Brain-Derived Neurotrophic Factor-Transduced Fibroblasts: Production of BDNF and Effects of Grafting to the Adult Rat Brain

CARRIE A. LUCIDI-PHILLIPI, FRED H. GAGE, CLIFFORD W. SHULTS, KEVIN R. JONES, LOUIS F. REICHARDT, AND UN JUNG KANG

Department of Neurosciences, University of California at San Diego, La Jolla, California 92093-0627 (C.A.L.-P., F.H.G., C.W.S.); Neurology Service, Veteran’s Administration Medical Center, San Diego, California 92093 (C.W.S.); Department of Neurosciences, University of California at San Francisco, San Francisco, California 94143 (K.R.J., L.F.R.); Departments of Neurology and Pharmacological and Physiological Sciences, Committee on Neurobiology, University of Chicago, Chicago, Illinois 60637 (U.J.K.)

ABSTRACT
Local delivery of brain-derived neurotrophic factor (BDNF) by genetically modified cells provides the unique opportunity to examine the effects of BDNF on adult dopaminergic and cholinergic neurons in vivo. Primary rat fibroblasts were genetically engineered to produce BDNF. Conditioned media from BDNF-transduced fibroblasts supported embryonic chick dorsal root ganglion neurons as well as rat fetal mesencephalic neurons. BDNF-transduced fibroblasts grafted to the rat brain survived and showed continued mRNA production for at least 2 weeks. The effects of BDNF-transduced fibroblast grafts on the dopaminergic and cholinergic systems were then assessed. BDNF-transduced fibroblasts grafted into the normal intact substantia nigra induced sprouting of tyrosine hydroxylase- and neurofilament-immunoreactive fibers into the graft. Fibroblast grafts implanted into the normal intact striatum and midbrain as well as the 6-hydroxodopamine-lesioned brain did not induce sprouting of dopaminergic fibers; neither did they affect drug-induced rotational behavior. BDNF-transduced fibroblasts did, however, significantly increase the homovanillic acid/dopamine ratio when grafted into the normal midbrain. Following transection of the fimbria-fornix, BDNF-transduced fibroblasts grafted into the septum were unable to rescue the septal cholinergic population, as did nerve growth factor-producing fibroblast grafts. Genetically modified fibroblast grafts may provide an effective, localized method of BDNF delivery in vivo to test biological effects of this factor on the central nervous system.

Indexing terms: cholinergic, dopaminergic, neurotrophic factor, NGF, transplantation

Neurotrophic factors are present in the central nervous system (CNS) and play important roles in neural development, differentiation, and survival (for reviews, see Barde, 1989; Thoenen, 1991). A family of related neurotrophic molecules, called neurotrophins, whose members affect overlapping as well as distinct populations of neurons, has been recently isolated. Members of the neurotrophin family include nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophic factor-3 (NT-3), and neurotrophic factors-4/-5 (NT-4/5). Extensive conservation between species and sequence similarity between members (over 50% homology in amino acid identity) suggest important roles for neurotrophins in the CNS (Leibrock et al., 1989; Maisonpierre et al., 1990; Berkemeier et al., 1991).

Neurotrophins exert their effects through specific binding to cell surface receptors that exist in both low (Kd ~ 10^-9) and high (Kd ~ 10^-11)-affinity forms (Meakin and Shooter, 1991). The low-affinity NGF receptor (p75NGFR)
comprises a 75 kD protein (Chao et al., 1986) and binds all known members in the neurotrophin family. Specificity in binding is afforded by high-affinity binding sites on trk protooncogenes: trkA, trkB, and trkC. Neurotrophic factors appear to mediate their effects by inducing phosphorylation of tyrosine residues on trk molecules (Kaplan et al., 1991). However, there exists a great deal of controversy regarding what receptor components are necessary and sufficient to mediate signal transduction (for reviews, see Bothwell, 1991; Meakin and Shooter, 1992).

