The Journal of Neuroscience, February 1991, 11(2): 318–326

# NGF Gene Expression in Actively Growing Brain Glia

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**Previous work suggested that brain NGF acts locally on cells adjacent to sites of synthesis, in addition to any putative actions on distant, projecting perikarya. To define the basis of local action, we used a sensitive nuclease protection assay to identify cells expressing the NGF gene *in vivo* and *in vitro.* In addition to neurons, glia from a variety of developing brain areas synthesized NGF mRNA, suggesting that CNS glia exhibit a generalized capacity to express the gene. Expression was associated with active glial growth. Stimulation of growth with serum increased NGF message 2-fold in culture. Moreover, rapidly growing, low-density glial cultures exhibited 8-fold higher levels of NGF mRNA than quiescent, confluent cultures. The optic nerve, which contains all 3 major types of glia, expressed the message *in vivo* during neonatal development. In contrast, expression was barely detectable in the adult optic nerve. Transection, which induces glial proliferation, elicited *de novo* appearance of NGF mRNA in the adult nerve. Our observations suggest that active glial growth is associated with expression of the NGF gene and raise the possibility that actively growing glia in the developing or injured brain regulate neuronal growth through the elaboration of NGF.**

Neurotrophic factors play important roles in a wide variety of neural ontogenetic processes (Easter et al., 1985). Study of the prototype NGF has led to a generally accepted model of trophic actions. A central feature of the model involves elaboration of a trophic factor by specific targets, with selective survival of those receptive, afferent neurons that successfully form synaptic connections. Innervation of appropriate targets ensures access to trophic factor and selective survival. However, does target-derived trophic factor guide the migration of axons emanating from spatially dispersed brain nuclei? How do distant targets regulate navigation through diverse microenvironments? Do targets really construct and maintain standing gradients of trophic factors in the embryonic microenvironment? As an alternative to action at a distance, we have recently found that NGF is synthesized locally in a number of brain regions (Lu et al., 1989). We now extend this work and suggest that trophic factor action may also be local, potentially guiding axons through a sequence of local interactions prior to target innervation.

NGF is the most fully characterized trophic factor, regulating normal development of the PNS (Levi-Montalcini and Angeletti, 1968). NGF is necessary for normal survival of sympathetic and sensory neurons (Thoenen and Barde, 1980). Moreover, the trophic agent may also guide neuritic growth in the periphery, thereby serving a tropic function (Campenot, 1977; Gundersen and Barret, 1979). In contrast, central actions of NGF are largely undefined. Although the factor and its receptor (NGF-R) have been localized to diverse brain areas, function during normal development and maturity remains to be elucidated (for review, see Thoenen et al., 1987; Whittemore and Seiger, 1987; Dreyfus, 1989). Assessment of any potential role in brain axon guidance, for example, requires definitive cellular localization during ontogeny. However, information regarding localization is limited and controversial.

Different studies suggest that different populations express the NGF gene. In the adult hippocampus *in vivo,* NGF mRNA has been localized exclusively to neurons by *in situ* hybridization (Rennert and Heinrich, 1986; Ayer-Lelievre et al., 1988; Whittemore et al., 1988). In contrast, *cultured* glia synthesize NGF protein and mRNA (Norrgren et al., 1980; Furukawa et al., 1986a; Yamakuni et al., 1987). It is unclear, at present, whether expression of the NGF gene by different populations is stage specific.

In the present study, we employed combined *in vitro* and *in vivo* approaches to define cellular expression of the NGF gene. A sensitive nuclease protection assay was used to detect NGF mRNA expression. Our results indicate that CNS glia express the gene *in vivo* and in culture. Expression appears to be a function of growth status of these cells: Active growth is associated with high levels of expression. Our observations suggest that local glial elaboration of NGF may play a role in neuron survival and axon guidance.

## Materials and Methods

### *Tissue dissection*

Time-mated pregnant Sprague–Dawley rats were killed by $CO_2$ asphyxiation. The day of discovery of a vaginal plug was considered as embryonic day 1 (E1), and the day of birth as postnatal day 0 (P0). The brains of fetuses or pups were dissected in ice-cold PBS under sterile conditions. Particular care was taken to avoid contamination from neighboring brain areas, blood vessels, and meninges during dissection.

### *Dissociated culture of hippocampus*

The dissected brain tissues were minced into small pieces in the plating medium and dissociated by mechanical trituration. Cells were counted

Received Apr. 12, 1990; revised July 20, 1990; accepted Aug. 15, 1990.

We thank Dr. Scott Whittemore for the rat NGF cDNA clone. This work was supported by NIH Grants HD23315 and NS10259 and by a grant from the Alzheimer's Disease and Related Disorders Association, Inc. I.B.B. is a recipient of a McKnight Foundation Research Project Award.

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and plated on 100-mm Petri dishes coated with poly-D-lysine (0.1 mg/ml). The cultured cells were maintained in a 37°C incubator with 100% humidity and 5% $CO_2$. For pure neuronal culture, E18 hippocampus was dissected and plated at $10^7$ cells/dish in a serum-free medium (SFM). SFM consisted of a 1:1 (v/v) mixture of Ham's F-12 and Eagle's minimum essential medium (MEM) and supplemented with insulin (25 μg/ml), transferrin (100 μg/ml), putrescine (60 μM), progesterone (20 nM), selenium (30 nM), glucose (6 mg/ml), and penicillin-streptomycin (0.5 U/ml, 0.5 μg/ml, respectively). For glial cell cultures, P0–P2 hippocampus, basal forebrain, and cerebellum tissues were dissociated and plated in a nutrient medium (NM). NM consisted of Eagle's MEM plus 10–20% heat-inactivated fetal calf serum (FCS), glucose (6 mg/ml), and penicillin-streptomycin (0.5 U/ml, 0.5 μg/ml, respectively). The cultures were grown for at least 10 d and became fully confluent. The confluent cultures were then detached with trypsin (2.5 mg/ml) and/or EDTA (0.2 mg/ml) and replated in NM at a low concentration. Replating 2 or 3 times significantly decreased the proportion of nonglial cells (fibroblasts and neurons, as monitored by immunocytochemistry). Glial cultures of different densities were generated in 2 ways: (1) Cells were detached and replated at the same low, initial density ($10^5$ cells/dish) and harvested after growing in culture for different amounts of time until they reached low, medium, and high densities. Conditions in which the distance between cells was approximately less than 1 cell diameter were defined as low density. In medium density cultures, cells just started to contact each other. High density cultures were those with very little intercellular space. (2) Cells were replated at different initial densities ($10^5$, $10^6$, and $10^7$ cells/dish, respectively) and harvested in 2 d.

