PROPERTIES OF THE β-NERVE GROWTH

FACTOR RECEPTOR IN DEVELOPMENT

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ABSTRACT

The cell surface receptor for β-nerve growth factor was used as a probe to study the development of embryonic chick sensory ganglia. The ganglia were shown to lose their responsiveness to nerve growth factor in vitro between 14 and 16 days of embryonic age. This loss occurred by a decrease in the magnitude of the maximum biological response, not by a shifting of the response to higher concentrations. Binding assays for the β-nerve growth factor receptor, using ^{125}I-radiolabelled β-nerve growth factor, were performed with cells from sensory ganglia 8, 12, 14, 16, 18, and 21 days of age. The assays revealed a twofold increase in the number of receptor sites per ganglion between 8 and 14 days and a sixfold drop between 14 and 16 days of embryonic life. Neither increase nor decrease was accompanied by a large change in the affinity of the receptor for the protein. Together with the results of the bioassay, the data show that the loss of biological responsiveness is correlated with and may be due to a loss of the cells' ability to bind β-nerve growth factor. Correlation of the results of the binding assays with the known ontogeny of the chick embryo provides a hint at the role of nerve growth factor in normal development.

Nerve growth factor (NGF) is a protein endowed with the ability to stimulate the hypertrophy and hyperplasia of responsive sympathetic and embryonic sensory ganglia. The protein chemistry of NGF has been extensively studied. This includes the elucidation of the primary amino acid sequence of mouse submaxillary gland NGF isolated either as 2.5S NGF (1, 2) or βNGF[1]. That NGF plays a role in peripheral nervous system development and maintenance is virtually certain (17, 20). NGF, for example, has been found in the serum of all

vertebrates tested, from teleost fish to man. Its biological potency from any one source is similar when measured with responsive tissues from various species. Furthermore, the targets of NGF action are very specific (i.e., sympathetic and embryonic sensory ganglia) and antiserum made to purified NGF destroys the developing sympathetic nervous system of newborn rats and mice and markedly impairs sympathetic function in adults.

Recent work has been aimed at elucidating the mechanism of action of NGF. Experiments with tissue grown in vitro have demonstrated that, with the exception of increasing glucose oxidation, most cellular anabolic processes are stimulated over

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[1] DeJong, W. W. W., D. Straus, J. Ohms, W. C. Mobley, and E. M. Shooter. Unpublished data.

levels found in controls (17). The NGF response requires protein synthesis but not RNA synthesis (24). At the molecular level, it is now known that the first interaction of NGF with responsive cells is with a cell-surface receptor, the latter being defined by that fraction of [^{125}I]NGF which binds to cells or membrane fractions and which is displaceable by native NGF.

Frazier et al. (9) have shown that Sepharose-bound NGF (Sepharose, Pharmacia Fine Chemicals, Inc., Piscataway, N. J.) is capable of eliciting fiber outgrowth from sensory ganglia. Herrup and Shooter (16) measured directly the binding of [^{125}I]βNGF to its receptor on dissociated sensory ganglion cells. This work demonstrated that both the levels of binding of [^{125}I]βNGF and the biological response of the cells varied with [^{125}I]-βNGF concentration in the same way. Chemical modification of the protein resulted in a parallel loss of both the biological activity and the affinity of the derivative for the receptor. In addition, the specific binding of [^{125}I]βNGF could not be displaced by proteins such as cytochrome c or proinsulin, nor could it be found on cells unresponsive to NGF such as liver cells. Banerjee et al. (3) have observed binding of a similar nature to a microsomal fraction from the sympathetic ganglia of rabbits. More recently, Frazier et al. (10), using a solid-phase iodination technique to prepare [^{125}I]NGF, presented evidence for high affinity binding of NGF to a membrane and cellular fraction from sympathetic ganglia of chick, rat, or rabbit and sensory ganglia from chick embryos. However, the specific binding they observed did not show saturation at concentrations up to 10^{-8} M, indicating populations of receptors of lower affinity, and it was also inhibited up to 20% by appropriate concentrations of insulin. Although the steps subsequent to binding are not known, the NGF receptor does not operate through modulation of cAMP concentration (11).

