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Expression of the β-nerve growth factor gene correlates with the density of sympathetic innervation in effector organs

(trophic factors/RNA blot hybridization/gene regulation/brain/denervation)

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ABSTRACT **Although β-nerve growth factor (NGF), a protein necessary for survival and development of sympathetic neurons, is believed to be a trophic factor that is produced by sympathetic effector organs, its synthesis by these tissues has never been conclusively demonstrated. Using an assay capable of detecting 10 fg of mRNA, we measured the level of NGF mRNA in tissues innervated by sympathetic neurons. NGF mRNA was detected unambiguously in each tissue at a level that appeared to be more than enough to account for the low levels of NGF protein previously detected. Tissues that were densely innervated had comparatively high levels of NGF mRNA, while those with sparser innervation had lower levels. There was a strong positive correlation between the NGF mRNA level and norepinephrine content, a measure of the density of sympathetic innervation. NGF gene expression in one of these tissues, the iris, was shown to be induced by denervation. NGF mRNA was also found in other areas, including elements of the adult peripheral nervous system—the sciatic nerve and the sympathetic and sensory ganglia. In the central nervous system, levels of NGF mRNA were found that are too high to be attributed entirely to the vasculature, suggesting a role for NGF in adult central nervous system function.**

During development, neurons respond to a variety of extrinsic signals that affect many aspects of neuronal differentiation, including neuronal survival. The observation that naturally occurring neuronal cell death can be influenced by changing the volume of a target tissue has led to the proposal that organs synthesize and secrete trophic factors that are required for the survival of the neurons that innervate them (see ref. 1).

β-Nerve growth factor (NGF) is the only trophic factor known to affect neurons that has been purified, characterized, and shown to be important *in vivo*, where it is required for survival of embryonic sensory and sympathetic neurons (see ref. 2). Thus, appropriately timed treatment of animals with antibodies to NGF results in almost complete destruction of both classes of neurons (e.g., refs. 3 and 4). Several observations support the proposal that NGF is synthesized by target tissues, where it functions to regulate survival and differentiation of sympathetic and sensory neurons. First, surgical or chemical axotomy of neonatal sympathetic neurons also results in neuronal death (5, 6). Second, administration of NGF is able to prevent both experimentally induced and normally occurring cell death (5, 6). In addition, NGF applied to sympathetic or sensory nerve terminals is bound to specific receptors, internalized, and transported retrogradely to the cell body (7), resulting in a variety of changes, including induction of transmitter enzymes (reviewed in ref. 2). Finally, experiments *in vitro* show that NGF is effective in circumstances where it is available only to axons and terminals and not to other areas of the neuronal surface (8).

A major feature of this model is that the trophic substance be synthesized by the target organ. In the case of NGF, it only recently has been possible to demonstrate, with a sensitive two-site antigen assay, that NGF is present in target tissues (9, 10). Although synthesis of NGF does appear to occur in a variety of explanted tissues (11, 12), it has not yet been demonstrated in innervated sympathetic effector organs.

Fortunately, there are several exocrine tissues, most notably the male mouse submaxillary gland, that contain large amounts of NGF (see ref. 2). While these sources have no known function in neuronal development (see ref. 2), they have made it possible to purify and characterize this protein. Recently, NGF cDNA clones have been isolated by using cDNA libraries prepared with mRNA from the male mouse submaxillary gland (13, 14).

Using one of these cDNAs as a probe (13), we have developed an assay using RNA blot hybridization transfers which can detect <10 fg of NGF mRNA. We report here that many mammalian sympathetic and sensory effector organs do express the NGF gene and, therefore, have the ability to synthesize NGF.

MATERIALS AND METHODS

Sources. Sprague–Dawley rats, New Zealand White rabbits, Swiss Webster mice, dogs, and cow eyes were obtained from local suppliers. Nitrocellulose paper (0.45 μm, BA85) was obtained from Schleicher & Schuell; restriction enzymes, Klenow fragment, and primer, from New England Biolabs; oligo(dT)-cellulose, from Collaborative Research; and [^{32}P]dCTP (3000 Ci/mol; 1 Ci = 37 GBq), from Amersham. All other chemicals were reagent grade.

