Nerve Growth Factor Receptors on Chick Embryo Sympathetic Ganglion Cells: Binding Characteristics and Development

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NGF is essential for the development and maintenance of sympathetic and certain sensory neurons. The NGF receptors on the surface of sympathetic ganglion cells from chick embryos were characterized; they consist of high-affinity receptors with a dissociation constant of about 10^{-11} M, and low-affinity receptors with a dissociation constant of about 10^{-9} M. There are more than 10 times as many low-affinity as high-affinity receptors per cell. The heterogeneity of NGF binding is not due to negatively cooperative interactions among the receptors. The high- and low-affinity components of NGF binding defined at steady state correspond to slowly and rapidly dissociating components of bound NGF seen in kinetic experiments. In addition, a very slowly dissociating component of bound NGF was observed; this component was a small fraction of binding at low concentrations of NGF but increased to 20–60% of bound NGF at the highest NGF concentrations examined. This very slowly dissociating component of bound NGF accounts for several peculiarities in the binding data not accounted for by steady-state binding of NGF to its high- and low-affinity receptors.

Developmental studies showed that both high- and low-affinity NGF receptors were present on chick embryo sympathetic ganglion cells from 6.5 to 20 d *in ovo*. No significant differences in the numbers or affinities of the receptors were seen with cells from ganglia at 9, 11, or 15 d of development. Cultured non-neuronal cells from sympathetic ganglia had only low-affinity NGF receptors.

NGF is a polypeptide hormone involved in many aspects of the development and maintenance of sympathetic and sensory neurons (Greene and Shooter, 1980; Thoenen and Barde, 1980). The first step in the interaction of NGF with these neurons is its binding to specific plasma membrane receptors (Yankner and Shooter, 1982). Frazier et al. (1974) described heterogeneity of NGF binding to chick embryo sympathetic and sensory ganglion cells, and they attributed it to negatively cooperative interactions among receptors. More recently, Sutter et al. (1979a, b) showed that the NGF receptors on chick sensory ganglion cells did not interact in a negatively cooperative manner. Instead, 2 major classes of NGF receptors with dissociation constants (K_d) of about 10^{-11} and 10^{-9} M were defined. These 2 classes of NGF receptors have also been seen in membrane preparations from sensory ganglia (Riopelle et al., 1980), and

they seem to correspond to polypeptides of 2 different molecular weights identified in cross-linking experiments with ^{125} I- β NGF (Hosang and Shooter, 1985). Olender and Stach (1980) also demonstrated these 2 classes of NGF receptors on sympathetic ganglion cells from chick embryos and found that some of the bound NGF is very slowly dissociable or "sequestered" in these cells. Here, we characterize in detail binding to the high- and low-affinity NGF receptors on embryonic chick sympathetic ganglion cells by a variety of steady-state and kinetic measurements, and we show that the heterogeneity of binding is not due to negatively cooperative interactions among NGF receptors. We also demonstrate a very slowly dissociating component of bound NGF and propose that this component is generated in large part from NGF initially bound to low-affinity receptors.

Although subpopulations of chick embryo sympathetic ganglion neurons depend on different growth factors, including NGF, for survival in culture (Edgar et al., 1981), all neurons cultured from 7, 12, and 16 d embryonic ganglia possess high-affinity NGF receptors, as shown by autoradiography of bound ^{125} I- β NGF (Rohrer et al., 1983). In order to determine if both high- and low-affinity NGF receptors are present on chick embryo sympathetic ganglion cells throughout their development, and if the numbers or affinities of these receptors change during development, we examined steady-state binding of ^{125} I- β NGF to cells from ganglia at days 6.5–20 of incubation. A preliminary report of these results has been made (Godfrey and Shooter, 1979).

Materials and Methods

Materials

Fertilized chicken eggs (DeKalb-Webster Sex-linked Red) were obtained from A&M Hatchery (Santa Rosa, CA), and were incubated at 39°C in a humidified incubator. Embryos were staged according to Hamilton (1952). Protamine sulfate and lactoperoxidase were obtained from Sigma, and Na ^{125} I (carrier-free, pH 8–11) was from Amersham/Searle. The β subunit (β NGF) was prepared as described by Moore and Shooter (1975). Centrifo CF50A filters were obtained from Amicon Corp. Trypsin (TRTPCK), DNase (DP), and soybean trypsin inhibitor (SI) were purchased from Worthington, fetal calf serum from Irvine Scientific, and BSA (Pentex fraction V) from Miles.

Iodination of β NGF

Iodination of β NGF was carried out by the lactoperoxidase method as described by Sutter et al. (1979a). Specific radioactivity of the preparations used was 58.8 ± 13 cpm/pg. In fresh preparations, acid precipitability was 92–98%. Each preparation was used for 4 weeks or less; during this time acid precipitability fell to about 85%.

