Walker, *Proc. Natl. Acad. Sci. U.S.A.* **83**, 4499 (1986).
31. S. Kent and I. Clark-Lewis, *Synthetic Peptides in Biology and Medicine*, K. Alitalo, P. Partanen, A. Vaheri, Eds. (Elsevier, New York, 1985), pp. 29–58.
32. E. A. Emini, B. A. Jameson, E. Wimmer, *Nature* **304**, 699 (1983).
33. Principal support for this work was provided by a Marshall Field's Award from the American Foundation for AIDS Research, grant No. 00465R (S.B.H.K.). The cell fusion assays were supported by AI25784 (G.S. and B.H.). We thank P. Rieber for MT151, D. Wernicke-Jameson for discussions, P. Dervan for the use of computer facilities, S. Perry, E. McAnaney, and L. Martin Selk for technical assistance, C. Elkins for manuscript typing, and D. Noskowitz for figure preparation.

18 February 1988; accepted 18 April 1988

Expression of the β-Nerve Growth Factor Gene in Hippocampal Neurons

CHRISTIANE AYER-LELIEVRE,* LARS OLSON, TED EBENDAL, ÅKE SEIGER, HÅKAN PERSSON

In situ hybridization with complementary DNA probes for nerve growth factor (NGF) was used to identify cells containing NGF messenger RNA in rat and mouse brain. The most intense labeling occurred in hippocampus, where hybridizing neurons were found in the dentate gyrus and the pyramidal cell layer. The neuronal identity of NGF mRNA–containing cells was further assessed by a loss of NGF-hybridizing mRNA in hippocampal areas where neurons had been destroyed by kainic acid or colchicine. RNA blot analysis also revealed a considerable decrease in the level of NGF mRNA in rat dentate gyrus after a lesion was produced by colchicine. This lesion also caused a decrease in the level of Thy-1 mRNA and an increase in the level of glial fibrillary acidic protein mRNA. Neuronal death was thus associated with the disappearance of NGF mRNA. These results suggest a synthesis of NGF by neurons in the brain and imply that, in hippocampus, NGF influences NGF-sensitive neurons through neuron-to-neuron interactions.

NERVE GROWTH FACTOR (NGF) IS essential in the development and maintenance of sympathetic and sensory peripheral neurons (*1*). In the central nervous system, cholinergic neurons of the septum-basal forebrain respond to exogenous NGF by increasing the levels of choline acetyltransferase (*2*), and the target areas of these neurons (hippocampus and cortex) contain the highest levels of NGF mRNA in the brain (*3*). Septum-basal forebrain neurons also carry NGF receptors (*4*), can retrogradely transport exogenous NGF injected into the hippocampus (*5*), and can be prevented from dying after axonal transection by infusion of NGF (*6*).

The structure of the NGF precursor protein has been deduced from DNA sequence analysis of cloned NGF genes (*7–9*). NGF mRNA has been detected in the brain of several species, including rat (*3*), but the cell types that synthesize NGF in the central nervous system are not known. In the study described here, we used in situ hybridization and RNA blot analysis in normal rats and in rats with experimental lesions to identify NGF mRNA–containing cells in the rodent brain.

In situ hybridization was performed with a ^{32}P-labeled 900-bp Pst I fragment from a mouse NGF cDNA clone. This fragment shows 97% DNA sequence homology between mouse and rat (*9*) and hybridizes strongly to rat NGF mRNA under stringent conditions (*3*). Under the conditions we used, a strong labeling occurred over NGF mRNA–containing cells in male mouse submandibular glands (*10*). In rat and mouse brain, the NGF cDNA probe revealed NGF mRNA in the dentate gyrus and in the hippocampal pyramidal cell layer in areas CA1 to CA4 (Fig. 1A). The same hybridization pattern was also seen when the NGF probe was mixed with an excess of unlabeled pUC9 DNA to eliminate possible nonspecific labeling over the hippocampus. Furthermore, a nonoverlapping NGF cDNA fragment, covering the 3′ untranslated region, showed an identical hybridization pattern, thus providing evidence against a nonspecific cross-hybridization of the probes with mRNA other than NGF mRNA.