### *Immunocytochemistry and cell count*

Six small dishes of cultures were used for each immunocytochemical staining and cell count. The culture dishes were rinsed in PBS and fixed with 4% paraformaldehyde in 0.1 M $NaPO_4$ buffer (pH, 7.4) for 1 hr at room temperature. The dishes were then rinsed with PBS and stored in the cold before staining. Immunocytochemical staining was performed using the avidin/biotin peroxidase technique (Taniuchi et al., 1986, 1988; Distefano and Johnson, 1988). Antisera to neural-specific enolase (NSE, 1:1000), glial fibrillary acidic protein (GFAP, 1:1000), galactocerebrocide (Gal C, 1:300), and fibronectin (FN, 1:500) were used to define neurons, astrocytes, oligodendrocytes, and fibroblasts, respectively. Cells from approximately 3% of the total dish area from each dish were counted. For glial cultures stained for NSE and FN, confluent cultures were used. A sister culture was harvested, and a drop of cell suspension was counted in a hemacytometer to provide the total number of cells in the dish. Lower-density ($1.6 \times 10^5$ cells/dish) glial cultures were used for GFAP and Gal C staining to facilitate glial cell counting. Neuronal cultures stained for GFAP, Gal C, and FN were counted in the same way. For neuronal NSE staining with or without 5-fluorodeoxyuridine (FDUR) treatment and glial GFAP staining, both positive and negative cells were counted.

### *Lesion, dissection, and dissociated culture of optic nerve*

*Lesion.* Adult female rats were anesthetized with 5% Fluothane. Incision was made just behind the left eyeball, and consequently, the eyeball was removed. There was very little bleeding, which was easily stopped by suppressing with sterile swabs. The right side was used as a nonlesion control. After the operation, the rats were housed in clean cages with free access to food and water for 1, 2, 4, and 6 d, respectively.

*Dissection.* Upon removal of the globes, the optic nerve was easily retracted from the skull with the whole brain. The optic nerves were dissected from just behind the globes to 1 mm before the optic chiasma, placed in PBS to clean any attached connective tissues, then frozen immediately on dry ice and stored in −70°C.

*Culture.* Optic nerves were cultured as described (Raff et al., 1975). Briefly, P5–P8 neonate optic nerves were dissected under sterile conditions and cut into small pieces in PBS. The nerve pieces were then incubated for 20 min at 37°C in 1 ml of solution A (MEM with 0.02 M HEPES buffer, pH 7.4) and 1 ml of solution B (2.5 mg/ml trypsin, 0.2 mg/ml collagenase, Tris-buffered saline, pH 7.4). The digestion procedure was followed by incubating the tissues for 20 min at 37°C in 1 ml solution B with 0.2 mg/ml EDTA. The tissues were then dissociated by trituration in solution C (40 μg/ml DNAase and 50 μg/ml trypsin inhibitor in MEM). The resulting cell suspension was passed through a fine nylon mesh to remove debris. Cells were cultured on polylysine-coated 35-mm Petri dishes in Dulbecco's modified Eagle's medium (DMEM) containing 10% FCS at $5 \times 10^5$ cells/dish. A few days later, when the cultures reached confluence, cells were replated to 100-mm Petri dishes with appropriate densities.

### *RNA extraction*

Total RNA was extracted by the method of Chirgwin et al. (1979) and purified through a CsCl gradient. For the optic nerve, the tissue was homogenized in a glass dounce homogenizer with lysis buffer (4 M guanidinium thiocyanate, 25 mM Na citrate (pH, 7.0), 0.5% sarcosyl, 0.1 M β-mercaptoethanol). For dissociated cultures, the lysis buffer was added to the culture dishes after removing the medium and scraping with an RNase-free rubber policeman. The lysates were immediately loaded onto a CsCl gradient and spun overnight at 35,000 rpm with an SW40 or SW50.1 rotor. The pellets were ethanol precipitated twice. The quality of the RNAs was monitored by the ratio of UV absorbance at 260 and 280 nm and by RNA denaturing minigel.

### *Ribonuclease protection assay*

Ribonuclease protection experiments were performed as described (Lu et al., 1989). Briefly, a $^{32}P$-labeled antisense NGF riboprobe was generated by *in vitro* transcription from a rat cDNA construct, pBSrNGF. Total RNA samples were hybridized with the probe, and single-stranded sequences were digested by RNase T2. A 411-bp NGF-specific protected fragment was resolved on polyacryamide gel, which was dried and exposed to x-ray film with an intensifying screen at −70°C. The consistent results were presented in either gel pictures or densitometric graphs. This assay has been demonstrated to be sensitive, quantitative, and specific for the NGF message (Lu et al., 1989).

