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Developmental and regional expression of β nerve growth factor messenger RNA and protein in the rat central nervous system

(trophic factors/brain/RNA hybridization/fetal nerve growth factor)

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ABSTRACT **The presence of nerve growth factor (NGF) mRNA and protein in the rat central nervous system is documented. Blot-hybridization analysis showed an abundance of NGF mRNA in the hippocampus, cerebral cortex, and olfactory bulb. Enzyme immunoassay confirmed significant levels of a NGF-like protein in the hippocampus and cerebral cortex. Bioassay of a NGF-like immunoaffinity-purified protein from these regions was physiologically indistinguishable from NGF. Immunohistochemistry revealed a widespread distribution of NGF-like reactivity in the adult brain, preferentially in fiber tracts. NGF mRNA accumulation began at birth, with adult levels reached 3 weeks postnatally. Enzyme immunoassay detected the presence of a NGF-like protein in the embryonic rat brain. Postnatally, the level of NGF-like protein reached a maximum at 3 weeks. Additionally, a distinct fetal form of NGF may exist.**

β nerve growth factor (NGF) is essential for the development and maintenance of sensory and sympathetic neurons in the peripheral nervous system (PNS) (1, 2). However, both the presence and potential physiological function of NGF in the central nervous system (CNS) has remained elusive (3). The characteristic responses of peripheral adrenergic neurons following NGF or anti-NGF antibody injection have not been observed after similar application into the CNS (4–11).

In the PNS, NGF is taken up by adrenergic terminals and also retrogradely transported to the cell body (2). In the CNS, injected NGF was specifically retrogradely transported from the hippocampus to the medial septum and the diagonal band of Broca (9) and also from the neocortex to the nucleus basalis (12), presumably in cholinergic neurons. NGF induced choline acetyltransferase in neonatal rat cortex, hippocampus, septum (10), and striatum (11), but not in adult hippocampus (13).

Thus, NGF may have a physiological function in the CNS, and affect cholinergic neurons. Initial indication of endogenous NGF in the CNS was obtained by immunohistochemistry in the fetal rat (14, 15). NGF cDNA clones have been isolated from male mouse submaxillary glands (16, 17), and Shelton and Reichardt (18) recently detected NGF mRNA in the rat brain. Subsequently, NGF mRNA and an NGF-like protein have been described in some regions of the adult rat brain (19).

In the present study, we demonstrate the developmental time course as well as the adult regional distribution of both NGF mRNA and protein in the adult rat brain. Finally, using a bioassay, we show that the adult brain contains nerve growth-stimulating activity typical of purified NGF. Our results demonstrate that NGF is expressed in the adult rat CNS with regional specificity and suggests the existence of a fetal form of NGF.

MATERIALS AND METHODS

Preparation of RNA and Blot-Hybridization Analysis. Total RNA from the brains of Sprague–Dawley rats (Alab, Stockholm, Sweden) was prepared (20), and poly(A)$^+$ RNA, isolated by oligo(dT) chromatography (21), was separated on 1% agarose gels containing 0.7% formaldehyde and transferred to nitrocellulose filters. The double-stranded DNA probe used to detect β-NGF (hereafter referred to as NGF) was a 900-base-pair (bp) *Pst* I fragment derived from a NGF cDNA clone (16); the probe to detect c-myc was 1.3-kilobase (kb) *Cla* I–*Eco*RI fragment from the 3′ exon of the human *MYC* gene (22); and the probe to detect α-actin was a 1.5-kb *Pst* I DNA fragment (23). Purified DNA fragments were nick-translated to a specific activity of $\approx 10^9$ cpm/μg, hybridized to the blotted nitrocellulose filters overnight, and washed at high stringency. Filters were exposed on Kodak XAR-5 x-ray film at −80°C. Known dilutions of poly(A)$^+$ RNA from male mouse submaxillary glands were always included as standards. Autoradiograms were quantified by densitometry [Shimadzu (Kyoto, Japan) CS-390].

