rified PCR products (Quiagen) were sequenced with an automated sequence analyzer (Applied Biosystems). The sequences of the fragments for MHC class I (spanning positions 155 to 1138) and β_2-microglobulin (spanning positions 18 to 329) agreed with the published sequence of Lewis rat RT1.A^l and rat β_2-microglobulin, respectively.

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10. Primer sequences were for rat GAPDH (GenBank-EMBL accession number X02231) [5′-CCCACG-GCAAGTTCAACGG-3′ (position 220, plus strand); 5′-CTTTCCAGAGGGGCCATCCA-3′ (position 628, negative strand)]; for rat β_2-microglobulin (accession number Y00441) [5′-CGGTGACCGTGATCTTTCT-GGT-3′ (position 18, plus strand); 5′-GGTGACG-GTTTTGGGCTCCTT-3′ (position 329, negative strand)]; and for Lewis rat MHC class I (accession number L26224) [5 -TGCAGAGAGACTCAGGGC-CTACC-3′ (position 537, plus strand); 5′-TGC-CAGCTCAGGGAGATGTCA-3′ (position 714, negative strand) and 5′-TCGGCTACGTGGACCACACG-3′ (position 155, plus strand); 5′-TGCCAGCTCAGG-GAGATGTCA-3′ (position 714, negative strand) and 5′-TGCAGAGAGACTCAGGGCCTA- CC-3′ (posrtion 537, plus strand); and 5′-GACTCTGGGTGTCACAG-GAGAGACC-3′ (position 1163, negative strand)]. MHC class I, β_2-microglobulin, and the constitutively expressed enzyme GAPDH were always amplified simultaneously. PCR products (10 μl) from multiplex PCR were digested at 37°C with the enzymes Bgl I, Bgl II, and Apa I (Boehringer Mannheim), which cut the PCR fragments of GAPDH, β_2-microglobulin, and MHC class I, respectively, at a single internal side.
11. Mixed hippocampal cell cultures were treated with IFN-γ (100 U/ml) and TTX for 72 hours; we labeled the cultures with monoclonal antibody OX-18 specific for MHC class I by adding antibodies 10 μg/ml; Serotec) to the culture for 30 min. Cells were washed with phosphate-buffered saline and fixed with 4% paraformaldehyde. After blocking with 2% goat serum and 2% bovine albumin, cells were visualized with fluorochrome Cy3–conjugated goat antibodies to mouse immunoglobulin (Dianova; 10 μg/ml). Cells permeabilized with 0.1% Triton X-100 were intracellularly stained with mouse monoclonal antibodies specific for MAP2 (Sigma; 10 μg/ml) followed by fluorochrome (dichlorotriazinyl) aminofluorescein-conjugated goat antibodies to mouse immunoglobulin (Dianova; 10 μg/ml). Twenty optical sections along the *z* axis were recorded by confocal laser scanning microscopy (Zeiss 410 and Leica). Baseline labeling levels were determined by parallel staining with irrelevant monoclonal antibodies (10 μg/ml) followed by fluorochrome Cy3–conjugated goat antibodies to mouse immunoglobulin.
12. We thank Ch. Czoppelt for technical assistance on the confocal laser microscopy, E. Hansert for statistical advice, M. Schwab for critically reading the manuscript, and G. Garotta (Hoffmann–La Roche) for rat IFN-γ and IFN-γ–specific antibodies. H.N. was holder of a scholarship from the Deutsche Forschungsgemeinschaft.

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Monitoring Release of Neurotrophic Activity in the Brains of Awake Rats

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Intracerebral microdialysis of awake rats was used to monitor the possible release of neurotrophic factors from brain cells in response to injury and excitation. Perfusates were tested with ganglia bioassays and enzyme immunoassay. Trophic activity was released after implantation of the microdialysis probe into the hippocampus but not into the striatum, as assessed by increased nerve fiber outgrowth from Remak's ganglion. Kainic acid treatment significantly increased the release of trophic activity from hippocampal sites. These findings suggest that the brain responds to mechanical injury as well as to certain excitatory stimuli by regional extracellular release of neurotrophic activity that is not identical to the actions of known neurotrophic factors.