Hybridization signals were assigned arbitrary numerical values based on densitometric scanning of autoradiograms. The results represented comparisons of relative NGF mRNA expression levels, rather than absolute values, among different conditions. The following measures were taken to ensure that the densitometric values faithfully reflected the relative levels and trends of message expression: (1) To reduce the potential error within each repetition of a particular experiment, at least 3–7 100-mm dishes of cells were combined, and tissues from a large number of animals were pooled for each RNA extraction. (2) Each experiment was repeated at least 3 times with independent RNA preparations, except for the experiment shown in Figure 6, which was repeated twice due to the extremely large demand of animals. The relative levels of NGF mRNA observed in these experiments were entirely consistent among repeats.

## Results

### Both neurons and glia express the NGF gene in culture

To define specific cell type(s) synthesizing NGF, a useful approach is to study the gene expression in isolated, homogeneous neuronal and glial populations. We therefore grew virtually *pure* hippocampal neuronal and glial dissociated cultures separately and examined NGF mRNA.

#### *Establishment of neuronal cultures*

Pure neuronal cultures were established by taking advantage of the fact that (1) the *embryonic* brain contains predominantly neurons with very few glia, and (2) non-neuronal cells do not survive in the absence of serum. Accordingly, E18 hippocampi were dissociated and cultured in SFM. Cell types in the cultures were characterized using a number of specific immunocytochemical markers. Over 91% of the hippocampal cells in 7-d-old cultures expressed the neuronal marker NSE (Fig. 1, top). Approximately 1% of the total cells were stained for FN (a marker for fibroblasts) and Gal C (an oligodendrocyte marker), respectively. Only 8% of the cells expressed GFAP (a marker for astrocytes; Table 1). The percentage of non-neuronal cells was further reduced by addition of FDUR ($10^{-4}$ M), a mitotic inhibitor, to the cultures. FDUR treatment for 5 d eliminated virtually all the GFAP-positive cells and yielded over 99% NSE-

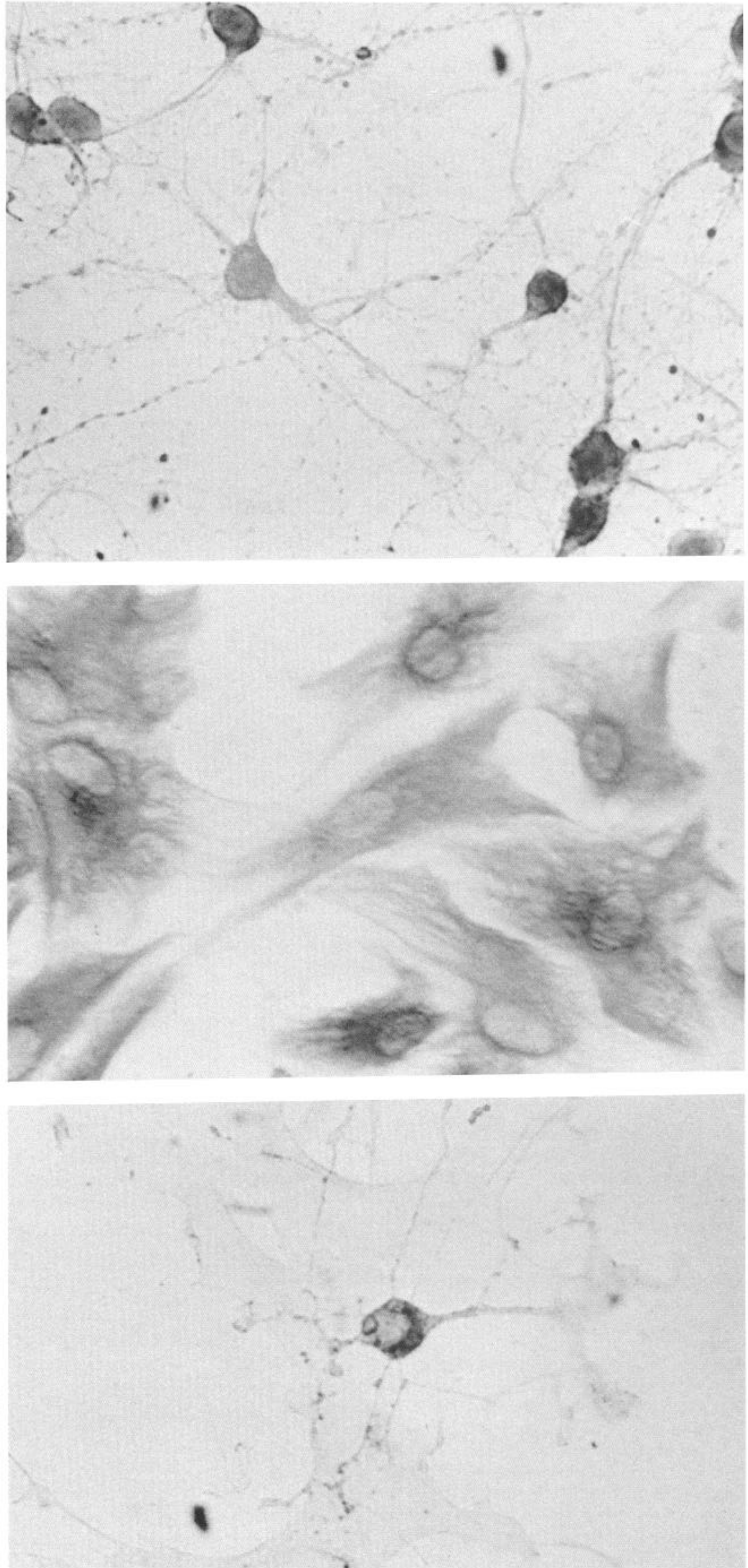


*Figure 1.* Staining of cultures with neuronal and glial markers. The hippocampal neuronal and glial cultures were prepared in the same ways as described in Table 1. *Top,* Neurons stained with antiserum (1:1000) against NSE, a neuronal marker. *Middle,* Astrocytes labeled with GFAP antiserum (1:1000). *Bottom,* The same culture as shown in the middle panel, visualized with antiserum (1:300) against Gal C to show oligodendrocytes.