Dissociation of ganglia

Sympathetic ganglia were removed from the lumbosacral region of 6.5–20 d chick embryos. Ganglia were kept in ice cold Dulbecco's PBS (Dulbecco and Vogt, 1954), with 1 g/liter glucose without Ca or Mg for up to 3–4 hr, then cleaned of adhering connective tissue with forceps and counted. Ganglia were then exposed to 0.01% trypsin (0.1% for

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Figure 1. Steady-state binding of ^{125}I - βNGF to sympathetic ganglion cells from 11 d chick embryos. Cells ($1.1 \times 10^6/\text{ml}$) were incubated (37°C , 45 min) with 3.4×10^{-12} to 3.3×10^{-9} M ^{125}I - βNGF . Bound and free ^{125}I - βNGF were separated by rapid centrifugation as described in Materials and Methods. The concentration of free ^{125}I - βNGF was corrected for its acid precipitability (10% trichloroacetic acid, 15 min, 4°C). A, Binding of ^{125}I - βNGF as a function of free ^{125}I - βNGF concentration. Total ($\bullet$), specific ($\circ$), and nonspecific ($\blacktriangle$) binding were measured in triplicate; bars indicate SD. B, Scatchard plot of data shown in A.

developmental studies and for embryos older than 11 d) and 0.001% DNase in PBS without Ca or Mg for 10 min at 37°C . Fetal calf serum (to 5% vol/vol) and soybean trypsin inhibitor (0.08 mg/ml) were then added, and the ganglia were disrupted by forcing the suspension vig-

orously through a 9 inch Pasteur pipette 30–40 times, until most visible pieces had disappeared. The cell suspension was then filtered through a double layer of Nylon mesh cloth (50 μm pore size) to remove clumps. The yield of cells increased from 10,000/ganglion at day 8 to 40,000/ganglion at day 14, then fell to 14,000/ganglion at day 20; at day 11, about 25,000 cells/ganglion (about 95% viable by Trypan blue exclusion) were obtained. Mechanical dissociation alone (day 9 ganglia) yielded 10–20% fewer cells than trypsin dissociation, but cell viability fell to about 65%.

Binding assays

The assays were similar to those used by Sutter et al. (1979a). Briefly, cells were centrifuged ($250 \times g$, 10 min) and resuspended in PBS with 1 mg/ml BSA, then added to ^{125}I - βNGF diluted into the same solution in polystyrene tubes (Falcon). All incubations were carried out at 37°C unless otherwise stated. Cell concentrations were generally $1\text{--}2 \times 10^6/\text{ml}$. Steady-state measurements were made after 45 min incubation. Bound and free ^{125}I - βNGF were separated by centrifugation ($10,000 \times g$, 1 min) of 0.1 ml portions of cell suspension through a 2-step sucrose gradient (0.225 ml) in 400 μl tubes in a Beckman Microfuge (Herrup and Shooter, 1973). Tubes were then frozen in dry ice-ethanol, tips were cut off with a razor blade, and both tips and tops of tubes were counted in a Beckman Gamma 300 counter. Nonspecific binding was measured in the presence of a 100-fold or greater excess of unlabeled βNGF ; a similar amount was added to initiate dissociation. "Tight" binding (see Results) was determined by measuring the amount of ^{125}I - βNGF remaining bound to cells after dissociation in the presence of excess unlabeled βNGF at 0.5°C for 20–40 min (Landreth and Shooter, 1980). For "chase versus dilution" experiments, cells were incubated with ^{125}I - βNGF until steady state was reached. Dissociation was then initiated by diluting 0.1 ml portions of cell suspensions into 1.4 ml PBS with 1 mg/ml BSA (with or without unlabeled βNGF) in 1.5 ml Microfuge tubes. Samples were taken at various times by centrifuging cells in the Microfuge ($10,000 \times g$, 1 min), aspirating the supernatant, cutting off the tip of the tube, and counting it. Some association experiments were carried out in modified F12 culture medium (Spooner, 1970) containing 25 mM Na-HEPES, 5 mM NaHCO_3 , and 1% fetal calf serum. Results did not differ from those obtained when PBS and BSA was used. All binding assays were done in triplicate.

Non-neuronal cells were obtained by allowing cells from dissociated ganglia to attach to plastic tissue culture dishes coated with calf skin collagen (Calbiochem) for 45 min. Adherent cells were then cultured for 1–4 weeks in modified F12 medium (Spooner, 1970) with 5% fetal calf serum with or without 20 ng/ml βNGF . Medium was changed twice per week, and cells were subcultured in plastic dishes, following trypsin dissociation, when confluency was reached. Non-neuronal cells were subcultured 2–4 times before use for binding assays. They were removed from culture dishes by incubation with Ca-, Mg-free PBS (sometimes with 2 mM EDTA) for 1–2.5 hr, followed by a combination of pipetting and scraping with a rubber policeman.