Probe Preparation. For initial work we used a double-stranded probe prepared from the NGF cDNA described in ref. 13. The 0.9-kilobase (kb) *Pst* I fragment complementary to the coding sequence and to the 5′ untranslated region was labeled by nick-translation to a specific activity of *ca.* 2×10^9 cpm/μg (15). To create a single-stranded cDNA probe, this fragment was cloned into the *Pst* I site of phage M13 mp8 (16). Labeled probe was synthesized on a phage DNA template hybridized with a 15-base primer. Phage DNA (700 ng; 80 ng of NGF insert) was boiled for 2 min with a 10-fold molar excess of primer in 4.0 μl of 20 mM Tris chloride, pH 7.4/10 mM $MgCl_2$/1 mM dithiothreitol/0.2 mM $CaCl_2$ and slowly cooled to room temperature. The final reaction volume was 20 μl of the same solution containing 200 μCi of [^{32}P]dCTP (dried) and 250 μM each of unlabeled dATP, dGTP, and TTP. Synthesis, initiated by the addition of 10 units of Klenow fragment of *Escherichia coli* DNA polymerase I, proceeded for 5 min at 22°C and 90 min at 37°C. After

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Abbreviations: NGF, β-nerve growth factor; DRG, dorsal root ganglion; SCG, superior cervical ganglion; kb, kilobase.

unincorporated dCTP (<10%) was removed by gel filtration through Sephadex G-50, DNA was precipitated with 2 vol of ethanol, dissolved in water, and diluted with an equal vol of 100 mM NaOH/2 mM EDTA. The sample was boiled for 2 min, chilled on ice, and loaded directly on a 1.5% "low melting temperature" agarose gel equilibrated with 40 mM Tris acetate, pH 7.5/2 mM EDTA. After electrophoresis, the labeled probe band was excised, melted at 68°C, and added directly to the hybridization solution.

RNA Preparation and Assay. Animal tissues were homogenized in 5 M guanidinium thiocyanate/5% 2-mercaptoethanol/10 mM EDTA/50 mM Na Hepes, pH 7.4. RNA was isolated either by sedimentation through 5.7 M CsCl and extraction with phenol/chloroform (17) or by precipitation with 5 vol of 4 M LiCl and extraction with phenol/chloroform (18). All solutions used after the initial homogenization were treated with 0.05% diethyl pyrocarbonate and were autoclaved. All glassware was baked for 4 hr at 200°C. Poly(A)$^+$ RNA was selected by two cycles of chromatography on oligo(dT)-cellulose (19). Selected RNA was denatured with formaldehyde, separated on formaldehyde-containing 1.5% agarose gels (20), and transferred to nitrocellulose paper with 3 M NaCl/0.3 M Na citrate, pH 7.0, without presoaking the gel (21). Blots were then treated with a prehybridization solution that contained 50% deionized formamide, 0.1% NaDodSO$_4$, denatured salmon sperm DNA at 200 μg/ml, 5× Denhardt's solution (1× Denhardt's is 0.02% each of Ficoll 400, polyvinylpyrrolidone, and bovine serum albumin), 0.75 M NaCl, 5 mM EDTA, and 50 mM sodium phosphate (pH 7.7) for at least 8 hr at 50°C. Hybridization was carried out for 48 hr at 50°C in the same solution with Denhardt's solution reduced to 1× and either 2.5 ng of double-stranded probe or 8 ng of single-stranded probe per ml. The filter was then washed four times with 0.3 M NaCl/0.1% NaDodSO$_4$/0.03 M Na citrate, pH 7.0, and four times with 15 mM NaCl/0.1% NaDodSO$_4$/1.5 mM Na citrate, pH 7.0, both at room temperature, and twice with the latter solution at 60°C. After drying, the paper was exposed to preflashed Kodak XAR-5 film with a DuPoint Cronex intensifying screen at −80°C. The autoradiogram was used to locate hybridizing bands on the filter, which were excised, dissolved in 1 ml of 2-ethoxyethanol, and assayed for radioactivity in Aquasol (New England Nuclear) on a scintillation counter. *Hin*dIII fragments of phage λ DNA were end-labeled with [^{32}P]dCTP, denatured with formaldehyde, and used as molecular weight standards. Serial dilutions of male mouse submaxillary gland poly(A)$^+$ RNA and standard curves of phage DNA from the M13 subclone were run on every gel.