**Table 1. Percentages of different cell types in hippocampal neuronal and glial cultures**

| Culture | NSE | GFAP | Gal C | FN | NSE-negative | GFAP-negative | NSE + FDUR |
|---|---|---|---|---|---|---|---|
| Neurons | 91.2 ± 2.84 | 8.2 ± 0.64 | 0.9 ± 0.19 | 1.1 ± 0.12 | 8.8 ± 2.17 | — | 99.7 ± 0.07 |
| Glia | 0.3 ± 0.05 | 97.7 ± 3.63 | 2.4 ± 0.50 | 0.5 ± 0.09 | — | 2.3 ± 0.78 | — |

Six 35-mm dish cultures were used for each immunocytochemical staining, and cells from approximately 3% of the total dish area from each dish were counted. The data represent average percentages of positive cells ± SD. Neuronal culture: E18 hippocampi were dissociated and cultured in SFM at a plating density of $2 \times 10^5$ cells/dish for 7 d and stained with NSE, Gal C, GFAP, and FN antisera. The last column represents NSE staining from sister neuronal cultures treated with FDUR ($10^{-4}$ M) for 5 d. Glial culture: P2 hippocampal dissociates were cultured in NM until confluence. The cultured cells were detached and replated 2 more times. The consequent confluent glial cultures ($4 \times 10^5$ cells per 35-mm dish as detached and counted under hemacytometer) were stained with antisera against NSE and FN. To facilitate glial cell counting, lower-density ($1.6 \times 10^5$ cells/dish) glial cultures were used for GFAP and Gal C staining. For neuronal cultures stained for NSE and glial cultures stained for GFAP, both positive and negative cells were counted, and the percentages of negative cells are given in columns 6 and 7.

positive cells in 7-d-old hippocampal neuronal cultures (Table 1).

*Establishment of glial cultures*

The neonatal hippocampus contains large numbers of glia. It is known that serum stimulates the growth and proliferation of glia *in vitro.* We established pure glial cultures by growing early postnatal hippocampal dissociates (P2–P4) in serum-containing medium, with subsequent multiple replatings. These cultures contained very few neurons and fibroblasts, as assessed by NSE and FN staining (Table 1). Over 97% of the cells were GFAP-positive astrocytes, with less than 3% Gal C-positive oligodendrocytes (Fig. 1, middle, bottom; Table 1). These glial cultures are thus referred to as astrocytic.

*Expression of the NGF gene*

A sensitive ribonuclease protection assay was employed to examine NGF expression in the pure neuronal and glial cultures. RNA from cultures was hybridized with an NGF riboprobe, followed by RNase $T_2$ digestion and polyacryramide gel electrophoresis. A 411-bp NGF-specific protected fragment (Fig. 2, lane 4) could be easily detected in hippocampal neurons cultured in SFM for 7 d. Elimination of non-neuronal cells in the neuronal cultures with FDUR did not change the level of NGF mRNA (arbitrary densitometric units: 205 in control, 214 in 5-d FDUR-treated cultures). Thus, hippocampal neurons expressed the NGF gene in culture. These results suggest that neurons contribute to NGF synthesis in the hippocampus, consistent with previous *in situ* hybridization experiments (Rennert and Heinrich, 1986; Ayer-Lelievre et al., 1988; Whittemore et al., 1988).

To determine whether glia also potentially synthesize NGF, we measured the message in pure glial cultures. Confluent hippocampal astrocytes cultured under serum-free conditions for 7 d produced 10 times as much NGF mRNA as neuronal cultures (Fig. 2, cf. lanes 2, 4). We examined the glial gene expression in further detail to explore the potential underlying mechanism(s).

## Expression of NGF mRNA correlates with the stage of glial growth

The absence of NGF expression by glia in the adult *in vivo* may simply reflect developmental "down-regulation" of NGF synthesis. Because glia undergo active growth and differentiation during early ontogeny, we examined the relationship of glial growth to NGF gene expression. Stimulation of the growth of astrocytes by serum resulted in a 2-fold increase of NGF mRNA in confluent cultures (Fig. 2, cf. lanes 2, 3). Thus, NGF synthesis may be associated with growth status of glia: Actively growing astrocytes produced higher levels of mRNA than did quiescent cells.

To further assess whether NGF gene expression truly correlates with glial growth, we measured message levels under different growth conditions. The effect of cell density on NGF gene expression was examined, because it is known that low density elicits rapid growth in these cultures. To achieve different cell densities, sparse cultures were plated and grown for different periods of time before harvest. In fact, levels of NGF mRNA correlated with glial density (Fig. 3). The lowest-density culture, in which astrocytes grew most rapidly, exhibited the highest expression. As density increased with time, levels of NGF mRNA decreased progressively. High-density (confluent) cultures expressed the lowest level of NGF mRNA. Inhibition of growth by withdrawal of serum further reduced gene expression (Fig. 3).

To exclude the possibility that the level of NGF expression was merely a function of time in culture itself, an alternative approach was adopted. Glia were plated at different initial densities ($10^5$, $10^6$, and $10^7$ cells/dish, respectively), and harvested in 2 d. Similar results were obtained in this experiment: Rapidly growing, low-density cultures exhibited higher levels of NGF mRNA than did high-density cultures (Fig. 3, inset). In sum, expression of the NGF message by actively growing glia in culture raises the possibility that CNS glia may preferentially synthesize NGF during early development.