Results

Steady-state binding

The concentration dependence of the binding of ^{125}I - βNGF to cells dissociated from sympathetic ganglia of 11 d chick embryos is shown in Figure 1A. Cells were exposed at 37°C to ^{125}I - βNGF at concentrations of 3.4 pM to 3.3 nM for 45 min, at which time

Table 1. NGF receptors of 11 d chick embryo sympathetic ganglion cells: steady-state and kinetic binding constants

Constant ^a	High-affinity receptors	Low-affinity receptors	n
K_d (concentration for half-maximal saturation, M)	$1.0 \pm 0.5 \times 10^{-11}$	$8.9 \pm 3.4 \times 10^{-10}$	5
R_0 (binding sites/cell)	2100 ± 980	$27,000 \pm 7500$	5
k_{+1} (association rate constant, $\text{M}^{-1} \text{sec}^{-1}$)	$8.7 \pm 2.3 \times 10^7$	$10^7\text{--}10^{8b}$	2
k_{-1} (dissociation rate constant, sec^{-1})	$6.7 \pm 1.5 \times 10^{-4}$	10^{-1b}	10

^a The steady-state constants shown are averages ($\pm$ SD) of constants derived by computer-fitting binding data (bound vs. free ^{125}I - βNGF) with binding isotherms representing 2 classes of receptors according to the algorithm of Marquardt. Association rate constants were obtained by plotting association data as described in the legend of Figure 3B. Dissociation rate constants for the high-affinity receptors were calculated from linear least-squares fits to semilog plots (e.g., Fig. 2) of dissociation data obtained after preloading cells for 45 min with 7.7×10^{-12} M ^{125}I - βNGF , a concentration at which $\geq 90\%$ of the binding was tight ($\geq 80\%$ high affinity).

^b Estimates; association and dissociation of ^{125}I - βNGF and low-affinity receptors occur too rapidly to measure.

steady state had been reached at all concentrations. Nonspecific binding (usually 10–20% of total binding at these concentrations) was subtracted, and the data were replotted according to the method of Scatchard (1949), as shown in Figure 1*B*. The biphasic Scatchard plot approaches linearity at both extremes of ^{125}I - βNGF concentration, suggesting the presence of 2 classes of receptors with affinities for ^{125}I - βNGF that differ by 2 orders of magnitude. The lower-affinity receptors are about 13-fold more numerous than the higher-affinity receptors (Table 1). Similar affinity constants and site numbers were obtained when binding reached steady state at 0.5°C instead of 37°C. Binding sites for ^{125}I - βNGF on these cells were not completely saturated (Fig. 1*A*), even at ^{125}I - βNGF concentrations as high as 7.7×10^{-9} M. At this concentration (about 9 times the K_d for low-affinity receptors), one would expect nearly complete saturation of the receptors. Unfortunately, the increasing contribution of nonspecific binding prevented accurate assessment of binding at even higher concentrations of ^{125}I - βNGF . A small amount of degradation of ^{125}I - βNGF occurred at the lowest concentrations of ligand used; only 5–15% specific degradation (Layer and Shooter, 1983) was seen after 1 hr incubation of cells with 8×10^{-12} M ^{125}I - βNGF at 37°C. This did not significantly affect the concentration of free ligand and has thus been disregarded in our calculations, as have similarly small decreases in steady-state binding and cell viability during the first hour of incubation.

A 10 min incubation of the ganglia in 0.01% trypsin was necessary to obtain the maximal number of viable dissociated cells. The effects of this trypsin treatment on the NGF receptors were assayed in 2 ways. First, cells were dispersed either by mechanical disruption or by trypsin treatment of ganglia from 9 d embryos. The number of receptors per cell was 50–200% larger in the preparations of mechanically dispersed cells. This was probably due both to the lower cell yield from mechanical dispersion (which probably produced more membrane fragments) and to the trypsin lability of both classes of receptors. However, the affinities (K_d s) and the ratios of high- and low-affinity sites per cell were not significantly different in the cells from the 2 preparations. Second, ganglia from 11 d embryos were dissociated using trypsin, and the cells further incubated in trypsin before inhibitors were added. The number of both high- and low-affinity NGF receptors fell 30–40% with a further 10 min trypsin treatment after dissociation. These data should not be confused with those of Landreth and Shooter (1980), who showed that high-affinity receptors on PC12 pheochromocytoma cells are preferentially protected from degradation by trypsin, but only following binding by NGF. While both classes of NGF receptors on sympathetic ganglion cells appear to be inactivated by trypsin, the rates of inactivation are similar for the 2 sites, and thus the ratio of high- and low-affinity receptors measured on cells exposed to trypsin should be correct. Furthermore, estimates of receptor numbers on these cell bodies are probably only 30–40% below actual values *in vivo*.

Kinetics of dissociation and association

Dissociation of ^{125}I - βNGF from its receptors was observed after addition of a large excess of unlabeled βNGF to a suspension of cells and ^{125}I - βNGF after steady-state binding had been reached. At low concentrations of ^{125}I - βNGF , binding should occur mainly at high-affinity sites. As expected, dissociation curves at these low concentrations consisted almost entirely of a slowly dissociating component (half-time for dissociation of about 17 min) with simple first-order kinetics (Fig. 2, filled circles). The dissociation rate constant for the high-affinity receptors was $6.7 (\pm 1.5) \times 10^{-4} \text{ sec}^{-1}$. Division of this value by the average association rate constant for this site (Table 1, Fig. 3) yielded a K_d of 7.7×10^{-12} M, in close agreement with the value (1.0×10^{-11} M) derived from steady-state measurements.

