Transplants of NGF-Secreting Fibroblasts Restore Stimulus-Evoked Activity in Barrel Cortex of Basal-Forebrain-Lesioned Rats

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INTRODUCTION

Nerve growth factor (NGF) and the family of neurotrophins play a role in protection and regeneration of CNS neurons (Levi-Montalcini 1987). NGF is implicated specifically in the protection and maintenance of cholinergic neurons in the basal forebrain. It has long been known that NGF improves neuronal survival and increases sprouting of cholinergic neurons in vivo and in vitro (Dekker et al. 1991; Hefti et al. 1985). Moreover, NGF protects cholinergic neurons against toxicity and damage (Hefti 1986; Hefti et al. 1989; Montero and Hefti 1989; Tuszynski and Gage 1990; Wilcox et al. 1995). These and many other observations suggest a potential therapeutic role for NGF and other neurotrophins in disease states that involve cholinergic neuronal atrophy, such as Alzheimer’s and other dementia. Although examination of NGF as a wonder drug in clinical trials of patients with Alzheimer’s disease has yielded mixed results (Eriksson Jonhagen et al. 1998; Seiger et al. 1993), many studies show that neurotrophins play a much wider role in physiological functions. For example, in addition to neuroprotective roles, NGF influences cells throughout the endocrine and immune systems (Levi-Montalcini et al. 1996). Neurotrophins may also be involved in higher processes such as information processing and cortical plasticity (Berardi and Maffei 1999). Despite the large number of studies fueled by the therapeutic potential of NGF, we still lack a clear understanding of the mechanisms, sites of action, and the role of NGF and neurotrophins in various CNS systems.

Because of the well-known role of NGF in protecting cholinergic basal forebrain cells from destruction, we began a series of experiments that delivered NGF to rats concurrent with ipsilateral lesions of the basal forebrain expecting to improve the survival of these cells. We knew from previous studies that unilateral lesions of the basal forebrain lead to decreased functional activity evoked in the somatosensory barrel cortex in response to whisker stimulation (Jacobs et al. 1991; Ma et al. 1989). In an attempt to “rescue” basal forebrain cholinergic neurons and concomitantly improve functional activity in the lesioned hemisphere, we delivered intraventricular injections of NGF to rats. Despite dramatic improvement of functional responses after NGF treatment, there was no increase in cholinergic innervation of cerebral cortex, which remained more than 50% depleted of ACh after the basal forebrain lesion (Rahimi et al. 1999).

The data from Rahimi et al. (1999) suggest that NGF may act directly on cortical neurons to restore functional activity toward normal, but the precise site and mechanism of action is unclear. Treatment using intraventricular injections of neurotrophins is not ideal because acute delivery of large doses of NGF limits the duration and the amount of NGF reaching target sites throughout the CNS, while increasing the possibility of deleterious side effects (Taglialatela et al. 1997). In addition, NGF may diffusely act on multiple locations within the CNS following intraventricular injections. In the study reported here, we used a different form of delivery by transplanting fibroblasts genetically engineered to secrete NGF into...
basal-forebrain-lesioned rats. To precisely define the site of NGF action and deliver more physiologic amounts of neurotrophin, we inserted NGF+ fibroblasts in various loci most likely to be affected by NGF, including the acetylcholine (ACh)-depleted neocortex, the lateral ventricle, and the basal forebrain. Our experiments revealed that NGF+ fibroblasts grafted into the neocortex ipsilateral to the basal-forebrain-depleted neocortex, the lateral ventricle, and the basal forebrain. The transplants were delivered immediately following the lesion and the second group received transplants into the lateral ventricle. Animals survived for 1, 2, or 4 wk after transplantation. Functional activity was assessed using [3H]2-deoxyglucose (2DG, ARC, St. Louis, MO) uptake in barrel cortex during whisker stimulation. Brain tissue was processed for cytochrome oxidase (CO) and acetylcholinesterase (AChE) activity, immunohistochemistry to identify the transplant, as well as 2DG autoradiography.

A fourth group of animals was used for electrophysiological experiments after we determined the efficacy of the fibroblast transplants on 2DG uptake. This group included rats that received a basal forebrain lesion and NGF+ fibroblast transplant, followed by electrophysiological recording 4 wk later. Control rats included animals with basal forebrain lesion only and no transplant.

**Basal forebrain lesion**

Each rat received a unilateral basal forebrain lesion as previously described (Jacobs et al. 1991; Rahimi et al. 1999). Briefly, rats were anesthetized using ketamine (60 mg/kg im) and rompun (4 mg/kg im) and placed in a stereotaxic apparatus. A small hole was drilled 4 mm anterior and 2.3 mm lateral to the bregma. 192-IgG-saporin (0.087 μg/μl, Chemicon, Temecula, CA) was delivered using a Hamilton syringe according to previously determined coordinates: 35° from vertical and 12° from lateral (Jacobs et al. 1991). The syringe penetrates the brain medially and moves laterally in a rostral to caudal direction, approaching the basal forebrain over the olfactory bulb while preserving the parietal cortex. These coordinates target the nucleus basalis, the substantia innominata, and the ventromedial globus pallidus. Immunoconjugate (1.0 μl) is injected at two sites in 0.5 μl volumes, the first at 8.0 mm below dura and the second at 7.0 mm below dura. This procedure results in consistent depletion of the cholinergic cells of the basal forebrain, while leaving other structures such as cerebral cortex and thalamus intact.

**Bioassay**

We performed a bioassay to examine the biological viability of the NGF secreted from genetically modified fibroblasts. Sympathetic ganglia were extracted from chick embryos at embryonic day 8. Neuronal suspensions were prepared and cultured into four groups. Each group received one of the following media conditions and subsequently incubated for 48 h at 37°C and 10% CO₂. Dulbecco’s modified Eagle’s medium (DMEM, Irvine Scientific, Santa Ana, CA) was used as control media, neuronal cultures were assessed for survival.

