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Studies on the expression of the β nerve growth factor (*NGF*) gene in the central nervous system: Level and regional distribution of NGF mRNA suggest that NGF functions as a trophic factor for several distinct populations of neurons

(RNA blot hybridization)

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ABSTRACT **β nerve growth factor (NGF), a target-derived protein necessary for survival and development of sympathetic and sensory neurons, can also affect subpopulations of neurons in the central nervous system (CNS). Using a blot hybridization assay capable of detecting 10 fg of mRNA, we measured the levels of NGF mRNA in the major brain regions, including those innervated by NGF-responsive neurons. NGF mRNA was detected unambiguously in each major region of the CNS. The levels were comparable to those in sympathetic effector organs. Discrete areas contained very different amounts of NGF mRNA. Up to 40-fold differences were seen, a range comparable to the differences between richly and sparsely innervated sympathetic effector organs. The highest concentrations of NGF mRNA were found in the cortex and hippocampus, which are the major targets of the NGF-responsive cholinergic neurons of the basal forebrain nuclei. Significant amounts of NGF mRNA were also found in areas that contain the central processes of NGF-responsive sensory neurons, such as the pons, medulla, and spinal cord. The presence of NGF mRNA in these areas suggests that brain NGF may act as a target-derived trophic factor for both populations of neurons. NGF mRNA was also found in the striatum, suggesting that locally derived NGF may act there as a trophic factor for a recently identified population of NGF-responsive cholinergic local circuit neurons. However, high levels of NGF mRNA were also found in some regions, such as the diencephalon, that have no relation to any identified population of NGF-responsive neurons. This suggests that there may be additional populations of NGF-responsive neurons in the CNS that have not yet been discovered.**

During development, neurons respond to extrinsic signals that affect many aspects of their development. Prominent among these are trophic factors that, in many cases, appear to be synthesized by the target tissues innervated by responsive neurons (see ref. 1).

β nerve growth factor (NGF) is the only identified trophic factor that has been shown to be important *in vivo*, where it is required for the survival and development of embryonic sympathetic and sensory neurons (see ref. 2). Its importance *in vivo* was demonstrated with antibodies to NGF, which can cause almost complete destruction of both classes of neurons (cf. refs. 3 and 4). Several observations support the proposal that these neurons obtain NGF from their target tissues. First, interrupting the connections of the neurons with their peripheral targets has similar effects as treatment with antibodies to NGF (cf. refs. 5–7). Second, responsive neurons have NGF receptors on their surfaces and are able to transport NGF from their terminal fields to their cell bodies (see ref. 2). More recently, low amounts of endogenous NGF have been detected in sympathetic effector organs (8). This endogenous NGF is transported retrogradely to sympathetic ganglia where maintenance of normal levels of NGF requires intact connections with the periphery (9). The NGF in target tissues also appears to be synthesized there. First, sufficient NGF mRNA has been detected in all targets examined to account for local synthesis of the NGF present (10, 11). Second, the amount of NGF mRNA in a given target correlates with the amount of NGF and density of sympathetic innervation (10–12).

In addition to modulating neuronal survival, NGF also regulates several facets of neuronal development. In particular, NGF increases the levels of appropriate neurotransmitters and neurotransmitter biosynthetic enzymes in both sympathetic and sensory ganglia (13–15). NGF remains able to regulate the level of substance P in postnatal sensory ganglia, which no longer appear to require NGF for survival (4, 15).