The specific binding assay is a direct measure of the cell's ability to bind NGF to its receptor (a molecular form of "recognition") and it has been used in the current study to explore the development of chick sensory ganglia. It is known that, as the embryo develops, the sensory ganglia lose their responsiveness to NGF in tissue culture (20). The work described here demonstrates that the cells'

loss of responsiveness to βNGF is accompanied by a loss of the cells' ability to bind [^{125}I]βNGF.

MATERIALS AND METHODS

Buffers

All binding assays were done in a modified version of Gey's balanced salts solution (GBS) (12) in which the concentrations of phosphate salts were raised to those of a 25 mM buffer (pH 7.4). The NaCl content (8.0 g/liter in the original) was reduced to 6.35 g/liter to maintain constant ionic strength. Dextrose and all other salts were maintained at their original concentrations. This new buffer is referred to as phosphate-buffered Gey's (PBG).

NGF and Other Proteins

βNGF was isolated according to the methods of Varon et al. (27) and Smith et al. (25). High specific activity [^{125}I]βNGF was prepared as described previously (16). It was stored at 4°C in 0.2% acetic acid with 1.0 mg/ml bovine serum albumin (Metrix Instrument Co., Houston, Texas) to protect it from radiation damage.

Collagen was extracted from rat tail tendons and used to coat 22 × 22-mm coverslips by a modification of the methods of Ehrman and Gey (8) and Bornstein (5).

Animal Materials

Fertilized eggs were obtained from Kimber Farms, Fremont, Calif. Eggs were from White Leghorn chickens and were stored in an incubator (Model no. 252, James Mfg. Div., Fort Atkinson, Wis.) at 38°C with turning every 2 h.

Mice, from whose submaxillary glands the βNGF was isolated, were obtained from Simonsen Laboratories, Gilroy, Calif. The mice were 60-day old males of the Swiss Webster strain.

Bioassay for NGF Activity

The assays were performed as described by Herrup and Shooter (16). Dorsal root ganglia were removed from chick embryos incubated 8, 12, 14, 16, and 18 days and stored separately in plastic petri dishes in chilled GBS. A single set of dilutions of βNGF in GBS was prepared, ranging in concentrations from 0.3 to 300 ng/ml. For each concentration, 15.0 ml of dilution were made up and 3.0 ml were dispensed to each of five plastic petri dishes (no. 3001, Falcon Plastics, Div. of BioQuest, Oxnard, Calif.) with a collagen-coated coverslip in the bottom. One set of dilutions was used for each age. Five ganglia were removed from the pool and added to each dilution of the set. After 60 min at room temperature, the ganglia had settled and begun attachment. The dishes were then transferred to a 37°C humidified incubator and incubated overnight in air. The following day, the growth of neurites from the ganglia was scored on an arbitrary scale from 0 to 5.

