Biosynthesis of β Nerve Growth Factor in Mouse Submaxillary Glands*

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The biosynthesis of β nerve growth factor (βNGF) was studied in isolated mouse submaxillary glands incubated with L-[35S]cystine. Sodium dodecyl sulfate gels of anti-βNGF immunoprecipitates from labeled gland homogenates showed a single major peak of radioactivity, which co-migrated with purified βNGF. This species was nearly completely precipitated by the addition of equivalent amounts of anti-βNGF, but was absent from immunoprecipitates obtained by the addition of ferritin plus anti-ferritin. The cystine-containing tryptic peptides of the labeled species appeared identical with those of purified βNGF.

In submaxillary glands from adult male mice, labeling of βNGF represented approximately 0.2% of the trichloroacetic acid-precipitable radioactivity. Castration reduced this value to one-third, while testosterone treatment of castrated animals restored the relative βNGF synthesis to normal or more. No βNGF synthesis could be detected in glands from female animals.

Several tissues were examined for their ability to synthesize βNGF in culture. Only the submaxillary gland incorporated detectable amounts of radioactivity into βNGF. Labeling of βNGF could also be obtained by direct injection of isotope into the submaxillary gland in vivo. The results are discussed in terms of the integration of βNGF synthesis into neuronal development and maintenance.

The development of the nervous system requires strict temporal and spatial coordination of cell growth, differentiation, and survival. The discovery and isolation of nerve growth factor, a protein capable of modulating neuronal morphology and biochemistry, promised new insight into the molecular nature of such regulatory processes (1). NGF has now been detected in a variety of vertebrates (1, 2) and is generally recognized to play an important role in the development and maintenance of the sympathetic nervous system, and in the development of the sensory ganglia as well (1).

In the male mouse, the bulk of the NGF is found in the submaxillary gland (3) in the form of specific high molecular weight complex called 7 S NGF (4, 5). The complex can be dissociated into three classes of subunits (α, β, and γ) which have been purified (6). All the nerve growth-stimulating activity resides in the β subunit (βNGF), which is a dimer of two identical noncovalently associated chains (M, 13,209) (7–9) whose amino acid sequence has been determined (9). The γ subunit is a potent arginyl esterase (10) and it has been proposed that it may function as a cleaving enzyme in the processing of a βNGF precursor (9, 11). Support for this hypothesis has recently been obtained (12). No enzymatic activity has yet been found for the α subunit.

Purified βNGF will elicit profound biochemical and morphological effects from responsive neurons. The well known stimulation of axonal outgrowth from cultured ganglia has been useful as a bioassay for the factor (13), and βNGF has also been reported to influence the directionality of fiber outgrowth (14–17). A variety of metabolic processes may be enhanced in the presence of βNGF, including cell enlargement and proliferation, glucose metabolism, RNA and protein synthesis, lipid synthesis (11), and uptake of [3H]monophosphate production (Ref. 18, but also see Refs. 19 and 20). Uptake of certain small molecules (21), and assembly of neurotubules and neurofilaments (22). Despite this plurality of effects, it remains unclear how these diverse functions are integrated into the overall pattern of neuronal development and maintenance. Our knowledge of βNGF synthesis is particularly deficient, and the role of this factor cannot be fully ascertained without a precise description of where and when it is produced and how its synthesis and processing are regulated. In this report, we show that isolated submaxillary glands synthesize βNGF de novo, and that the synthesis is regulated by steroid hormones. Preliminary versions of this work have been published (23, 24).

EXPERIMENTAL PROCEDURES

Materials

L-[35S]cystine (20 to 60 Ci/mmol), Na111 (carrier-free), and iodo[3H]acetic acid (207 mCi/mmol) were purchased from New England Nuclear. Horse ferritin and rabbit anti-ferritin antisera were generous gifts from Marvin Wickens (Stanford University).