Immunoaffinity Chromatography of Rat Brain NGF. Cortex and hippocampus (100 g wet weight) from 100 adult female Sprague–Dawley rats were homogenized in 0.1 M Tris·HCl, pH 8.0/0.5 M NaCl and centrifuged. The supernatant was applied to a 3-ml column of affinity-purified anti-mouse NGF antibodies coupled to CNBr-activated Sepharose (Pharmacia). The column was washed extensively, and bound NGF was eluted with 0.1 M glycine·HCl (pH 2.5) containing 0.02% bovine serum albumin, concentrated to 4 ml in the culture medium used for bioassay by pressure dialysis, sterilized by filtration, and tested for NGF activity.

Quantitation of NGF in Rat Brain. *Explanted ganglia bioassay.* Chicken sympathetic ganglia (day 9 embryos) were explanted into a collagen matrix, and the rat brain immunoelutant was tested for NGF activity (24, 25). Cultures were examined with dark-field microscopy and scored for fiber outgrowth.

Enzyme-linked immunoassay for NGF. Immunoplates (96-well black Microfluor plates, Dynatech, Alexandria, VA) were coated with affinity-purified antibodies against mouse NGF (25–27), and nonspecific binding was blocked with 1% bovine serum albumin. Brain samples in TBS buffer (0.02 M Tris·HCl, pH 7.5/0.5 M NaCl) containing aprotinin, 0.1 mM phenylmethylsulfonyl fluoride, 0.1% bovine serum albumin, 10 mM EDTA, and 0.5% Tween 20 were added to the wells

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Abbreviations: NGF, nerve growth factor; PNS, peripheral nervous system; CNS, central nervous system; bp, base pair(s); kb, kilobase(s).

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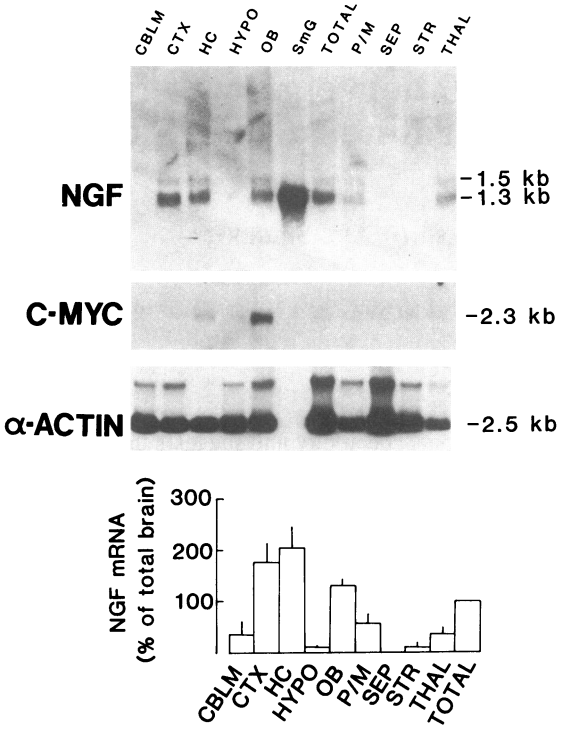

FIG. 1. Regional expression of NGF mRNA in the adult CNS. (*Upper*) Poly(A)$^+$ mRNA (15 μg) from the indicated adult brain regions and 80 ng of male mouse submaxillary gland poly(A)$^+$ RNA were probed with NGF, c-*myc*, and actin DNA probes. The same filters were used for all three probes. (*Lower*) Quantitation of relative amounts of NGF mRNA in adult brain (mean ± SEM of 3–6 independent experiments). CBLM, cerebellum; CTX, cerebral cortex; HC, hippocampus; HYPO, hypothalamus; OB, olfactory bulb; SmG, male mouse submaxillary gland; TOTAL, whole brain; P/M, pons–medulla; SEP, septum; STR, striatum; THAL, thalamus.

and incubated overnight at 4°C. Purified mouse NGF was used as a standard. Plates were then washed overnight at room temperature. To measure bound NGF, an anti-NGF antibody-β-galactosidase conjugate and 4-methylumbelliferyl-β-galactoside as a fluorigenic substrate were used. The reaction was followed in a Dynatech Microfluor plate reader. The subtracted background was the enzyme activity measured in parallel wells coated with the NGF antiserum immunoglobulins not absorbed by the NGF-Sepharose.