Specific Binding Assay

All assays were done in PBG which was filtered through a 0.2-μm filter (Nalge Co., Nalgene Labware Div., Rochester, N. Y.) to remove any particles that might bind [^{125}I]βNGF. Ganglia were dissected as for the bioassay, except that sterility was not observed. The pooled ganglia were washed twice in 5.0 ml PBG and resuspended a third time in only enough liquid for the assay and cell counts. The ganglia were dissociated by drawing them in and out of a 5.0-ml pipet. Care was taken during this procedure to avoid bubbling air through the cell suspension, as this created a froth which invariably led to high levels of cell damage. The use of enzymes or chelating agents to aid in dissociation of the ganglia was specifically avoided. The cell suspensions thus obtained contained, almost entirely, single cells. Cell debris remaining after dissociation was minimal and was removed by centrifugation at 500 rpm for 5 sec. The nature of this debris is important since if certain cell populations are selectively lost during dissociation, artifacts may be obtained which would obscure the experimental results. At all ages, a small percentage of the ganglia resist dissociation, usually when a length of the distal or proximal root is dissected with the ganglia. Most of the ganglia (90–95%), however, are completely dissociated to single cells. The debris which is removed is thus usually whole ganglia and not small pieces of all ganglia. On this basis, the cell suspensions used for these experiments are representative of the composition of the ganglia of different ages and not enriched with one population of cells or another. This is borne out by phase-contrast examination of the cell suspension which reveals no selective loss of any one class of cells. After removal of the debris, cell counts were done in a hemocytometer. Cell viability, as determined by trypan blue (Grand Island Biological Co., Grand Island, N. Y.) exclusion, was 85–100%. Cell concentrations ranged from 2 to 15 × 10^6 cells/ml. The binding assay was performed as described previously (16). After incubation of [^{125}I]-βNGF and cells, the latter were freed from unbound [^{125}I]βNGF by sedimentation through a two step sucrose gradient in a Beckman microfuge tube (Beckman Instruments Inc., Spinco Div., Palo Alto, Calif.). Both pellet and supernate were counted and the results expressed as pg βNGF bound per 10^5 cells. For each concentration, total and nonspecific binding, the latter determined in the presence of 10 μg/ml nonradioactive βNGF, were measured. Only specific binding values, calculated as the difference between total and nonspecific binding, are reported here. Nonspecific binding was approximately of the same order of magnitude for the cells from all the ganglia of different ages.

RESULTS

The Biological Response of Sensory Ganglia to NGF during Development

Though there is agreement that the ability of the sensory ganglia to respond to NGF disappears in older chick embryos, the exact time limits of this phenomenon have not been well defined. Winick and Greenberg (30) reported that no neurite outgrowth could be detected in sensory ganglia removed from embryos younger than 7 days or older than 13 days of incubation. In contrast, Banks et al. (4) found responses from ganglia as young as 5 days and as old as 16 days of embryonic life. Larrabee et al. (as quoted by Jacobson [18]) observed no response of the ganglia to NGF after 16–17 days. Some of this variation might stem from differences in incubation conditions or the initial condition of the eggs. The effects of embryo age on NGF responsiveness have therefore been reexamined using dorsal root ganglia removed from chicks incubated 8, 12, 14, 16, and 18 days under the conditions used to obtain the ganglia for the binding assays. All the bioassays were done using aliquots from a single set of βNGF dilutions. The results of these assays are shown in Fig. 1.

Ganglia from 8- to 14-day old embryos showed peak neurite outgrowth in the concentration range between 3 and 10 ng/ml βNGF. Ganglia from
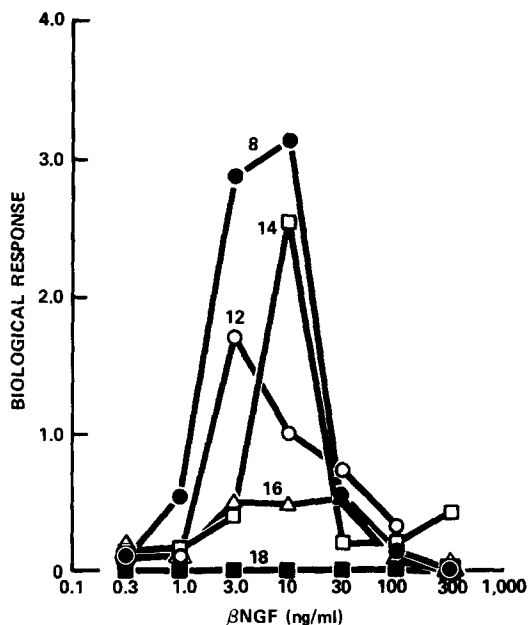

FIGURE 1 Bioassay of dorsal root ganglia from embryos of various ages. Dorsal root ganglia from 8- (●), 12- (O), 14- (□), 16- (△), and 18- (■) day old chick embryos were grown on collagen-coated coverslips at 37°C in the presence of various concentrations of βNGF in GBS. After 24 h, the resulting halo of nerve fibers was scored on an arbitrary scale from 0 to 5. Each point represents the mean of five ganglia.