RESULTS

Sequence Homology of Mouse NGF cDNA with the NGF Gene in Other Mammals. In order to test the sequence homology of the mouse cDNA with NGF sequences from other mammals, genomic DNA was digested with *Hin*dIII and analyzed by the Southern technique (22). After hybridization to the mouse single-stranded probe and autoradiography, single bands of approximately equal intensity were found for mouse, rat, rabbit, dog, and cow DNA (Fig. 1). The hybridization and washes were done under stringent conditions (see Fig. 1 legend), and when transfers were washed sequentially with 0.015 M NaCl/0.0015 M sodium citrate, pH 7.0, at increasing temperatures, the bands of various species decreased and disappeared approximately coincidentally (data not shown). Thus, the mouse cDNA is highly homologous to the NGF sequences of genomic DNA from each of these mammals and, therefore, is expected to hybridize to their NGF mRNAs (see also ref. 14).

Development of a Sensitive Assay for NGF mRNA. Since NGF is present in minute quantities in targets of sympathetic neurons (9), we expected to detect correspondingly low levels of NGF mRNA. Therefore, we felt it necessary to show that any hybridizing RNA was of the appropriate size and attempted to develop a more sensitive assay with RNA blot transfers (21).

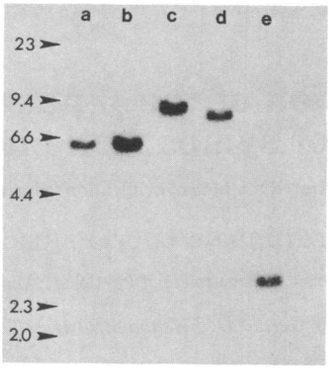

FIG. 1. Hybridization of NGF [^{32}P]cDNA to genomic DNA. High molecular weight DNA samples were prepared as described in ref. 23, digested with *Hin*dIII, and analyzed by electrophoresis, transfer, and hybridization to a single-stranded [^{32}P]DNA probe. Samples (0.5 μg) were run on 0.8% agarose gels and transferred to nitrocellulose (22). Pretreatment and hybridization were done at 42°C, and final washes were performed at 50°C in conditions otherwise the same as for RNA assays. The positions and sizes in kilobase pairs of phage λ DNA *Hin*dIII fragments are indicated by arrowheads and numbers. Lanes: a, mouse; b, rat; c, rabbit; d, cow; e, dog.

At the time this work was initiated, no assay of RNA transferred to nitrocellulose claimed a sensitivity of <0.5 pg of specific mRNA (24). Using serial dilutions of a rich source of NGF mRNA, poly(A)$^+$ RNA from the male mouse submaxillary gland, we examined several parameters of this assay. We tried both nick-translated and single-stranded probes, various conditions of electrophoresis, transfer, hybridization, and washing in attempts to increase sensitivity.

A standard curve obtained with our optimized method with a single-stranded probe is shown in Fig. 2. The radioactivity varied linearly with applied RNA or DNA over at least 3 orders of magnitude. The absolute detection limit of DNA varied somewhat but was always <10 fg of hybridizing sequence. Assuming equal hybridization efficiency to NGF

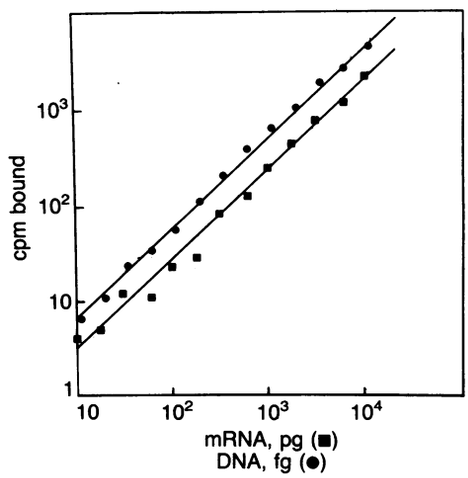

FIG. 2. NGF DNA and RNA standard curves. Two dilution series were performed, one with poly(A)$^+$ male mouse submaxillary gland RNA (■) and one with M13 phage DNA containing the 900-base-pair *Pst* I fragment of the NGF cDNA (●). Each sample was counted for at least 40 min. The background has been subtracted from each point.

mRNA, this corresponds to about 12,000 mRNA molecules. Each gel lane had a capacity of at least 5 μg of poly(A)$^+$ RNA, giving the ability to detect <2 parts in 10^9 by weight. By this assay, NGF mRNA appeared to be 0.08% ± 0.03% of the total poly(A)$^+$ RNA in the male mouse submaxillary gland.