Although the role of neurotrophic factors in shaping the developing nervous system by their presence in limited amounts in target tissues has been best established (Barde, 1989), neurotrophic factors have also been implicated in maintaining adult neuronal populations. Neurotrophin mRNA and protein as well as receptors for neurotrophins are present in the adult nervous system (Korsching et al., 1985; Shelton and Reichardt, 1986), and adult neurons are able to transport retrogradely 125I-NGF (Schwab et al., 1979; Seiler and Schwab, 1984) and 125I-BDNF (Wiegand et al., 1991; DiStefano et al., 1992). Neurotrophic molecules promote neuronal survival when exogenously administered in amounts that exceed normal physiological levels. NGF, the best-characterized member of the neurotrophin family, has been extensively studied for its role in the cholinergic septohippocampal model, where administration of exogenous NGF to the axotomized septohippocampal projection rescues up to 90% of the transected septal cholinergic neurons (Hefti, 1986; Williams et al., 1986; Kromer, 1987; Gage et al., 1988). A decrease in neurotrophic factors during aging has also been hypothesized to precipitate degenerative disorders such as Alzheimer’s disease (AD) and Parkinson’s disease (PD; Appel, 1981; Hefti, 1983), where the loss of dopaminergic neurons of the substantia nigra pars compacta (SNC) is correlated with the major motor disability of PD, and the loss of cholinergic neurons in the basal forebrain is associated with memory loss in AD (Bernheimer et al., 1973; Olton, 1990). Although the etiology and mechanisms underlying the selective degeneration of cholinergic and dopaminergic neuronal populations are unclear, the action of neurotrophic substances in protecting and regenerating damaged neurons has recently received attention for its potential therapeutic role (see Phelps, 1989).

BDNF promotes the survival and differentiation of fetal cholinergic neurons of the basal forebrain in vitro (Knusel et al., 1991; Nonomura and Hatanaka, 1992) as well as adult cholinergic neurons in vivo following septohippocampal transection (Knusel et al., 1992; Morse et al., 1993). BDNF also promotes the survival of fetal dopaminergic neurons and protects them from MPP+ and 6-hydroxydopamine (6-OHDA) toxicity in vitro (Hyman et al., 1991; Beck et al., 1992; Spina et al., 1992; Hyman et al., 1994). When applied to grafted fetal dopaminergic neurons, BDNF enhances the functional effect of the grafts in reducing amphetamine-induced rotation behavior but does not enhance graft survival (Sauer et al., 1993). The effect of BDNF on adult dopaminergic neurons is not well characterized but, when infused above the SNC following transection of the medial forebrain bundle (MBB), BDNF is unable to protect tyrosine hydroxylase (TH)-positive neurons from degenerative changes (Knusel et al., 1992). On the other hand, BDNF induces rotational behavior in the normal adult rat when infused into the SNC (Altar et al., 1992; Martin-Iverson et al., 1994; Shults et al., 1994), indicating a role for BDNF in the adult dopaminergic system.

Methods of examining the role of BDNF in vivo involve infusing the protein intraventricularly or intraparenchymally. It is thought that an abundance of truncated forms of the trkB receptor (Klein et al., 1990; Middlemas et al., 1991), however, limit the diffusion of BDNF in vivo. To circumvent this problem, we employed a technique involving the use of genetically engineered cells. Dermal fibroblasts were genetically altered to produce the neurotrophic factor of interest and subsequently grafted to the brain, where they act locally as sources of the trophic substance (Gage and Fisher, 1991; Gage et al., 1991). NGF, introduced to the brain by both genetically modified fibroblast cell lines (Rosenberg et al., 1988) and primary fibroblasts (Kawaja et al., 1992), is successful in rescuing cholinergic neurons following fimbria-fornix transection. We hypothesized that very discrete administration of BDNF using genetically modified fibroblasts would provide more localized and sustained delivery of BDNF than that of cannula infusion. Therefore, we tested the effects of localized BDNF delivery on adult dopaminergic and cholinergic neurons after chemical or mechanical lesions.

Intracerebral grafting of genetically modified fibroblasts also affords the unique opportunity of studying the ability of trophic molecules to promote neurite extension in vivo. With this approach, we can monitor axonal arborization and sprouting induced by localized production of trophic molecules in vitro. Grafs of NGF-producing fibroblasts (FF/NGF) implanted into the septum or striatum encourage the ingrowth of cholinergic fibers (Kawaja and Gage, 1991) and promote the regeneration of acetylcholinesterase (AChE)-positive axons when grafted into the lesioned fimbria-fornix cavity in a collagen matrix (Kawaja et al., 1992). Neurite extension into the grafts, although promoted by NGF, is thought to be mediated by astrocytic processes that provide a permissive substrate for axonal ingrowth. Therefore, delivery of neurotrophic factors by fibroblast grafts provides substrates for growth as well as trophic factors for neurite extension. We hypothesized that grafts of BDNF-transduced fibroblasts (FF/BDFN) implanted into the striatum or midbrain would analogously induce dopaminergic fiber ingrowth. Because BDNF binding is conferred by p75NGFR, which is expressed by cholinergic neurons of the basal forebrain sensitive to BDNF, we also investigated cholinergic ingrowth into FF/BDFN grafts in vivo.