NGF Immunohistochemistry. Coronal sections of adult brain (2 mm) were fixed by immersion for 2 hr in 0.2% parabenzoquinone/2% formaldehyde/0.05 M phosphate buffer and stored in 0.05 M phosphate buffer/10% sucrose at 4°C until 15-μm-thick coronal sections were cut on a cryostat. Indirect immunofluorescence with the same antibodies as those used in enzyme-linked immunoassay was used (28). Controls included exclusion of the first antibody, specimens incubated with anti-mouse NGF preabsorbed with NGF and adult male mouse submaxillary gland sections as internal controls for immunoreactivity.

Fimbria Transections. Adult animals were anesthetized with halothane, and the fimbria were stereotactically transected bilaterally.

RESULTS

Detection of NGF-Specific mRNA. The 900-bp *Pst* I fragment encoding male mouse submaxillary gland NGF (17) hybridized to a 1.3-kb poly(A)$^+$ RNA species in both the mouse submaxillary gland and the rat brain (Fig. 1). No hybridization to the NGF probe was seen in the poly(A)$^-$ fraction when equal amounts of brain poly(A)$^+$ and poly(A)$^-$ RNA were analyzed. An additional band was seen at 1.5 kb only in the rat brain, which comprised 20–25% of the total hybridization signal. We isolated a rat genomic NGF clone from a Charon 4A phage library, and we used as a hybridization probe a 1.2-kb *Bgl* II–*Pvu* II fragment encoding the major part of the rat NGF mRNA. The hybridization pattern, both in the adult regions and developmentally in the total brain did not change when this homologous DNA probe was used (data not shown). Thus, this 1.5-kb band appeared to be specific for NGF and was included in the quantitation of NGF mRNA expression.

Expression of NGF mRNA and Protein in Adult Rat Brain. The differential expression of NGF mRNA in the adult rat brain is shown in Fig. 1. All values are normalized to the levels found in adult total brain, which contained NGF mRNA at 1/450th that in the male mouse submaxillary gland.

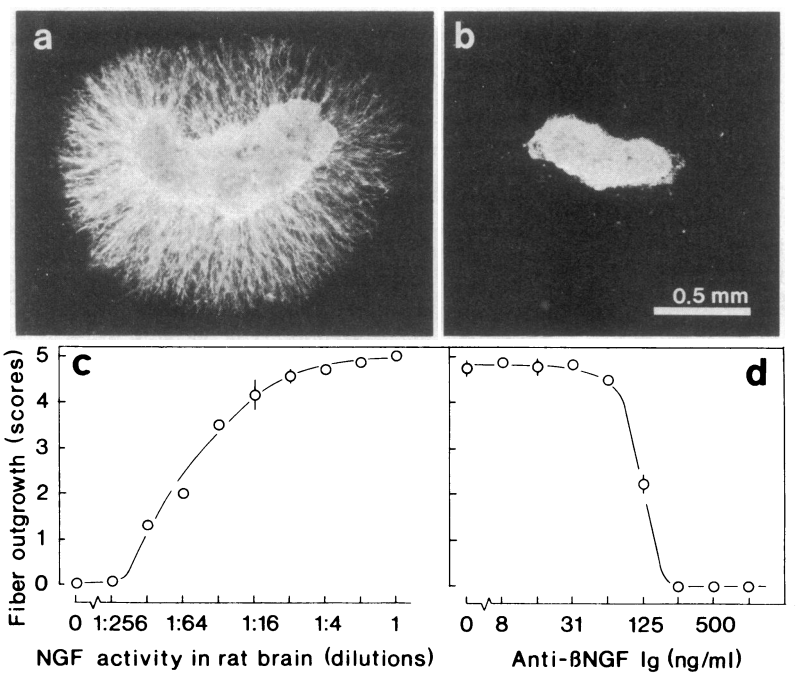

FIG. 2. Detection of endogenous NGF in the adult rat CNS. (*a*) Dark-field micrograph of sympathetic fiber outgrowth evoked by an immunopurified extract of hippocampus and cortex after 2 days in culture. (*b*) Same concentration of extract as in *a* with the addition of affinity purified antibodies against mouse NGF at 500 ng/ml. (*c*) Blind scoring of fiber density as a function of dilution of the extract. The highest score (5) is slightly denser than that seen in *a*. (*d*) Inhibition of the fiber response as a function of the concentration of anti-NGF antibodies. Means ± SEM from two determinations are shown in *c* and *d*.