The observation that neuronal cell death occurs normally in the central nervous system (CNS) and can be increased by interrupting neuronal connections has made it seem probable that trophic factors are also important in regulating development of central neurons (see ref. 1). In the past few years, exogenous NGF has been shown to affect several subpopulations of neurons in the CNS, suggesting that endogenous NGF might serve as a trophic factor in the brain. Prominent among these are the cholinergic cells of the basal forebrain nuclei. The neurons of the septal nuclei and the nucleus of the diagonal band send a massive cholinergic projection to the hippocampus (16), and the neurons of the nucleus basalis have a widespread projection to the cortex (16, 17). This set of neurons is reported to be important in memory acquisition and is known to degenerate in Alzheimer disease (reviewed in ref. 18). These neurons are able to retrogradely transport exogenous NGF from injection sites in the hippocampus and cerebral cortex (19, 20), and treatment of them with NGF either *in vitro* (21, 22) or *in vivo* (23, 24) leads to an increase in their choline acetyltransferase (CATase; acetyl-CoA:choline *O*-acetyltransferase, E.C. 2.3.1.6) activity. However, NGF has never been shown to affect the survival of these neurons (22, 23). Other regions of the CNS also contain NGF-responsive neurons. The cholinergic neurons of the neonatal striatum also respond to exogenous NGF by increasing their level of CATase activity (25). The spinal cord and hindbrain also contain projections from neurons that are

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Abbreviations: NGF, β nerve growth factor; CNS, central nervous system; CATase, choline acetyltransferase; CBFN, cholinergic basal forebrain nuclei; kb, kilobase(s).

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responsive to NGF, the central processes of the spinal sensory ganglia, and the neural crest-derived cranial sensory ganglia (26, 27). Spinal sensory ganglia clearly obtain trophic support, possibly including NGF, via their central processes (28).

Although these results suggest that endogenous NGF may act as a trophic factor in the CNS, there has been little information available on its distribution there. We previously reported the existence of high levels of NGF mRNA in brain (10, 11, 29). We detected 20-fold more NGF mRNA in brain than in other tissues with similar densities of sympathetic innervation, such as kidney and liver. It thus seemed likely to have functions in addition to maintaining sympathetic innervation of the vasculature.

Since the vasculature and its accompanying innervation are quite evenly distributed throughout the CNS (30), while quite limited and discrete populations of CNS neurons have been found to respond to NGF (cf. ref. 19), it seemed probable that determining the regional distribution of NGF mRNA would be helpful for clarifying the possible trophic functions of NGF in the CNS. The results of our examination are reported here. The level of NGF mRNA varies dramatically in different brain regions. There appears to be sufficient NGF mRNA to promote the synthesis of physiologically significant amounts of NGF in the projection fields of all of the identified NGF-responsive neurons in the CNS. The results thus support the possibility that brain NGF affects the development of these populations of neurons.

Our preliminary results indicating regional variation in NGF mRNA have been reported in abstract form (29). Since the completion of the work in this paper, another paper reporting the detection of NGF mRNA in several regions of the rat brain has appeared (31).

MATERIALS AND METHODS

Materials. Adult Sprague–Dawley rats obtained from Bantin–Kingman (Fremont, CA) were used for all experiments. Enzymes were purchased from New England Biolabs. [α-^{32}P]dCTP (3000 Ci/mmol; 1 Ci = 37 GBq) was purchased from Amersham. All other supplies were reagent grade or the best commercially available.

Methods. Initial experiments showed that there were no obvious differences between males and females in the level of NGF mRNA in brain regions, so data from both sexes have been pooled. Rats were sacrificed by cervical dislocation, and the brain and spinal cord were quickly removed to ice-cold saline. The following regions were then dissected from the brains while they were maintained on ice: olfactory bulb, cerebellum, medulla, pons, midbrain, diencephalon, the septal area, striatum, hippocampus, and cortex. The cortex was further divided along the rhinal fissure, that part dorsal of the fissure being called neocortex and that part ventral of the fissure being called pyriform–entorhinal. The regions were homogenized in 5 M guanidinium thiocyanate/5% 2-mercaptoethanol/10 mM EDTA/50 mM Na Hepes, pH 7.4, as they were dissected. The procedures for preparing RNA, purifying poly(A)$^+$ RNA by two cycles of chromatography on oligo(dT)-cellulose, separating the poly(A)$^+$ RNA in formaldehyde-containing agarose gels, transfer of RNA to nitrocellulose, and hybridization have been described (10). As described in ref. 10, a ^{32}P-labeled single-stranded cDNA probe, derived from the mouse NGF cDNA characterized in ref. 32, was used to detect NGF mRNA. In a modification of our previous procedure (10), a densitometer was used to scan autoradiograms of the hybridized blots. Comparative levels were determined by comparison to standard curves of male mouse submaxillary gland poly(A)$^+$ RNA that were run on the same gel. Absolute levels were calculated from the relative levels by adjusting for the fraction of this RNA standard that was NGF mRNA. This standard RNA contained 0.08% ± 0.03% NGF mRNA, as determined by comparison to cloned NGF single-stranded cDNA (10). To confirm this, known quantities of single-stranded NGF cDNA were also run on these gels and were used to construct similar standard curves.

