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14. This work was supported by grant 5-R01-NS14703 from the National Institutes of Health. We thank Philip R. Kennedy for help with electromyography.

16 February 1982; revised 13 April 1982

Brain Injury Causes a Time-Dependent Increase in Neuronotrophic Activity at the Lesion Site

Abstract. *A cavity was made in the brain (entorhinal cortex) of developing or adult rats, and a small piece of Gelfoam was emplaced to collect fluid secreted into the wound. The neuronotrophic activity of the fluid was assayed with sympathetic and parasympathetic neurons in culture. The results show that wounds in the brain of developing or adult rats stimulate the accumulation of neuronotrophic factors and that the activity of these factors increases over the first few days after infliction of the damage.*

The potential of central nervous system (CNS) tissue to recover from injury depends on the substances and processes that support neuron survival (neuronotrophic factors), promote the sprouting of neurites, and guide the neurites to their targets. The existence of substances with neuronotrophic, neurite-promoting, and guiding activities has been known for about 30 years. The best characterized of these molecules is nerve growth factor, which exhibits all three types of activity (*1*). The search for factors with trophic effects on central as well as peripheral cholinergic neurons has led to the description of a variety of such factors present in tissue extracts or secreted by cells in culture (*2*).

It has been suggested that, following injury to the nervous system, neurite-promoting and neuronotrophic factors are made available to facilitate repair (*3*). Evidence supporting the existence of such factors has been either rare, as in the case of the peripheral nervous system (*4*), or indirect, as in the case of the CNS (*5*).

Recently, however, a new system for nerve regeneration was used to demonstrate that neuronotrophic factors appear when the adult rat peripheral nervous system is damaged. The fluid that accumulated between the two stumps of a resected sciatic nerve and bathed the regenerating nerve structure contained trophic factors for sensory, motor, and sympathetic neurons, the contributors to the sciatic nerve (*6*). Lewis and Cotman (*7*) showed that the survival and growth of embryonic striatal tissue implanted in a cavity made in the entorhinal or occipital cortices of 3-day-old rats was much increased if the implant was emplaced 3 to 6 days after infliction of the wound. These observations suggest that factors accumulate in brain wounds which aid neuron survival and growth.

We tested this possibility by collecting the fluid secreted into brain wound cavities and measuring its ability to support neuron survival in culture. A cavity of 2 to 4 mm^3 was made in the entorhinal or occipital cortex of Sprague-Dawley rat pups (3 days old) or young adults (45 to 60 days old), and the cavity was filled with Gelfoam (Upjohn) moistened in isotonic saline solution. At various times after the operation the Gelfoam was removed and extracted with cell culture medium. The extract was tested for its ability to support the survival of dissociated neurons from ciliary (cholinergic) ganglia or sympathetic (noradrenergic) ganglia from chick embryos in the 8th or 12th day of development, respectively. The extracts were serially diluted and placed in the wells of microtiter plates. Neurons were added and the cultures were incubated for 24 hours, at which time they were fixed in 2 percent glutaraldehyde. The neurons were then counted under a phase microscope (*8*). The assay is set up so that in the absence of exogenous trophic factors, neuron survival is less than 15 percent of the maximum values obtained in the presence of nerve growth factor (sympathetic ganglia) or eye-derived neuronotrophic factor (ciliary ganglia) (*9*).

All implant surgery was performed on day 0. Extracts from the excised tissue had little or no neuronotrophic activity (Fig. 1). In the developing animals low neuronotrophic activity appeared on day 1 and increased to very high levels by days 3 and 6. In the young adults neuronotrophic activity was also present in the fluid by day 6 (little or no activity was evident earlier). The Gelfoam itself and the rat serum had no trophic activity. The extract was usually more active on sympathetic than ciliary ganglia neurons.