Table 1. NGF-like protein in the rat CNS as determined by enzyme-linked immunoassay

Adult tissue	NGF, ng/g of tissue	*N*
Hippocampus	1.01 ± 0.12	10
Cortex	0.27 ± 0.02	31
Whole brain	0.26 ± 0.04	13

Data represent the mean ± SEM of the number of experiments (*n*). The median detection limit for 15 experiments was 1 fg/ml.

Cortex and hippocampus were highest in NGF mRNA, having 2-fold higher levels than those seen in total brain. Significant levels of NGF mRNA were also found in the olfactory bulb, while it was undetectable in the hypothalamus, striatum, and septum. Low NGF mRNA levels were observed in the cerebellum, thalamus, and pons–medulla.

Two control probes were used to ensure that the regional variation of NGF mRNA levels was specific: c-*myc*, a protooncogene that encodes a nuclear DNA binding protein correlated with cell proliferation (29, 30), and α-actin, a major structural protein common to all cells. Low levels of c-myc mRNA were found in most regions of the adult rat brain (Fig. 1 *Upper*). Higher levels were seen in the olfactory bulb, possibly reflecting turnover of neurons (31). In contrast to the regional variation of NGF and c-myc RNAs, actin mRNA was detected in all regions at nearly constant levels.

Enzyme-linked immunoassay demonstrated the presence of a NGF-like protein in the rat brain. The hippocampus contained three times as much NGF-immunoreactive material as in the cerebral cortex or total brain (Table 1).

Adult cortices and hippocampi were homogenized and applied to a NGF-immunoaffinity column. Near optimal fiber outgrowth in sympathetic ganglia was elicited by the eluted material (Fig. 2*a*). The induction of neurite outgrowth indicated a titer of 0.3–0.8 ng of NGF/g of tissue (25, 32) in agreement with the enzyme-linked immunoassay. The effect of brain NGF could be blocked by antibodies raised against mouse NGF (Fig. 2 *b* and *d*), with total inhibition at 250 ng of antibodies per ml, as seen for mouse salivary gland NGF (25, 26). Similar results were obtained with another independent preparation.

NGF-like immunoreactivity was widespread in the adult brain in major nerve fiber bundles and fiber tracts (Fig. 3). Networks of finer fiber-like structures also were seen in the cerebral cortex, running parallel and perpendicular to the cortical surface above the corpus callosum (Fig. 3*a*), whereas in the hippocampus a network of positive fibers surrounded the pyramidal cell bodies, with strongly fluorescent fibers in the cingulum, alveus, and dorsal parts of stratum oriens (Fig. 3*b*). The immunoreaction was considerably weaker than in the male mouse submaxillary gland. No specific immunoreactivity was seen with preabsorbed antibodies.

Developmental Expression of NGF mRNA and Protein in the CNS. NGF mRNA was observed on postnatal day 1 (P1), at 5% of the adult level, whereas it was undetectable prenatally (Fig. 4 *Left* and *Upper Center*). Adult levels were reached at 3 weeks, with half-maximal levels occurring between 1 and 2 weeks; 47-week- and 17-month-old total brains contained 90% and 80%, respectively, of the NGF mRNA level seen in adult brain (Fig. 4 *Right*).

Expression of c-*myc* began prenatally with maximal levels seen 1 week postnatally, coinciding with the peak of glial cell mitosis (33). In contrast to the developmentally regulated NGF and c-*myc* expression, actin mRNA was expressed at the same level at all developmental stages (Fig. 4 *Left* and *Right*).