RESULTS

The assay for NGF mRNA, which is based on hybridization of RNA blots to a ^{32}P-labeled single-stranded cDNA probe (10, 29), has been modified by using densitometry of autoradiograms to provide more convenient and reliable measurements of low levels of NGF transcripts. A standard curve obtained with the modified assay is shown in Fig. 1. The standard curves obtained by densitometry of serial dilutions of poly(A)$^+$ RNA were linear for at least an order of magnitude for each autoradiogram, and values were always determined from exposure times that yielded signals within this range. Since there was 0.08% NGF mRNA in the submaxillary gland RNA preparation used for this standard curve (cf. ref. 10), the limit of sensitivity for the overnight exposure shown in Fig. 1 is ≈30 fg of NGF mRNA. Longer exposures gave better sensitivity. The lower limit that was consistently quantifiable was ≈8 fg. This corresponds to ≈10,000 molecules of NGF mRNA.

When poly(A)$^+$ RNA was prepared from different regions of the rat CNS and assayed, NGF mRNA was unambiguously detected in each area examined. A typical autoradiogram obtained after an overnight exposure is shown in Fig. 2. The major band detected in poly(A)$^+$ RNA from each region of the rat brain had an apparent size of 1.3 kilobases (kb) and migrated at the same position as NGF mRNA from male mouse submaxillary gland and NGF mRNA from peripheral sympathetic targets (10). A 1.7-kb band, which was previously described in peripheral sympathetic effector organs of the Sprague–Dawley rat (10), was also detected in all rat brain regions that had very dense 1.3-kb bands. In several measurements, this more slowly migrating band averaged ≈15% of the hybridization signal of the 1.3-kb band, but it was not included in calculations of NGF mRNA levels.

The amounts of NGF mRNA in different regions of the rat CNS were measured and the results are presented in Fig. 3. Although NGF mRNA was found to be present in all brain regions tested, its level varied over a 40-fold range. In general, cortical areas had the highest concentration of NGF mRNA—at least 90 fg per μg of poly(A)$^+$ RNA. The level was

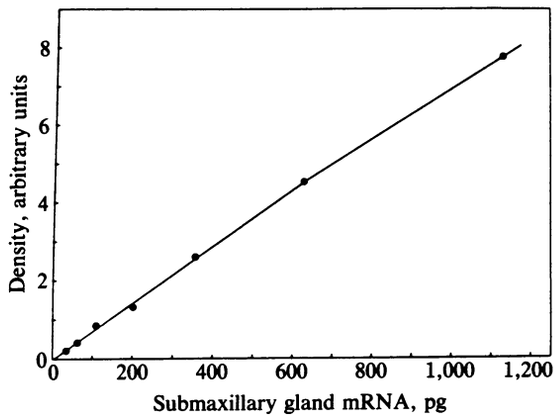

FIG. 1. Example of standard curve of NGF mRNA assay obtained by densitometry of autoradiogram. Autoradiographic density was obtained by an integration of a scan covering the band of interest. Line was drawn by eye. Of the total poly(A)$^+$ RNA in male mouse submaxillary gland, 0.08% is NGF mRNA (10).