It has been suggested that neurons in the brain may respond to different forms of stress by increased synthesis and release of neurotrophic or neuroprotective factors. However, there is no evidence that such stress-induced release to the extracellular compartment of the brain occurs in vivo. Although the presence of mRNA coding for three of the four known mammalian neurotrophins (*1*) [nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), and neurotrophin-3 (NT-3)] has been demonstrated in neurons at the mRNA level by in situ hybridization (*2*), only two of the corresponding proteins, NGF and BDNF, have actually been demonstrated in vivo in the brain (*1*). In order to obtain information about the regional presence of neurotrophins in the brain, one possibility is to develop specific antibodies for immunohistochemical localization, a technique that has allowed cellular localization of BDNF protein in the brain (*3*). A second possibility is to use bioassays, taking advantage of the unique patterns of fiber growth responses seen, for instance, with different members of the neurotrophin family and with several different ganglia (*4*).

In the present study, we tested a new microdialysis probe with a membrane capable of dialyzing proteins the size of neurotrophins. We used this probe in vivo to monitor neurotrophic bioactivity in the hippocampus and striatum of rats and tested the effects of two different epileptogenic treatments. Released bioactivity was analyzed by addition of the perfusates to different ganglia and by enzyme immunoassay (EIA).

The dialysis probe was tested by in vitro dialysis of NGF solutions (*5*). NT-3 protein was measured with an EIA (*6*) that used a recently characterized antibody (*7*). The bioassay was done as described (*8*), using chick embryo spinal, sympathetic, ciliary, and nodose ganglia, as well as the unique Remak's autonomic ganglion from the dorsal mesorectum (*4*).

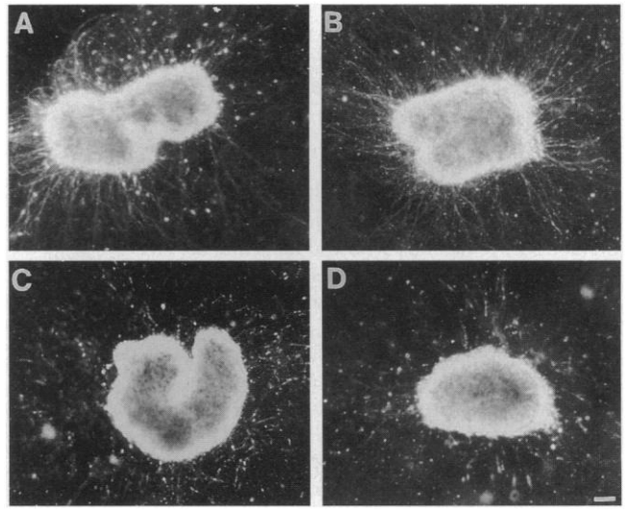

Fig. 1. Nerve fiber outgrowth from Remak's ganglion (**A** and **B**) and sympathetic ganglion (**C** and **D**) tested with hippocampal perfusates. After implantation of the microdialysis probe into the dorsal hippocampus (A and C), perfusates significantly stimulated nerve fiber outgrowth from Remak's ganglion (A) but not from the sympathetic ganglion (C). Kainic acid–induced seizures (B and D) markedly enhanced nerve fiber outgrowth from Remak's ganglion (B) but not from the sympathetic ganglion (D). Scale bar, 150 mm. (A) and (B) are examples of ganglia scored as positive; (C) and (D) are examples of ganglia scored as negative. There is a certain migration of cells from the explanted ganglia in (C) and (D), but no or almost no neurite extension.

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Stereotaxically guided implantation of microdialysis probes (9) into the hippocampus revealed no or very little trophic activity of the perfusates on sympathetic [0 to 0.02 biological units (BU), Fig. 1C] and spinal (0 to 0.1 BU) ganglia and no detectable activity on ciliary and nodose ganglia. When Remak's ganglion was used, a small degree of spontaneous outgrowth of nerve fibers, scored as 0.11 ± 0.02 (n = 48), was found when only sterile Ringer solution was tested. When the dialysis probe was inserted into the hippocampus, the presence of trophic activity in the perfusate was significantly increased (0.44 ± 0.12, n = 28) at 0 to 2 hours, decreasing continously at 2 to 8 hours after probe insertion (Figs. 1A and 2A). Perfusates from striatal sites did not evoke any growth responses from Remak's ganglia (0.02 to 0.15, Fig. 2B).