**Culture and transplantation of fibroblasts**

Following the lesion procedure, cell suspensions of genetically engineered host and control fibroblasts were grafted at either of three loci ipsilateral to the lesion. The genetically modified fibroblast cell line (Fischer rat skin) was generously donated to us by Dr. F. Gage (The Salk Institute, La Jolla, CA). The fibroblasts were grown in vitro using standard tissue culture procedures (Kawaja et al. 1992). Briefly, fibroblasts were cultured using DMEM (Irvine Scientific) with the following additives: 10% fetal bovine serum, 2.5 μg/ml fungizone, 2 mM glutamine, and 50 μg/ml gentamycin. G418 (400 μg/ml, Geneticin, Life Technologies, Grand Island, NY) was used as an additive only for the NGF+ fibroblasts that were also genetically altered to be resistant to this antibiotic, ensuring the purity of the NGF+ fibroblast population. Cultures were maintained using standard culture conditions at 37°C in 10% CO₂. Fibroblasts were fed once every 3–4 days with fresh conditioned media. Once confluent (approximately once per week), the cells were passaged using trypsin-EDTA solution (ATV solution, Irvine Scientific). NGF+ and control fibroblasts were used at six to seven passages or less for CNS grafting. The cells harvested for grafting were at resting state (postconfluence). Fibroblasts were dislodged from culture plates using trypsin-EDTA solution and collected using grafting media (grafting PBS and 2% rat serum). Grafting PBS consisted of sterile PBS supplemented with 1 μg/ml MgCl₂, and CaCl₂ and 0.1% glucose. After centrifugation at 70 g for 5 min, the cells were washed with 10 ml of fresh grafting PBS, counted, and examined for viability using an acridine orange-ethidium bromide solution (0.3% solution). Only fibroblast cell suspensions with a viability of 90% or better were used for transplantation. The suspension was subsequently centrifuged at 200 g and transferred to an eppendorf tube to yield a final concentration of 1.0–1.5 × 10⁵ cells/μl. Higher concentrations of cells may be transplanted with no deleterious effect on the brain (personal communication, Dr. F. Gage). Using a 10 μl Hamilton syringe, 2.5 or 5.0 μl of the cell suspension (in grafting PBS solution) was injected into the CNS. An injection rate of 1 μl/min was used. The surgical procedure and transplant locations were similar to those described previously (Jacobs et al. 1994; Rahimi et al. 1999). Coordinates for cortical and transplant locations were similar to those described previously (Jacobs et al. 1994; Rahimi et al. 1999). Coordinates for cortical transplant included three sites along an axis 1.1 mm lateral to midline at 0.1, 1.1, and 2.1 mm posterior to bregma, and 1.1 mm below dura (7.5 μl). Intraparenchymal grafts were made at coordinates 0.5 mm posterior to bregma, 1.1 mm lateral to midline, and 3.5 mm below dura (about 5 μl of cell suspension). Fibroblast grafts into the lesioned basal forebrain were performed in the same manner as the lesion procedure described in the preceding text (about 6 μl of cell suspension).

**2DG experiments**

Functional responses were assessed in all rats 1, 2, or 4 wk after the grafting procedure. A 2DG experiment was performed on each rat, and the area of stimulus-evoked metabolic uptake in barrel cortex was measured as previously described (Rahimi et al. 1999). Briefly, each rat was lightly anesthetized using isoflurane. All whiskers were removed except three or four pairs of matched vibrissae on the rat’s face. Nonadjacent whiskers used for stimulation included whiskers from B or D rows. Each vibrissa was fitted with a piece of mu-metal, an alloy of iron and 75–80% nickel, 3 mm long and 350 μm in diameter, using glue (Type 201 Aron Alpha, Borden, Columbus, OH). The mu-metal was placed at equal distances, approximately 1.0–1.2...
cm away from the face on each remaining whisker. Following recovery from anesthesia (1–1.5 h), each rat was placed in a cylindrical cage, 16 cm in diameter, surrounded by an electromagnetic coil. Our whisker stimulator system is similar to the “Lausanne whisker stimulator” developed by Melzer and colleagues (Melzer et al. 1985). A Grass stimulator attached to the coil generates magnetic field bursts every 50 ms. The magnetic field mechanically moves the whiskers with attached mics-metals at 20 Hz while the rat is moving freely in the cylinder. Stimulation began 5 min prior to injection of [14C]2-deoxy-D-glucose (20 μCi/100 g ip) and continued for 1 h following injection. Each rat was then injected with an overdose of pentobarbital sodium (50 mg/kg ip) and perfused intracardially with saline followed by 0.1 M phosphate buffer containing paraformaldehyde with sucrose (4%). Each brain was then quickly removed and frozen in isopentane at −35°C and stored at −80°C until processed.

### Tissue processing

Brains were cut into 30-μm-thick coronal sections using a cryostat at −19°C; alternate sections were saved for 2DG autoradiography, CO, AChE histochemistry, and immunohistochemistry. The procedures for tissue processing were previously described (Jacob et al. 1991, 1994). Sections saved for 2DG autoradiography were collected using 2% gelatin-coated slides and placed on a hot plate (60°C) for immediate dehydration. These slides were exposed to X-ray film (Kodak MR-1; Sigma, St. Louis, MO) with 14C methylacrylate standards (2 nCi; Amersham, Piscataway, NJ) for 3–5 days and subsequently developed using standard procedures. Sections were later used for staining to identify Nissl substance. AChE and CO histochemistry were performed according to previously described protocols (Jacobowitz and Creed 1983; Rahimi et al. 1999; Wong-Riley 1979).