Hybridization with a 900-bp Pst I fragment from the chicken NGF gene, corresponding to the 900-bp fragment of mouse cDNA, gave only weak labeling on male mouse submandibular gland (*10*) and no labeling on sections of rat brain. This result is consistent with previous RNA blot analysis that showed limited cross-hybridization between mouse NGF mRNA and chicken NGF probes under high stringency conditions of hybridization (*8*). No hybridization was observed when pUC9 DNA was used as a hybridization probe or after treatment of sections with ribonuclease A (RNase A). Furthermore, under the same hybridization conditions, unrelated cDNA probes for genes expressed in rat and mouse brain gave

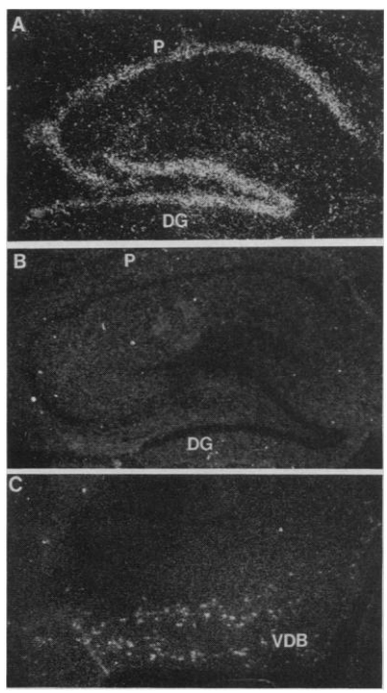

Fig. 1. In situ hybridization to sections of rat hippocampus. The DNA probes were labeled with [α-^{32}P]deoxycytidine 5′-triphosphate (dCTP) by nick translation to a specific activity of approximately 10^9 cpm/μg, and 5 to 10 ng of probe were added to each section. The conditions for in situ hybridization were as described (*10, 23*). The slides were dehydrated and dipped in 50% Ilford K5 nuclear emulsion and exposed for 9 days. The following probes were used: (**A**) A 900-bp Pst I fragment from a mouse NGF cDNA clone (*7*) and (**B** and **C**) a 400-bp Bst EII fragment derived from a rat NGF receptor cDNA clone (*24*). (A and B) Sagittal sections of rat brain at hippocampus level. (C) Transverse section through the ventral part of vertical limb of diagonal band of Broca (VDB). The sections are shown in dark-field illumination (×18). Abbreviations: P, pyramidal cell layer, and DG, granular cell layer of dentate gyrus.

C. Ayer-LeLievre, Department of Medical Chemistry, Laboratory of Molecular Neurobiology, and Department of Histology and Neurobiology, Karolinska Institutet, Box 60400, S-104 01 Stockholm, Sweden.
L. Olson, Department of Histology and Neurobiology, Karolinska Institutet, Box 60400, S-104 01 Stockholm, Sweden.
T. Ebendal, Department of Developmental Biology, Uppsala University, P.O. Box 587, S-751 23 Uppsala, Sweden.
Å. Seiger, Department of Neurological Surgery, University of Miami School of Medicine, Miami, FL 33136.
H. Persson, Department of Medical Chemistry, Laboratory of Molecular Neurobiology, Karolinska Institutet, Box 60400, S-104 01 Stockholm, Sweden.

*To whom correspondence should be addressed at the Department of Histology and Neurobiology, Karolinska Institutet, Box 60400, S-104 01 Stockholm, Sweden.

expected hybridization patterns that were different from those seen with the NGF cDNA probe. This is exemplified by using a probe for the rat NGF receptor that showed no hybridization to hippocampal formation (Fig. 1B), whereas strongly labeled cells were seen in septum-basal forebrain areas, such as the diagonal band of Broca (Fig. 1C).

To obtain better cellular resolution, we also performed hybridization with ^{3}H-labeled NGF cDNA probes. The labeling was then concentrated around nuclei of cell bodies that showed the typical shape, dimensions, and localization of hippocampal pyramidal neurons (Fig. 2A). In the dentate gyrus a high grain density was observed over cell bodies, presumably of granular neurons (Fig. 2B). Hippocampal cells were not labeled when control DNA probes were used (Fig. 2C), or when sections were treated with RNase A. Measurements obtained with a computerized image analysis system (*11*) showed that the density of labeling on hippocampal neurons with the NGF probe ranged from 11 to 66 times that of the background level, with a mean value of 34. The intensity of specific labeling varied from strongly positive neurons to negative ones, presumably reflecting differential NGF synthesis by individual neurons.