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**Abbreviations**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ChAT</td>
<td>choline acetyltransferase</td>
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<tr>
<td>CM-β-gal</td>
<td>β-gal conditioned media</td>
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<tr>
<td>CM-BDNF</td>
<td>brain-derived neurotrophic factor-conditioned media</td>
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<tr>
<td>CM-FP</td>
<td>uninfected fibroblast conditioned media</td>
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<tr>
<td>FF</td>
<td>fibroblasts</td>
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<td>FF/β-gal</td>
<td>β-gal-transduced fibroblasts</td>
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<tr>
<td>FF/BDFN</td>
<td>brain-derived neurotrophic factor-transduced fibroblasts</td>
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<tr>
<td>FF/NGF</td>
<td>nerve growth factor-transduced fibroblasts</td>
</tr>
<tr>
<td>FN</td>
<td>fibronectin</td>
</tr>
<tr>
<td>GFAP</td>
<td>glial fibrillary acidic protein</td>
</tr>
<tr>
<td>NF</td>
<td>neurofilament</td>
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<tr>
<td>p75NGFR</td>
<td>low-affinity NGF receptor</td>
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<td>TH</td>
<td>tyrosine hydroxylase</td>
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MATERIALS AND METHODS

Construction of the plasmid including the BDNF gene

The gene encoding human BDNF was isolated by screening genomic clones with degenerate oligonucleotides derived from regions of amino acid sequence conserved among pig BDNF and NGF from several species. Sequence analysis identified a clone with near identity to pig BDNF, which was subsequently confirmed to be the gene for human BDNF (Jones and Reichardt, 1990). The entire coding region for human BDNF is present in one exon. An expression cassette was constructed by adding restriction enzyme sites and cloning into Bluescript vector.

Retroviral vector construction

The retroviral vector was derived from the Moloney murine leukemia virus in which the structural genes gag, pol, and env were replaced by the selectable marker aminoglycoside phosphotransferase (neomycin). Transcription is controlled by an internal Rous sarcoma virus (RSV) promoter (LRNL). The 830 bp EcoRI-BamHI fragment containing the entire coding region of human BDNF was ligated into the vector LRNL downstream from the viral 5' long terminal repeat (LTR; Fig. 1: LBdnfRNL). The plasmid LBdnfRNL was then lipofected into the Ψ-2 ecotropic packaging cell line by Lipofectin (BRL/Gibco). Conditioned medium was then used to infect the amphotrophic cell line, PA-317. Clones of PA-317 cells were selected in the presence of 400 μg/ml of Geneticin (G-418 Sulfate; BRL/Gibco Laboratories), and the conditioned medium was used to infect Rat 1 cell lines to assay for virus titer. PA-317 clones showing the highest titer were selected and used to infect primary rat fibroblasts.

Primary culture of rat dermal fibroblasts

Skin biopsies were obtained from inbred Fischer 344 rats as previously described (Kawaja et al., 1991). Primary fibroblasts were grown in Dulbecco’s minimal essential medium (DME) supplemented with 10% fetal bovine serum, 0.3 mg/ml (2 mM) glutamate, 2.5 μg/ml amphotericin, and 40 μg/ml gentamycin (supplemented DME) in a 10% CO2 incubator. After reaching confluence, fibroblasts were passaged by trypsinization and propagated by replating at a density of 1:4. Passage number was carefully monitored. Primary fibroblasts (FF) were infected with the recombinant retrovirus containing the BDNF minigene by incubation in conditioned medium from the packaging cell lines in 4 μg/ml polybrene. After 24 hours, successfully transfected fibroblasts (FF/BDNF) were selected in the presence of 400 μg/ml G-418 and grown to confluence. FF/NGF- and β-galactosidase-transfected fibroblasts (FF/β-gal) engineered from identical biopsies served as control grafts. The retroviral vector and genetic modification of these cells have been previously described (Rosenberg et al., 1988).

Conditioned media were sometimes obtained from transduced and unmodified fibroblasts. Five milliliters of media (without G418; supplemented DMEM for chick DRG cultures, defined media for mesencephalic cultures) were conditioned by preconfluent (75%) fibroblasts in a T75 flask for 24 hours. The media incubated with the fibroblasts were filtered with a 0.45 pm filter to remove cell debris and obtain BDNF, β-gal, or uninfected fibroblast conditioned media (CM-BDNF, CM-β-gal, or CM-FF).