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The abbreviations used are: NGF, nerve growth factor; SDS, sodium dodecyl sulfate.
pNGF band, the corresponding position on the sliced gels was
analyzed by counting the radioactivity at the βNGF position on SDS
gels and the dye fronts were noted. The gel slices were incubated
overnight at 37° in 10 ml of scintillation fluid containing per liter of
toluene 4 g of 2,5-diphenyloxazole, 0.05 g of 1,4-bis(2-5-phenylox-
azo)benzene, 5 ml of protosol (New England Nuclear), and 6 ml
of H2O. Slices were counted on a liquid scintillation counter. For
analysis of immunoprecipitates containing 125I-labeled βNGF, gel
slices were counted on a well-type gamma counter. Aliquots (20 μl)
of the gel samples were also counted to determine recovery of
radioactivity after precipitation and washing, determined by the recovery of tracer 125I-
labeled βNGF from separate aliquots of tissue extracts. Net recov-
eries ranged between 71 and 83% and an average yield of 75% was
used in all calculations (see "Results," Table I; (b) the fraction of
the βNGF radioactivity which appeared in the βNGF peak gel
series was normalized relative to wet weight of tissue or to incorpora-
tion into total trichloroacetic acid-precipitable radioactivity.

Preparative SDS Gel Electrophoresis - SDS gel electropho-
resis was used to isolate the major radioactive species ob-
served in immunoprecipitates from labeled submaxillary gland
extracts. The system of Laemmli (27) modified as described above
was used. Electrophoresis was performed with an apparatus de-
scribed by Furlong et al. (30) using a resolving gel (5 x 2.4 cm) at 14
mA. The elution chamber below the gel surface was continuously
flushed with electrode buffer at 36 ml/h. Fractions (2.6 ml) were
collected once the bromphenol blue dye front reached the bottom of
the gel which was stained with Coomassie blue, and from the Rf of
the βNGF, the corresponding position on the sliced gels was deter-
determined.

Trichloroacetic Acid Precipitation - Aliquots (10 μl) of submax-
illary gland supernatants were spotted (in triplicate) on Whatman
GF-C filter discs. Filters were soaked batchwise (5 ml/filter), twice
in 10% trichloroacetic acid at 4° (30 min), once in 66% ethanol (5
min), and finally once in anhydrous ether (5 min). Filters were
dried at room temperature and counted.

Quantitation of βNGF Synthesis - βNGF synthesis was quanti-
tated by counting the radioactivity at the βNGF position on SDS
gels of washed immunoprecipitates obtained from labeled tissue
extracts. Very short exposure times were used to visualize the im-
munoprecipitates and washing, determined by the recovery of tracer 125I-
labeled βNGF from separate aliquots of tissue extracts. Net recov-
eries ranged between 71 and 83% and an average yield of 75% was
used in all calculations (see "Results," Table I; (b) the fraction of
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collected once the bromphenol blue dye front reached the bottom of
the gel which was stained with Coomassie blue, and from the Rf of
the βNGF, the corresponding position on the sliced gels was deter-
determined.
acid was added to 20 mM, and alkylation was allowed to proceed for 15 min in the dark. The reaction was terminated by addition of β-mercaptopethanol to 200 mM.

Purified βNGF was alkylated with iodo[3H]acetic acid as follows: 3.25 mg of βNGF (1.5 μmol of total SH) in a volume of 1.1 ml were dialyzed against 0.5 M Tris-HCl, pH 8, containing 8 M urea and 2 mg/ml of EDTA. Then 30 μmol of dithiothreitol were added and the sample was continuously flushed with nitrogen for 4 h at room temperature. A mixture of 4.8 μmol of iodo[3H]acetic acid (1 mCi) plus 70 μmol of unlabeled recrystallized iodoacetic acid were added, and the sample was covered with aluminum foil and incubated 15 min at room temperature. The reaction was stopped by the addition of 700 μmol of β-mercaptopethanol, and the sample was dialyzed first against the original dialysis buffer and then against 0.2% acetic acid. Analysis indicated that the 3H-labeled alkylated βNGF contained 640 cpm/μg.

The tryptic map procedures are described in the legend to Fig. 5.

Protein Determination—Protein content was determined by the method of Lowry et al. (31).