### Localization of transplants

The fibroblast transplants were localized during processing of the tissue using several different methods. The NGF+ transplants were identified using antibodies directed against NGF or fibronectin to localize the fibroblasts. The control NGF− transplants were visualized using immunoreactivity against fibronectin or by labeling with a fluorescent dye. Appropriate sections were sectioned at 30 μm, and immunoreactivity against fibronectin or by labeling with a fluorescent dye for 15 min at 37°C. The PBS with dye was removed and cells resuspended in fresh PBS, and incubated for 30 min. Cell suspensions were washed with gassing PBS and used for transplantation.

### Measurement of AChE distribution

Cholinergic depletion following basal forebrain lesion was measured using AChE histochemistry. Staining for AChE, the catabolic enzyme for acetylcholine, is not a direct measure of acetylcholine but is useful as a tool to assess cholinergic depletion (Hohmann and Coyle 1988; Jacobs et al. 1994; Ma et al. 1989). Optical-density measurements were used to determine AChE reactive fiber density as previously described (Jacobs and Juliano 1995; Jacobs et al. 1994). The density of AChE-positive fibers was assessed and quantified in both hemispheres of all rats using the sections adjacent to 2DG-labeled autoradiographs that contained activated barreels. Optical-density levels were measured within a rectangular region of 1.0 × 0.5 mm, which extended from layer II–V. An average value of optical density was derived across sections assessed from each hemisphere. This allowed us to quantify and compare AChE staining in the treated barrel cortex to that of the control, untreated cortex. Optical-density values in the lesioned hemisphere were calculated and represented as a percentage of the density in the untreated, normal cortex, set at 100%.

### Barrel-associated metabolic uptake

A video-based imaging system was used to visualize and quantify metabolic uptake in the activated barreels revealed on 2DG autoradiographs. Each autoradiograph was digitized using a previously described protocol (Jacobs and Juliano 1995). Radioactive standards were used to convert optical density values to color or gray scales. Variability of the 2DG label was quantified in each section by measuring optical-density values for specific regions and expressed as a percent above background, where background is set to optical density of white matter. Using a digitizing tablet, layer IV of the frontoparietal cortex within each 2DG autoradiograph was flattened and represented in two dimensions using software that partitions and designates regions of cortex into vertical and tangential arrays of high resolution (Tommardahl et al. 1985). Bins (50 μm) spanning layers II–V are generated and collapsed to a single point containing the density values. Files from all sections are aligned and displayed as unfolded maps that display evoked-metabolic uptake in barreels as specific foci of high activity. Values 1.5 SD above the average density of the entire map were set to black in the unfolded map. Areal measurements were obtained from the barrel-associated foci and comparisons made between the treated, experimental cortex and the untreated, normal cortex.

### Electrophysiological experiments

Neuronal response characteristics following whisker stimulation were measured using extracellular recordings of single units within layer IV of ACh-depleted barrel cortex. Recordings were obtained from neurons located in matched barreels from both hemispheres. Both spontaneous activity and response magnitude of single units were assessed. Recording experiments were done in a “blind” fashion, that is, the experimenter did not know the nature of the animal treatment.

Rats were anesthetized with a ketamine (60 mg/kg) and rompun (4 mg/kg) mixture (injected intramuscularly) and maintained on this mixture for the duration of the recording experiment. Each rat was monitored regularly during the procedure and supplemental doses of anesthesia were administered to ensure a uniform state. Each rat was placed in a stereotaxic apparatus, and an opening was made in the skull overlying the postero medial barrel subfield. The dura was removed and the brain covered with mineral oil for protection. An opening was also made in the cisterna magna to drain cerebrospinal fluid and reduce pulsations and swelling of the brain.

Response characteristics were assessed in layer IV of the cortical representation for each designated whisker. Cortical single units corresponding to at least two different matched whisker barreels were located (e.g., B2–3, D2–3, C2–3 whiskers), using tungsten-in-glass recording electrodes (0.5–1.0 MΩ). The electrode was lowered to the surface of the cortex at the specific stereotaxic coordinates of a designated whisker. The cortical representation of
each whisker was confirmed electrophysiologically by locating the cortical site from which single units responded most vigorously to displacement of the designated vibrissae. The electrode was lowered through the cortex as single or multiple units were encountered in response to stimulation of the designated or surrounding whiskers. Recorded signals were subsequently amplified, filtered, and displayed while simultaneously sent to a personal computer equipped with the BrainWave data-acquisition and -analysis software (DataWave Technologies, Long Mont, CO). The location and depth of each recording site was noted, and response activity was recorded in multiple units and subsequently sorted into single units off-line. Neuronal activity was recorded during 30 sweeps of 1-s epochs. The first 500 ms of each sweep provided spontaneous activity of the unit recorded. A uniform stimulus was applied to the appropriate whisker using a switching device (whisker displacement apparatus) fitted with a glass pipette. The switching device delivered a stimulus that consisted of a motion that deflects the whisker by 1 mm (1.14°) in the upward direction. The whisker was trimmed to 15-mm length and fitted inside the glass pipette attached to the switching device, approximately 10 mm away from the skin. Cortical units displayed optimal response to this excision as reported by Simons and colleagues (Simons 1978). The response properties and spontaneous activity of each unit was recorded and averaged over 30 stimulus sweeps. The electrode was then advanced to other sites of responsive neuronal units, and the recording procedure repeated. At the end of each penetration site, we made a small electrolytic lesion by a passing current through the electrode (2 mA, anodal current; 5–8 s) to identify the electrode track. Single-unit recordings in the same layer were assessed in matched barrels of the opposite, otherwise normal hemisphere. At the end of the recording session, each animal was deeply anesthetized and perfused intracardially with paraformaldehyde (4%) and sucrose (4%). Brains were removed and immediately frozen using isopentane at −35°C. All brains were stored at −80°C until processed. Brains were cut on a cryostat at 30 μm, and three series of sections were collected for Nissl substance staining, CO activity, and AChE histochemistry. The laminar location of each recording site was verified using depth of the recording electrode and the site of the electrolytic lesion identified in Nissl-stained sections.