NGF-like protein detected by enzyme-linked immunoassay showed a bimodal developmental expression (Fig. 4

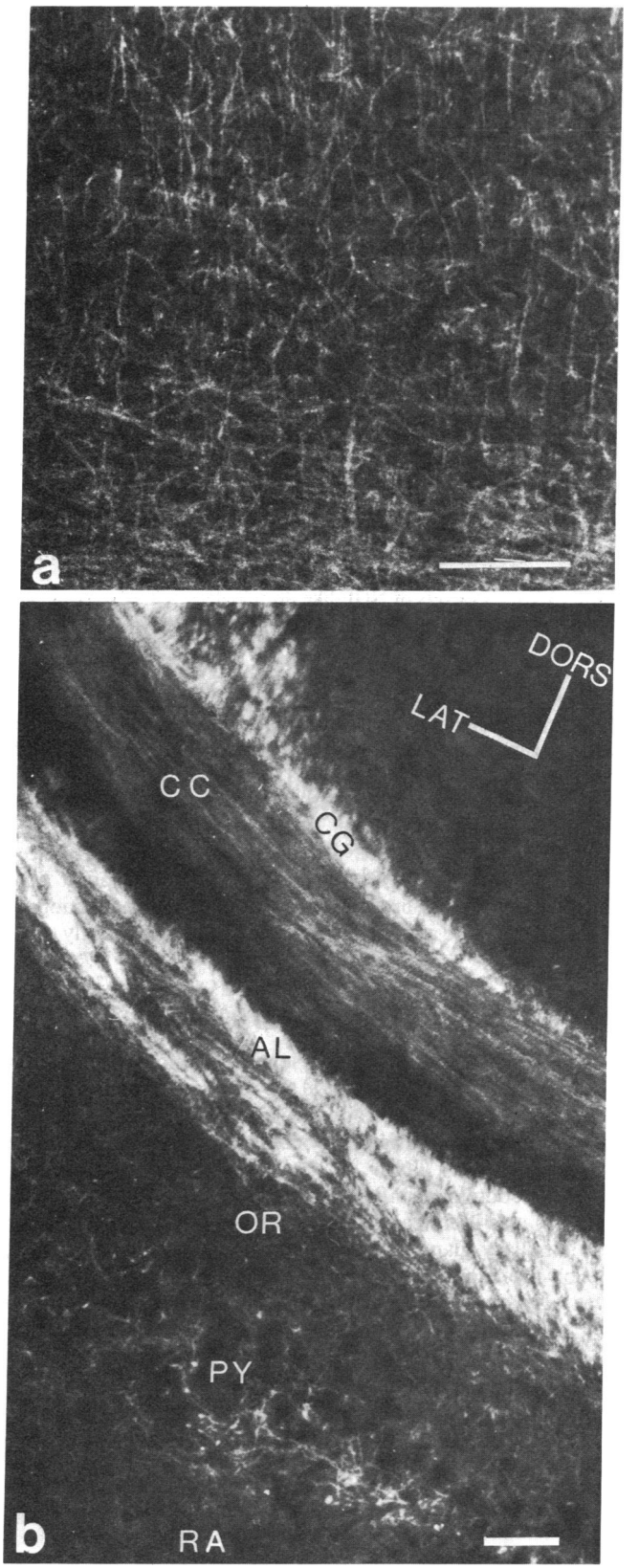

FIG. 3. Immunohistochemical localization of NGF-like material in the rat brain. (*a*) Dorsal cerebral cortex. (Bar = 75 μm.) (*b*) Transverse section through part of the hippocampal CA1 area and overlying structures. Orientation is indicated in the top right corner. (Bar = 50 μm.) Slides processed in parallel with NGF antibodies preabsorbed with NGF were devoid of specific staining. CC, corpus callosum; CG, cingulum; AL, alveus; OR, stratum oriens; PY, stratum pyramidale; RA, stratum radiatum; DORS, dorsal; LAT, lateral.