Figure 2. Dissociation of ^{125}I - βNGF from chick sympathetic ganglion cells. Cells ($1.6\text{--}3.2 \times 10^6/\text{ml}$) from 11 d chick embryos were incubated (37°C, 45 min) to steady state with 1.1×10^{-11} M (●), 5.2×10^{-11} M (○), or 2×10^{-9} M (▲) ^{125}I - βNGF . Dissociation was initiated by addition of 1.9×10^{-8} M (●, ○) or 1.9×10^{-7} M (▲) unlabeled βNGF . Samples were taken in quintuplicate immediately before unlabeled βNGF was added (t_0), and in triplicate thereafter. Bars indicate SD.

At higher ^{125}I - βNGF concentrations, a second, very rapidly dissociating component became more apparent (Fig. 2, open circles and triangles). This rapid dissociation was complete in 15 sec and, therefore, it was not possible to obtain an accurate dissociation rate constant. The estimate given in Table 1 (10^{-1} sec^{-1}) is a minimum value, assuming a half-time for dissociation of 7 sec. The dissociation rate constant for the low-affinity site is at least 2 orders of magnitude greater than that for the high-affinity site, corresponding to the approximately 100-fold difference in the K_d of the 2 sites. The fraction of rapidly dissociating ^{125}I - βNGF increased with increasing concentration of ^{125}I - βNGF used for binding, and thus with increasing occupancy of low-affinity receptors. Therefore, the rapidly dissociating component corresponds to ^{125}I - βNGF bound to low-affinity sites.

Two other aspects of dissociation were observed upon closer examination of plots such as that shown in Figure 2. First, there was a very slowly dissociating component of bound ^{125}I - βNGF . This component was most easily observed as a plateau in dissociation plots of ^{125}I - βNGF bound at high (2×10^{-9} M) concentrations (triangles, Fig. 2). We refer to this component of bound ^{125}I - βNGF as “sequestered” (Olender and Stach, 1980; Olender et al., 1981), but we do not wish to imply, by using this term, that it is necessarily inaccessible to the external medium (see Discussion). When ^{125}I - βNGF was bound to cells at this

Figure 3. Association of a low concentration (2.3×10^{-11} M) of ^{125}I - β -NGF to sympathetic ganglion cells. Cells ($1.5 \times 10^6/\text{ml}$) from 11 d chick embryos were incubated with ^{125}I - β -NGF at 37°C for various times. **A**, Total ($\bullet$), tight ($\circ$; see text for definition), and nonspecific ($\blacktriangle$) binding were measured in triplicate; bars indicate SD. **B**, Tight binding data from **A** were replotted to obtain k_{+1} for the high-affinity site according to the equation (Shiu and Friesen, 1974) $\omega = k_{+1}t = [\ln(\beta(2x + \gamma)/\gamma(2x + \beta))]/\sqrt{Q}$ where $Q = D^2 - 4ab$, $\beta = D + \sqrt{Q}$, $\gamma = D - \sqrt{Q}$; $D = (-a - b - c)$; a is the initial concentration of ^{125}I - β -NGF (22.9 fmol/ml); b the initial concentration of high-affinity receptors (R_0 from Table 1, 5.25 fmol/ml); $c = (a - x_e)(b - x_e)/x_e$ ($x_e = ^{125}\text{I}$ - β -NGF bound at equilibrium, 4.10 fmol/ml); t the time of association (min); and x the ^{125}I - β -NGF tightly bound at time t (fmol/ml). The slope yields k_{+1} ; in this experiment $1.03 \times 10^8 \text{ M}^{-1} \text{ sec}^{-1}$.

concentration, 20–60% of the bound radioactivity was found in the sequestered compartment. Further, the fraction of bound ^{125}I - β -NGF that was sequestered was highly variable but increased with increasing concentration of ^{125}I - β -NGF. At low ^{125}I - β -NGF concentrations (1×10^{-11} M; Fig. 2, filled circles), sequestered ^{125}I - β -NGF accounted for less than 10% of total binding and therefore did not interfere with the determination of the dissociation rate constant for the high-affinity site (Table 1)

performed at this concentration. Second, the fraction of slowly dissociating or “tightly bound” ^{125}I - β -NGF (defined in these experiments as the ordinate intercept of the semilog dissociation plot) was greater than that predicted (Catt et al., 1976) for high-affinity binding, using the constants obtained from the steady-state data. At the 3 concentrations of ^{125}I - β -NGF used for pre-equilibration in the experiment of Figure 2, 93% (1.1×10^{-11} M), 72% (5.2×10^{-11} M), and 46% (2.6×10^{-9} M) tight binding was observed, compared to the predicted values of 75, 56, and 10% for high-affinity binding at the respective concentrations. The increase in the tight binding component over predicted values was greatest at higher concentrations, and corresponded to the larger fraction of sequestered material seen after binding at higher concentrations of ^{125}I - β -NGF.