Multiple-unit recordings were analyzed off-line and isolated into single units of activity using the BrainWave program. Single units were recognized by their waveform characteristics and isolated. Each unit was distinguished using conservative and consistent criteria and represented by peri-stimulus time histograms (PSTHs). The spontaneous firing rate for each single unit was also determined and represented using PSTHs. Spontaneous activity of an individual neuron was the firing rate from a 500-ms prestimulus epoch of each stimulus trial, averaged over 30 trial sweeps. The response magnitude of individual neurons was represented on PSTHs as the number of spikes/stimulus for the 50-ms period following a whisker stimulus. Statistical analysis was used to compare evoked activity in the treated hemisphere to that of the untreated, otherwise normal hemisphere.

RESULTS

Immunotoxin lesions of cholinergic basal forebrain deplete the cortical distribution of AChE

Similar to our previous experiments, lesions of the basal forebrain produced persistent depletion of the cortical cholinergic input from basal forebrain nuclei. Correct placement of the lesion was verified using CO histochemistry and AChE reactivity. Immunotoxin lesions using 192-IgG-saporin specifically target and destroy cholinergic neurons that express the low affinity NGF receptor, p75NTF. In these experiments, injections of immunotoxin destroyed cholinergic neurons within the basal forebrain confined to the nucleus basalis magnocellularis, substantia innominata, and ventromedial globus pallidus, while sparing other systems (Fig. 1).

We compared the density of AChE staining in sections adjacent to stimulus-activated barrels in normal and treated hemispheres. The number of animals in all the experimental groups can be seen in Table 1. All rats receiving a unilateral basal forebrain lesion displayed reduced levels of AChE fiber staining irrespective of graft placement. The AChE depletion was homogenous throughout the cortical layers, ranging from 85 to 95% below the AChE fiber staining within the normal cortex (Fig. 2). That is, despite whether the NGF producing fibroblasts were placed in the cortex, the lateral ventricle, or basal forebrain ipsilateral to the lesion, AChE reactivity remained significantly reduced in comparison to the control, otherwise normal, hemisphere (Fig. 3). Although we generally found few differences related to the survival time after the transplant, in animals with cortical grafts of NGF+ fibroblasts
that survived for 4 wk following treatment, slight increases in AChE-positive fibers were present surrounding the area of the graft. This increase in AChE fiber staining, however, was limited to the site of the graft and did not extend significantly into surrounding cortical regions. In addition, the optical density measurements did not show significant overall increases in the density of AChE fiber staining in the treated hemispheres in these animals compared with the animals with transplants of shorter duration. Other animals with similar treatment and shorter survival periods, i.e., 1 or 2 wk survival, following cortical transplantation did not show increases in AChE fiber staining.

Secretion of biologically active NGF from genetically engineered fibroblasts

To ensure that the genetically altered fibroblasts produced and secreted biologically active NGF in our hands, prior to grafting into CNS loci, we used conditioned media obtained from the fibroblast cultures in a bioassay. Neuronal cultures were prepared from embryonic sympathetic neurons, which are dependent on NGF for survival. The cultured sympathetic neurons were then incubated for 48 h with conditioned media obtained from fibroblast cultures. The neurons were grown either with conditioned media from NGF+ fibroblast cultures or from NGF− fibroblast cultures. The conditioned media was extracted from cultured fibroblasts after 5 days of active fibroblast growth. Positive and negative controls included sympathetic neurons cultured with control media containing synthetic NGF additives or no additives. Our results show that the sympathetic cultured neurons survived when incubated with media from NGF+ fibroblasts or media with NGF additives but not when cultured with control media or conditioned media from the control fibroblasts. Figure 4 demonstrates typical healthy sympathetic neurons after conditioned media culture obtained from NGF+ fibroblasts and nonneuronal cells (i.e., no surviving neurons) seen after culture with conditioned media obtained from the control fibroblasts.

Localization of transplants

The fibroblast transplants remained near the injection site and were viable for up to 4 wk post transplantation. The immunoreactivity for NGF peptide or fibronectin revealed labeled cells at the site of the transplant and at distances up to 500 \( \mu m \) away. Positive NGF immunoreactivity was seen in animals that survived up to 4 wk following treatment (Fig. 5). Fibronectin immunoreactivity in a control transplant was observed at the site of transplant. The final method for identifying the fibroblasts, using fluorescent dye, CFDA SE cell tracer, can be seen in Fig. 6, which shows labeled cells in a transplant placed in the basal forebrain.

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<th>Table 1. Number of rats analyzed for each treatment and duration</th>
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All animals received a unilateral basal forebrain lesion. Nerve growth factor (NGF)-secreting fibroblast grafts in cortex (NGF+ cortex), normal fibroblast grafts in cortex (NGF− cortex), NGF-secreting fibroblast grafts in lesioned basal forebrain (NGF+ BF), normal fibroblast grafts in basal forebrain (NGF− BF), NGF− secreting fibroblast grafts in lateral ventricle (NGF+ LV), normal fibroblast grafts in lateral ventricle (NGF− LV).

FIG. 2. Example of acetylcholinesterase (AChE) staining in an animal that sustained a unilateral lesion of the basal forebrain. Top: staining in the barrel region of somatosensory cortex in lesioned (right) and otherwise normal hemisphere (left). Middle: the approximate site of the lesion and the barrel region. Bottom: an example of AChE staining in the basal forebrain on the lesioned side (right) and the normal side (left), demonstrating that AChE reactive neurons are greatly reduced or absent on the lesioned side. Cortical layers are indicated with numbers. Scale = 200 \( \mu m \).
2DG uptake

The metabolic uptake, in response to whisker stimulation, occurred in column-like patches that corresponded to locus of the individual barrels, as we reported previously (e.g., Ma et al. 1989). The 2DG patches were compared with adjacent sections that were reacted for CO activity and observed to coincide with the individual barrels (Fig. 7A). We also found that the dimension of the barrels, measured by CO, did not change after basal forebrain lesion as we have reported previously (Jacobs and Juliano 1995; Jacobs et al. 1991; Ma et al. 1989).