Injection of the convulsive glutamate agonist kainic acid (*10*) 4 hours after probe insertion into the hippocampus significantly increased trophic activity recovered in the hippocampal perfusates, as shown by an increased nerve fiber outgrowth from Remak's ganglion in both the first (0.77 ± 0.24, n = 13) and the second (0.94 ± 0.44, n = 9) 2-hour period after drug treatment (Figs. 1B and 2A). Injection of the γ-aminobutyric acid A antagonist pentylenetetrazol (*10*) did not elicit similar changes (Fig. 2A). No effects of implantation of the probe or of kainic acid on trophic activity were found in striatal perfusates [P = 0.7, one-way analysis of variance (ANOVA), Fig. 2B]. Hippocampal perfusates collected after kainic acid–induced seizures did not stimulate sympathetic (Fig. 1D), spinal, ciliary, or nodose ganglia.

NGF was not detectable in any of the hippocampal perfusates by the high-sensitivity EIA. Similarly, when the EIA was used for NT-3 (*7*), NT-3–like protein was below the detection limit in hippocampal perfusates. Injection of kainic acid did not elicit any increases in the concentration of NT-3, as measured by EIA (Fig. 3C).

The ganglia used for the bioassay were selected to differentiate between different neurotrophins and to indicate specific family members by an exclusion strategy (*4*, *8*). Our dialysates increased nerve fiber outgrowth from Remak's ganglion but not from any of the other ganglia. Although NT-3 (*8*) and glial cell line–derived neurotrophic factor (GDNF) (*11*) both stimulate neurite formation from Remak's ganglion, the lack of effect of the dialysates on sympathetic and dorsal root ganglia appears to exclude all neurotrophins and GDNF. Thus NGF, BDNF, NT-3, and NT-4 all evoke responses in dorsal root ganglia, and NGF and GDNF also stimulate sympathetic ganglia, whereas NT-3 only stimulates Remak's ganglion. Moreover, the bioassay data suggest that concentrations of NT-3 in the 100 to 150 pg/ml range would have been needed in the dialysates for the observed effects to be accounted for by NT-3. However, the NT-3 EIA failed to detect any NT-3, which suggests first, that NT-3 could not be present in concentrations greater than approximately 1 pg/ml, and second, that none of the treatments had any effect on NT-3 concentration. From the above and from extensive bioassays of ciliary neurotrophic factor, as well as members of the insulin-like growth factor, platelet-derived growth factor, and fibroblast growth factor families, none of which show the present pattern of effects in ganglia (*12*), we conclude that another, yet unidentified trophic factor, which could be released from the hip-

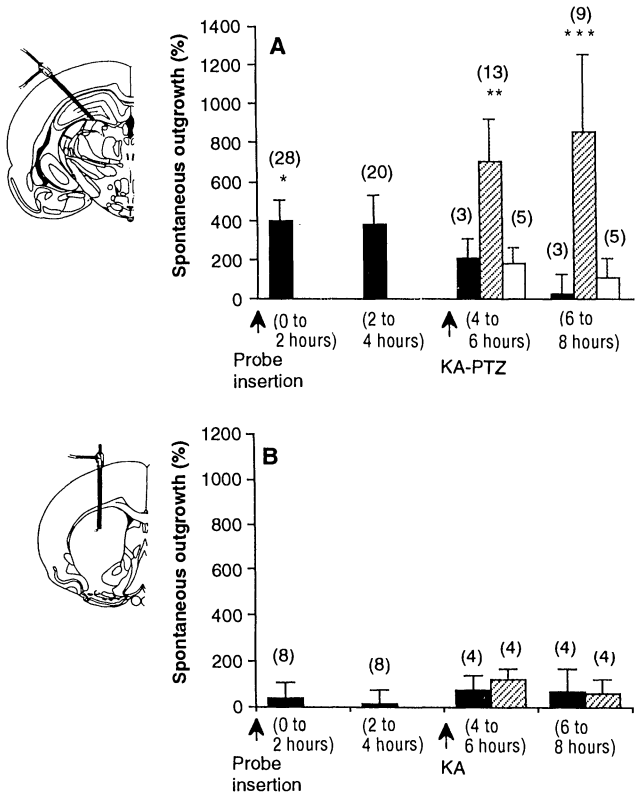

Fig. 2. Fiber outgrowth scores obtained with Remak's ganglion in the presence of brain dialysates. Microdialysis probes were implanted into the hippocampus (**A**) or striatum (**B**), as indicated on the left. Scores are given as percentage of spontaneous outgrowth seen with Remak's ganglion in the absence of dialysates. Numbers of animals are given in parentheses above each bar. Solid bars indicate outgrowth 2 to 8 hours after probe insertion. Hatched bars indicate scores after injection of kainic acid (KA). Open bars indicate scores after injection of pentylenetetrazol (PTZ). No effects were found in the striatum either after implantation of the microdialysis probe or after injection of kainic acid (B). The kainic acid–induced activity had the same pattern of bioassay activity as that seen in response to implantation. *P <0.05, **P <0.01, and ***P <0.001.