The labeling of cell layers in hippocampus by in situ hybridization is consistent with RNA blot analysis showing that hippocampus is the brain region with the highest levels of NGF mRNA (*3*). A similar labeling pattern of mouse hippocampus was reported after in situ hybridization with a ^{32}P-labeled NGF cRNA probe (*12*), but the resolution was such that the cells containing NGF mRNA could not be identified. With our ^{3}H-labeled NGF probes, we observed that pyramidal neurons in areas CA1 to CA4 and granular neurons in the dentate gyrus contained numerous silver grains around their nuclei.

Labeled neurons were also observed in other brain regions, including the cerebral cortex, although the density of autoradiographic grains was lower in cortical than in hippocampal neurons. Complete identification of dispersed cells containing NGF mRNA will require further investigation. No labeling was seen in granular cells of the cerebellum, although the cell density in the granular layer of the cerebellum is as high as in hippocampus. However, the nonspecific background was sometimes slightly enhanced in the cerebellum (*13*). These results are in agreement with RNA blot analysis (*3*)

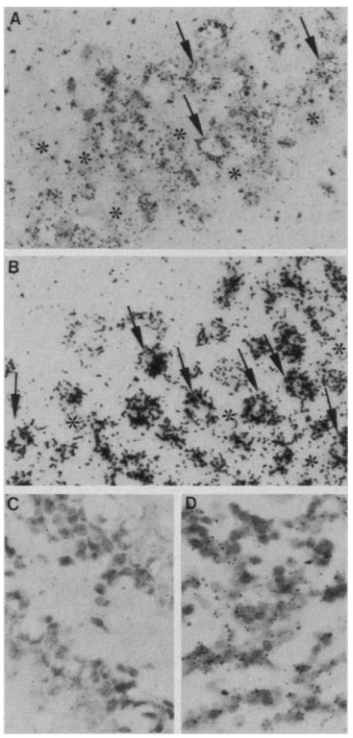

Fig. 2. Identification of NGF-producing cells in rat hippocampus. Conditions for in situ hybridizations were as described in the legend to Fig. 1, except that the 900-bp NGF cDNA fragment (**A** to **C**) and the pUC9 DNA (**D**) were labeled with [α-^{3}H]dCTP to a specific activity of approximately 10^{7} cpm/μg. The slides were dipped in Kodak NTB2 nuclear emulsion and exposed for 3 (A, C, and D) or 10 (B) months. The sections were lightly counterstained with cresyl violet. (A) Labeled pyramidal cells in the CA3 area (×280). (B) Granular layer of dentate gyrus (×440). (C) No labeling of hippocampal cells with control plasmid probe (×280). (D) No labeling on cerebellar granular layer (×280). Arrows indicate labeled cells; asterisks indicate localization of unlabeled cells.