In order to study association of ^{125}I - β -NGF to kinetically defined high- and low-affinity receptors, a simple method for separating rapidly dissociating from tightly bound ^{125}I - β -NGF was used (Landreth and Shooter, 1980). Portions of cell suspensions containing ^{125}I - β -NGF were removed from incubation mixtures at 37°C at various times and added to a large excess of unlabeled β -NGF at 0.5°C . Under these conditions, the rapidly dissociating NGF was removed in less than 5 min, but the fraction of tightly bound material was unchanged and became virtually nondissociable (half-time of 4 hr). Therefore, in these experiments tight binding was defined as ^{125}I - β -NGF remaining bound after 20–40 min of dissociation at 0.5°C . This value was not more than 10% lower than that obtained from extrapolation to the ordinate of the line fit to the slowly dissociating binding component. This method conveniently measures tightly bound NGF and does not appreciably distort the values obtained from dissociation data at 37°C .

Association of ^{125}I - β -NGF to its receptors on chick sympathetic ganglion cells and the development of the tightly bound component were followed using the assay just described. At low concentrations of ^{125}I - β -NGF (2.3×10^{-11} M), most of the binding was tight, as expected from the steady-state results. Steady state was reached between 20 and 50 min of incubation, followed by a slight decrease in both total and tight binding (Fig. 3A). Since sequestration of bound ^{125}I - β -NGF was minimal at this low concentration, tight binding was used as an approximation of binding to the high-affinity site. Analysis of tight binding data using the integrated form of the second-order reversible rate equation (Shiu and Friesen, 1974) yielded a linear plot through the origin (Fig. 3B) and an average value for the association rate constant of $8.7 \pm 2.3 \times 10^7 \text{ M}^{-1} \text{ sec}^{-1}$. Such a large value implies that only diffusion limits the binding of ^{125}I - β -NGF to its high-affinity receptor.

Association to the low-affinity receptor was impossible to measure. If the dissociation rate constant for this site is about 10^{-1} sec^{-1} , and the K_d is about 10^{-9} M, then the association rate constant should be about $10^8 \text{ M}^{-1} \text{ sec}^{-1}$. At the high concentrations of ^{125}I - β -NGF needed to assure that most binding occurs at the low-affinity site, one would predict that binding to both classes of receptors would be virtually complete within 1 min. Such an initial rapid phase of association was seen when 1.5×10^{-9} M ^{125}I - β -NGF was bound to cells (Fig. 4A). However, a slower phase of association in both total and tight binding was also seen, which was not complete for about 50 min. When total, tight, or rapidly dissociable binding was replotted according to the second-order reversible rate equation as in Figure 3B, fairly linear plots were obtained that intersected the ordinate at points considerably above the origin. Such a plot (for total binding) is shown in Figure 4B. These plots confirm the biphasic nature of association to both tight and rapidly dissociating components at high ^{125}I - β -NGF concentrations. The slope of the second phase in these derived plots yielded values of k_{+1} of 10^4 – $10^5 \text{ M}^{-1} \text{ sec}^{-1}$. These values are 2–3 orders of magnitude too low to account for association to either of the 2 sites observed

Figure 4. Association of a high concentration ($1.5 \times 10^{-9} \text{ M}$) of ^{125}I - βNGF to sympathetic ganglion cells. Cells ($1.4 \times 10^6/\text{ml}$) from 11 d chick embryos were incubated with ^{125}I - βNGF at 37°C for various times. *A*, Total (●), tight (○; see text for definition), and nonspecific (▲) binding were measured in triplicate; bars indicate SD. *B*, Total binding data from *A* were replotted as in Figure 3.

in steady-state experiments, and are thus likely to reflect a secondary process subsequent to binding. Association proceeded at similar rates at 0.5°C , and proportions of tight and rapidly reversible binding similar to those shown in Figure 4*A* were seen (data not shown).

Data from another kind of experiment also revealed 2 components of tight binding. Tight binding was measured, as described for the association experiments above, after steady state had been reached at various concentrations of ^{125}I - βNGF . Scatchard plots of total, tight, and rapidly dissociable binding were then made. Tight binding data yielded curvilinear Scatchard plots (Fig. 5, open circles) with 2 components having slopes similar to those of the high- and low-affinity sites seen with Scatchard plots of total binding (Fig. 5, closed circles). Rapidly reversible binding (Fig. 5, triangles) was almost entirely low affinity; the small component that appears to be high affinity is probably an artifact due to the small amount of dissociation of tightly bound NGF that occurs at 0.5°C .

Figure 5. Scatchard plots of total (●), tight (○), and rapidly dissociable (▲) binding at steady state. Cells ($1.1 \times 10^6/\text{ml}$) from 11 d embryos were incubated (37°C , 45 min) with 3.4×10^{-12} to $3.3 \times 10^{-9} \text{ M}$ ^{125}I - βNGF . Aliquots of the incubation mixtures were then assayed for total and tight binding (see Materials and Methods). Tight binding was measured after dissociation for 20 min at 0.5°C in the presence of a 50–5000-fold excess of unlabeled βNGF . Rapidly dissociable binding was defined as the difference between total and tight binding.