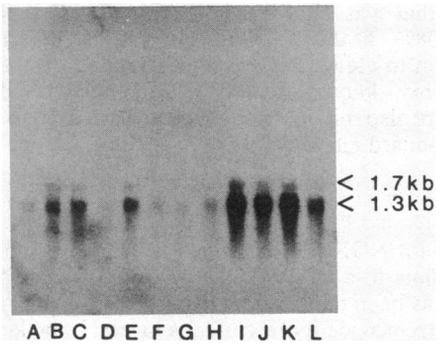

FIG. 2. Autoradiogram of RNA blot hybridization to regions of the rat CNS. Arrows and numbers indicate the molecular size of the hybridizing bands. Region run in each lane with amount of poly(A)$^+$ RNA in parentheses is as follows: lane A, spinal cord (6.0 μg); lane B, medulla (6.1 μg); lane C, pons (7.9 μg); lane D, midbrain (10.2 μg); lane E, diencephalon (10.4 μg); lane F, cerebellum (19.9 μg); lane G, septal area (4.1 μg); lane H, striatum (8.2 μg); lane I, hippocampus (7.4 μg); lane J, pyriform-entorhinal (9.1 μg); lane K, neocortex (13.8 μg); lane L, olfactory bulb (11.6 μg).

highest in hippocampus (archaecortex), followed, in order, by pyriform–entorhinal cortex, and neocortex. The level of NGF mRNA found in the hippocampus is about two-thirds that found in the rat heart atrium, which has a dense sympathetic innervation (10, 11). The pons, medulla, diencephalon, and olfactory bulb had levels around 50 fg/μg, whereas the spinal cord and striatum contained $\approx$20 fg/μg. The midbrain, septal area, and cerebellum contained the least NGF mRNA of any region examined. The cerebellum had the lowest observed content—5 fg/μg. Given the high level of NGF mRNA and the relatively large size of the region, cortical areas contain >75% of the NGF mRNA in the CNS, with neocortex alone accounting for >40% of the total NGF mRNA in brain (Fig. 4).

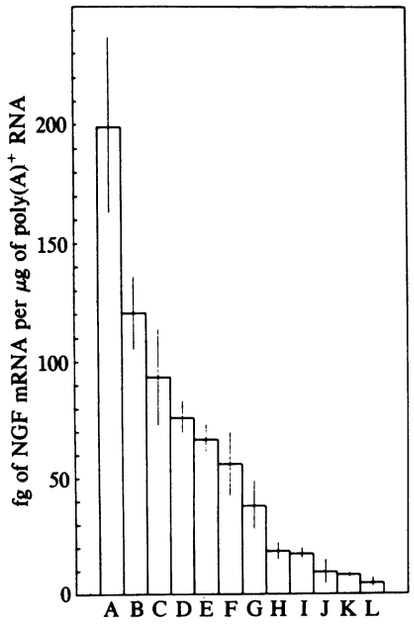

FIG. 3. Levels of NGF mRNA in regions of the CNS. The amount of NGF mRNA per μg of poly(A)$^+$ RNA is indicated in femtograms. Bars are the means of determinations done on two independent sets of dissections and RNA preparations. The vertical lines are the ranges of those determinations. A, hippocampus; B, pyriform entorhinal; C, neocortex; D, pons; E, medulla; F, diencephalon; G, olfactory bulb; H, spinal cord; I, striatum; J, midbrain; K, septal area; L, cerebellum.