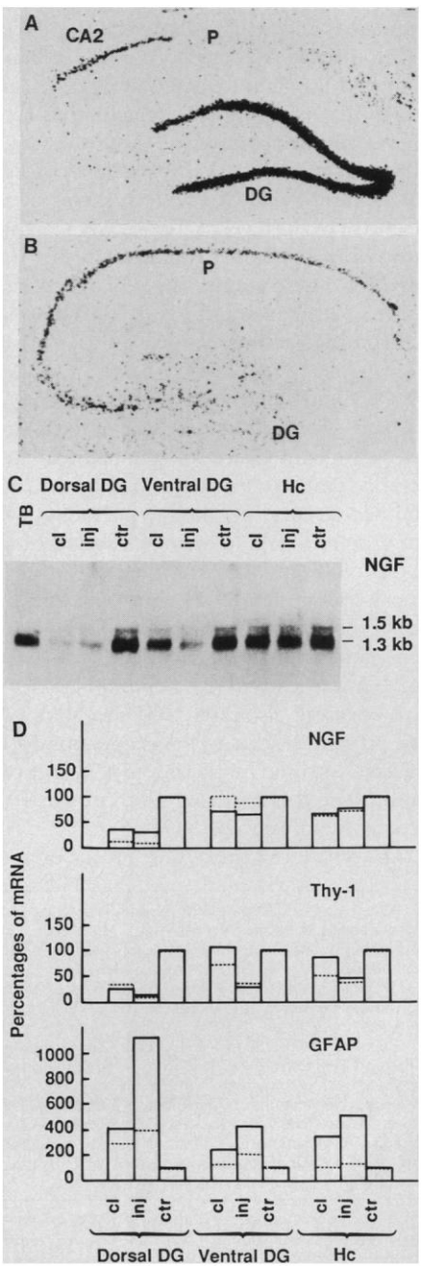

Fig. 3. Disappearance of NGF hybridizing cells after lesion of the rat hippocampus. (**A**) Kainic acid injection (0.3 μg in 1 μl of saline; 1.5 mm lateral and 0.3 mm caudal to bregma, and 4.8 mm below pial surface). The brain was fixed 7 days after injection and prepared for in situ hybridization (*10, 23*). Sections were lightly counterstained with cresyl violet. No hybridization can be seen in areas CA1, CA3, and CA4, where neurons were destroyed. CA2 pyramidal (P) neurons and dentate gyrus (DG) are labeled (×20). (**B**) Colchicine injection (6 μg in 1 μl of saline; 1.8 mm lateral and 3.3 mm caudal to bregma, and 3.3 mm below pial surface). The brain was fixed 9 days later and prepared for in situ hybridization. Destruction of granular neurons in DG abolished hybridization to the NGF probe in this area (×20). (**C**) Colchicine was injected in 16 rats (5 weeks old). Hippocampi were dissected 7 days later into dorsal DG, ventral DG, and remaining hippocampus (Hc) from the injected (inj) and contralateral (cl) sides. The same parts were also dissected from eight normal (control) animals (ctr). Total RNA was prepared from these samples and from total brain (TB). RNA was then separated by electrophoresis in formaldehyde-containing agarose gel (25 μg per lane) and blotted onto a nitrocellulose filter for hybridization as described (*25*). The filter was hybridized with a ^{32}P-labeled, 900-bp Pst I fragment from a mouse NGF cDNA clone (*7*). (**D**) Same experiment as in (C). The filter was additionally hybridized with a 250-bp Pvu II fragment from a mouse Thy-1 genomic clone (*19*), a 600-bp Pst I DNA fragment from a human GFAP cDNA clone (*21*), and (not shown) a ^{32}P-labeled, 1.5-kb Pst I DNA fragment from a mouse α-actin cDNA clone. Between consecutive hybridizations, the previous probe was removed by boiling the filter in 1% glycerol. The mRNA levels were quantified by densitometry of autoradiograms. The levels of NGF, Thy-1, and GFAP mRNA in each lane were calculated relative to the levels of actin mRNA and expressed as percentages of the levels of the same mRNA in the corresponding control areas. Similar results obtained in a second independent experiment are shown by dashed lines.

that showed relatively high levels of NGF mRNA in rat cerebral cortex but very low levels in cerebellum.

No labeling occurred in brain regions rich in oligodendrocytes such as corpus callosum. These results do not, however, exclude the possibility that NGF is synthesized by oligodendrocytes at levels below the detection limit of our technique or by different glial cells in other brain areas. The synthesis of NGF in glial cells might, for instance, be enhanced under certain conditions; expression of NGF by cultured primary glial cells and glia-derived cell lines has been reported (*14*). Similarly, NGF synthesis in the septum after a fimbria-fornix lesion (*15*) could be due to stimulation of this synthesis in astroglial cells surrounding cholinergic cell bodies that are deprived of their normal access to neuronally produced NGF in the hippocampus.

