF1	—	—	55.8 ± 10.1
NGF2	—	—	49.8 ± 10.0
NGF3	—	—	51.6 + 9.8
11 NGF1	49.6 ± 8.4	52.9 ± 9.4	54.7 + 10.4**
NGF2	49.0 + 6.8	50.8 ± 8.2	57.5 + 10.8
NGF3	—	—	58.7 + 10.5
NGF4	52.6 + 10.1	53.3 + 7.5	57.9 + 9.1
12 NGF1	—	—	62.6 + 9.9**
NGF2	—	—	60.8 ± 8.9

*not measured

$p < 0.005$ against normal values

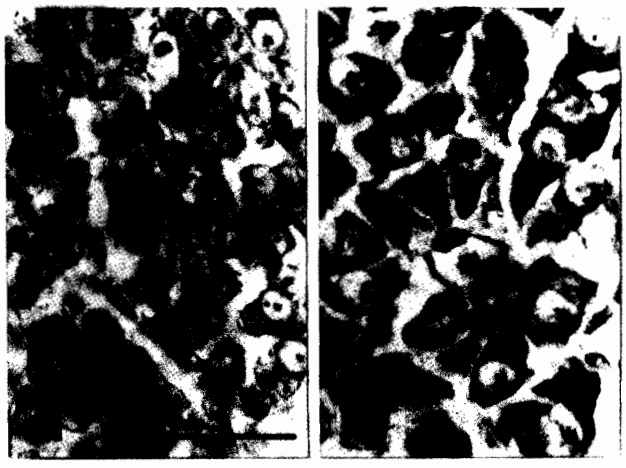


Fig. 7. Microphotographs of pyknotic neurons (arrowheads) in the proximal IX and X ganglion (a) of a 7 day old embryo and in the distal IX ganglion (b) of an 11 day old embryo. Bar in (a) is 50 μm applies also to (b).

and 12th (45 + 21) day of incubation did not differ significantly between NGF treated and normal ganglia; however, it is possible that the length of the pyknotic cycle was shortened. In the distal X ganglion, the neuronal nuclear size increased following NGF treatment, and was more marked on the 11th and 12th day of incubation (Table 1). This effect is shown by the shift of nuclear size distribution towards the larger nuclear regimes (Fig. 6).

Discussion

The results of the present investigation confirm previous observations (Narayanan and Narayanan, 1980; D'Amico-Martel, 1982; D'Amico-Martel and Noden, 1983) that neurons of distal IX and X ganglia are generated between the 2nd and 5th day and neurons of proximal IX and X ganglion are generated somewhat later, from the end of the 3rd to the 7th day of incubation. Furthermore, our quantitative autoradiographic study shows that irrespective of the time-scale of cell generation, over 80% of all neurons in the three ganglia are born within a short

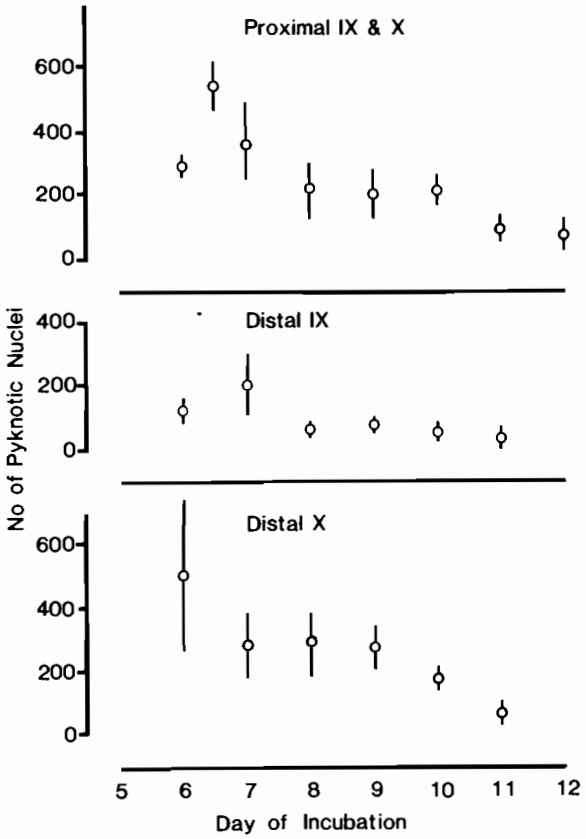


Fig. 8. Histogram showing the number of pyknotic neurons (means and + SD) between the 5th and 12th day of incubation in the IX and X ganglia.