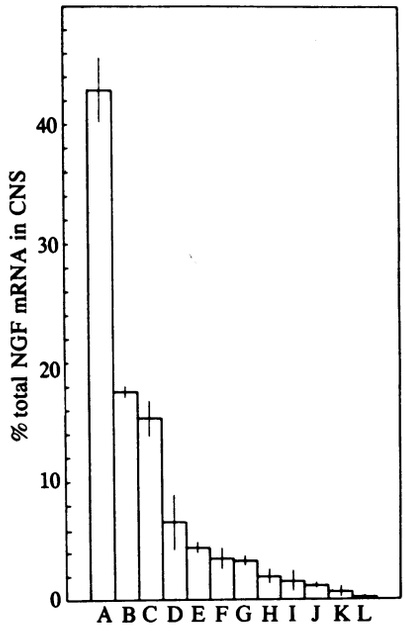

FIG. 4. Contribution of regions to the total NGF mRNA content of the rat CNS. Content of each region of the CNS is displayed as a percentage of the total content of NGF mRNA in the entire CNS. Bars and vertical lines are as in Fig. 3. In the two preparations, the total CNS content was 3.4 and 2.3 pg. A, neocortex; B, pyriform entorhinal; C, hippocampus; D, diencephalon; E, pons; F, spinal cord; G, medulla; H, olfactory bulb; I, cerebellum; J, striatum; K, midbrain; L, septal area.

The data presented substantiate the finding that there is expression of the gene for NGF in rat brain and indicate that the level of expression varies widely in different brain regions.

DISCUSSION

Assay for NGF mRNA. In this paper, we report a modification of our earlier RNA blot assay for NGF mRNA (10, 29), which permits more reliable detection of low levels of NGF transcripts. This assay has a detection limit of 8 fg of NGF mRNA, corresponding to $\approx$10,000 NGF transcripts (Fig. 1). This assay appears to detect authentic NGF mRNA in each brain region examined for the following reasons. First, the hybridization and washing conditions were very stringent. Under conditions of similar stringency, Southern blots of rat genomic DNA digested with several different restriction enzymes showed a single hybridizing band, implying that there is only one gene capable of hybridizing under these conditions (10, 11). Second, the major band of RNA that hybridized under these conditions appears to be the same size as authentic NGF mRNA from male mouse submaxillary gland. This result argues that the major NGF mRNA transcript in brain is very similar to the major transcript in male mouse submaxillary gland.

In addition to a 1.3-kb transcript of approximately the same size as the NGF mRNA in the mouse submaxillary gland, a 1.7-kb transcript that is purified by chromatography on oligo(dT)-cellulose and hybridizes to ^{32}P-labeled NGF cDNA probes has been detected in many rat sympathetic effector organs and brain regions (Fig. 2; see also ref. 10). The amount of this transcript is $\leq$25% that of the 1.3-kb transcript. It has only been observed in tissues of the Sprague–Dawley rat and not in those from mouse, rabbit, cow, or dog (10, 11). Even though it has not been included in our measurements of NGF mRNA levels, it almost certainly is a transcript derived from the *NGF* gene. First, analysis of rat genomic DNA indicates

that there is only one gene capable of hybridizing with the NGF cDNA under the conditions used (10, 11). Furthermore, the 1.3- and 1.7-kb bands appear to be coordinately regulated in the rat iris during the large increase in NGF mRNA level that is observed after the culture of explants (10, 11). Thus, it seems likely that the two transcripts are generated by selective processing of the same primary transcript.

NGF mRNA in Brain Regions. The major result of this paper is the demonstration that there is an uneven regional distribution of the extremely high levels of NGF mRNA in brain that we reported earlier (10, 29). As discussed earlier (10), this NGF mRNA cannot reflect sympathetic innervation of the pineal, since this gland was removed from the brain. It is also not likely to be associated exclusively with the vasculature or its associated sympathetic innervation. First, the vasculature is relatively evenly distributed in the brain (30), while NGF mRNA varies greatly between different regions (Fig. 3). Second, >20-fold more NGF mRNA is found in brain than in peripheral organs with similar densities of sympathetic innervation (10, 11). The NGF mRNA detected in the brain is therefore likely to function in some aspect(s) of development and maintenance of the CNS.