Levels of NGF mRNA in Different Tissues. To determine if NGF mRNA was present in sympathetically innervated tissues, poly(A)$^+$ RNA was prepared from major sympathetic targets and assayed as described. In addition, we measured NGF mRNA in tissues with sparse sympathetic innervation and in nerves and ganglia containing elements of the sensory and sympathetic nervous systems.

A summary of NGF mRNA levels in tissues tested is shown in Fig. 3, and an example of an autoradiogram obtained with this technique is shown in Fig. 4. All tissues in which NGF mRNA was detected in mouse, rabbit, dog, and cow showed a single hybridizing band of *ca.* 1.3 kb, the same size as NGF mRNA in the male mouse submaxillary gland. All rat tissues tested had higher levels of NGF mRNA of this molecular size than in the same tissues from the other species. Also, in some but not all tissues from the rat, an additional hybridizing band was detected, with a molecular size of ≈1.7 kb. This band was always less intense than the 1.3-kb band, accounting for no more than 20% of the total amount of hybridization and was not included in the data in Fig. 3.

There is a correlation between the level of NGF mRNA and the level of sympathetic innervation. Iris had the highest level of NGF mRNA of any tissue in all species tested (with the exception of male mouse submaxillary gland and rabbit prostate, two exocrine tissues with anomalously high NGF levels). Other tissues with heavy sympathetic innervation, such as heart and spleen, also contained comparatively high levels of NGF mRNA. Tissues with little sympathetic innervation, such as skeletal muscle and thymus, had much lower levels of transcription.

In addition, some tissues were subdivided into regions with different levels of sympathetic innervation. For example, heart atrium and ventricle, both of which receive a dense sympathetic innervation, were both shown to contain comparatively high levels of NGF mRNA. The innervated spleen capsule was shown to contain essentially all of the NGF mRNA in the spleen and had at least a 10-fold higher level of NGF mRNA than the uninnervated splenocytes. The thymus receives very little sympathetic innervation, and neither thymocytes nor thymic capsule had high levels of NGF mRNA.

To determine how closely the level of sympathetic innervation correlated with the level of NGF mRNA in different tissues, we compared the NGF mRNA level to the previously reported norepinephrine content for each tissue (25). There was a strong positive correlation between these two values (Fig. 5).

Elements of the peripheral nervous system—sympathetic ganglia from dog and dorsal root ganglia and sciatic nerve from rabbit—also contained readily detectable NGF mRNA. Surprisingly, brain, which has only a sparse sympathetic innervation of the vasculature, had a relatively high level of NGF mRNA.

Regulation of NGF mRNA Levels. Many tissues accumulate higher levels of NGF after being explanted or denervated than are normally present *in situ* (11, 12). To test whether these increases in NGF reflect increases in NGF gene expression, rat irides were assayed for NGF mRNA after explantation and culture at 37°C in Dulbecco's modified Eagle's medium with 0.4% glucose, 10% newborn calf serum, and 10% horse serum for 5–10 hr; ≈1 μg of total RNA was isolated per iris (17). Individual determinations of NGF mRNA were made with 2–8 μg of RNA; control irides contained 25 and 61 fg (mean, 43 fg per iris), and explanted irides contained 297 and 333 fg (mean, 315 fg per iris). Thus, there was a large induction of NGF mRNA in cultured irides leading to a *ca.* 7-fold increase in the total amount of NGF mRNA per iris even though the total RNA content decreased in explanted irides.