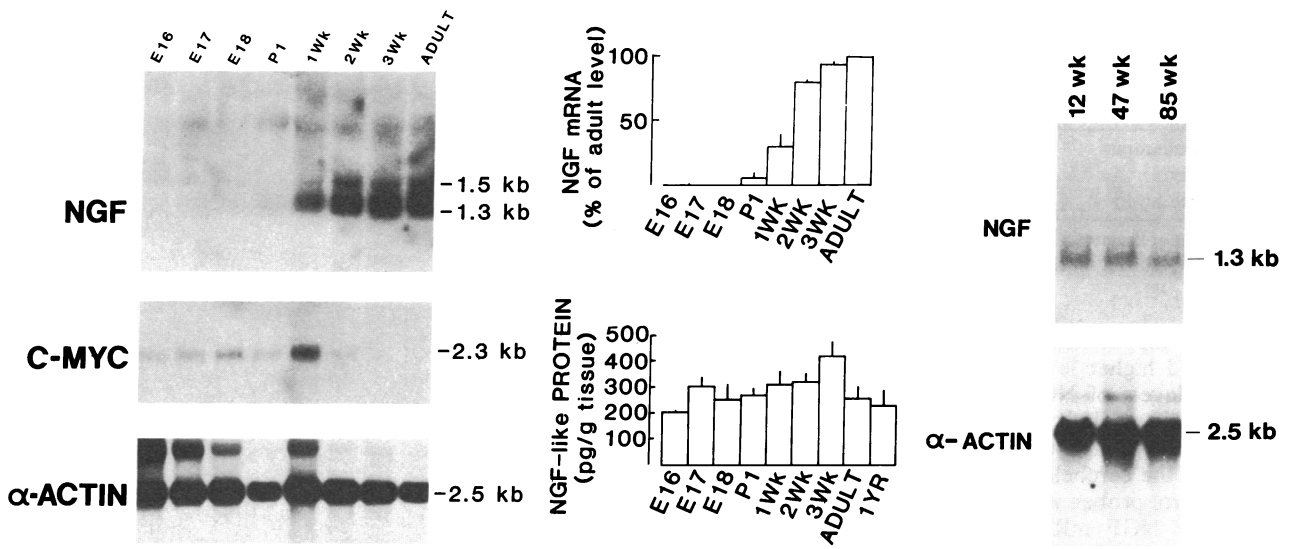

FIG. 4. Developmental expression of NGF mRNA and NGF-like protein. (*Left*) Poly(A)$^+$ RNA (15 μg) from whole brains of the ages indicated were probed as in Fig. 1 *Upper*. (*Upper Center*) Mean ± SEM of 3–6 independent experiments, with results normalized to adult brain. (*Lower Center*) Levels of NGF-like protein detected by enzyme immunoassay (mean ± SEM of 2–15 separate experiments). (*Right*) Poly(A)$^+$ RNA (10 μg) from whole brains of the indicated ages were probed with NGF and actin DNA probes using the same filters. E16–E18, embryonic days 16–18; P1, postnatal day 1; 1–85 Wk, 1–85 weeks.

Lower Center). Levels were 0.21 ng/g of tissue at embryonic day 16, increased 50% at embryonic day 17, and then dropped slightly through postnatal day 1. Subsequently, the levels of NGF-like protein increased to a maximum of 0.42 ng/g of tissue 3 weeks postnatally, again leveling off to the values found for adult brain (Table 1; Fig. 4 *Lower Center*).

Hippocampal NGF mRNA Levels After Fimbria Transection. NGF mRNA levels did not change in the adult hippocampus either 15 or 30 days after complete transection of the fimbria, while actin mRNA did increase significantly (Fig. 5).

DISCUSSION

We present data at the mRNA level from blot-hybridization assays and at the protein level from enzyme immunoassay, immunohistochemistry, and bioassay that form strong evidence for the synthesis of physiologically active NGF in the adult mammalian CNS.

The predominant mRNA species for NGF in the rat brain (1.3 kb) was identical in size to that seen in the male mouse submaxillary gland. An additional RNA species of 1.5 kb, which represents 20–25% of the total hybridization signal, was also observed, as described previously (18). It is expressed concomitant with the predominant 1.3-kb mRNA species, suggesting similar regulation of the two NGF mRNAs. The 1.5-kb mRNA may represent an unspliced precursor RNA, suggesting that the rat NGF gene has a different intron–exon structure than does the human gene (17). Alternatively, it may use a different promoter and/or polyadenylylation site. S1 endonuclease analysis with a homologous rat DNA probe should distinguish between these possibilities.