<table>
<thead>
<tr>
<th>Experiment</th>
<th>FF/BDNF vs. Control (n)</th>
<th>Lesion</th>
<th>Rotational test</th>
<th>Histology or biochemistry</th>
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<tbody>
<tr>
<td>Graft in normal striatum (Fig. 5)</td>
<td>3 vs. 3</td>
<td>None</td>
<td>None</td>
<td>1, 2, 3, and 6 weeks</td>
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<tr>
<td>Graft in normal striatum with subsequent 6-OHDA lesion in MFB</td>
<td>11 vs. 11</td>
<td>8–13 days postgrafting</td>
<td>2, 4, 6, 8, and 15 weeks after lesion (apomorphine); 3, 5, 7, and 16 weeks after lesion (amphetamine)</td>
<td>10 (n = 6 each) and 16 weeks (n = 5 each) after lesion</td>
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<tr>
<td>Graft in normal midbrain (Fig. 6)</td>
<td>10 vs. 8</td>
<td>None</td>
<td>2 and 4 days</td>
<td>4 days</td>
</tr>
<tr>
<td>Graft in normal midbrain</td>
<td>10 vs. 10</td>
<td>None</td>
<td>4 and 7 days</td>
<td>1 week</td>
</tr>
<tr>
<td>Graft in normal midbrain (Fig. 7)</td>
<td>11 vs. 10</td>
<td>None</td>
<td>1, 4, and 6 weeks</td>
<td>6 weeks</td>
</tr>
<tr>
<td>6-OHDA lesion (Fig. 9)</td>
<td>11 vs. 10</td>
<td>2 weeks postgrafting (after 2 week rotation test)</td>
<td>1.5 and 2 weeks after grafting; 2.5, 3.5, and 4.5 weeks after lesion</td>
<td>8 weeks after grafting</td>
</tr>
<tr>
<td>Graft in septum following fornix-fornix lesion (Figs. 10, 11)</td>
<td>5 vs. 5</td>
<td>At time of grafting</td>
<td>None</td>
<td>4 weeks after grafting</td>
</tr>
</tbody>
</table>

1Boldface indicates data illustrated in this paper.
Fetal mesencephalic culture

Fetal mesencephalon was dissected from the mesencephalic flexure of El5 rat fetuses and dissociated in papain solution. Dissociated cells were plated at a concentration of 100,000 cells/cm² on poly-D-lysine-coated plates in chemically defined medium after a 24 hour incubation in defined media supplemented with 10% fetal calf serum. Mesencephalic cultures were then incubated in defined media alone, CM-BDNF, CM-FF, or media containing rhBDNF at 50 ng/ml. Two parallel cultures were fixed at 3 and 5 days with 4% paraformaldehyde and immunostained with an antibody directed against TH (Boehringer-Mannheim; 1:200). Tyrosine hydroxylase-immunoreactive (TH-IR) cells and total cells were counted.

Surgical procedures: Grafting, 6-OHDA lesions, and fimbria-fornix transections

Female Fischer 344 rats (150-200 g) were anesthetized with a mixture of ketamine (75 mg/kg), acepromazine (0.75 mg/kg), and rompun (4 mg/kg) and placed in a Kopf stereotaxic apparatus for all surgical procedures. FF/BDNF, FF/NGF, and FF/β-gal were washed, trypsinized, and suspended in Dulbecco's phosphate-buffered saline (PBS) at a concentration of 1 x 10⁵ cells/ml. Two or three microliters of the fibroblast suspension were stereotaxically implanted at one or more sites in the normal intact striatum (A/P, +0.2; LAT, 2.0; D/V, 4.5; and A/P, 1.2; LAT, 2.0; D/V 4.5) or midbrain (A/P, -5.4; LAT, 1.5; D/V, 7.5) at a rate of 1 µl/minute (all coordinates given with reference to Bregma; Paxinos and Watson).

One week following grafting into the striatum, unilateral lesions of the substantia nigra ipsilateral to the graft site were made by stereotaxic injection (A/P, 4.2; LAT, 1.5; D/V, 7.8) of 12 mg 6-OHDA dissolved in 4 µl saline containing 0.5 mg/ml ascorbic acid. The efficacy of the lesion was tested by observing rotational behavior.

Complete unilateral aspirative lesions of the fimbria-fornix and supracollosal striae were made under visual inspection as previously described (Gage et al., 1983). Aspirative lesions, conducted with the aid of a surgical microscope, were made on the right side of the brain ipsilateral to the graft site. A summary of the grafting experiments discussed in the text is included in Table 1.