In all brain regions examined, there was a readily identifiable band of hybridizing RNA that migrated with authentic NGF mRNA from mouse submaxillary gland (Fig. 2), permitting the unambiguous measurement of NGF mRNA content in the major areas of the CNS (Fig. 3). The fraction of total poly(A)$^+$ RNA that encodes NGF mRNA varies by at least 40-fold between different brain regions (Fig. 3). The levels of NGF transcripts in brain are similar to those seen in peripheral sympathetic effector organs of the rat (10). The 40-fold range in values between different brain regions is also similar to the 30-fold difference seen between richly and poorly innervated peripheral sympathetic effector organs (10). The lowest level of NGF mRNA in the CNS was found in the cerebellum (Fig. 3). The level detected there is very close to that predicted from the density of the sympathetic innervation of brain vasculature (discussed in ref. 10), suggesting that NGF may have little function in the cerebellum except to maintain a normal density of sympathetic innervation of the blood vessels. In most other brain regions, however, there are levels of NGF mRNA that appear to be much higher than needed to maintain sympathetic innervation. This suggests that NGF may function as a trophic factor in many regions of the brain.

After these experiments were completed, a paper appeared that extended our initial reports of NGF mRNA in brain (10, 29) with measurements of NGF mRNA in hippocampus and neocortex and estimates of NGF mRNA in a few other areas (31). These authors also detected comparatively high levels of NGF antigen in the hippocampus and neocortex. The reported measurements of NGF mRNA in hippocampus and neocortex agree reasonably well with our observations (Fig. 3). The reported estimates of NGF mRNA in other areas differ significantly from our measurements, but seem to be at or below the detection limit of the assay. As reported, the assay used in ref. 31 appears to be more than an order of magnitude less sensitive than the assay used in this paper. This probably explains why NGF mRNA was not detected in all regions in ref. 31. In contrast to the conclusions in ref. 31, our use of the more sensitive assay has shown that the distribution of NGF mRNA is not limited to regions of the CNS that contain the projection field of the cholinergic basal forebrain neurons. These findings extend the range of possible trophic functions for NGF in the brain (Table 1).

NGF as a Trophic Factor for the Neurons of the Cholinergic Basal Forebrain Nuclei (CBFN). Cells of the CBFN respond to NGF by increasing CATase activity (23, 24) and are also capable of retrograde transport of exogenous NGF administered to their terminal fields in either the hippocampus or cortex (19, 20). The hippocampus and cerebral cortex have especially high levels of NGF mRNA (Fig. 3; Table 1) and NGF antigen (31), comparable to those seen in densely innervated peripheral sympathetic targets (10, 11), and so may be serving as a source of NGF for these CBFN neurons *in vivo*. There are comparatively high levels of NGF antigen, but not NGF mRNA in the brain regions containing the cholinergic neurons (Fig. 2; ref. 31). Recently, it has been reported in abstract form that these cholinergic cells die after separation from their targets in the hippocampus and that this effect can be prevented by application of NGF (37).

Table 1. Possible roles for NGF in the CNS

Possibly regulated neurons	Target	Target NGF mRNA level, fg/μg	Refs.
Cholinergic basal Forebrain neurons	Hippocampus	200	19, 20, 23, 24
	Neocortex	95	
	Olfactory bulb	40	
Cholinergic striatal neurons	Striatum	20	25, 33
Spinal sensory neurons	Spinal cord	20	34, 35
Cranial sensory neurons	Pons	75	27, 36
	Medulla	65	
?	Diencephalon	55	

Indirect evidence that the hippocampus and cortex may serve as endogenous sources of NGF has been obtained by examining the effects of lesions of the CBFN. After such lesions, sympathetic fibers associated with the brain vasculature sprout into the neuropil of the hippocampus and cortex (38, 39). This outgrowth of processes by sympathetic neurons is similar to that induced by exogenous NGF (40), and antiserum to NGF has been reported to block the sympathetic sprouting seen in the hippocampus after lesions of the septal nuclei (41).