In situ hybridization was also performed on sections of hippocampus from lesioned animals. Pyramidal and granular neurons were destroyed by injections of kainic acid or colchicine, respectively (*16, 17*). Injections of kainic acid close to the dorsal hippocampus caused a complete ipsilateral destruction of dorsal pyramidal neurons in areas CA3 and CA4 and a partial destruction in area CA1. No cells were labeled with the NGF probes in the areas where the pyramidal neurons had been destroyed (Fig. 3A) in spite of local gliosis. However, specific hybridization still occurred in areas where pyramidal neurons survived (CA2 and part of CA1) as well as in the dentate gyrus granular cell layer. Local injection of colchicine completely destroyed granular neurons in the dentate gyrus close to the injection site and abolished hybridization to NGF probes in the same area (Fig. 3B), whereas pyramidal cell labeling in areas CA1, CA2, and CA3 was unaffected. After injection of colchicine we also observed destruction of neurons in ventral ipsilateral and dorsal contralateral dentate gyrus. The concomitant disappearance of the in situ hybridization signal and of neurons in hippocampus after these different chemical lesions were produced strengthens the view that NGF mRNA in this area is present in neurons.

We also determined the levels of NGF mRNA in different areas of hippocampus of normal and colchicine-lesioned rats by RNA blot analysis (Fig. 3, C and D). After injection of colchicine close to the dorsal right dentate gyrus, NGF mRNA in the corresponding areas decreased to less than 30% of normal (Fig. 3D). Similarly, Thy-1 mRNA, used as a marker for neurons (*18, 19*), also drastically decreased, reflecting a massive neuronal death. In contrast, the levels of glial fibrillary acidic protein (GFAP) mRNA, a marker for astrocytes (*20, 21*), were 4 to 11 times that in the control, reflecting a high percentage of glial cells in the neuron-deprived areas and local gliosis. In addition, the neuronal lesions caused by diffusion of the toxin to the dorsal contralateral and ventral ipsilateral dentate gyrus resulted in a decrease of Thy-1 and NGF mRNA, and an increase of GFAP mRNA compared to control dorsal and ventral dentate gyrus (Fig. 3, C and D). However, limited lesions may stimulate complex regulation mechanisms. A general gliosis in hippocampus after colchicine injection was confirmed by increased immunoreactivity of astrocytes with antibodies against GFAP, especially in the lesioned dentate gyrus. These results indicate a close correlation between NGF mRNA expression and degree of integrity of the neuronal cell population, as opposed to the glial cell population in hippocampus.

Consequently, these results support the data obtained by in situ hybridization, strongly arguing for a production of NGF by neurons in hippocampus. They also suggest that the terminal fields of the septo-hippocampal cholinergic projections are maintained by a continuous trophic supply of NGF from the target neurons themselves. Such neuron-to-neuron interactions could result in the establishment of a balance between the intensity of input (fiber density and neurotransmitter release) from septal to hippocampal neurons and the level of NGF provided by hippocampal neurons. Disturbances in this balance may underlie pathological alterations involved in aging (*22*) and cerebral diseases.