## Astrocytes from diverse brain areas synthesize NGF in culture

NGF gene expression by hippocampal glial cultures may be unique to that brain area, where the highest levels of NGF mRNA have been detected *in vivo.* To determine whether CNS glia are generally capable of expressing the gene, we examined the NGF message in glia derived from different brain areas. Cultured astrocytes from all brain areas expressed high levels of the message (Fig. 4). NGF mRNA was highest in hippocampal glia. Moreover, glial culture from the cerebellum, which produces NGF only during the postnatal period, exhibited a slightly lower expression of the gene. Surprisingly, glia from the basal forebrain, an area that normally synthesizes little NGF message in the adult *in vivo,* also expressed high levels of the message in culture. Thus, CNS astroglia exhibited a general capacity to synthesize NGF in culture.

Although glial cultures exhibited high message levels, potential neuronal contamination could have resulted in stimulation of NGF synthesis. To examine this possibility, we used the optic nerve, which contains all 3 glial types, but no neuronal perikarya (Raff, 1989). Similar to brain glia, dissociated optic nerve culture expressed high levels of NGF mRNA. Gene expression also

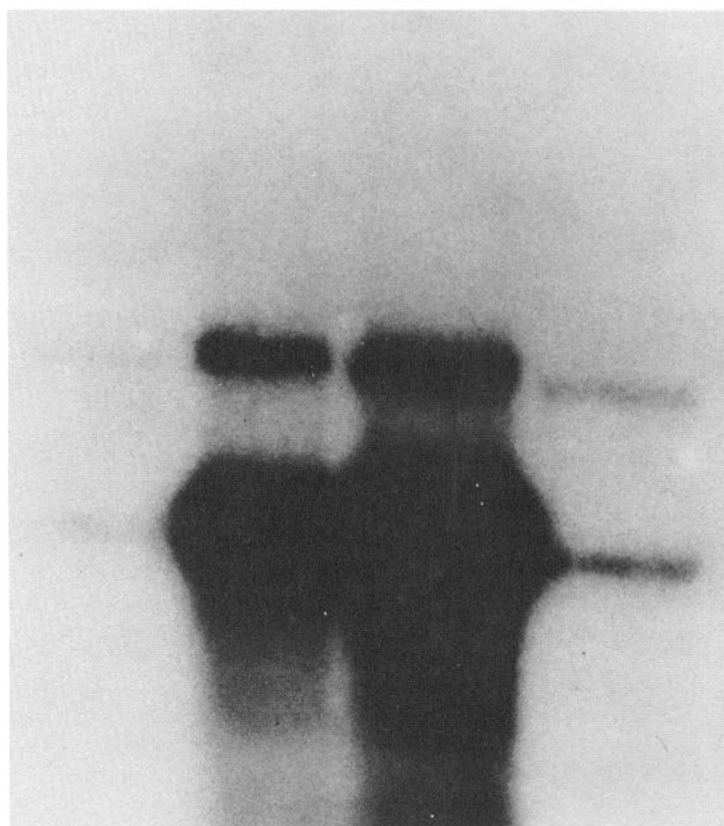



*Figure 2.* Expression of NGF mRNA in hippocampal neuronal and glial cultures. Cell cultures, RNA extraction, and ribonuclease protection assay were performed as described in the text. Eighty μg of total RNA were assayed in all lanes. *Lane 1,* E18 hippocampal (*Hi*) tissue. *Lane 2,* Confluent hippocampal glial (*gHi*) culture switched to SFM for 7 d. *Lane 3,* A sister culture grown in serum-containing medium for 7 d. *Lane 4,* E18 hippocampal neurons (*nHi*) cultured in SFM for 7 d. *Arrow* points to a 411-bp NGF-specific protected fragment. The *top band* is residual undigested riboprobe.

appeared to correlate with glial growth: Confluent, quiescent cultures exhibited reduced NGF mRNA expression (Fig. 5).

## Optic nerve glia produce NGF *in vivo* during development and after injury

To determine whether CNS glia normally express the NGF gene, mRNA was examined *in vivo.* While it is technically difficult to separate neurons and glia in the brain, the optic nerve offers a uniquely pure glial preparation. In contrast to results in culture, NGF mRNA was barely detectable by sensitive ribonuclease protection in the adult optic nerve *in vivo* (Fig. 5). Neonatal optic nerve (P10), on the other hand, expressed the gene, though the level was still lower than in confluent optic nerve cultures (Fig. 5). We did not examine mRNA in optic nerves from younger rats, because it was virtually impossible to obtain tissue for RNA extraction. Nevertheless, these initial data suggest that glial expression of the NGF gene *in vivo* also correlates with growth.

Lesions elicit a rapid glial growth response in the central optic nerve (Skoff, 1975) and induction of NGF expression in the peripheral sciatic nerve (Heumann et al., 1987a). It was natural, consequently, to determine whether glial cells in the optic nerve express the NGF message after axotomy. The optic nerve was transected immediately posterior to the left globe, and the right optic nerve was used as a nonlesion control. The lesion elicited a marked increase of NGF mRNA (Fig. 6). Injury-induced NGF mRNA expression was restricted to the early period after transection. One day after surgery, the induction was most dramatic, after which time, the effect decreased. By the sixth day, the lesion effect was reduced to a minimum (Fig. 6). Interestingly, the time course of expression paralleled that reported for the glial growth response after transection (Skoff and Vaughn, 1972). These results suggest that glia may synthesize NGF not only during development, but also during the degeneration/regeneration process.