It is not known which cell types in the hippocampus and cortex contain NGF mRNA. The sympathetic sprouting caused by the cholinergic denervation of the hippocampus occurs in the absence of either hippocampal pyramidal cells or granule cells (42), suggesting that non-neuronal cells may be one source of NGF mRNA in the brain. Furthermore, cultured astrocytes appear to produce NGF (43). The results from experiments done *in vitro* should be interpreted with caution, however, as large changes in the content of NGF mRNA occur within hours of explant of at least one tissue, the rat iris (10, 11). Since the NGF mRNA content of this tissue increases by over an order of magnitude *in vitro*, the same cell types may not contain NGF mRNA *in vivo* and *in vitro*. Whatever cell types contain the NGF mRNA, the levels detected in hippocampus and cortex almost certainly correspond to considerably less than one molecule of NGF message per cell. Although we have not measured the number of poly(A)$^+$ RNA molecules per cell in the hippocampus and cortex, conventional estimates are on the order of 2×10^5 molecules per cell (44). Using this estimate, one copy of NGF mRNA per cell would correspond to 5000 fg of NGF mRNA per μg of poly(A)$^+$ RNA, ≈25-fold higher than the level seen in the hippocampus. This suggests that, at any one time, only a small subset of cells in the hippocampus and other brain regions contains NGF mRNA. It is not known whether a small percentage of cells express the NGF gene constantly, or if many cells express the gene for short periods of time.

NGF as a Trophic Factor for Other Cells in the CNS. The presence of comparatively high levels of NGF mRNA in the

hippocampus and cortex (Fig. 3) suggests that these tissues are synthesizing equivalent levels of NGF protein. Recently, levels of NGF protein equivalent to those seen in richly innervated sympathetic effector organs have been detected in both of these brain areas (31). This supports the possibility that NGF acts as a trophic factor for the cells of the cholinergic basal forebrain nuclei. However, the large amounts of NGF mRNA detected in other areas of the CNS argue that the functional role of NGF in the brain may not be limited to this system. There are two other systems at least partly in the CNS that are known to respond to applications of NGF, and one area of the brain that contains a high level of NGF mRNA but is unrelated to any known NGF-responsive cells (listed in Table 1).

In a manner similar to the neurons of the CBFN, the cholinergic neurons of the neonatal rat striatum also show an increase in the levels of CATase activity after the administration of exogenous NGF *in vivo* or *in vitro* (25, 33). These cells appear to be local circuit neurons (45), so if endogenous NGF is involved in the control of this enzyme *in vivo*, the NGF must be locally available. The level of NGF mRNA in the adult rat striatum is 3- to 4-fold greater than that in the cerebellum, but it is not high relative to many other brain areas (Fig. 3; Table 1). It will be interesting to see if the level of NGF mRNA in the striatum is higher early in development when the neurons are known to be sensitive to the presence of NGF (25). It is not known if adult striatal neurons respond to NGF.

There are populations of peripheral neurons known to respond to NGF whose processes invade regions of the CNS. For instance, sensory neurons respond to NGF throughout life and are capable of retrograde transport of NGF via their central processes in the spinal cord (34, 35). Furthermore, it has been shown that sensory neurons require trophic support from their central projections, although the molecule(s) mediating this support remains unknown (28). The finding that the spinal cord has significant quantities of NGF mRNA (Fig. 3; Table 1) implies that it may be synthesizing NGF *in vivo* and thus might serve as an endogenous central source of NGF for the spinal ganglia. It is possible that this centrally supplied NGF may underlie at least some of the trophic support that sensory ganglia obtain from their central processes. Other centrally supplied factors may also be required by sensory neurons (46). The NGF mRNA that is present in the pons and medulla may likewise supply the central projections of the cranial ganglia, which are known to have NGF receptors (27, 36). The pons and medulla have higher levels of NGF mRNA than the spinal cord, so it is possible that these hindbrain areas are also serving as a source of NGF for other populations of neurons.

The NGF mRNA that is present in some areas of the brain does not correspond to the projection field of any neurons that are known to respond to NGF (Table 1). For example, the diencephalon has large amounts of NGF mRNA, yet it does not receive a described projection from the cholinergic basal forebrain neurons (16, 17), the NGF-responsive cholinergic cells of the striatum (45), or primary sensory neurons. Considering the limited effects of NGF and anti-NGF upon neurons in the cholinergic basal forebrain neurons, it seems likely that there are populations of NGF-responsive neurons in the CNS that remain unknown. The knowledge of the regional localization of NGF mRNA in the brain may prove helpful in their discovery.

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