Behavioral analysis

Following subcutaneous injection of 0.1 mg/kg apomorphine or 5 mg/kg amphetamine, animals were placed in an automated rotameter, and the number of rotations/hour were recorded. Complete (360°) rotations were recorded for both clockwise and counterclockwise directions for 60 minutes. Rotational behavior was monitored at time points of 2, 4, and 7 days in intact adult rats with striatal grafts and 1, 4, and 6 weeks following grafting into the MFB. Behavioral analysis was also performed at weekly intervals in the 6-OHDA-lesioned rat beginning 2 weeks after grafting into the striatum. Apomorphine and amphetamine were used in alternate weeks.

Biochemistry: High-pressure liquid chromatography

Four days following grafting, rats were killed by rapid decapitation, and brains were immediately removed. The striata were dissected on ice, immediately frozen in liquid nitrogen, and stored at −80°C. For high-pressure liquid
Fig. 3. CM-BDNF were examined for biological activity in E15 fetal mesencephalic cultures. Photographs show tyrosine hydroxylase (TH)-immunoreactive (IR) mesencephalic neurons cultured for 5 days in control (N2) media (A), CM-FF (B), and CM-BDNF (C). Scale bar = 100 μm.
day mesencephalic cultures. Cultures incubated with CM-BDNF contained more TH-IR cells than control cultures incubated with CM-FF at 3 and 5 days. The effect of media conditioned by FFiBDNF was similar to that of adding 50 ng/ml of rhBDNF to FF conditioned media. *P < .05; **P < .01 (ANOVA).

**Histological analysis**

Rats were anesthetized (as described above) and transcardially perfused with 50 ml saline followed by 250 ml ice cold 4% paraformaldehyde at various time points following grafting (1, 2, 3, and 8 weeks for the intact striatum; 8 weeks for the 6-OHDA-lesioned brain; 4 weeks for the fimbria-fornix-lesioned brain). Brains were removed, post-fixed overnight, and transferred to 30% sucrose until equilibration. Forty micrometer sections were cut on a freezing sliding microtome and stored in cryoprotectant. Sections were stained with thionin to obtain a qualitative assessment of cells within the graft and host tissue, stained with picrofuchsin to visualize graft-derived collagen (Sheehan and Hrapchak, 1973), or processed for immunohistochemistry using the following procedure. Endogenous peroxidases were first blocked with 0.06% H2O2, and sections were permeabilized with 0.25% Triton X-100 and blocked with 10% horse serum (monoclonal antibodies) or 10% goat serum (polyclonal antibodies). Sections were incubated with either anti-rat TH monoclonal antibody (Boehringer-Mannheim), anti-rat neurofilament (NF; 200 kD) monoclonal antibody (Boehringer-Mannheim), anti-mouse p75NGFR monoclonal antibody, anti-rat glial fibrillary acidic protein (GFAP) monoclonal antibody (Boehringer-Mannheim), anti-rat OX-42 monoclonal antibody (Serotec), anticholine acetyltransferase (ChAT) polyclonal antibody (a generous gift of David Armstrong), or anti-rat fibronectin (FN) polyclonal antibody (Telios) at dilutions of 1:200, 1:25, 1:100, 1:500, 1:7,500, or 1:500, respectively. The antigen-antibody complex was reacted with horse antimouse or goat anti-rabbit secondary antibody coupled to biotin. Staining was developed by the avidin-biotin method (Vector Laboratories) with nickel intensification using 3,3-diaminobenzidine as the chromogen.

**In situ hybridization**

Cells expressing mRNA for BDNF were identified by in situ hybridization. Complementary antisense RNA probes were transcribed from the linearized plasmid using T7 polymerase in the presence of 35S-CTP. Forty micrometer sections were mounted directly onto slides and hybridized overnight at 56°C. Sections were processed for emulsion autoradiography and counterstained with thionin.

**RESULTS**

**Transcription of the BDNF transgene in vitro: Northern blot analysis**

FF/BDNF have a doubling time of 48 hours in culture and exhibit contact inhibition, as do FF. To determine if BDNF mRNA is transcribed from the transgene in vitro, we performed Northern blot analysis on the cultured cells (Fig. 2). The hybridized blot shows message of 4.8 kb, which is the expected size of the retroviral transcript containing the BDNF minigene, RSV promoter, and neomycin resistance gene. No hybridization occurred in the lane representing FF. This result was repeatable using several different infections and biopsies.