The fibroblast transplants were placed in three different locations, for both the control and the NGF+ cells. In the animals that received control fibroblasts, 2DG uptake following whisker stimulation remained diminished on the side of the lesion, regardless of transplant placement, including cerebral cortex (e.g., Fig. 8A). Basal-forebrain-lesioned animals with NGF+ fibroblast transplants placed either in the basal forebrain (Fig. 8C) or the lateral ventricle (Fig. 8D), also demonstrate reduced activity in response to whisker stimulation. When an NGF+ transplant was placed in the cerebral cortex of a basal-forebrain-lesioned animal, the 2DG uptake elicited in response to whisker stimulation was similar to that in the normal hemisphere (Fig. 8B).

When the activity was viewed as two-dimensional digitized maps, 2DG uptake appears as foci of stimulus-evoked activity that correspond to activated barrels. An example of the relation between the foci of 2DG uptake as represented in the two-dimensional maps and the CO staining of the barrels is indicated in Fig. 7B. The maps in this figure were prepared by indicating the boundaries of the cytochrome oxidase-stained barrels, using the sections adjacent to the 2DG autoradiographs, and making a separate map of the CO barrel boundaries. The map of the CO boundaries was then superimposed on the 2DG map, indicating the correspondence between the 2DG foci and the CO-stained barrels.

Illustrated in the two-dimensional maps of 2DG activity are examples of several experimental conditions (Figs. 9 and 10). The only condition that led to improved functional responses was the transplantation of NGF+ fibroblasts into the cerebral cortex. In this condition, the regions of 2DG uptake produced in the transplanted hemispheres are similar in dimension to those in the untreated, normal hemisphere (Fig. 9). In all other experimental conditions, whether receiving control NGF− transplants, or NGF+ grafts into the basal forebrain or lateral ventricle, the barrel-associated 2DG uptake remained diminished, similar to that observed in rats with basal-forebrain-lesioned hemisphere alone (Fig. 10C). Shown in Fig. 10 are examples of maps generated from control NGF− fibroblast transplants (Fig. 10A) and from animals receiving NGF+ fibroblast transplants ipsilateral to the lesioned hemisphere, placed either into the basal forebrain or the lateral ventricle (Fig. 10, B and D). In these conditions, foci of evoked activity are smaller in dimension to that evoked in the normal hemisphere.

To quantify these observations across all animals, the area of each region of barrel-associated activity was measured and

FIG. 3. Quantification of the density of AChE staining in the somatosensory cortex. The optical density of AChE staining in barrel cortex was averaged across several sections and expressed as a ratio of the density of staining in the opposite, untreated hemisphere, which was considered to be 100%. The ratios were averaged across all animals in each group; the number of animals for each condition can be seen in Table 1. All the experimental groups illustrated here are significantly reduced in the density of AChE staining compared with the density in the opposite normal hemisphere ($P < 0.00086$, $t$-test). The bars equal SE.

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FIG. 4. Examples of cultured sympathetic neurons maintained in the presence of conditioned media (A) obtained from nerve growth factor (NGF)+ fibroblasts, which were maintained in culture for 5 days. These neurons appear healthy after 48 h in culture. B: a view of similar cultures maintained with conditioned media obtained from control, NGF− fibroblasts. There were no surviving neurons following 48 h in culture. Scale = 50 μm.
compared between the treated and the untreated, otherwise normal hemisphere and displayed as a ratio. Figure 11 demonstrates that the only experimental condition resulting in improved functional activity was that of NGF+ fibroblasts placed in the cerebral cortex. Other placements of NGF+ fibroblasts and control NGF− transplants led to 2DG uptake that was significantly reduced from that evoked in the normal hemisphere.

FIG. 5. Immunoreactivity directed against NGF. This reaction was used to examine the presence of NGF peptide following NGF+ fibroblast grafts into CNS. Shown are several examples of tissue immunoreacted against NGF from one animal treated with a cortical graft of NGF+ fibroblasts. The region boxed in A, depicting NGF immunoreactivity at transplant site, is shown in higher power in B. C: a different section from the same transplant also stained for NGF immunoreactivity. *, the penetration site of the syringe. The boxed region is shown in higher power in D. E: a higher magnification of NGF immunoreactivity from the boxed region in D. Scale in A and C = 2 mm; scale in B and D = 1 mm; scale in E = 100 μm.
We examined the effects of the length of NGF treatment in ACh-depleted rats. The results presented in the preceding text included all survival times. Rats with unilateral basal forebrain lesions survived for 1, 2, or 4 wk following transplantation of either control NGF− or NGF+ fibroblasts at various loci. Deficits in functional activity persisted in rats that received control or NGF+ grafts in either the lateral ventricle or basal forebrain, despite the duration that the fibroblasts remained in situ. Since restoration of stimulus-evoked activity was restricted to rats with NGF+ grafts placed in the ACh-depleted cortex, we compared short- and long-term effects of NGF treatment in this group (Fig. 12). Shown in Fig. 12 are the combined data for animals with NGF+ fibroblasts in the cerebral cortex for 1, 2, or 4 wk. The mean ratio of the response for the treated: untreated hemisphere was plotted for each survival time. Although the magnitude of the activated regions was slightly larger after 1 wk of survival, after 4 wk of survival, the magnitude of uptake in the treated hemisphere was still similar to that in the normal hemisphere (i.e., a ratio slightly more than 1.0). In addition, the dimensions were not significantly different from one another (Mann-Whitney test), suggesting that cortical grafts of NGF+ fibroblasts were effective in restoring functional activity as long as 4 wk following transplantation.