## Discussion

We have characterized NGF gene expression in the brain *in vivo* and *in vitro* to gain clearer insights into the physiological role of the trophic protein. In particular, we sought to identify brain populations that express the gene, and the stages during which gene expression occurs. Our studies indicate that brain glia as well as neurons express the gene. The capacity for expression is apparently a general property of glia, because the message is elaborated by glia from a wide variety of brain areas. However, expression of the gene is stage specific: Actively growing glia express high levels of the message, whereas NGF mRNA is barely detectable in quiescent glia. Thus, for example, glial growth stimulated by serum or low density in culture, or by optic nerve transection *in vivo,* elicits high levels of NGF gene expression. Our observations suggest that actively growing glia synthesize NGF locally in a variety of brain areas, and that local actions

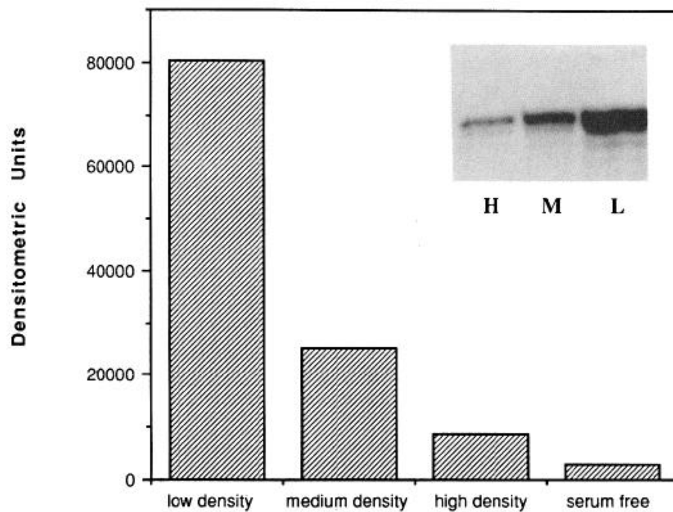



*Figure 3.* Density effect on NGF mRNA expression in hippocampal glial cultures. The ribonuclease protection experiments were performed using an equal amount of total RNA isolated from a pool of at least 3 dishes of glial cultures in all conditions. Representative densitometric analysis and autoradiogram of 4 independent repeats are given here for relative comparison. *Densitometric bar graph,* Glial cultures of different densities generated by growth for different amounts of time. Hippocampal glia were plated at $10^5$ cells per 100-mm dish and harvested after growing for 2, 5, and 9 d in serum-containing medium to obtain low-, medium-, and high- (confluent) density cultures according to the criteria described in Materials and Methods. Separate confluent hippocampal glial cultures grown in SFM for 7 d were assayed for comparison. *Autoradiograph inset,* Glial cultures of different densities generated by growing at different initial densities. Hippocampal glia were plated at $10^7$, $10^6$, and $10^5$ cells/dish, respectively, and harvested in 2 d to obtain high- (*H*), medium- (*M*), and low- (*L*) density cultures for NGF mRNA assay.

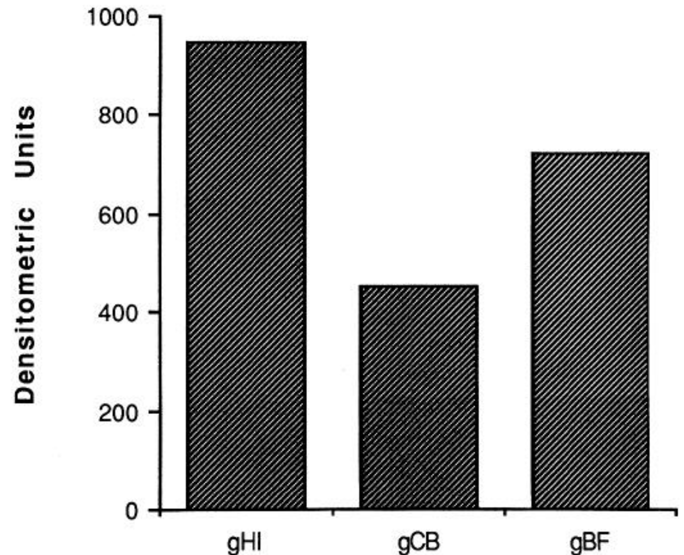



*Figure 4.* Expression of NGF mRNA by cultured glia from diverse brain areas. Confluent cultures of glial cells prepared from P2 hippocampus (*gHI*), cerebellum (*gCB*), and basal forebrain (*gBF*) were assayed for NGF message. Similar to gHI, gCB and gBF cultures contain predominantly (over 95%) astrocytes. Equal amounts of total RNA were used. This experiment was repeated 3 times, and variability among experiments was within 20%.

may play a critical role during development and after injury. In turn, local glial NGF may be necessary for neuronal survival and guidance of axon growth.

*Brain astrocytes and neurons express the NGF gene*

Cellular localization of NGF synthesis in the brain has been controversial (for review, see Thoenen et al., 1987). Apparently conflicting results have been derived from *in vivo* and *in vitro* studies of cells of different ages. In culture, CNS glia synthesize and release the protein (Lindsay, 1979; Norrgren et al., 1980; Furukawa et al., 1986a; Assouline et al., 1987). Moreover, NGF mRNA has been detected in glial cultures (Yamakuni et al., 1987). During the preparation of this manuscript, Spranger et al. (1990) published a paper that demonstrated that NGF mRNA expression in CNS glial culture was confined to astrocytes. Finally, many fiber tracts contain NGF-immunoreactive glia in the embryonic mouse brain (Finn et al., 1987). In contrast, *in situ* hybridization studies in the adult hippocampus indicated that NGF mRNA is restricted to neurons (Ayer-Lelievre et al., 1988; Whittemore et al., 1988).