Cooperativity

Frazier et al. (1974) proposed that the heterogeneity of binding of ^{125}I - βNGF to chick embryo sympathetic and sensory ganglion cells was due to negatively cooperative interactions among the receptors. According to this hypothesis, increased occupancy of receptors produces a decreased affinity for the ligand. Assuming association rate constants are diffusion limited and do not change with receptor occupancy, this decreased affinity would be reflected in an increasing dissociation rate at increasing occupancies of the receptor. The experimental procedure typically used to detect such an increase in dissociation rate is the “chase versus dilution” experiment. In such an experiment, labeled ligand is allowed to bind to cells until steady state is reached. Dissociation of the labeled ligand is then initiated, either by dilution into ligand-free medium (“dilution” conditions) or by dilution into medium containing a large excess of unlabeled ligand (“dilution plus chase” conditions). Under dilution conditions, the receptor occupancy would not increase, but under dilution plus chase conditions, the large excess of unlabeled ligand would cause a rapid increase in occupancy as it saturated the receptors. If binding is negatively cooperative, the dissociation rate should be accelerated under dilution plus chase conditions, and the magnitude of the increase should be similar to that of the difference in apparent K_d at low and high ligand concentrations.

Results of chase versus dilution experiments are shown in Figure 6. Cells were incubated to steady state with $5 \times 10^{-12} \text{ M}$ ^{125}I - βNGF , then diluted 15-fold into buffer with or without unlabeled βNGF . This dilution factor was sufficient to prevent significant rebinding of dissociated label during the initial phases of dissociation. When a large excess (1500-fold) of unlabeled βNGF was used in chase plus dilution conditions, the dissociation rate was increased (Fig. 6*A*). However, a similar, though smaller, acceleration of dissociation was also seen when the concentration of unlabeled βNGF in chase plus dilution conditions was lower than that of the ^{125}I - βNGF used for preloading (Fig. 6*B*). In other words, the rate of dissociation was increased

Figure 6. Dissociation kinetics of bound ^{125}I - βNGF from chick sympathetic ganglion cells under dilution and dilution plus chase conditions. **A**, βNGF chase concentration was greater than ^{125}I - βNGF preloading concentration. Cells ($3.0 \times 10^6/\text{ml}$) from 11 d chick embryos were incubated with 5.2×10^{-12} M ^{125}I - βNGF for 45 min at 37°C . Total and nonspecific binding were then measured in quadruplicate without dilution (t_0 values). Dissociation was initiated by dilution of 0.1 ml portions of cell suspension into 1.4 ml PBS plus 1 mg/ml BSA at 37°C , with (○) or without (●) 7.7×10^{-9} M unlabeled βNGF . Quadruplicate samples were taken; bars indicate SD. **B**, βNGF chase concentration was less than ^{125}I - βNGF preloading concentration. Cells ($2.2 \times 10^6/\text{ml}$) were incubated with ^{125}I - βNGF as in **A**. Dissociation was followed after dilution (as in **A**) either with (○) or without (●) 1.5×10^{-12} M unlabeled βNGF . Arrows along the ordinates indicate amounts tightly bound after addition of a large excess of unlabeled βNGF (chase) at 0.5°C without any dilution.

by unlabeled βNGF , and this occurred whether the receptor occupancy was increased or decreased. Furthermore, the increase in dissociation rate (a doubling with a 1000- to 1500-fold excess of unlabeled βNGF) is insufficient to account for the 100-fold difference in apparent K_d . Thus, negative cooperativity cannot explain the heterogeneity of ^{125}I - βNGF binding seen in both steady state and kinetic experiments.

Cellular and developmental aspects

Development of NGF receptors was examined using sympathetic ganglion cells from embryos at 6½–20 d of incubation. Scatchard plots of steady-state binding data revealed both low- and high-affinity components at all 11 stages examined. Several experiments were performed at each of the following ages: 9, 11, and 15 days *in ovo*. No significant difference ($p > 0.1$) was found in K_d or number of receptors per cell, for either of the 2 sites, at these stages (Table 2).

Cultures of non-neuronal cells from sympathetic ganglia were obtained from cells adhering to collagen-coated dishes during a preplating step, as described in Materials and Methods. The cells that attached to the preplates were almost exclusively non-neuronal (i.e., they were not phase bright and did not have long, thin processes). Any remaining neurons were lost after subculturing these cells 2–4 times. Scatchard plots of binding data obtained with these non-neuronal cells showed no detectable high-affinity component, although a large number of low-affinity receptors ($>10^5$ per cell) were present (data not shown). Competition curves using 7.7×10^{-10} M ^{125}I - βNGF and increasing concentrations of unlabeled βNGF also showed a low-affinity site with a K_d of about 10^{-9} M on these non-neuronal cells (data not shown). The binding data were similar if the non-neuronal cells had been cultured in the presence of βNGF (7.7×10^{-10} M).