We also assessed whether the distance of the activated barrel from the transplant influenced the dimension of the 2DG uptake in the animals receiving NGF+ fibroblasts into the cerebral cortex. This information is presented in Fig. 13, which plots the size of the barrel-associated spots of label as a function of the distance from the transplant. Although there is a trend for the dimension of the uptake to reduce with increasing distance from the transplant, at the greatest distance examined, the ratio of the dimension of uptake for the treated: untreated hemisphere remains close to 1.0. In addition, statistical analysis reveals that there is no correlation between the distance from the transplant and the size of evoked activity ($R^2 = 0.16$). It is likely, however, that if the activated regions were of greater distance from the transplant, we would have observed a decrease in the area of the activated region with increasing distance from the transplant.

**DISCUSSION**

Our results show that NGF delivered to the ACh-depleted barrel cortex using modified fibroblasts improves functional responses in rats with unilateral basal forebrain lesions. The restoration of activity was dependent on the placement of the fibroblast grafts. Animals with cortical transplants of NGF+ fibroblasts showed significant improvements in evoked activ-
ity, while those animals receiving NGF+ transplants in the basal forebrain or lateral ventricle or control transplants in any site did not show improvements. Restoration of functional activity was not dependent on the duration of survival following transplantation of NGF+ fibroblasts.

Rationale for study of NGF

Many studies emphasize the influence of ACh on neuronal response properties in sensory cortex. Emerging hypotheses suggest that the cholinergic neurons, in combination with the inhibitory input from the basal forebrain, exert a tuning mechanism to enhance and strengthen relevant sensory stimuli at the level of the neocortex (Dykes 1997). Numerous studies show that alteration in cholinergic input to the sensory cortices changes the physiological and behavioral processing of sensory information. Previous reports from this laboratory indicate that unilateral basal forebrain lesions, which produce significant reductions in neocortical cholinergic innervation, result in reductions of stimulus-evoked activity (Jacobs and Juliano 1995; Jacobs et al. 1991). Similarly, other studies show that manipulation of cortical cholinergic innervation influences sensory processing in cerebral cortex. For example, Kilgard and Merzenich recently found that the frequency distributions in auditory cortex were significantly altered after pairing a sound stimulus with basal forebrain stimulation (Kilgard and Merzenich 1998). In a different study, Dykes and colleagues demonstrated that increased cortical responses due to a whisker-pairing paradigm do not occur during blockade of muscarinic receptors (Maalouf et al. 1998). If ACh is applied directly to sensory regions of cortex, increases in neuronal response magnitude or increases in the size of a given receptive field occur (Lamour et al. 1988; Metherate 1998). These and many other studies emphasize the importance of the cholinergic influence originating from the basal forebrain on cerebral cortex function.

Because of the importance of cholinergic innervation to sensory processing, we aimed to ameliorate the effect of basal forebrain lesion and subsequent cholinergic depletion in our model. Initially our experiments delivered NGF to basal-forebrain-lesioned rats in an attempt to ameliorate the destruction of the cholinergic neurons in the lesioned site. The protective role of NGF for the cholinergic neurons of the basal forebrain and its ability to guard the cholinergic (and noncholinergic) neurons of the basal forebrain from damage and to rescue them...
from lesion-induced atrophy is well documented (Hefti 1986; Kromer 1987; Williams et al. 1986). In addition, NGF offsets cognitive and behavioral deficits attributed to cholinergic neuronal atrophy (Fischer et al. 1987, 1991). Findings such as these encouraged us to use intraventricular injections of NGF in an attempt to rescue basal forebrain cholinergic neurons from lesion, restore neocortical cholinergic innervation, and improve functional responses that were diminished in our earlier studies. Although we previously observed dramatic increases in functional responses after NGF treatment, to our surprise, no improvement in cholinergic innervation of neocortex occurred. This suggested that NGF might directly influence the cerebral cortex independent of cholinergic input (Rahimi et al. 1999). Because the NGF was delivered intraventricularly in the previous study and we could not determine the precise site of action, the current experiment aimed to determine the site of action by insertion of NGF + fibroblasts in CNS regions likely to mediate a positive effect. The fibroblasts in this study also delivered more physiologic amounts of NGF compared with the relatively large injections into the lateral ventricle.

**Localized delivery of NGF via genetically engineered fibroblast transplants**

Ex vivo gene manipulations of biological cell lines such as fibroblasts allow for a continuous, biologically active delivery of neurotrophins (Gage et al. 1990; Kawaja et al. 1991, 1992). Fibroblasts survive following CNS grafting and can continue peptide secretion for periods up to 6 mo (Tuszynski et al. 1994). Rossner and colleagues showed 30- to 100-fold increases of NGF in cerebrospinal fluid following grafts of NGF + 3T3 mouse fibroblasts (Rossner et al. 1996). In vitro analysis of the NGF + fibroblasts used in the present study, as reported by Gage and colleagues, produce an average of 160 pg of NGF · h⁻¹ · 10⁷ cells (Kawaja et al. 1992). On the other hand, although several studies describe relatively longer-term survival and activity of NGF + fibroblasts, others indicate a reduction of NGF peptide production after periods of transplantation. For example, Frim and colleagues examined the role of NGF + fibroblasts in an excitotoxic model of Huntington’s disease. They showed that preimplantation of NGF +...
fibroblasts protected striatal neurons against excitotoxic lesions by 80%. Despite a robust biological effect, after 7 or 18 days following implantation, few cells within the transplant site stained positively for NGF peptide or for mRNA of the transfected NGF gene (Frim et al. 1993).