The present results may help resolve the apparent conflict. Our studies indicate that both neurons and glia exhibit the capacity to express the NGF gene. However, neuronal and glial populations express the gene at *different times.* Actively growing glia express far higher levels of mRNA than neurons in culture. In contrast, quiescent glia in the adult brain *in vivo* exhibit barely detectable levels of the message. These observations, in conjunction with the foregoing previous results, suggest that brain neurons and glia express the gene, but at different stages of life. Glia express the NGF gene, and presumably synthesize the trophic protein, only with active growth, during development or after injury. Normally, glial expression ceases in the adult brain, and neurons become the predominant population synthesizing the protein. Consequently, local glial elaboration of NGF may serve important functions during development or recovery from injury. Target neuronal production of NGF, conversely, may assume importance later in development and during adulthood.

*Astrocytes from diverse brain areas express the NGF gene*

NGF gene expression was not restricted to glia from a single brain area. Rather, glia from multiple areas exhibited the capacity to express the gene. Astrocytes from the hippocampus, basal forebrain, and cerebellum expressed high levels of NGF mRNA in culture (Fig. 4). Because these regions contain neurons that differ anatomically, biochemically, and functionally, it is apparent that astrocytes in the microenvironment of heterogeneous neuronal populations may elaborate the trophic factor.

NGF message was also expressed by cultured optic nerve (Fig. 5), which contains all 3 major types of glia (Raff, 1989), without neurons. In aggregate, our results indicate that glia from widely differing brain areas express the NGF gene, raising the possibility that the trophic molecule acts in microenvironments in widespread brain areas.

*Actively growing glia express the NGF gene*

A number of manipulations that stimulate glial growth promoted expression of the NGF gene in culture. Exposure to serum, which is known to evoke glial growth, increased levels of NGF mRNA (Fig. 2). Conversely, withdrawal of serum, and decreased glial growth, decreased expression. Moreover, rapidly growing, low-density glial cultures exhibited 8-fold higher levels of NGF message than quiescent, confluent cultures (Fig. 3). As

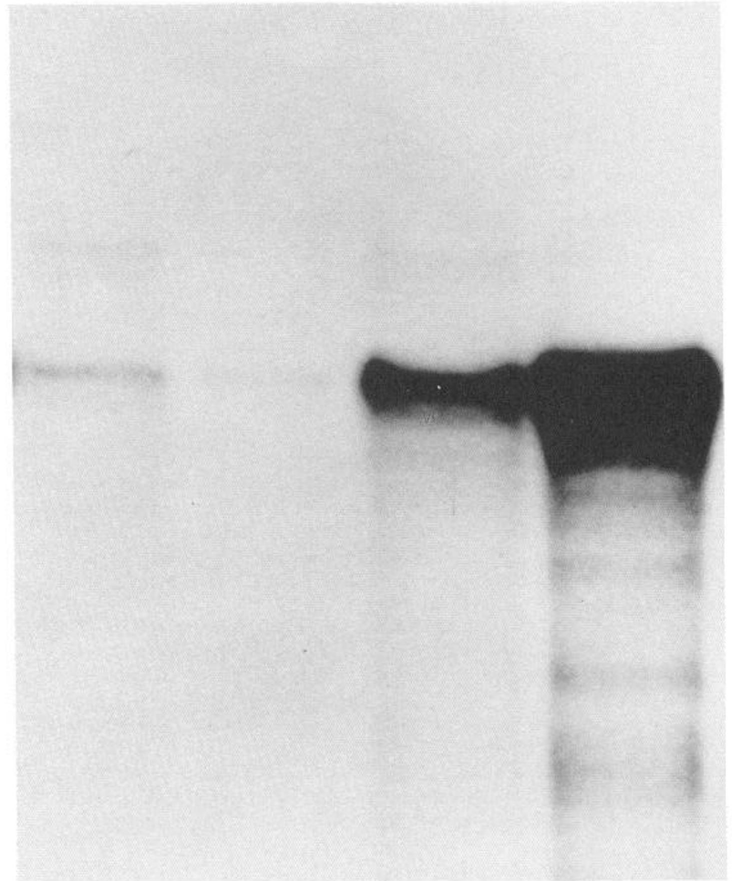
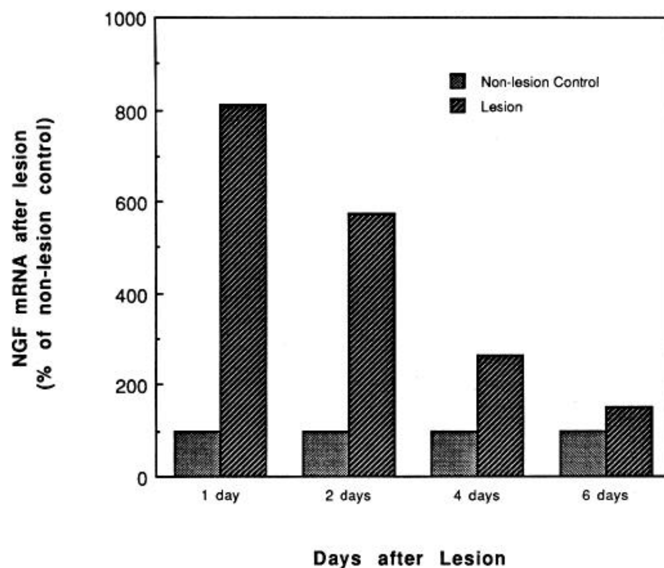



*Figure 5.* NGF mRNA expression in optic nerve. Fifty μg of total RNA was used in all lanes. *P10,* RNA isolated from optic nerves from 7 P10 litters. *Adult,* Optic nerve RNA from 20 adult rats. *HD,* RNA from 4 dishes of high-density (confluent) optic nerve cultures. *LD,* RNA from 7 dishes of low-density (approximately 5 × $10^5$ cells per 100-mm dish) optic nerve cultures. The autoradiogram represents 3 identical experiments with independent RNA preparations.




time in culture progressed, density increased, growth decreased, and NGF mRNA also progressively decreased. Control experiments indicated that growth status, not time in culture, critically regulated NGF expression.