2 day period, whereas the beginning and the end of the proliferative period furnish relatively few neurons. This may indicate that the duration of the mitotic cycle is much shorter at the peak of cell proliferation than at the times when cell generation is set at a lower level. The timetables of a number of developmental parameters were different for the two neuron groups. Estimation of peak cell number, nuclear cross-sectional area and volume of the ganglia correlated well with the period of neuron generation in the proximal **IX** and **X** and in the distal **IX** and **X** ganglia.

Nuclear pyknosis was observed in the **IX** and **X** ganglia from the 5th to the 12th day of incubation, partly overlapping the period of cell generation. This result is similar to that seen in the dorsal root ganglia (Carr and Simpson, 1978). Although the size of neuron pools in these ganglia is substantially different, about 45% of the originally generated neurons in each of the ganglia were eliminated. The observed magnitude of cell loss corresponds well with published data on the Gasserian ganglion (Straznicky and Rush, 1985a) and dorsal root ganglia (Carr and Simpson, 1978) in chick embryos and in guinea pig cranial nerve sensory ganglia (Pearson el al., 1983). Ganglion cell death and the formation of peripheral connections have been shown to have a similar time course (Rogers and Cowan, 1973; Noden, 1980; Hiscock and Straznicky, 1986). It is thus likely that naturally occurring neuron loss serves the purpose of matching up the size of the originally generated neuron pool with the size of the available target tissue (Oppenheim, 1981). The number of surviving dorsal root ganglion cells depends upon the size of the target. Supernumerary limb transplatation or limb ablation in chick embryos brings about a corresponding increase or decrease in the number of surviving sensory neurons (Hamburger and Levi-Montalcini, 1949). Since sensory fibres do not form synapses with the peripheral target, competition for a general trophic maintenance factor produced by the target tissue is more likely than competition for synaptic termination.

Sympathetic and sensory neurons of neural crest origin have been shown to respond to NGF. The main effects of NGF on developing dorsal root ganglion cells are (i) reduction of naturally occurring cell death, (ii) cellular hypertrophy and (iii) promotion of neurite outgrowth (Levi-Montalcini and Hamburger, 1951; Levi-Montalcini and Angeletti, 1968; Hamburger et al., 1981; Davies and Lindsey, 1984; Straznicky and Rush, 1985b). Recent experiments have demonstrated that neurons of the Gasserian ganglion, of dual neural crest and placodal origin (Hamburger, 1961), are dependent on NGF for survival (Pearson et al., 1983; Straznicky and Rush, 1985a).

NGF administration in the present study did not have any measurable effect on the neural crest derived neurons of the proximal **IX** and **X** and the placodal derived distal **IX** ganglia. In contrast, neurons of the distal **X** ganglion, the derivatives of the placodal material, responded to NGF treatment by increased survival and nuclear hypertrophy. Although NGF was administered from the 5th day of incubation ganglion cell death was affected only from the 10th day onwards. This is particularly shown by the fact that neuron numbers on the 9th day of incubation in NGF treated embryos were not significantly different from controls.

We have shown that neural crest derived mesencephalic trigeminal neurons do not have NGF sensitivity (Straznicky and Rush, 1985a). These and the present observations challenge the prevailing view that the embryonic origin of cranial sensory neurons unequivocally determine their requirement for a particular trophic maintenance factor, NGF for the neural crest derived cells. Because of the diverse targets of cranial sensory neurons it is unlikely that each of these target tissues would produce NGF as the trophic maintenance factor for neuronal survival. Hence, some of the cranial sensory neurons of neural crest origin, i.e. neurons of the proximal **IX** and **X** ganglion may require trophic maintenance other than NGF. Similarly, for some of the placodal neurons in the Gasserian and distal X ganglia the target derived trophic maintenance factor may be NGF. Consequently, these neurons respond to exogenous NGF administration by increased survival. The notion that the trophic requirement of the cranial sensory neurons is determined by the nature of the maintenance factor the target tissue produces and not the embryonic source of origin, appears to be supported by observations on the presence of NGF-like trophic maintenance factor in the heart for sensory neurons (Ebendal, 1979) and the concomitant respon siveness of distal X neurons to NGF observed both *in vivo* and in tissue culture (Hedlund and Ebendal, 1980).

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