16-day old embryos also responded with a peak of activity in the same concentration range, but the level of response was much reduced. Of 48 ganglia assayed from 16-day embryos, only three showed neurite outgrowth which rated scores of 1.0 or more. By 18 days of incubation, the ganglia showed no response to the presence of βNGF. These results confirm the reports of the loss of sensory responsiveness of NGF. In addition, they establish that this loss occurs at around 16 days of embryonic life under the conditions used in this investigation.

Specific Binding of βNGF During Development

In light of these results the binding assay for the βNGF receptor was used to examine the receptor properties during this developmental sequence. The ability to measure, directly, the binding of [^{125}I]βNGF to the cells allows the determination of whether the loss of responsiveness in the bioassay is correlated with a change in the cells' ability to bind βNGF or whether there is some other mechanism, one or several steps subsequent to the initial binding step, which is blocked.

The binding assays were performed in a manner identical to that used previously (16). Ganglia from embryos of several of the different ages were assayed twice. In these instances a different preparation of [^{125}I]βNGF was used as well as a different set of eggs. The close coincidence of these duplicate experiments demonstrates the reproducibility of the binding assay. The results are compiled in Fig. 2.

The binding curve for 8-day embryo ganglion cells saturated at a level of specific binding around 30 pg [^{125}I]βNGF per 10^5 ganglion cells (Fig. 2 A). This number is the total specific binding capacity of the cells. Half-saturation was reached at 7–8 ng/ml which corresponds to the apparent dissociation constant, K_D, and is a measure of the affinity of the receptor for [^{125}I]βNGF.

The binding curve for 12-day embryo ganglion cells (Fig. 2 B) was virtually identical to that for 8-day cells. The concentration at which half-saturation was reached was unchanged and the total specific binding capacity was around 26 pg/10^5 cells. The binding curve for cells from 14-day embryo ganglia (Fig. 2 C) was slightly, though not significantly, different. In two experiments, the K_D varied from 5.4 to 7.7 × 10^{-10} M (14–20 ng/ml). This is two to three times the values for 8- and 12-day embryo ganglion cells. The total specific

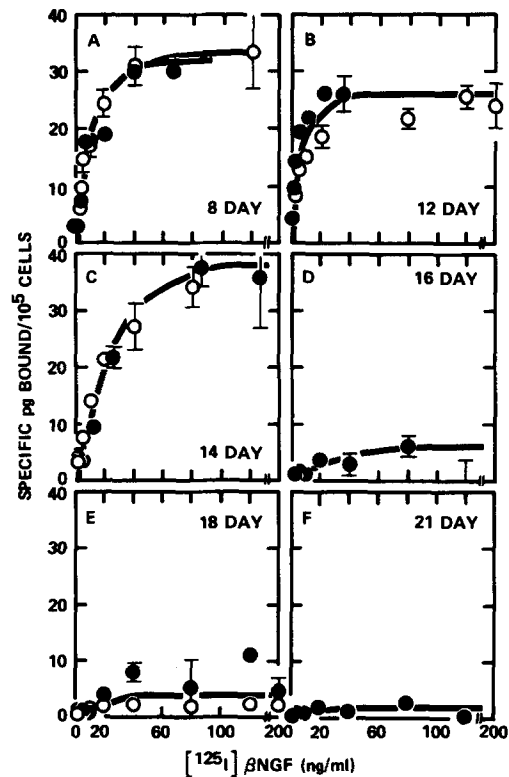FIGURE 2 Effects of embryo age on the saturation curve of the βNGF receptor. The specific binding of [^{125}I]βNGF to dissociated dorsal root ganglion cells was measured as a function of concentration using ganglia from 8-, 12-, 14-, 18-, and 21-day embryos. For several of these age groups, two experiments were done. These are distinguished by open (O) or closed (●) circles. All points represent means of three experiments. Bars represent one SEM.

binding capacity also increased to 36 pg/10^5 cells, about one-third higher than at the younger ages. Neither of these changes was significant statistically, given the errors indicated in Fig. 2.