Discussion

Three major conclusions can be drawn from the results presented here. First, there are at least 2 classes of receptors for NGF on chick sympathetic ganglion cells; these 2 classes are similar to those found on chick sensory ganglion cells and differ in their affinity for NGF by about 2 orders of magnitude. Second, in addition to the reversible binding to these 2 classes of receptors, some of the NGF bound at high concentrations is very slowly dissociable. Third, there is little change in the steady-state binding characteristics of NGF receptors on sympathetic ganglion cells during development from 9 to 15 d of incubation; both high- and low-affinity receptors are present from 6.5 to 20 d of development.

The simplest and most consistent interpretation of the steady-state and kinetic binding data presented here is that there are 2 classes of NGF receptors on cells of chick embryo sympathetic ganglia. Scatchard plots of steady-state data are biphasic, and plots of bound versus free ligand concentration can be fit well by curves generated from a 2-site model. Kinetic data confirm this interpretation. Dissociation of bound ^{125}I - βNGF was essentially a biphasic process. The fraction of rapidly dissociable (low-affinity bound) ^{125}I - βNGF increased with increasing concentrations of ^{125}I - βNGF . The association and dissociation rate constants obtained from the kinetic data agree well with the affinity constants obtained from fitting the 2-site model to the steady-state data. Despite the presence of a substantial, very slowly dissociating component of bound NGF ("sequestered" NGF) at high preloading concentrations, the 2-site model provides a useful, consistent first approximation to describe these NGF receptors (see below).

Negatively cooperative interactions between NGF receptors (Frazier et al., 1974) cannot explain the heterogeneity of NGF binding. Biphasic dissociation kinetics are inconsistent with such

Table 2. Steady-state binding constants during development

Age of embryos ^a (d)	High-affinity receptors ^b		Low-affinity receptors ^b		n
	Sites per cell	Affinity constant (M)	Sites per cell	Affinity constant (M)	
9	2500 ± 2200	4.9 ± 4.1 × 10 ⁻¹¹	67,000 ± 47,000	4.4 ± 2.2 × 10 ⁻⁹	2
11	4000 ± 3500	3.0 ± 3.5 × 10 ⁻¹¹	38,000 ± 27,000	1.8 ± 1.8 × 10 ⁻⁹	7
15	2800 ± 1050	1.1 ± 0.4 × 10 ⁻¹⁰	57,000 ± 28,000	3.3 ± 3.1 × 10 ⁻⁸	5

^a Ganglia from 9 and 11 d embryos were dissociated with 0.01% trypsin; those from 15 d embryos required 0.1% trypsin for dissociation (see Results). Steady-state binding constants are averages (±SD) of constants derived from computer fits of a 2-site model to steady-state binding data (see legend of Table 1).

^b Constants obtained from experiments with 9, 11, and 15 d embryos are not significantly different (Student's *t* test, *p* > 0.1).

an interpretation. Both phases were present at all concentrations of ¹²⁵I-βNGF used for preloading, and the proportions of the 2 phases depended on the preloading concentration and not on the concentration of unlabeled NGF present during dissociation (Figs. 2, 6). In addition, only a 2-fold increase in the dissociation rate was seen under dilution plus chase conditions, as compared to dilution alone. This increased dissociation rate cannot account for the 100-fold difference in apparent *K_d*; furthermore, the increase was seen whether receptor occupancy during dissociation was increased or decreased. Thus, dissociation rates did not depend on receptor occupancy, ruling out the negative cooperativity model.

Closer examination of the kinetic data reveals additional complexity in the system. In addition to the 2 classes of receptors that bind NGF reversibly, there is a component of NGF bound at high preloading concentrations that is very slowly dissociable or "sequestered." At least 4 observations could reflect this component of bound NGF. First, there was a plateau in dissociation plots (Fig. 2, triangles). The fraction of bound ¹²⁵I-βNGF that was sequestered increased with increasing ¹²⁵I-βNGF concentration, being minimal (<10%) at concentrations up to 1.5 × 10⁻¹⁰ M but reaching values as high as 20–60% at an ¹²⁵I-βNGF concentration of 1.5 × 10⁻⁹ M. Second, the fraction of tightly bound NGF was larger than predicted from the steady-state binding constants. Third, association kinetics of tightly bound material were monophasic at low ¹²⁵I-βNGF concentrations, but a second, slower, phase appeared at high preloading concentrations—conditions under which low-affinity binding becomes significant. Fourth, Scatchard plots of *tight binding* were *biphasic* (Fig. 5), with a prominent low-affinity component that probably corresponds to sequestered bound NGF. Similar results have been seen with sensory ganglion cells from chick embryos (Sutter, 1981). All these observations have led us to propose that the very slowly dissociating, sequestered component of bound NGF initially binds largely to low-affinity receptors. This hypothesis explains the concentration dependence of sequestration and the presence of the low-affinity component of tight binding on Scatchard plots.