In our study, we found that the NGF+ fibroblasts were effective in restoring functional responses when transplanted into the cerebral cortex. In addition, the bioassay demonstrated that only the NGF+ fibroblasts supported sympathetic neuronal cultures. We also found that the NGF+ fibroblasts continued to secrete immunologically detectable NGF for periods up to 4 wk following transplantation into CNS. This was observed in the finding of positive NGF immunoreactivity in the fibroblasts after 4 wk in situ (e.g., Fig. 5) and secondarily in the increases of 2DG uptake after a 4-wk survival. The transplants also displayed immunoreactivity directed against fibronectin in the case of the control fibroblasts after 4 wk in vivo.

Our results suggest direct action of NGF on the cerebral cortex since placement of the fibroblast transplants into the lesioned basal forebrain or lateral ventricle were not effective in resorting functional activity, while direct placement into the cerebral cortex resulted in increased functional responses. Other studies show that the biological effects of NGF secretions from grafted fibroblasts are dependent on the proximity of the NGF source and target cells (Frim et al. 1993). Our previous study found that direct injections of NGF into the lateral ventricle generated improved stimulus-evoked activity, but the transplants of NGF+ fibroblasts used in this study were not effective when placed in the lateral ventricle. This may be due to the difference in dose delivered by several bolus injections into the lateral ventricle versus the more physiologic NGF levels delivered by the fibroblasts. The same may be the case with the fibroblast transplants into the basal forebrain. The combination of the amount of NGF delivered by the fibroblasts and the amount of damage as a result of the immunotoxin may be too high to expect restoration of the damaged cells. The NGF delivered by the fibroblasts may only be effective locally by direct action on cortical cells.

On the other hand, we did not see significant differences in the magnitude of effect related to the distance of an activated barrel from the fibroblast neocortical transplant. In general, the barrel associated 2DG uptake closer to the edge of an NGF+ transplant was only slightly larger than regions of activity located farther away. For the most part, however, the active barrels were relatively close to the transplants (i.e., within 1.8 mm), which may be within a reasonable range to expect an intracortical transplant to be effective (Jacobs et al. 1994).

**Does NGF act directly on neocortical cells?**

Our data suggest that NGF acts at the level of neocortical neurons to alter cortical function because only transplants directly into the neocortex led to positive results and improved functional responses. In addition, cholinergic innervation of the lesioned hemisphere was not improved, implying a noncholinergic mechanism of action on the part of the NGF+ cells. Neurotrophins act using specific high affinity tyrosine kinase receptors (trk A, B, C) and the low-affinity receptor p75NTF. NGF is particularly associated with actions on the trkA and the p75NTF receptor. The idea that NGF acts directly on cortical neurons is somewhat problematic because it is generally believed that adult cortex lacks the high affinity receptors specific for NGF (Chao 1994; Holtzman et al. 1995; Sobreviela et al. 1994). Several studies have recently reported the presence of these receptors across cortical fields, however, suggesting that neocortex may be able to respond directly to NGF through high-affinity trkA receptors (Cellerino and Maffei 1996; Prakash

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**FIG. 10.** A: maps generated from an animal that received a cortical transplant of control (NGF−) fibroblasts. The location of the transplant is indicated with a black line in the bottom. Three spots of 2DG uptake are seen in the distributions generated from each hemisphere following stimulation of 3 whiskers. The regions of uptake in the hemisphere with a lesion of the basal forebrain plus control transplant are smaller than those evoked in the normal hemisphere (top). B: maps generated from a rat with a basal forebrain lesion that received an NGF+ transplant directly in the basal-forebrain-lesioned site. The regions of uptake in the lesioned-transplanted hemisphere (bottom) are smaller than those seen in the normal hemisphere (top). C: maps contain 4 barrel-associated spots evoked by stimulation of matching whiskers in an animal with a basal forebrain lesion only. The dimensions of the spots of label in the basal-forebrain-lesioned hemisphere are smaller than those labeled in the normal hemisphere. D: maps from an animal with a unilateral basal forebrain lesion and NGF+ fibroblast transplant into the lateral ventricle ipsilateral to the lesioned hemisphere. Four matching pairs of whiskers were stimulated bilaterally. Foci of high activity in the treated hemisphere (bottom) are smaller when compared with matching foci of stimulus-evoked activity in the normal hemisphere (top). Scale = 1 mm.
Kolb and colleagues showed that exogenous delivery of NGF influenced dendritic branching and spine density (Kolb et al. 1997). Such growth of dendrites might be a mechanism for mediating the increases in cortical responses seen in this study.

Specific neurotrophic influences on cortical function have also been demonstrated. During a critical period of postnatal development, exogenous delivery of NGF to the neocortex can prevent cortical alterations due to monococular deprivation in visual cortex, thus not allowing plastic rearrangements to occur (Berardi et al. 1993; Maffei et al. 1992). In dark-reared rats, NGF administration to visual cortex also prevents changes in cortical responses due to deprivation (Pizzorusso et al. 1997). Other studies show that blockade of NGF function prevents normal postnatal development of the visual cortex. Berardi and colleagues blocked endogenous NGF activity by implanting hybridoma cells that produced antibodies directed against NGF in the lateral ventricle. They found that visual acuity and
dendritic growth were reduced compared to controls.
Binocularity of cortical neurons in visual cortex were significantly altered (Berardi et al. 1994). Inhibition of endogenous NGF also prolongs the period of sensitivity of visual cortex to monocular deprivation (Domenici et al. 1993).

The ability of NGF to acutely affect cortical function in barrel cortex was demonstrated by Prakash and colleagues (Prakash et al. 1996). These researchers found that topical applications of NGF revealed rapid but transient increases in the dimension of a functional activity associated with a barrel, while brain-derived neurotrophic factor (BDNF) produced an opposite effect, reducing barrel-associated area of activity for longer periods. Although the precise site of action for NGF cannot be determined from the preceding studies, taken together, these data suggest a direct modulatory role for NGF at the level of the neocortex in activities that involve active sensory input and stimulation.