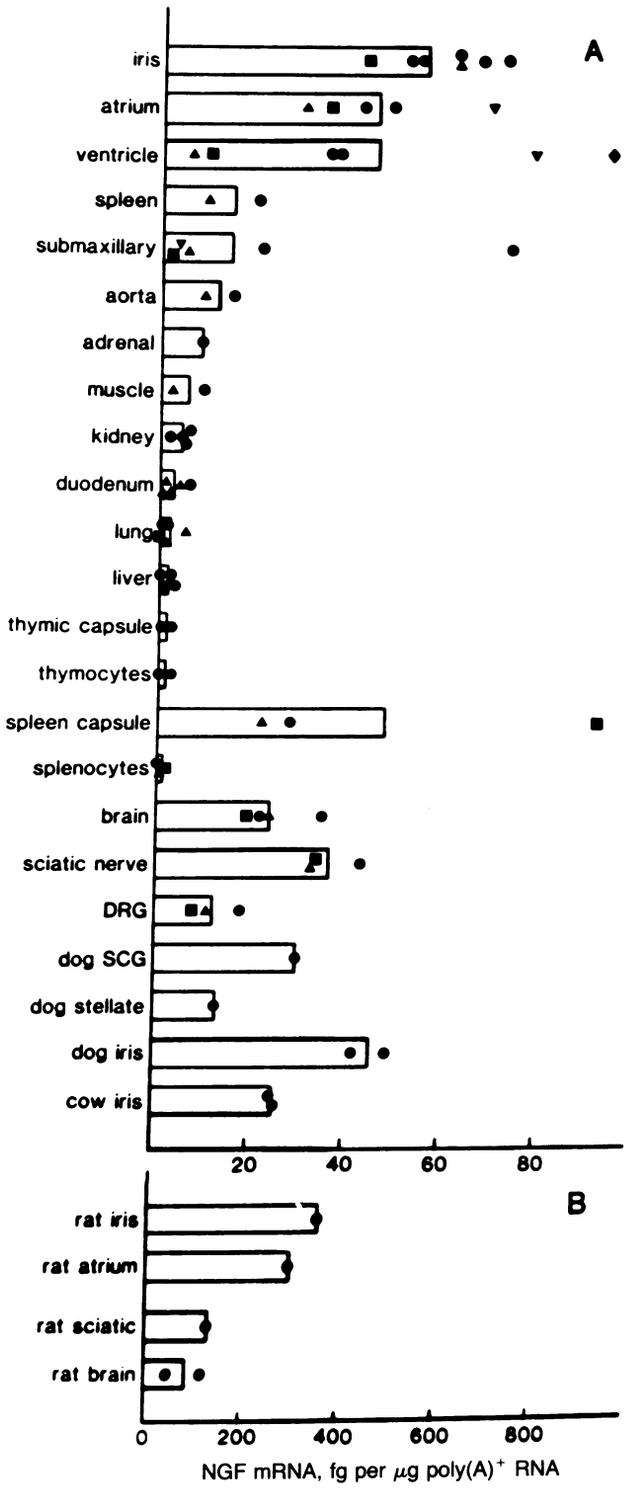

FIG. 3. NGF mRNA content of various mammalian tissues. Unless otherwise specified, organs are from rabbits. Bars represent either single determinations or the means of all preparations for a given organ. Individual points represent individual determinations. Different symbols mark different preparations, while identical symbols represent independent determinations done on the same preparation. Note the change in scale between *A* (rabbit, dog, and cow) and *B* (rat).

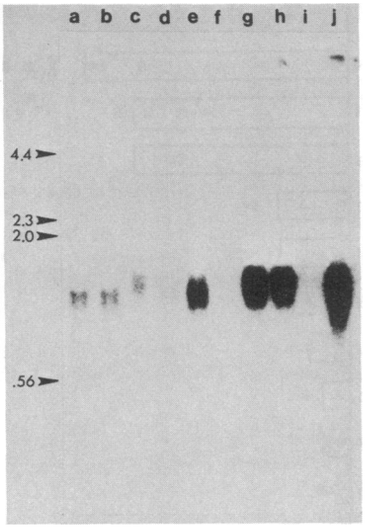

FIG. 4. Autoradiogram of a RNA blot hybridization. Poly(A)$^+$ RNA samples are from the rabbit unless otherwise specified. The positions of standards are indicated by arrows, and numbers are their sizes in kb. Lanes: a, cow iris (2 μg); b, dog iris (1.5 μg); c, dorsal root ganglion (2.6 μg); d, kidney (5 μg); e, iris (2 μg); f, duodenum (5 μg); g, heart ventricle (2.6 μg); h, heart atrium (2.6 μg); i, lung (5 μg); j, spleen capsule (5 μg).