RESULTS

Equivalence Point of βNGF Antiserum—The equivalence point of the anti-βNGF antiserum was determined by titrating increasing volumes of antiserum against a constant amount of purified βNGF containing tracer 125I-labeled βNGF. As shown in Fig. 1, the titration curves obtained by monitoring precipitation of either radioactivity or total protein were very similar. This finding agrees with a previous report demonstrating that 125I-labeled βNGF and unlabeled βNGF have similar avidities for this antiserum when tested in a solid state radioimmune assay (29). Identical titration curves were obtained at pH 7, although 0.5 M NaCl was not required (not shown). Using freshly prepared 125I-labeled βNGF, up to 90%
of the radioactivity could be immunoprecipitated at equivalence. (The residual 10% remained soluble even after the addition of more βNGF plus a large volume of antiserum and thus probably represents βNGF molecules whose antigenicity was altered by the iodination procedure.) The extent of precipitation of tracer amounts of 125I-labeled βNGF is therefore a valid measure of the precipitation of total βNGF and can be used as a probe to determine the volume of antiserum required for quantitative precipitation of βNGF from tissue homogenates, and to calculate the recovery of βNGF on immunoprecipitation and washing.

Comparison of Washing Procedures for Immunoprecipitates Obtained from Labeled Tissue Extracts—Fig. 2A indicates that anti-βNGF antiserum added to a 35S-labeled submaxillary gland 105,000 x g supernatant precipitates a major peak of radioactivity which co-migrates on SDS gels with purified βNGF. There is, however, considerable radioactivity in other gel fractions, particularly in regions corresponding to the major labeled peaks in a trichloroacetic acid precipitate (Fig. 3). This radioactivity most likely represents nonspecific contamination by labeled submaxillary gland proteins, and its proximity to the βNGF position prevents accurate determination of the radioactivity in βNGF. As shown in Fig. 2 and Table I, the background can be markedly reduced by (a) dissolving the immunoprecipitate in NaOH and then reprecipitating by neutralization followed by the addition of buffer and additional carrier βNGF plus antiserum, and (b) the use of siliconized tubes and the transfer of the immunoprecipitate to a fresh tube prior to centrifugation during the final wash. The reduction in background contamination is accomplished with only a minor sacrifice of βNGF recovery (Table I), and this washing method was therefore employed in the experiments described below. It should be noted that even with this procedure, a considerable portion (30%) of the total radioactivity in the immunoprecipitate in nonspecific, and therefore accurate determination of βNGF synthesis requires analysis of the precipitated label by gel electrophoresis.

Specificity of Immunoprecipitation—The results shown in Fig. 4A provide further evidence that the labeled peak at the βNGF position is in fact βNGF. When an immunoprecipitate was obtained from the supernatant of the first precipitation by the addition of carrier βNGF plus equivalent antiserum, little radioactivity was observed at this position. The peak in the initial immunoprecipitate, therefore, represents a molecule which was nearly completely removed by the first addition of antiserum. This agrees with the calculated completeness of βNGF immunoprecipitation (90%) determined by the precipitation of tracer 125I-labeled βNGF immunoprecipitated from a separate aliquot of homogenate, and provides further confirmation of the accuracy of the equivalence point determined by immunotitration of tracer 125I-labeled βNGF added to gland homogenates. In a second control, addition of ferritin plus anti-ferritin in amounts sufficient to give a larger immunoprecipitate than that obtained with anti-βNGF did not precipitate the labeled peak at the βNGF position (Fig. 4A).

When tracer 125I-labeled βNGF was added to a separate aliquot of gland supernatant and immunoprecipitated in ex-

### Table I

Comparison of wash procedures of labeled immunoprecipitates

Data were obtained from the experiment described in Fig. 2. The radioactivity in βNGF and in the background were computed from the SDS gels. Recovery of βNGF was monitored by performing the identical isolations with separate aliquots of homogenate to which had been added 3 ng of 125I-labeled βNGF.

| Wash procedures | Fraction of nonspecific trichloroacetic acid-precitable cpm in immunoprecipitate which are in βNGF | Recovery of 125I-labeled βNGF during wash |
|-----------------|-----------------------------------------------|-----------------------------------------------|
|                 | %                                           | %                                           |
| A               | 0.42                                        | 37                                          | 99                                          |
| B               | 0.27                                        | 45                                          | 89                                          |
| C               | 0.09                                        | 70                                          | 83a                                         |

* Since the yield during the initial immunoprecipitation was 90%, the net recovery of βNGF was 75%.