**Translation of BDNF mRNA in vitro**

Embryonic chick dorsal root ganglion assay. After establishing that the BDNF transgene was transcribed in vitro, we sought to determine whether the mRNA was...
translated into biologically active protein and, moreover, secreted by FF/BDNF. We relied on two bioassays that are standard for assessing the presence of BDNF: cultures obtained from embryonic chick DRG and fetal rat mesencephalon. Lack of specific antibody available to us prevented us from performing Western blot analysis or immunohistochemistry.

To obtain a qualitative indication of the biological activity of BDNF secreted by our fibroblasts, we cultured E9 chick DRG in the presence of CM-BDNF. Control cultures were incubated in supplemented DMEM alone, CM-β-gal, 100 ng/ml rhBDNF, or 100 ng/ml rhNGF. Neurite outgrowth from phase-bright cells was evident several hours after plating, and extensive processes were apparent the next day. After 48 hours, cultures were fixed and photographed.

Two days after plating, DRG cultures incubated in rhNGF, rhBDNF, and CM-BDNF contained phase-bright cells with extensive processes and few (<10%) nonneuronal cells. CM-β-gal also supported the survival of substantially fewer DRG neurons with shorter processes. Control cultures incubated in supplemented DMEM contained almost no phase-bright cells with long processes after 48 hours in vitro. CM-BDNF did not support the survival of E9 cultured sympathetic neurons (data not shown), indicating the specificity of BDNF protein produced by FF/BDNF.

**Fetal rat mesencephalic culture.** We assessed the effects of CM-BDNF on cultures derived from E15 rat mesencephalon. Cultures were incubated in defined media, CM-BDNF, CM-FF, or in the presence of defined media containing rhBDNF. After 5 days, cultures incubated in CM-BDNF contained more TH-positive neurons with more extensive processes (Fig. 3C) than cultures incubated in control media (Fig. 3A) or CM-FF (Fig. 3B).

To obtain a more quantitative indication of the biological activity (and, therefore, of BDNF protein levels), we determined the number of TH-positive neurons in mesencephalic cultures. After 3 days in culture with control media, the percentage of TH-IR cells was 1.6% ±0.1%; SEM and 1.3% ±0.06%; SEM) after 5 days. Cultures incubated with media conditioned by FF/BDNF showed a significant in-
A. control grafts (CONT-IPSI). B: group (BDNF-CONTRA) and compared to the ipsilateral sides of the homovanillic acid (HVA)/dopamine in the striatum ipsilateral to FFI
dopamine turnover as demonstrated by the ratio of the metabolite (DOPAC)/dopamine ratios in the striatum, however, were not significantly elevated. *P < 0.05 (Tukey’s multiple comparison). BDNF-IPSI, FF/BDNF ipsilateral; BDNF-CONTRA, FF/BDNF contralateral; CONT-IPSI, FF/β-gal ipsilateral; CONT-CONTRA, FF/β-gal contralateral.

Effects of BDNF-transfected fibroblast grafts in vivo

Transcription of the BDNF transgene in vivo: In situ hybridization. To investigate whether the BDNF transgene was transcribed in vivo, we performed in situ hybridization using riboprobes on tissue sections containing fibroblast grafts. Fisher 344 rats received bilateral grafts of FF/BDNF and FF/β-gal into the striatum. Thionin staining (Fig. 5A,B) depicts bilateral grafts in the same rat. One week following implantation, cells in the BDNF graft (Fig. 5C), but not in the contralateral β-gal graft (Fig. 5D), hybridized with the BDNF probe. The expected pattern of hybridization was observed in the hippocampus of the same rat. We observed hybridized cells within the grafts at 1 week following grafting, and hybridization was also seen (although to a lesser extent) at 2 weeks.

Effects on the intact dopaminergic system. Having confirmed that FF/BDNF express BDNF mRNA in vitro and in vivo and that biologically active protein is secreted from the cells, we investigated the effects of FF/BDNF grafts on dopaminergic neurons in vivo. Grafts were stereotaxically placed in the target field striatum of normal adult rats. Because FF/NGF grafts encourage the ingrowth of cholinergic fibers (Kawaja and Gage, 1991), we analogously monitored the effect of the FF/BDNF grafts on dopaminergic neurite sprouting. Histological analysis of the grafts at time points covering 1–8 weeks showed good survival and placement of the fibroblast grafts but a lack of TH-IR processes within the grafts. Neurofilament staining was likewise negative (data not shown).