Cells from 16-day embryo ganglia showed a marked change in the characteristics of the binding curve (Fig. 2 D). The total specific binding capacity dropped to 6 pg/10^5 cells, a four- to sixfold drop compared to the cells from earlier embryos. This change is revealing since these ganglia also are the first to show a decrease in βNGF responsiveness in the bioassay (Fig. 1). The binding curve for 18-day embryo ganglion cells (Fig. 2 E) was similar to that for cells from 16-day embryos with a total specific binding capacity of 4 pg/10^5 cells. By 21 days, the specific binding of the ganglion cells was close to zero, with the total specific binding capacity down to about 2 pg/10^5 cells (Fig.

2 F). The specific binding capacities of the sensory ganglion cells at saturation therefore change during the course of development. The data have been plotted as a function of embryonic age, using both the total specific binding per 10^5 cells (Fig. 3 A) and per ganglion (Fig. 3 B). The binding per 10^5 cells falls about sixfold from its average value up to 14 days of embryonic age to the value at 16 days of age. The total specific binding per ganglion increases about twofold between 8 and 14 days and then falls about fourfold between 14 and 16 days of embryonic age. The decrease in binding capacity occurs at the same stage of development as the
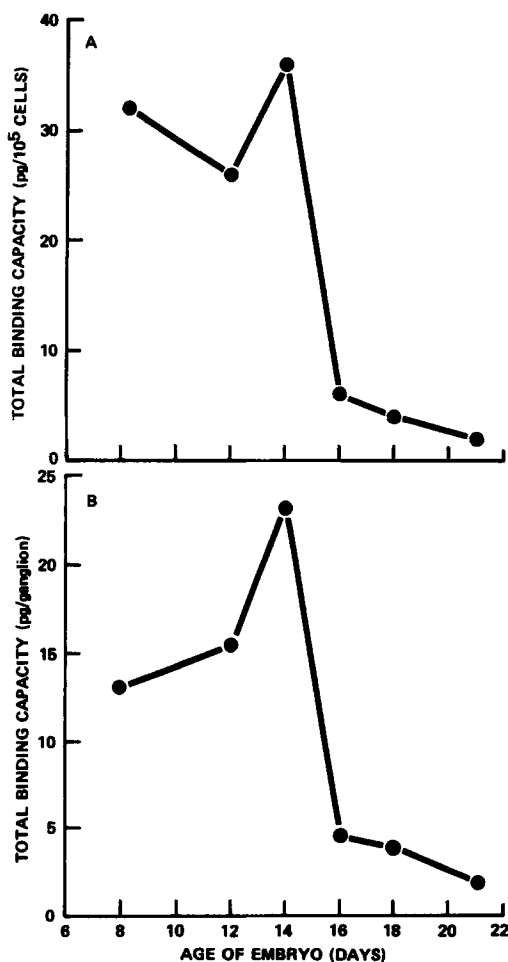

FIGURE 3 Total specific binding capacity of dorsal root ganglion cells as a function of embryo age. The saturation levels of the curves in Fig. 2, normalized to 10^5 ganglion cells (Fig. 3 A) or to one dorsal root ganglion (Fig. 3 B), are plotted here for each age of embryo examined.

decrease in NGF responsiveness of the sensory cells (Fig. 1).