![Fig. 3. SDS gel pattern of immunoprecipitates from submaxillary gland homogenate. Submaxillary glands were labeled with L-[35S]cysteine, and immunoprecipitates were washed and analyzed as described under "Methods." A. Immunoprecipitate obtained from 0.1 ml of gland supernatant using anti-βNGF; C — C, immunoprecipitate obtained from the supernatant of the first anti-βNGF immunoprecipitation by the addition of unlabeled βNGF (2.5 μg) and an equivalent amount of anti-βNGF; A — A, immunoprecipitate obtained from 0.1 ml of gland supernatant by the addition of ferritin (5 μg) and an equivalent amount of anti-ferritin. B, 3 ng of 125I-labeled βNGF was added to 0.1 ml of gland supernatant, and the washed immunoprecipitate obtained using anti-βNGF.](http://www.jbc.org/)

![Fig. 4. SDS gel pattern of immunoprecipitates from submaxillary gland homogenate. Submaxillary glands were labeled with L-[35S]cysteine, and immunoprecipitates were washed and analyzed as described under "Methods." A. Immunoprecipitate obtained from 0.1 ml of gland supernatant using anti-βNGF; C — C, immunoprecipitate obtained from the supernatant of the first anti-βNGF immunoprecipitation by the addition of unlabeled βNGF (2.5 μg) and an equivalent amount of anti-βNGF; A — A, immunoprecipitate obtained from 0.1 ml of gland supernatant by the addition of ferritin (5 μg) and an equivalent amount of anti-ferritin. B, 3 ng of 125I-labeled βNGF was added to 0.1 ml of gland supernatant, and the washed immunoprecipitate obtained using anti-βNGF.](http://www.jbc.org/)
of 8 M urea, and centrifuged to remove insoluble material. Then continued for 3 h. The mixture was lyophilized, dissolved in 0.3 ml 50% (v/v) glycerol, 10% (v/v) Triton X-100, and 4 mg/ml of cyto-
additional 40 ~1 of trypsin solution was added and the incubation solution of (tosyl+phenylalanine chloromethyl ketone&treated trypsin was added and the mixture was incubated for 1 h at 37°. An
0.12 ml of supernatant was added to 0.013 ml of 2 rnM HCl containing
peak gel slices at the /3NGF position (Fig. 4B). A small but
exactly the same way, 90% of the radioactivity appeared in four
the presence of L-[35S]cysteine (2 mCi/ml). The radioactivity in
was determined by 3D3 gel electrophoresis of the washed immunoprecipitates from the gland homogenates (see "Methods").
remove free L-[35S]cysteine. Immunotitration indicated that the total quantity of /3NGF in the medium was less than 10% of that found in the gland supernatant, and the radioactivity in
betaNGF, determined by immunoprecipitation and SDS-gel electrophoresis, did not exceed 4% of that found in the gland homogenate. We conclude that the majority of the /3NGF,
including the newly synthesized material, is not released into the culture medium during the labeling period.
Hormonal Regulation of /3NGF Synthesis — The /3NGF content of the submaxillary gland and the serum are regulated by androgens. Thus, the levels in male mice rise sharply
during puberty and by adulthood greatly exceed those in females (3, 32). Castration of adult males produces a sharp drop in /3NGF levels (29, 33). Kinetic analyses suggest that

testosterone acts at the level of synthesis. Testosterone treatment of castrated animals boosts the synthetic level to slightly above normal. /3NGF synthesis is below detectable limits in submax-
illary glands from adult females.

\( \beta \)NGF Synthesis by Various Tissues — In order to test whether the \( \beta \)NGF synthesis observed in the submaxillary gland is merely a base-line level typical of all tissues or is truly a specialized function of the submaxillary gland, we measured synthesis in a variety of isolated tissues by the same methods. Of all the tissues examined, \( \beta \)NGF synthesis could only be detected in the submaxillary gland under the conditions employed (Table III).