REFERENCES AND NOTES

1. R. Levi-Montalcini and P. U. Angeletti, *Physiol. Rev.* **8**, 534 (1968); H. Thoenen and Y. A. Barde, *ibid.* **60**, 1284 (1980).
2. P. Honegger and D. Lenoir, *Dev. Brain Res.* **3**, 229 (1982); F. Hefti, A. Dravid, J. Hartikka, *Brain Res.* **293**, 305 (1984); W. C. Mobley, J. L. Rutkowski, G. I. Tennekoon, K. Buchanan, M. V. Johnston, *Science* **229**, 284 (1985); H. J. Martínez, C. F. Dreyfus, G. M. Jonakait, I. B. Black, *Proc. Natl. Acad. Sci. U.S.A.* **82**, 7777 (1985).
3. S. Korsching, G. Auberger, R. Heumann, J. Scott, H. Thoenen, *EMBO J.* **4**, 1389 (1985); S. R. Whittemore *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **83**, 817 (1986); D. L. Shelton and L. F. Reichardt, *ibid.*, p. 2714.
4. P. M. Richardson, V. M. K. Verge Issa, R. J. Riopelle, *J. Neurosci.* **6**, 2312 (1986); M. Taniuchi, J. B. Schweitzer, E. M. Johnson, Jr., *Proc. Natl. Acad. Sci. U.S.A.* **83**, 1950 (1986); G. Raivich and G. W. Kreutzberg, *Neuroscience* **20**, 23 (1987); J. E. Springer, S. Koh, M. W. Tayrien, R. Loy, *J. Neurosci. Res.* **17**, 111 (1987).
5. M. E. Schwab, U. Otten, Y. Agid, H. Thoenen, *Brain Res.* **168**, 473 (1979).
6. B. Will and F. Hefti, *Behav. Brain Res.* **17**, 17 (1985); L. R. Williams *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **83**, 9231 (1986); L. F. Kromer, *Science* **235**, 214 (1987).
7. J. Scott *et al.*, *Nature* **302**, 538 (1983); A. Ullrich, A. Gray, C. Berman, T. J. Dull, *ibid.* **303**, 821 (1983); R. Meier *et al.*, *EMBO J.* **5**, 1489 (1986).
8. T. Ebendal, D. Larhammar, H. Persson, *EMBO J.* **5**, 1483 (1986).
9. S. Whittemore *et al.*, in *Life Sciences* (NATO Advanced Study Institutes Series, Springer-Verlag, Berlin, in press).
10. C. Ayer-LeLievre, L. Olson, T. Ebendal, Å. Seiger, H. Persson, *Histochem. J.*, in press.
11. Using a computerized image analysis system (IBAS, Zeiss/Kontron), we evaluated the labeling of cells as the percentage of area covered by silver grains over defined cell bodies or, for the background, outside specifically labeled cell bodies. In each section area, 18 to 24 measurements were made and repeated in four different experiments.
12. P. D. Rennert and G. Heinrich, *Biochem. Biophys. Res. Commun.* **138**, 813 (1986).
13. M. Schalling *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **83**, 6208 (1986).
14. A. M. Longo and E. E. Penhoet, *ibid.* **71**, 2347 (1974); A. M. Longo, *Dev. Biol.* **65**, 260 (1978); R. M. Lindsay, *Nature* **282**, 80 (1979); G. Norrgren, T. Ebendal, M. Belew, C.-O. Jacobson, J. Porath, *Exp. Cell Res.* **130**, 31 (1980).
15. G. Weskamp, U. E. Gasser, A. R. Dravid, U. Otten, *Neurosci. Lett.* **70**, 12 (1986).
16. J. V. Nadler, B. W. Perry, C. W. Cotman, *Nature* **271**, 676 (1978).
17. R. B. Goldschmidt and O. Steward, *Proc. Natl. Acad. Sci. U.S.A.* **77**, 3047 (1980).
18. R. Morris, *Dev. Neurosci.* **7**, 133 (1985).
19. T. Seki *et al.*, *Science* **227**, 649 (1985).
20. A. Bignami, L. F. Eng, D. Dahl, C. T. Uyeda, *Brain Res.* **43**, 429 (1972).
21. P. Rataboul *et al.*, *J. Neurosci. Res.*, in press.
22. L. Lärkfors *et al.*, *Mol. Brain Res.* **3**, 55 (1987).
23. B. Bloch *et al.*, *Regul. Pept.* **8**, 345 (1984).
24. M. J. Radeke, T. P. Misko, C. Hsu, L. A. Herzenberg, E. M. Shooter, *Nature* **325**, 593 (1987).
25. H. Persson, H. E. Gray, F. Godeau, *Mol. Cell. Biol.* **5**, 2903 (1985).

26. Supported by the Swedish National Board for Technical Development, The Swedish Medical Council (14X-03185, 14X-06555, 16X-07175, and B-BU-4024-116) and National Science Research Council (1948-112), Bank of Sweden Tercentenary Foundation, the Convention d'Échanges et d'Accord de Coopération Scientifique Franco-Suédoise, and R. Ayer (C.A.L.); and the Miami Project Foundation (Å.S.). We thank S. R. Whittemore for helpful discussion, J. Scott for the mouse NGF cDNA clone, J. Silver for the mouse Thy-1 genomic clone, J. Mallet and P. Rataboul for the human GFAP DNA clone, E. M. Shooter for the rat NGF receptor cDNA clone, M. Ingvar for setting up the IBAS program, and K. Lundströmer and I. Engqvist for technical assistance and secretarial help.

25 November 1987; accepted 7 April 1988