It is unlikely that the decrease in total specific binding capacity after 14 days of embryonic age is the result of a change in receptor affinity as measured by the concentration needed to achieve half-saturation of the receptor sites (K_D). Although the curves in Fig. 2 D-F have been drawn to saturate within the range of concentrations examined and to reach half-saturation at concentrations comparable to those in Fig. 2 A-C, the data are not sufficiently precise to rule out curves with a positive slope which saturate at higher concentrations. If, however, the saturation curve for 18-day embryo ganglion cells were to actually reach levels of 30 pg/10^5 cells instead of the observed 4 pg/10^6 cells, Michaelis-Menton kinetic assumptions indicate that the K_D would be no less than 1,500 ng/ml. Binding of this nature would not be detected with the current assay, due to the high ratio of nonspecific to specific binding. The fact that the observed change in binding is not due to such a change in K_D is supported by the results of the bioassay. An increase in K_D would mean that, at sufficiently high concentrations of NGF, maximum fiber outgrowth could be stimulated in the cultured ganglia. That this is not so is shown in Fig. 1. The peak of biological response flattens with increasing developmental age (as would be expected from a decreasing number of receptors with a constant K_D) rather than shifting to higher concentrations (as would be expected for a constant number of receptors with increasing K_D's).

One difference between the binding assays and the bioassay is the presence of residual specific binding to the 18- and 21-day old embryonic sensory cells at ages where the whole ganglia show no neurite outgrowth in response to added NGF. The morphology of the older ganglia, however, provides a clue to this discrepancy. As the embryo develops, the sensory ganglia contain more connective tissue and are enveloped in a tough capsule. It is possible that, if the neurite outgrowth in the older ganglia is restricted to only a few neurons, the growing fibers would be unable to penetrate this capsule. Experiments in which ganglionic dissociates in culture are used (13, 29) are required to resolve this point.

DISCUSSION

The identification and characterization of the cell surface receptor for NGF (3, 10, 16) have provided new evidence on the mechanism of action of NGF.

A comparison of the data in these reports demonstrates that NGF prepared from mouse submaxillary gland displays similar high affinity binding to receptors on chick, rat, or rabbit sympathetic and chick sensory cells. This extends the observations of the interspecies potency of NGF and speaks to the constancy of the essential properties of both the receptor and the NGF protein in evolution. The present study demonstrates that the NGF receptor itself offers a sensitive probe for the exploration of the development of the peripheral sensory nervous system.

One important question which has to be considered in the interpretation of the binding data is the effect of the changing cellular composition of the ganglia during development. Fig. 3 A represents the effects of developmental age on the total specific binding capacity of 10^5 sensory ganglion cells. This method of normalization (per 10^5 cells) makes no allowance for the changing contribution which the cells of any one type make to the total binding. It is possible, for example, that the cells responsible for the specific binding of NGF are recovered in lower yields in the dissociates of the older ganglia compared to those of the younger ganglia. The work of Varon and Raiborn (28) has already emphasized that many factors affect the cell yield in the dissociation of chick embryo dorsal root ganglia. While the present data do not speak directly to this question, it should be noted that the total cell yields, 40,000; 64,000; 73,000; and 100,000 cells per ganglion for 8, 14, 16, and 18 day old embryos, were roughly comparable to those obtained in other studies (7, 28); that little cellular debris was present in the ganglionic dissociates and that the nature of this debris was such that no selective loss of any one cell type was obseved.

With respect to the sensory ganglion itself, Levi-Montalcini and Levi (21) have shown that there is first a hypertrophy of the cells. Whereas at 8 days in embryo most neurons in the ganglia average 5–10 μm in diameter, by 19 days most neurons are larger than 10–15 μm and over half are more than 20 μm in diameter. In addition, there is an increase in cell numbers. Neuronal division, however, slows by 8 days and is finished by 9 days (14). The increase in cell numbers, therefore, must be due entirely to nonneuronal elements (i.e., glial cells). This is in agreement with the work of Pannese et al. (23) who found that the number of glia associated with a neuron was directly proportional to the surface area of the neuron. These

developmental changes could greatly affect the interpretation of the binding assays. If, for example, the receptors for βNGF are found on the neurons, then the large drop seen in the total binding capacity per 10^5 cells between 14 and 16 days may merely be a reflection of a change in the percentage of neurons. Since the number of nerve cells per ganglion is not changing during the time period examined (14), recalculating the results of Fig. 3 A on the basis of pg total specific binding per ganglion should normalize the binding to a fixed number of neurons. These results are shown in Fig. 3 B. The data show that the drop in total binding capacity between 14 and 16 days is real and independent of the altered cellular composition.