DISCUSSION

Detection of NGF mRNA. To determine whether sympathetic (and sensory) effector organs are capable of synthesizing NGF, we developed an assay based on the RNA blot hybridization technique (21), which is capable of detecting extremely low levels of NGF mRNA. This assay appears to detect authentic NGF mRNA for the following reasons. First, the hybridization and washing conditions were very stringent. Second, the major bands of RNA that hybridized under these conditions comigrated with authentic NGF mRNA from male mouse submaxillary and rabbit prostate glands. Finally, under conditions of similar stringency, Southern blots of genomic DNA from the species used showed single hybridizing bands, implying that there is only one gene capable of hybridizing under these conditions. Although we have not used pure NGF mRNA to calibrate this assay, the values obtained for male mouse submaxillary gland mRNA agree closely with estimates obtained from the frequency of occurrence of NGF cDNA in a submaxillary gland cDNA library (13) and with the relative rate of synthesis of the protein in the same gland (26). The standard curves obtained using serial dilutions of DNA and RNA are linear over at least 3 orders of magnitude; therefore, the assay should give reliable estimates for the relative amounts of NGF mRNA in samples of poly(A)$^+$ RNA.

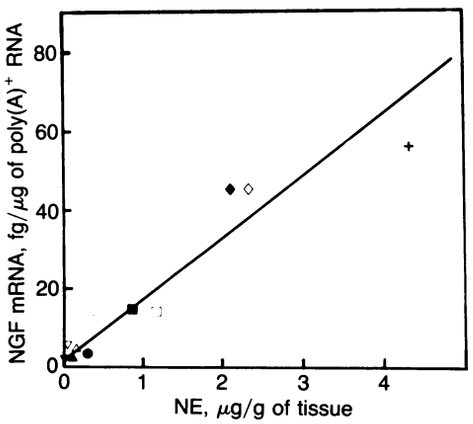

FIG. 5. Correlation of NGF mRNA content with norepinephrine (NE) content in organs (wet weight) of the rabbit. The line is drawn from a linear least-squares fit to the experimental data (r^2 = 0.95). The NE values are from ref. 25. In a few organs, the NE level has not been measured in the rabbit. In those cases, levels of rabbit NGF mRNA are plotted against means of NE levels in the same organs of other species, which are listed in parentheses. ▼, Lung; ▽, muscle (rat, dog, cat, and cow); ▲, liver; △, kidney; ●, duodenum; ○, aorta; ■, spleen; □, submaxillary gland (rat, dog, cat, and cow); ◆, heart ventricle; ◇, heart atrium; +, iris (rat and cat).

NGF mRNA in Sympathetic Effector Organs. The major result in this paper is the unambiguous detection of NGF mRNA in sympathetic effector organs, thereby extending earlier work with sensitive antigen assays that demonstrated the presence of NGF in some of these same tissues, retrograde transport of endogenous NGF, and substantial accumulation of NGF in the sympathetic ganglion (9, 10). In the rat atrium, *ca.* 1.0 ng of NGF was detected per g (wet weight) (9, 10). Assuming 10% of the atrium wet weight is protein, NGF is *ca.* 1×10^{-8} of the total protein. In contrast, NGF mRNA is *ca.* 3×10^{-7} of the total poly(A)$^+$ RNA in this tissue. Thus, the proportion of mRNA encoding NGF appears to be about 30-fold higher than the proportion of NGF. This may reflect the loss of NGF due to secretion. NGF mRNA levels in sympathetic effector organs seem to be high enough to direct the synthesis of all the NGF found in these tissues. Since NGF cannot be detected in normal serum (9), all the NGF in a target is probably synthesized locally.

Our results suggest that there is a correlation of NGF mRNA content with sympathetic innervation in a variety of tissues. To clarify this relationship, we sought some parameter that would indicate relative innervation density in the various organs. In most tissues of the body, norepinephrine is localized in the varicosities of sympathetic nerves and, therefore, provides an assay for the level of sympathetic innervation. The striking correlation between norepinephrine content and NGF mRNA content (Fig. 5) is unlikely to reflect simply a direct relationship *per se* because the adrenal gland, which has a high level of norepinephrine not due to sympathetic innervation, has a relatively low level of NGF mRNA. It seems most plausible that both the NGF mRNA content and the norepinephrine content parallel the density of sympathetic innervation.

The strong correlation between NGF mRNA levels and estimated densities of sympathetic innervation in sympathetic targets suggests that the level of NGF synthesis may limit the density of this innervation in adult tissues. In support of this possibility, systemic application of NGF is known to induce a general hypertrophy in sympathetic innervation (e.g., ref. 27). Furthermore, anti-NGF has been shown to inhibit, and NGF to promote, the growth of sympathetic fibers into transplanted sympathetic targets (27, 28). It should be interesting to see whether increases in expression of the NGF gene precede increases in sympathetic innervation during development. While NGF is likely to be important, it is clearly not the only factor important in regulating innervation (e.g., ref. 29).