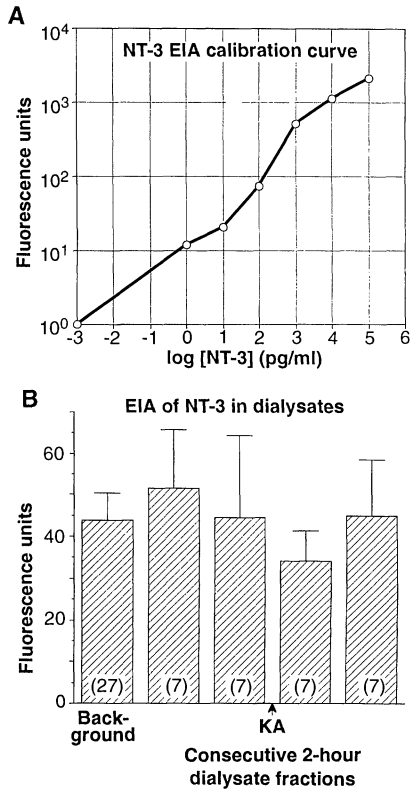

Fig. 3. EIA of NT-3. (**A**) Calibration curve illustrating the relation between average fluorescence readings from triplicate samples with added amounts of human recombinant NT-3. The linear relation obtained in the log/log diagram suggests optimal performance of the EIA. In this diagram, background fluorescence readings were deducted at each point. (**B**) Average concentrations (± SEM) of NT-3 immunoreactive material in four consecutive 2-hour hippocampal dialysates diluted to double volumes from seven rats. The leftmost bar shows the average (± SEM, n = 27) background fluorescence readings from this experiment. Background concentrations were obtained by addition of soluble antibody to NT-3 to parallel wells, blocking any NT-3 in the sample from binding to the anti–NT-3 coating of the wells (*7*). The four bars on the right represent average fluorescence readings from the four consecutive dialysates. Kainic acid (KA) was administered after 4 hours. There are no significant differences between background concentration and concentrations in any of the four dialysate fractions.

pocampus but not from the striatum, was responsible for the trophic effects on Remak's ganglion. The trophic activity in the dialysate seen in the first 2 hours after implantation of the probe into the hippocampus is probably due to the probe-induced mechanical damage of the tissue, affecting an already existing pool of trophic bioactivity in the hippocampus.

After injection of kainic acid, all animals exhibited the typical complex stages of convulsions, lasting the entire period of the experiment. Kainic acid significantly increased the same kind of trophic activity in hippocampal dialysates, as demonstrated by an increased nerve fiber outgrowth in Remak's ganglion. Again, the kainic acid effect was region-specific and thus not found in the striatum. In marked contrast to kainic acid, pentylenetetrazol did not increase trophic activity in hippocampal dialysates, despite the fact that animals exhibited massive generalized seizures 3 to 5 min after injection. The lack of ability of pentylenetetrazol to induce an increased activity in hippocampal dialysates might possibly be explained by the shorter excitatory stimulation and lack of neuronal degeneration. Alternatively, the two convulsant drugs might act through different mechanisms.

Taken together, our experiments demonstrate that neurotrophic activity can be captured from the extracellular space by in vivo dialysis in the hippocampus, but not striatum, of awake rats. The trophic activity is not compatible with that of known neurotrophins or other trophic factors, is regionally specific, and is increased by mechanical and certain convulsant treatments.