The results shown in Fig. 3 when compared to those obtained in the bioassay (Fig. 1) lead to the conclusion that the change of the chick embryo sensory cells from an NGF-responsive to an NGF-unresponsive state, between 14 and 18 days in embryo, is accompanied by a decrease in the specific binding of NGF to their cell surfaces. This decrease could result from a reduction in the number of functional NGF receptors on the cell bodies by increased receptor turnover or from a masking of a relatively constant number of receptors through changes in cell surface macromolecules. Other possibilities also exist. Whatever mechanism is involved, it is therefore possible that the cells cease to respond to NGF (in the bioassay and therefore perhaps also in vivo) because they either cease to bind it altogether or do so in insufficient amounts. The timing of this event can be compared to the known morphological development of the neurons in the sensory ganglion. The neuronal population is heterogeneous and has been divided, on the basis of morphology, into two groups: the large ventrolateral (V-L) neurons and the smaller medio-dorsal (M-D) cells (14). The patterns of development of these two cell types differ as well. A detailed description of this phenomenon has been given by Levi-Montalcini and Levi (21) and Hamburger and Levi-Montalcini (14). The differentiation of the large V-L cells begins at about 2.5 days of incubation. By 5 days• the tips of the growing axons have reached the dermis and by 8 days the cells form a homogeneous, fully differentiated group of neurons on the V-L aspect of the ganglion. After 8 days, these cells change very little except to gradually increase in size and assume their characteristic pseudo-unipolar form.

The M-D neurons do not begin to differentiate until late in development. At 8 days they are still small, undifferentiated cells, which will not stain with silver. It is only the presence of a diffuse Nissl substance and the morphology of the nucleus that allow these cells to be characterized as neuroblasts. Beginning at 9–10 days, silver staining is observed in these cells along with the outgrowth of neurofibrils. By 12 days, increasing numbers of small cells demonstrate this differentiation. From 12 days on, there is progressive differentiation of the M-D cells as well as a considerable increase in size. Both M-D and V-L cells grow conspicuously up to 15 days. From 15 days to hatching, it is no longer possible to recognize two distinctly separate populations. The decreased NGF binding to the cell bodies of the sensory ganglion cells therefore correlates almost precisely with the completion of the differentiation of the M-D neurons. This correlation strengthens the previous suggestion (20) that NGF functions in the organism, at least in part, in conjunction with the differentiation of these cells, and it is significant to note that the binding of NGF decreases after differentiation is complete, rather than soon after its beginning. The implication is that NGF may be needed throughout the differentiation process, not just at its initiation. At the moment, there is only indirect evidence that the receptors in the ganglion are on neuronal cell bodies. This comes from the finding (10) that the bindings of both NGF and α-bungarotoxin, the latter to the acetylcholine receptor, fractionate with the same membrane fragments in two separation systems. More direct evidence will require the use of cell separation techniques. It has been assumed that the NGF which reacts with the responsive cells in the sensory (and sympathetic) ganglia was derived from the circulation. The recent findings that mouse dorsal root ganglionic glial cells can replace NGF in supporting attachment and survival of neurons from the same ganglia (6) and that the causative agent supplied by the glia is functionally and antigenically similar to NGF (31) raise the interesting possibility that these cells also supply NGF to the ganglion.

Although the cell body NGF receptors in the chick sensory ganglia largely disappear or are masked by 21 days in embryo, NGF continues to reach the neurons in the ganglia by an alternative route. This route is by a retrograde flow from the nerve terminals back to the cell body (15). Retrograde transport of NGF has been demonstrated in

sympathetic and sensory fibers, and it continues in both in adult animals (26). The source of this NGF may be the effector organs themselves since it is known that rat irides (19) and fibroblasts (22) produce NGF. Little is yet known about the development of the receptor sites on or near the nerve terminals which presumably initiate this retrograde flow.

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