Following infusion of rhBDNF above the SNC, normal rats exhibit amphetamine-induced rotation behavior (Altar et al., 1992; Shultz et al., 1994). We therefore assessed behavioral effects of FF/BDNF grafts in the midbrain (near the rostral end of the substantia nigra) by monitoring amphetamine-induced rotations. Because transgene expression driven by the retroviral promoter appears to be highest shortly after graft implantation (Palmer et al., 1991; Kang et al., unpublished data), rotational behavior was monitored at 2 days, 4 days, and 1 week following grafting. No behavioral changes were noted. Despite the lack of significant difference in rotation behavior, biochemical measurement of the dissected striatum 4 days after grafting showed evidence for increased turnover of dopamine by a significant increase in the HVA/dopamine ratio when grafts were placed next to the MFB (Fig. 6). Another experimental group with graft placement near the MFB showed no behavioral changes at longer time points of 1, 4, or 6 weeks following grafting.

FF/BDNF grafts placed directly into the SNC in normal rats elicited sprouting of TH-IR and neurofilament immunoreactive (NF-IR) fibers when examined after 6 weeks (Fig. 7). Only those animals with FF/BDNF grafts placed directly in the SNC partially disrupting the dopaminergic neurons showed neuronal profiles observed within the graft (Fig. 7A,C). Similarly placed FF/β-gal grafts were devoid of TH-IR or NF-IR fibers (Fig. 7B,D). We assessed the viability of FF/BDNF grafts at 6 weeks following grafting, the longest time point shown in our study. Figure 8 depicts a typical FF/BDNF graft implanted into the
ventral mesencephalon at 6 weeks. Coronal sections taken through the level of the substantia nigra were stained with picrofuchsin (pink) followed by a thionin counterstain (Fig. 8A) to visualize graft-associated collagen. At 6 weeks, grafts are healthy, as indicated by intense pink staining within the graft. Figure 8B also demonstrates graft viability with immunostaining directed against FN within the borders of the graft. Immunostaining directed against GFAP (Fig. 8C) showed astrocytic migration into the graft that has been previously shown to support axonal ingrowth (Kawaja et al., 1991). Mild macrophage infiltration was also observed as noted by OX-42 immunoreactivity (Fig. 8D).

**Effects on the 6-OHDA-lesioned dopaminergic system.** BDNF has been shown to protect fetal dopaminergic neurons from the toxicity of MPP⁺ and 6-OHDA in vitro (Hyman et al., 1991; Spina et al., 1992). We therefore tested whether BDNF produced from FF/BDNF grafts placed in the striatum could protect against 6-OHDA toxicity. One
Effects on the intact basal forebrain cholinergic system.

Grafts of FF/NGF induce the ingrowth of p75NGFR and ChAT-immunoreactive fibers from the nucleus basalis when grafted into the intact striatum (Kawaja and Gage, 1991). Given that cholinergic neurons of the basal forebrain express p75NGFR (which binds BDNF) and respond to BDNF in vitro as well as in vivo, we monitored the sprouting of cholinergic fibers into striatal grafts. At time points covering 1–8 weeks, we observed no p75NGFR, ChAT, or NF-immunoreactive processes within the grafts (data not shown). Control (FF/β-gal) grafts were likewise devoid of neuronal fibers.

Fig. 8. Coronal sections at the level of the substantia nigra show a typical FF/BDNF graft 6 weeks following implantation into the ventral mesencephalon. Sections taken through one FF/BDNF graft are stained with picrofuchsin, followed by a thionin counterstain (A), and immunostained with antibodies directed against FN (B), GFAP (C), and OX-42 (D). Arrowheads in D indicate graft borders. Scale bar = 100 μm.

Fig. 9. Amphetamine (A)- and apomorphine (B)-induced rotation are plotted for animals that received 6-OHDA lesions in the medial forebrain bundle (MFB) 1 week following grafting of FF/BDNF or FF/β-gal into the striatum. Behavioral analysis showed no significant differences between the FF/BDNF-grafted group and the control group in either amphetamine- or apomorphine-induced rotation.