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5. For all release experiments, a microdialysis probe (Carnegie Medicine, Stockholm, Sweden) with a molecular cutoff of 100,000 daltons was used. To determine recovery in vitro, the probe was immersed in a range of concentrations of purified NGF and perfused with Ringer solution (147 mM NaCl, 4 mM KCl, and 2.3 mM $CaCl_2$) at a flow rate of 2 or 0.5 μl/min, starting with the lowest concentration of NGF. Samples were collected for 60 min on ice and subsequently frozen at −20°C. NGF concentrations were determined by bioassay using chick embryo spinal and sympathetic ganglia (*4*) or by EIA (*6*). At a flow rate of 2 μl/min, NGF activity was detected in the dialysate from stock solutions of 2 μg/ml and 300 ng/ml (0.35 to 0.08 scoring units), corresponding to a recovery of 3 to 5%. When the flow rate was lowered to 0.5 μl/min, NGF could be captured from stock solutions of 3 ng of NGF per milliliter, with a recovery of 12 to 35% as determined by EIA (*6*).
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8. Trophic activity was determined (in scored BU) in a bioassay with chick embryo spinal, sympathetic, Remak's, ciliary, and nodose ganglia (*4*). Ganglia were dissected from 8- to 9-day-old chicken embryos. Several ganglia were put in a culture dish in a 50-ml collagen matrix (*4*). Incubation was carried out for 2 days at 37°C under standard cell culture conditions. Fiber outgrowth was examined with dark-field or phase-contrast microscopy. Ganglia and fiber outgrowth patterns were drawn on a blind basis by one investigator. To score the different outgrowth intensities, the drawings were scored blindly by a second investigator on a scale of 0 to 3. This scale defines 3.0 as the maximal outgrowth seen in dorsal root or sympathetic ganglia in the presence of optimal concentrations of NGF. In the present experiments, maximal scores obtained with addition of brain dialysates to Remak's ganglion were 2.0. Most scores were in the 0.0 to 1.0 range. By counting of neurites or estimation of neurite densities when larger numbers had formed, or both, the scoring in this range could be done in increments of 0.2. All neurotrophins have been tested in the bioassay with different ganglia (P. Ernfors *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **87**, 5454, 1990).
9. To determine the in vivo release of trophic substances, microdialysis probes were implanted with the use of coordinates calculated from the atlas of Paxinos and Watson [S. Paxinos and C. Watson, *The Rat Brain in Stereotaxic Coordinates* (Academic Press, San Diego, CA, 1986)] Probes were placed in the hippocampus (bregma, −4.0; lateral, +5.0; angle, 45° to the midline; and depth, −5.0) or striatum (bregma, +0.5; lateral, +3.0; and depth, −5.0) and perfusion was started with a flow rate of 0.5 μl/min with sterile Ringer solution. All in vivo perfusion experiments were done under sterile conditions in a laminar flow bench. Perfusates were usually collected over 2-hour periods, generating four 60-μl 2-hour samples per animal. Samples were collected at room temperature and frozen. The animal setup, tubings, and the chosen flow rate allowed material in the probe to reach the collection vials in 20 min. Known neurotrophins are stable for long periods of time (weeks to months) at temperatures up to body temperature.
10. To induce release of trophic substances, the convulsive drugs kainic acid [12 mg per kilogram of body weight, intraperitoneal (i.p.); $n = 13$ rats] and pentylenetetrazol (50 mg per kilogram of body weight, i.p.; $n = 5$ rats) were injected 4 hours after implantation of the microdialysis probes. Statistical evaluations were carried out using one-way ANOVA with subsequent Fisher's protected least significant difference posthoc test comparison.
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Activation of Yeast PBS2 MAPKK by MAPKKKs or by Binding of an SH3-Containing Osmosensor

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The role of mitogen-activated protein (MAP) kinase cascades in integrating distinct upstream signals was studied in yeast. Mutants that were not able to activate *PBS2* MAP kinase kinase (MAPKK; Pbs2p) at high osmolarity were characterized. Pbs2p was activated by two independent signals that emanated from distinct cell-surface osmosensors. Pbs2p was activated by MAP kinase kinase kinases (MAPKKKs) Ssk2p and Ssk22p that are under the control of the *SLN1-SSK1* two-component osmosensor. Alternatively, Pbs2p was activated by a mechanism that involves the binding of its amino terminal proline-rich motif to the Src homology 3 (SH3) domain of a putative transmembrane osmosensor Sho1p.

A conserved MAP kinase cascade that functions in many eukaryotic signal transduction pathways consists of a MAP kinase (MAPK), a MAPK kinase (MAPKK), and a MAPKK kinase (MAPKKK) (*1*). The cascades of three kinases serve as intermediaries between cell-surface receptors and cytosolic and nuclear effectors. However, the functional significance of having three kinases in a cascade is not clear. Although the three kinases amplify a signal along the pathway, it is also possible that the series of protein kinases provides a mechanism for integrating signals from distinct receptors. Here, we demonstrate a case in which a single MAPKK integrates two distinct upstream signals. One of the activation mechanisms involves an interaction between the SH3 domain of a transmembrane molecule (a putative osmosensor) and a Pro-rich region in the Pbs2p MAPKK.

In the yeast *Saccharomyces cerevisiae*, ex-

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