**FIG. 14.** Illustrated are peri-stimulus time histograms from 2 representative animals. Data were recorded over 500-ms sweeps, and accumulated over 30 stimulus trials. Each bin is 2 ms. The stimulus (whisker deflection) is placed at the 0 time point. The cumulative number of spikes (number of action potentials) is indicated as “counts” on the y axis. A: representative recording from an animal with unilateral basal forebrain lesion and no other treatment. Top: data from normal hemisphere. Bottom: data from the treated hemisphere. The number of action potentials elicited in response to stimulation is markedly reduced in ACh-depleted somatosensory cortex. B: representative recording from an animal receiving a unilateral basal forebrain lesion and a NGF+ fibroblast transplant in ACh-depleted somatosensory cortex. Top: data obtained from the normal hemisphere. Bottom: the data from the treated hemisphere. The number of action potentials elicited in the ACh-depleted hemisphere with the NGF+ fibroblast transplant is not different from the neuronal responses in the normal hemisphere. See Fig. 15 for statistics for all trials. This illustrates that the NGF+ transplant is effective in improving neuronal responses in the cholinergically depleted hemisphere.

**FIG. 15.** This graph indicates the overall mean response evoked for animals with basal forebrain lesion only (n = 3) and for those with basal forebrain lesion plus ipsilateral transplants of NGF+ transplants into the neocortex (n = 3). The response magnitudes (spikes/stimulus averaged over 30 trials) were calculated for each hemisphere and presented as a mean value for all animals. The number of spikes included for each condition is indicated on the graph; the bar equals SE. These data demonstrate that in rats with basal forebrain lesions alone, the mean response is significantly reduced in the hemisphere with the lesion (Mann-Whitney test, asterisk, P = 0.003), whereas in animals with a basal forebrain lesion plus an NGF+ transplant, the mean evoked response in the treated hemisphere is not significantly different from that in the normal hemisphere (Mann-Whitney test).
Do NGF+ transplants act on remaining cholinergic fibers?

In the normal situation, cortical NGF is likely to act on cholinergic fibers originating from the basal forebrain. Although most of the cholinergic afferent fibers are destroyed in our study, the possibility exists that NGF may act on the remaining basal forebrain afferent fibers to increase cortical cholinergic activity through action on basal forebrain neurons. We cannot conclusively rule out this possibility, but the lack of effect after transplants in the basal forebrain, and the strength of the response after intracortical transplants suggests that NGF action was directly on cortical cells. In addition, the immuno- noles in this study reduced the density of cortical cholinergic fibers by 85–95%. This extent of reduction may diminish the ability of NGF to act on the remaining afferent cholinergic fibers in the neocortex. Winkler and colleagues recently showed that administration of NGF could not restore cholinergic activity in animals with an 81% reduction in cholinergic innervation following 192-IgG-saporin lesions, while it was capable of restoring cholinergic activity in animals with less severe lesions (Winkler et al. 2000). The ability of NGF to act on cortical cholinergic fields may therefore depend on levels of cholinergic damage, dose and duration of NGF action, and method of NGF delivery to cholinergic neurons of the basal forebrain.

Possible mechanisms of NGF action

Several studies indicate that, NGF can have direct effects on neurons. NGF can directly increase the release of glutamate in hippocampal and cerebral cortex (Knipper et al. 1994; Sala et al. 1998). Other studies show that NGF alters calcium storage by enhancing calcium entry through voltage dependent channels (Levine et al. 1995b). Although the mechanism for alterations in calcium levels is not fully understood, it implicates NGF in playing a role in plastic and morphologic modifications in CNS neurons.

Other studies show that neurotrophins influence synaptic transmission and synaptic efficacy by increasing postsynaptic responsiveness. BDNF, another member of the neurotrophin family, significantly and rapidly increases spontaneous firing rate, as well as the frequency and excitatory postsynaptic currents in cultured hippocampal neurons (Levine et al. 1995a). In vitro BDNF also regulates cortical excitability by regulating GABA-mediated inhibition (Rutherford et al. 1997). Other members of the neurotrophin family, including NGF, may affect neuronal excitability in a similar manner although there is limited direct evidence to support this idea.

Novel methods of neurotrophin function may also operate. Studies in developing chick brain suggest that neurotrophins travel anterogradely from cell body to axon, are subsequently released, and taken up by a second order neuron (von Bartheld et al. 1996). Neurotrophins have also been suggested to function in a paracrine or autocrine fashion (Acheson et al. 1995; Altar et al. 1997). In our study, exogenous NGF may augment these processes in neocortex and enhance endogenous neurotrophin action. In addition, NGF released from the graft site may act in a paracrine manner on cortical neurons of barrel cortex. Neurotrophins may also control their own release via regulated feed back loops (Canossa et al. 1997; Kruttgen et al. 1998). Other activity-dependent regulated release mechanisms may exist for NGF and BDNF, which are induced by stimulation of glutamate and muscarinic receptors and inhibited following GABAergic activation (da Penha Berzaghi et al. 1993; Zafra et al. 1990, 1991). More than likely, NGF action and expression on cortical cells involves complex interactions that are activity dependent and regulated by feed back loop.

Further studies are necessary to determine the precise mechanism of NGF action on cortical neurons. A better understanding of the site of production and release as well as the uptake mechanisms and physiological responses of neurons to NGF exposure in both short and long term will clarify the role of NGF in cortical function and processing of information. Recent studies indicate that trkA blockade affects the size and number of early postnatal basal forebrain neurons (Cattaneo et al. 1999). Functional blockade of trkA receptors using anti-receptor monoclonal antibodies may help decipher the role and mechanism of NGF action across cortical surfaces.

REFERENCES


Levi-Es, Dreyfus CB, Black IB, and Plummer MR. Brain-derived neurotrophic factor rapidly enhances synaptic transmission in hippocampal neurons via postsynaptic tyrosine kinase receptors. Proc Natl Acad Sci USA 92: 8074–8077, 1995a.