**FIG. 5.** Isoelectric focusing of tryptic peptides of \(^{3}H\)-labeled car-
boxymethylated \( \beta \)NGF and the carboxymethylated \(^{35}S\) unknown. A portion of the carboxymethylated \(^{3}S\)-labeled peak from the prepara-
tive SDS gel (10,600 cpn containing 200 µg of carrier bovine serum albumin) was combined with purified \( \beta \)NGF which had been carboxymethylated with iodo-[\(^{3}H\)acetic acid (84,500 cpn, 132 µg of
PGF). The mixture (total volume 0.16 ml) was dialyzed at 4° against 0.1 m ammonium bicarbonate, pH 8.2, 40 µl of a 1 mg/ml solution of (tosyl+phenylalanine chloromethyl ketone)-treated trypsin was added and the mixture was incubated for 1 h at 37°. An
additional 40 µl of trypsin solution was added and the incubation continued for 3 h. The mixture was lyophilized, dissolved in 0.3 ml of 8 M urea, and centrifuged to remove insoluble material. Then
0.12 ml of supernatant was added to 0.013 ml of 2 mM HCl containing
peak gel slices at the \( \beta \)NGF position (Fig. 4B). A small but
exactly the same way, 90% of the radioactivity appeared in four
the presence of L-[35S]cysteine (2 mCi/ml). The radioactivity in
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remove free L-[35S]cysteine. Immunotitration indicated that the total quantity of \( \beta \)NGF in the medium was less than 10% of that found in the gland supernatant, and the radioactivity in
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including the newly synthesized material, is not released into the culture medium during the labeling period.
Hormonal Regulation of \( \beta \)NGF Synthesis — The \( \beta \)NGF content of the submaxillary gland and the serum are regulated by androgens. Thus, the levels in male mice rise sharply
during puberty and by adulthood greatly exceed those in females (3, 32). Castration of adult males produces a sharp drop in \( \beta \)NGF levels (29, 33). Kinetic analyses suggest that

testosterone acts at the level of \( \beta \)NGF synthesis rather than output from the gland (29), yet a direct effect of steroids on synthesis has not yet been demonstrated.

**Table II** shows the synthesis of \( \beta \)NGF in cultured submax-
illary glands from animals under different hormonal states. Castration of adult male mice causes a marked reduction in relative \( \beta \)NGF synthesis, and testosterone treatment of castrated animals boosts the synthetic level to slightly above normal. \( \beta \)NGF synthesis is below detectable limits in submax-
illary glands from adult females.

**\( \beta \)NGF Synthesis by Various Tissues** — In order to test whether the \( \beta \)NGF synthesis observed in the submaxillary gland is merely a base-line level typical of all tissues or is truly a specialized function of the submaxillary gland, we measured synthesis in a variety of isolated tissues by the same methods. Of all the tissues examined, \( \beta \)NGF synthesis could only be detected in the submaxillary gland under the conditions employed (Table III).
TABLE III

βNGF synthesis in various isolated mouse tissues

| Tissue               | Fraction of trichloro-<br>acetate-acid-precipitable<br>cpm which are in<br>βNGF | Radioactivity in<br>βNGF<br>cpm/mg tissue wet<br>wt % |
|----------------------|-----------------------------------|-------------------|
| Castrated mice treated with testosterone | 0.233 | 588 |
| Submaxillary gland   | <0.002* | <2 |
| Liver                | <0.001 | <2 |
| Kidney               | <0.003 | <3 |
| Spleen               | <0.003 | <13 |
| Lung                 | <0.005 | <19 |
| Heart                | <0.002 | <2 |
| Adrenal*             | <0.007 | <24 |
| Brain*               | <0.004 | <8 |
| Spinal cord*         | <0.004 | <8 |

Sham-operated, untreated male mice

| Submaxillary gland | 0.210 | 555 |
| Testes*            | <0.002 | <4 |
| Vas deferens*      | <0.009 | <9 |

*Since only in the submaxillary gland immunoprecipitants were there discrete radioactive peaks at the βNGF position, the values presented for the other tissues represent overestimates of the true βNGF biosynthesis.

†The media were tested following the incubations by adding carrier βNGF plus antisera, and electrophoresing the washed immunoprecipitates. In no case was labeled βNGF detected in the medium.

βNGF Synthesis in Vivo—When L-[35S]cystine was injected directly into the submaxillary gland of a living mouse and the immunoprecipitate analyzed, radioactive βNGF was observed (data not shown). The label represented 0.46% of the trichloroacetic acid-precipitable radioactivity in the gland supernatant.