Trophic activity of the brain fluid was not lost by centrifugation at 250,000*g* or by dialysis but was destroyed by heating (90°C for 10 minutes) and by digestion with trypsin (incubation with 100 U for

Table 1. Effect of various treatments on the trophic activity of the Gelfoam extracts. Three to 6 days after infliction of the lesions, extracts of Gelfoam fragments from neonatal animals were pooled and 10-μl portions were titrated on the various test neurons after being subjected to a variety of treatments. Control samples were kept at 4°C for 60 minutes and then were diluted to 200 μl with modified Eagle's medium (*8*) before serial dilution and assay in the microtiter plates. For heat treatment, the portions were diluted to 200 μl, incubated for 10 minutes at 90°C, and returned to 4°C until assay time. Other portions were mixed with 50 μl of modified Eagle's medium, dialyzed against the medium for 2 hours at 4°C, and brought to 200 μl for assay. For trypsin treatment, samples were mixed with 10 μl of medium and 10 μl of trypsin stock (9700 U/mg; 1 mg/ml in medium), incubated for 60 minutes at 37°C, and mixed with 10 μl of Trasylol stock (100 U/ml) and 160 μl of medium. The final 200-μl mixture was kept at 4°C until being assayed. The same protocol was used for sample portions receiving trypsin and Trasylol at the same time. Antiserum to nerve growth factor (NGF) (1 μl blocks the activity of 5 trophic units of the factor) was added to other portions (20 μl of antiserum to 10 μl of CNS neural fluid), and the mixture was brought to 200 μl and kept at 4°C until being assayed. In this case, however, the subsequent serial dilution was carried out in the constant presence of antiserum to NGF (100 μl/ml). Trasylol or serum from unimmunized rabbits had no effect on trophic activity.

Treatment	Trophic activity (percentage of control)	
	Neurons from superior cervical ganglia	Neurons from ciliary ganglia
None	100	100
Heat	0	3
Dialysis	100	100
Trypsin	0	15
Trypsin plus Trasylol	100	100
Antiserum to NGF	100	100

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60 minutes at 37°C) (Table 1). Therefore, like other recently discovered neuronotrophic factors in the peripheral nervous system (*2*), these factors appear to be proteins. The trophic factors described here are different from nerve growth factor because their activity was not affected by treatment with an antibody that abolishes the biological activities of that protein (Table 1).

To our knowledge, neither the occurrence of neuronotrophic factors in the CNS nor their temporal increase after injury have been described previously. Such features may be part of the biochemical sequel to CNS injury. The hippocampus is widely used as a locus for brain implants (*10*) including cholinergic systems (*7, 10, 11*) and sympathetic ganglia (*12*). The survival of corpus striatum implants is enhanced if implantation is delayed after the initial surgery (*13*). In our study the optimal delay for graft implantation (*7, 14*) generally coincided with the time at which high levels of neuronotrophic activity first appeared in the Gelfoam. Therefore, induced trophic activity may have been responsible for the enhanced survival of the implants. The correlation between enhanced survival of CNS tissue in vivo and the presence of neuronotrophic activity in cell culture is important, from a methodological point of view, for evaluating the physiological significance of in vitro studies.

Our findings also have implications for studies of axon sprouting. There is extensive axon sprouting in the hippocampus following lesions to various types of afferents (*15*), including sympathetic fibers (*16*). Preliminary observations indicate that sprouting from the hippocampus can also be induced by injections of the Gelfoam extracts in vivo. In vivo, sprouting occurs more slowly with increasing age (*13*), a finding that may correspond to the slower appearance of neuronotrophic factors in adults in the present study. Gradual accumulation of these factors may also account for the accelerated sprouting seen after serial lesions. A small lesion in the entorhinal cortex of rats, made so that the lesion itself elicits little or no reactive growth in the dentate gyrus, causes a greatly accelerated growth response to a larger lesion made at a later time (*17*).

Brain implants are being explored as a means to aid in the restoration of functions lost through neurological disease or disorder. For example, implants rich in catecholamines can partially counteract movement disorders associated with damage to the nigrostriatal pathway (*18*). A major problem is that implants in adult brains survive and grow less successfully than implants in newborn animals (*19*). As neuronotrophic and other such factors are characterized and as these substances are made available in purified form, brain grafts in adult animals may become more successful. This research should provide a more detailed understanding of the humoral response to brain injury and may hold promise for therapeutic applications.