It is perhaps surprising that the level of NGF mRNA correlates so well with the density of sympathetic innervation, since virtually all of these tissues are also innervated by sensory neurons, some of which also have a high-affinity retrograde transport system for NGF (e.g., ref. 7). If neurons efficiently remove secreted NGF, the two neuronal populations should compete for available NGF. In this case, levels of NGF mRNA should correlate with the combined innervation density of these two neuronal types. Unfortunately, there is no way to measure the innervation density of the subset of sensory neurons that transports NGF.

The cell types that synthesize NGF in sympathetic targets

are currently not known. Although we have not measured the amount of poly(A)$^+$ RNA per cell in these tissues, conventional estimates are on the order of 2×10^5 poly(A)$^+$ RNA molecules per cell (cf. ref. 30). This suggests that only a small percentage of cells can contain NGF mRNA at any one time, even in a richly innervated tissue such as the rat atrium, where the NGF mRNA is about 3 parts in 10^7 of the poly(A)$^+$ RNA. It is unknown whether every cell expresses the NGF gene a small fraction of the time or whether a small percentage of the cells makes NGF all of the time. There is evidence suggesting that many cell types make NGF *in vitro* (see ref. 11).

Regulation of NGF Gene Expression. NGF accumulates in many tissues that are either denervated *in vivo* or explanted into culture medium (11, 12). Either increased synthesis or the elimination of the retrograde transport system, or both, provided plausible explanations for these results. In this paper, we demonstrate a *ca.* 7-fold increase in NGF mRNA levels in explanted rat irides. Preliminary experiments have detected an increase within 1 hr after explantation that persists for at least 40 hr (unpublished data), which suggests that the increased accumulation of NGF observed in denervated or explanted irides reflects, at least in part, an increase in its rate of synthesis.

NGF mRNA in Other Elements of the Peripheral Nervous System. Comparatively large amounts of NGF mRNA were found in peripheral elements of the nervous system. Sensory ganglia, sympathetic ganglia, and sciatic nerve appear capable of synthesizing some NGF in adult animals. Since these tissues have a small mass compared to that of sympathetic targets, this does not mean that sympathetic or sensory neurons normally derive much of their NGF content from the nerve or ganglia. Axotomy results in a large decrease in the level of NGF in the sympathetic ganglion (10), providing direct evidence that the bulk of this trophic factor is not derived from the vicinity of the ganglion. However, local synthesis of NGF may partially explain why treatments such as axotomy and chemical sympathectomy do not have irreversible effects on adult sympathetic neurons, even though they prevent retrograde transport from targets (e.g., ref. 5).

NGF mRNA in the Brain. Mammalian brains have surprisingly high levels of mRNA encoding NGF (Fig. 3), considering that antibodies to this trophic factor have not been reported to have effects on neurons in the central nervous system (e.g., ref. 4). This NGF mRNA cannot reflect sympathetic innervation of the pineal, since this gland was removed from the brain tissue. It is also not likely to be associated exclusively with the vasculature. The norepinephrine content of brain is low, comparable to that of the duodenum, and very little of that content is due to sympathetic innervation (cf. ref. 25). If all of the brain norepinephrine were due to sympathetic innervation, the results in Fig. 5 would predict *ca.* 7 fg of NGF mRNA per μg of poly(A)$^+$ RNA. In fact, 4-fold more NGF mRNA is seen. Moreover, preliminary experiments have detected large differences in the level of NGF mRNA in different brain regions (unpublished data). Interestingly, the hippocampus seems to have particularly high levels of NGF mRNA, and this is one area where there is evidence suggesting that NGF may have a physiological role (e.g., ref. 31).

Other NGF Transcripts. In several tissues of the rat, but not other mammalian species examined, [^{32}P]NGF probes reproducibly hybridize to transcripts of two different sizes. While the smaller transcript of *ca.* 1300 bases is the same size as the NGF mRNA detected in tissues of other species, the larger one of *ca.* 1700 bases (never >20% of the smaller band) does not correspond to any NGF transcript detected elsewhere. Similar amounts of this band are seen whether or not poly(A)$^+$ RNA is purified, so it is not likely to be ribosomal RNA. While it seems most likely to us that the larger RNA also represents a transcript of the NGF gene, further analysis will be needed to determine definitively the relationship between this transcript and the more common NGF mRNA.

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