DISCUSSION

The submaxillary gland of the adult male mouse has long been recognized for its high NGF content (3), yet the source of this NGF has not been directly determined. Several lines of evidence argue against NGF uptake by the gland from the serum. (a) NGF injected directly into the circulation does not accumulate in the submaxillary gland (29, 34), (b) radioactive amino acids injected unilaterally into one lobe of the submaxillary gland preferentially label the NGF from the injected lobe (35), (c) the NGF activity in venous blood effluent from the gland exceeds that in arterial blood affluent to the gland (33), and (d) daily injection of anti NGF does not prevent the testosterone-induced rise in submaxillary gland NGF content in female or young male mice (34).

It is often assumed on the basis of these criteria that submaxillary gland NGF is produced in situ, but more direct evidence has thus far been lacking and the question is still controversial (36, 37). Levi-Montalcini and Angeletti (35) have reported that addition of anti-NGF antisera to homogenates of submaxillary glands labeled in culture precipitates radioactivity, but neither analytical characterization of the labeled material nor controls for nonspecific precipitation were presented. In view of the relatively large amount of label precipitated (4 to 6% of the trichloroacetic acid-precipitable counts compared to our observations of approximately 0.2% for specific incorporation into βNGF), one is not convinced that the label in their immunoprecipitates represents NGF synthesized in vitro.

The findings presented herein provide the most conclusive evidence for de novo production of βNGF by mouse submaxillary glands, and the negligible levels of βNGF synthesis in other tissues examined (Table III) suggest that most if not all of the submaxillary gland βNGF is produced in situ. As anticipated from previous kinetic studies (29), the hormonal modulation of gland βNGF levels (3, 29, 32, 33) correlates with direct measurements of βNGF synthesis (Table II).

Still, the physiological function of the submaxillary gland βNGF remains unclear. The report of Hendry and Iverson (38) that sialectomy induces a transient drop in serum NGF levels suggested that the gland is the source of NGF for the circulation. However, Murphy et al. (39) were unable to detect this decline and proposed instead "that the serum factor arises from multifocal cellular secretion." In favor of this view is the observation that circulating NGF levels are eventually restored after sialectomy without concomitant regeneration of the gland (38), as well as the apparent ability of many primary (40-43) and transformed (43-47) cell types to produce NGF in culture. Clearly, the question of an endocrine role for the submaxillary gland in NGF production can be ascertained only when the effects of sialectomy on serum NGF levels are resolved. Alternatively, the possibility of an exocrine role for NGF secretion into the digestive tract has been considered (35) and given recent support by the finding of high NGF concentrations in mouse saliva (39, 40). It should be recalled that snake venom is another rich source of NGF (49), and that the venom gland is the phylogenetic homologue of the mammalian salivary gland.

Perhaps the most significant question concerning NGF synthesis is the mechanism by which the factor is delivered to those neurons which depend upon it for growth and survival. The problem is complicated not only by the multiplicity of potential synthesis sites, but also by the possibility of changing physiological sources at different stages of development. The suggestion of a subordinate role for circulating NGF has been put forth (21, 50), and alternate supply routes have been postulated. Mouse (40) and human (51) neuroblastoma can apparently produce the factor, and several workers have emphasized the potential importance of glial cells in NGF production (21, 44, 47, 52), although this has been vigorously challenged (53). We were unable to detect βNGF synthesis in mouse brain or spinal cord (Table III) but have not yet examined synthesis by sensory or sympathetic ganglia. A provocative alternative is the potential involvement of target organs in NGF production. It is clear that NGF can be specifically taken up by both sympathetic (54, 55) and sensory (55) nerve terminals and transported retrogradely back to the cell body, suggesting the possibility of a direct NGF supply between effector organ and innervating neurons. Consistent with this, NGF production has been reported in mouse adrenals (42) and rat irides (40) in vitro, although our methods failed to detect synthesis in cultured mouse adrenals or vas deferens (organs which receive sympathetic innervation). The question becomes particularly intriguing in view of the apparent ability of NGF to attract growing sympathetic and sensory fibers both in vivo (14, 16) and in vitro (15, 17), and it has been proposed that NGF synthesis and release by an effector organ might ultimately determine that organ's density and...
pattern of innervation (38, 55). It therefore becomes imperative to test directly whether tissues which ultimately become innervated by sympathetic or sensory fibers synthesize and release NGF during the period of innervation. A positive correlation would lend strong support to the concept of NGF as not only an essential growth and maintenance factor for responsive neurons, but also as a critical trophic messenger between target organ and growing axon.

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