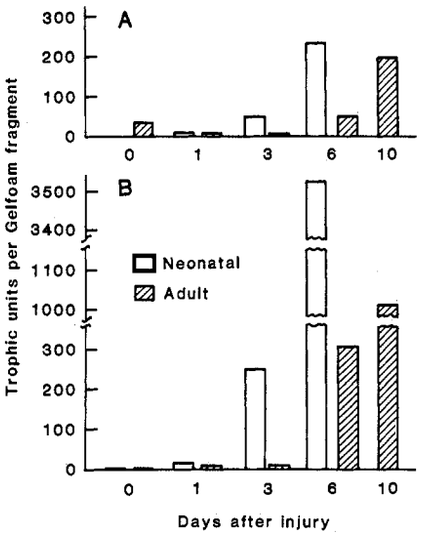

Fig. 1. Time course of appearance of neuronotrophic activity in a cortical wound assayed in cell culture with chick embryo ciliary (A) and sympathetic (B) ganglia neurons (*8*). Fragments of cortical tissue removed to make the wound cavity were assayed on day 0. One trophic unit is defined as the activity causing half-maximal neuronal survival when diluted to 1 ml of culture medium (*6, 8, 9*).

M. NIETO-SAMPEDRO
ELLEN R. LEWIS
CARL W. COTMAN
Department of Psychobiology, University of California, Irvine 92717
MARSTON MANTHORPE
STEPHEN D. SKAPER
GILLES BARBIN
FRANK M. LONGO
SILVIO VARON
Department of Biology, School of Medicine, University of California, San Diego, La Jolla 92093

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8. Approximately 2 to 4 mm^3 of entorhinal or occipital cortex was removed from 3-day-old or young adult rats, and the wound cavity was filled with a similar volume of Gelfoam. The Gelfoam was removed after remaining in the wound for the desired number of days. Pooled Gelfoam fragments from 4 to 12 animals for each time point were extracted with modified Eagle's medium [S. Varon, S. D. Skaper, M. Manthorpe, *Dev. Brain Res.* **1**, 73 (1981)] (100 μl per Gelfoam fragment) and the extract was centrifuged at 10,000*g* for 15 minutes. The supernatant fractions were tested for their ability to support the 24-hour survival of dissociated neurons from 8-day chick embryo ciliary ganglia and 12-day chick embryo sympathetic ganglia [M. Manthorpe, S. D. Skaper, S. Varon, *Brain Res.* **230**, 295 (1981); M. Manthorpe, S. D. Skaper, G. Barbin, S. Varon, *J. Neurochem.* **38**, 415 (1982)]. Falcon or Costar microtiter plates, each containing 96 6-mm-diameter wells, were treated for 30 minutes with polyornithine (0.1 mg/ml in 150 m*M* borate buffer, *p*H 8.4) and with polyornithine-binding neurite-promoting factor [M. Manthorpe, S. Varon, R. Adler, *ibid.* **37**, 759 (1981)] to provide a neurite-conducive substratum to facilitate recognition and counting of the test neurons. The plates were stored with 50 μl of modified Eagle's medium per well. At assay time the storage medium was replaced with the wound fluid solutions (50 μl per well). Collected factors were diluted at least tenfold in the medium and then serially diluted in the culture wells with medium containing 10 percent fetal calf serum. The cultures were initiated by adding 50 μl of the appropriate test cell suspensions. The cultures were incubated at 37°C for 24 hours fixed in 2 percent glutaraldehyde in medium, and analyzed for neuronal survival under a phase microscope.
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20. We are grateful to S. Bathgate for typing the manuscript. Supported by National Institute on Aging grant AG-00538 and National Institute of Mental Health grant MH-19691 to C.W.C. and National Institute of Neurological and Communicative Disorders and Stroke grant NS-16349 to S.V.

26 April 1982