The present studies confirm and extend recent work suggesting that NGF *secretion* is associated with glial growth in culture (Furukawa et al., 1986b). Indeed, our observations suggest that active glial growth is associated with NGF gene expression and trophic protein synthesis, which may form the basis for the increased secretion noted by Furukawa and colleagues. In con-

*Figure 6.* Effect of lesion on NGF mRNA expression in adult optic nerve *in vivo.* Optic nerve lesions were placed posterior to the left globe, and right optic nerves were used as nonlesion controls. The optic nerves from both sides were collected 1, 2, 4, or 6 d after lesion. Twenty-five nonpregnant adult female rats were used for each group. Identical results were obtained from 2 independent experiments. Autoradiograms were analyzed densitometrically, and numerical values were normalized to percentage of nonlesion controls.

junction, these studies suggest that increased gene expression attendant to growth is functionally important. This contention is supported by our *in vivo* studies.

NGF gene expression by brain glia *in vivo* also correlates with growth and development. Differentiated, quiescent glia in the adult optic nerve *in vivo* expressed extremely low levels of NGF mRNA. In contrast, the message was readily detectable in the developing, neonatal optic nerve, in which glia are actively growing and differentiating (Fig. 5). In aggregate, the *in vitro* and *in vivo* studies suggest that glial NGF gene expression, and synthesis of the protein, may be restricted to the active growth phase that normally occurs during early development. We also explored the possibility that glial growth induced at other times induces NGF gene *reexpression.*

*Injury-induced glial growth elicits NGF gene expression*

Our studies indicate that glial growth consequent to injury, as well as normal development, induces NGF gene expression. Optic nerve transection, which is known to elicit glial mitosis and growth (Skoff, 1975), evoked a marked increase of NGF mRNA expression in adults (Fig. 6). Moreover, the time course of expression faithfully reflected the glial growth response after transection (Fig. 5; Skoff and Vaughn, 1972). The present observations complement previous work indicating that brain astroglia elaborate trophic factors, some with NGF-like activities, after injury (Nieto-Sampedro et al., 1982). It may be concluded that glial growth during maturity as well as development fosters trophic-factor production. Consequently, brain astroglia apparently retain the capacity to express trophic-factor genes throughout life, depending on growth status.

Expression of NGF by brain astrocytes may be analogous to that of peripheral Schwann cells, a commonly employed model. While cultured Schwann cells contain high levels of NGF (Assouline et al., 1987), the message is virtually undetectable in adult peripheral glia (Bandtlow et al., 1987). The sciatic nerve, which consists mostly of Schwann cells, expresses high levels of NGF mRNA at birth, with rapid decreases in expression during development. NGF message is extremely low in the adult sciatic nerve (Bandtlow et al., 1987; Heumann et al., 1987b). However, axotomy, which induces Schwann cell proliferation (Heumann et al., 1987a,b), dramatically augments NGF content.

We conclude that growth-associated elaboration of NGF may be a ubiquitous glial mechanism, manifested in both the PNS and the CNS. In addition, then, injury-induced elaboration of NGF by glia may be widespread, potentially fostering regrowth and regeneration of neurons after illness or trauma. Indeed, reexpression of the NGF gene by astrocytes in the adult brain after injury suggests that neural repair may be a potential feature of the CNS as well as the PNS.

*Potential roles of glial NGF*

Previous work illustrated that mRNAs for NGF as well as its receptor are synthesized locally in a variety of brain areas during ontogeny (Lu et al., 1989). These observations raised the possibility that NGF acts locally during development, potentially exerting trophic and tropic actions. The present studies, indicating that local synthesis largely derives from actively growing glia, extends this formulation. Based on this work, we suggest that some actions of NGF are predominantly local, and that neurons initially interact with NGF elaborated by proximate glial "targets," which simultaneously foster survival and axon navigation to distant, definitive sites. Growth cones may be directed to distant targets through a series of interactions with NGF elaborated by actively growing glia, serving as "guideposts" that form a sequential pathway. After navigation to the microenvironment of the definitive neuronal target, NGF from that source may then become determinative. Glial NGF may thus be critical early in development, while neuronal NGF may assume importance at later stages during maturity. This formulation is consistent with the observations that NGF has been localized to developing brain glia, but only to neurons in the adult brain (see above).

This model for brain development is consistent with observations made in the periphery. Extensive study has suggested that NGF acts as a "chemotropic" factor for peripheral neurons, supporting and even guiding directional neurite outgrowth (Campenot, 1977; Menesini-Chen et al., 1978; Gundersen and Barret, 1979). Brain glial NGF may serve a tropic function, as well. During early CNS ontogeny, glia are commonly aligned, forming pathways that play critical roles in axon guidance (Silver et al., 1982). Sequential production of NGF by actively growing glia in a pathway may guide axons to appropriate distant targets.

Glial NGF may exert trophic and tropic effects after injury, as well. In the periphery, for example, sciatic transection induces glial proliferation and NGF synthesis in the proximal and distal stumps (Heumann et al., 1987a,b). Available evidence suggests that the NGF promotes directed regrowth from proximal to distal segments within the Schwann cell sheath (for review, see Heumann, 1987). Similarly, in the brain, optic transection rapidly elicits glial mitosis (Skoff, 1975) and NGF expression. Moreover, recent work indicates that injection of NGF into the globe after axotomy rescues retinal ganglion cells and their proximal axons (Carmignoto, 1989). Consequently, glial elaboration of NGF after optic damage may play an important role in retinal ganglion-cell regeneration.

In summary, the glial growth response during development and after injury may constitute a final common pathway that elicits NGF elaboration and guided neuronal growth.

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