The molecular basis and functional significance of sequestration are unclear. Internalization of NGF leading to its degradation in lysosomes has been demonstrated in both chick sensory ganglion cells and PC12 cells (Layer and Shooter, 1983; Sutter et al., 1979a). A small amount of degradation was also observed in chick sympathetic ganglion cells; thus, some of the bound ¹²⁵I-βNGF could have been internalized via pinocytosis or receptor-mediated endocytosis. Artifacts of handling could also have contributed to sequestration, since it was quite variable. Although every effort was made to standardize the preparation of the cell suspension, such factors as recovery from trypsinization and incubation in BSA-containing buffer could have contributed to this phenomenon. Olender and Stach (1980) suggest that sequestration is probably not internalization, since

they observed it at 4°C, a condition under which internalization should be blocked (Bernd and Greene, 1984). Although we did not measure sequestration after NGF binding at 0.5°C, we did not observe a decrease in the number of low-affinity binding sites or in the proportion of tightly bound NGF at that temperature, consistent with their findings. It is not possible to resolve the question of the localization of sequestered NGF on the basis of NGF binding data alone. Other approaches, such as electron microscope autoradiography and differential release of surface-bound NGF (e.g., Bernd and Greene, 1984), will be required to define the localization and function of the various components of bound NGF.

It is important to emphasize, first, that sequestration, although it can account for a large portion of NGF binding at high ¹²⁵I-βNGF concentrations, is probably not physiologically relevant, since it becomes significant only at concentrations above 10⁻¹⁰ M. These levels are above the saturation point for the high-affinity receptor, which is thought to mediate the initiation of neurite outgrowth from responsive neurons (Sutter et al., 1979b), and are probably never encountered by a developing sympathetic neuron *in vivo*. Second, this phenomenon is seen in a mixed population of cells; it is possible that the cells that sequester NGF are different from those that have the high- and low-affinity receptors. This could be tested using purified neuronal and non-neuronal cells. Third, the presence of this slowly dissociating component does not invalidate the use of a steady-state model to measure the 2 classes of cell surface receptors. Since it is seen only at high concentrations, it does not affect estimates of the number, affinity, or kinetic constants of the high-affinity receptors. It probably causes an overestimation of the number of low-affinity sites by as much as a factor of 2, due to the large amount of tightly bound NGF seen in the low-affinity component of Scatchard plots (Fig. 5), although the *K_d* for these sites is probably not distorted much, since the slope of the rapidly reversible low-affinity component is similar to that of the low-affinity portion of the Scatchard plot of total binding. This complexity indicates that the steady-state, 2-site model approximates but does not completely explain the interaction of NGF with sympathetic ganglion cells.

Both high- and low-affinity receptors were present on sympathetic ganglion cells from chick embryos at 6.5–20 d of development. These findings are consistent with those of Rohrer et al. (1983), who showed, by autoradiography of ¹²⁵I-βNGF bound to cultured cells, that all chick embryo sympathetic neurons had high-affinity NGF receptors at 7, 12, and 16 d of incubation. We were unable to define the time of appearance of high- and low-affinity NGF receptors on these cells, since we could not remove ganglia from embryos younger than 6.5 d because they were too small. The numbers and affinities of receptors on the cell bodies appeared to change little during the period of development we examined. This is not surprising, since neurite outgrowth from secondary sympathetic ganglia has

been observed *in vivo* as early as 5.5 d (Kirby and Gilmore, 1976), and monoaminergic processes were seen in target organs as early as day 8 (Enemar et al., 1965). It is likely that NGF receptors on the neurites are more numerous and more important for sustaining neurite outgrowth than those on the cell bodies (Campenot, 1982; Claude et al., 1982); however, we assume that binding data obtained with cell bodies reflect the characteristics of NGF receptors on neurites as well.

Non-neuronal cells from sympathetic ganglia were cultured with or without added β NGF in the medium. These cells possessed only the low-affinity NGF receptors. This result is similar to that seen with non-neuronal cells from chick sensory ganglia (Sutter et al., 1979b; Zimmermann and Sutter, 1983). It is assumed from the results with cultured non-neuronal cells that high-affinity NGF receptors are found only on neurons in the sympathetic ganglia *in vivo*, as is the case with chick sensory ganglia (Sutter et al., 1979b); however, this assumption was not tested.

The NGF receptors described here appear to be very similar to those described for chick sensory ganglion cells. The kinetic and steady-state data obtained by Sutter et al. (1979a) for sensory ganglion cells and the constants derived from the data are very similar to those obtained in the present study. More recent data (Olender et al., 1981) show that sequestration also occurs in sensory ganglion cells. The only known difference between the NGF receptors in these 2 types of NGF-responsive cells is in their development. In sensory ganglia, the number of NGF receptors per cell appears to be greatly reduced after embryonic day 16; this correlates well with a decreased responsiveness to NGF (Barde et al., 1980; Greene, 1977a; Herrup and Shooter, 1975). In chick sympathetic ganglia, however, the number of NGF receptors per cell does not appear to change appreciably during most of embryonic development. Similarly, some chick sympathetic neurons continue to require NGF for neurite outgrowth and survival through late embryonic life (Edgar and Thoenen, 1982; Greene, 1977b).

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