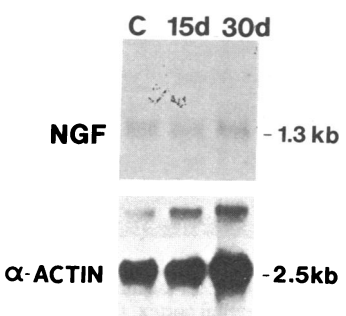

FIG. 5. Hippocampal expression of NGF mRNA after fimbria transection. Poly(A)$^+$ RNA (10 μg) from control (C), 15-day (15d), and 30-day (30d) postlesion were probed for NGF and actin mRNA. Similar results were obtained in two other experiments with RNA from the same preparation.

The NGF mRNA-rich cortex and hippocampus contain the terminal fields of those cholinergic neurons that have the ability to retrogradely transport NGF (9, 12). These regions also expressed significant levels of an NGF-like protein, as detected by enzyme-linked immunoassay, in close agreement with recent data (19).

Immunohistochemistry supports the presence of NGF-like material in the adult brain. Similar observations of NGF-like immunoreactivity in the fetal brain have been observed (14, 15).

The detection of a NGF-like protein by enzyme immunoassay was paralleled by NGF-like physiological activity. The immunoadsorbed protein from the brain was indistinguishable in activity from purified submaxillary gland NGF. No other growth factor than NGF tested to date can elicit this typical fiber outgrowth from sympathetic ganglia (2, 25).

Hippocampal NGF mRNA levels were unchanged after transection of the fimbria, the main cholinergic input into the hippocampus, despite the possible increase in NGF-like protein levels resulting from such denervation (34, 35). Thus, the exact role of NGF in regulating cholinergic function remains an open question.

NGF mRNA first appeared at birth, reaching adult levels 3 weeks postnatal, slightly earlier than the final maturation of synaptic connections in the cortex (36) and hippocampus (37). The appearance of NGF mRNA closely matches the development of cholinergic innervation but not other inputs into the dentate gyrus of the hippocampus (38). It would appear possible from our data that NGF plays a role in the development and maintenance of synapses and/or in maintaining adult axon pathways. We observed successive slight reductions in NGF mRNA in total brains of ages 47 weeks and 17 months. These decreases in NGF mRNA may reflect large deficits in trophic support within specific populations of

neurons at ages when the number of synapses in the CNS are decreased (39).

A peak of NGF-immunoreactive protein was detected by enzyme-linked immunoassay 3 weeks postnatally. As functional synapses form (36–39), NGF protein is perhaps removed by retrograde transport and/or degraded, accounting for the subsequent 40% decrease to adult levels.

The developmental appearance of NGF-like protein detected by enzyme-linked immunoassay contrasts with the blot-hybridization analysis. Previous immunohistochemical data (14, 15) indicated the presence of a NGF-like protein in the fetal brain, and the levels of NGF detected by enzyme-linked immunoassay in the adult cortex and embryonic brain are similar, such that NGF mRNA would be expected to be found fetally.

Immunoblotting of purified mouse NGF or crude submaxillary gland extract revealed only one band with a M_r of 14,000, as expected for the NGF monomer (15). The sensitivity of the immunoblotting technique (200 ng of NGF per ml) did not allow for detection of NGF in the brain samples. More importantly, no other cross-reacting protein in the fetal rat brain was detected (data not shown), suggesting that the antibodies were not cross-reacting with an unrelated protein in the fetal brain.

The turnover of NGF mRNA in the fetal brain may also be very rapid, resulting in steady-state levels of NGF mRNA below the detection limits of our hybridization assay.

An intriguing alternative possibility is that there exists a fetal NGF-like protein, possibly reaching maximal expression at embryonic day 18 (Fig. 4 *Lower Center*) that cross-reacts with anti-NGF antibodies but not with the more specific cDNA probe. If such a fetal NGF-like protein exists, the DNA sequence for this putative NGF-like gene has diverged to <70% homology with the adult NGF gene because a homologous rat DNA probe did not detect any message embryonically, even under lower hybridization stringency.

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