week following grafting, a time at which transgene expression appears to be high and BDNF message is produced by the grafts in vivo, 6-OHDA lesions were made in the MFB. We monitored rotation behavior beginning 2 weeks after grafting. Behavioral analysis showed no differences between the FF/BDNF-grafted group and the control group in either amphetamine- or apomorphine-induced rotation (Fig. 9). Immunocytochemistry directed against TH revealed severe cell loss in the substantia nigra and a complete lack of TH-positive fibers in the ipsilateral striatum in both experimental and control groups. No differences in TH-IR profiles were observed in the FF/BDNF-grafted animals, even directly surrounding the graft. Grafting into the midbrain with subsequent ipsilateral 6-OHDA lesioning at similar time points also showed no significant behavioral or histological effects (data not shown).
Effects on the fimbria-fornix lesioned basal forebrain cholinergic system. Cholinergic neurons of the medial septum primarily innervate the hippocampus via the fimbria-fornix, and transections of this afferent pathway result in the loss of cholinergic neurons. NGF, infused intraventricularly, is able to prevent the loss of approximately 90% of the cholinergic neurons. Intraventricular and intraparenchymal infusions of BDNF also rescue septal cholinergic neurons following lesions of the fimbria-fornix (Knusel et al., 1992; Morse et al., 1993). We investigated whether FFI-BDNF grafts placed directly in the septum could avert cholinergic neuronal degeneration following fimbria-fornix lesions, as do grafts of FFI-NGF (Rosenberg et al., 1988).

The effects of FFI-BDNF grafts were examined 4 weeks following unilateral fimbria-fornix lesions. FFI-NGF, which served as a positive control, grafted into the ipsilateral septum, encouraged the ingrowth of p75NGFR- and ChAT-immunoreactive fibers, but no immunoreactive profiles were seen in similarly placed FFI-BDNF grafts (data not shown). FFI/β-gal grafts, which served as a negative control, were similarly devoid of immunoreactive fibers. Figure 10 depicts septal cholinergic neurons immunoreactive for p75NGFR and ChAT in each of the grafting paradigms (FFI-BDNF, FFI-NGF, FFI/β-gal). Quantification of ChAT-positive cells in the medial septum revealed that about 50% of the septal neurons ipsilateral to the lesion were present in the animals grafted with FFI-BDNF and FFI/β-gal compared to the contralateral septum (Fig. 11). The cell savings elicited by the NGF grafts (approximately 80%) was significantly different from that of FFI/β-gal and FFI-BDNF grafted animals \( (P < 0.01) \). Quantification of p75NGFR-immunoreactive cells revealed a similar cell savings (over 90%) in the lesioned animals that received FFI-NGF grafts as a positive control (again, significantly greater than FFI/β-gal and FFI-BDNF grafted animals; \( P < 0.01 \)). FFI-BDNF grafts tended to increase the proportion of ipsilateral/
contralateral p75NGFR-positive cells above negative control values (68% vs. 60%), although not significantly.

**DISCUSSION**

**Production of BDNF by genetically modified cells**

In this study, we show that the genetic modification of fibroblasts to effect trophic factor delivery in vivo is a generalizable technique. Genetically engineered fibroblasts express mRNA corresponding to the BDNF transgene both in vitro and in vivo, as determined by Northern blot analysis and in situ hybridization. Like NGF, BDNF is transcribed in precursor form and subsequently proteolytically processed to yield the mature, biologically active protein (Jones and Reichardt, 1990). The BDNF minigene containing coding information for the precursor form is transcribed and apparently properly processed to secrete the mature, biologically active form. Our previous experience with NGF, which is encoded by a similar pre-pro sequence, suggests a similar capacity of primary fibroblast cells. Our evidence that NGF is secreted in the properly processed form is more direct, in that we can measure quantitatively amounts of secreted protein by ELISA. Lack of specific antibody available to us precluded similar quantitative demonstration for BDNF. Instead, we rely on data from bioassays to analyze conditioned media for the presence of biologically active BDNF.

BDNF supports the survival of embryonic chick DRG neurons and rat fetal mesencephalic neurons (Davies et al., 1986; Hyman et al., 1991). CM-BDNF likewise promote the survival of these neuronal populations. Moreover, the number of TH-IR cells in fetal mesencephalic cultures incubated in 50 ng/ml rhBDNF added to CM-FF was not different from that of cultures incubated in CM-BDNF (from 1 million preconfluent cells in a 24-hour time period in vitro; Fig. 4). Again, this is consistent with our previous experience with FF/NGF, where 10⁶ FF/NGF produce between 10 and 50 ng/ml NGF over 24 hours (unpublished data).

Importantly, we also observed expression of the BDNF transcript in vivo. Riboprobes specific to the BDNF transcript hybridize with fibroblast cells in FF/BDNF, but not FF/β-gal grafts, for at least 1 to 2 weeks following grafting. Moreover, grafts of FF/BDFN remain viable and integrate well within the host tissue at least 6 weeks following implantation (Fig. 7). Picrofuchsin staining indicates graft production of collagen, whereas immunostaining with FN, which recognizes a group of structurally related glycoproteins, also indicates the production of fibroblast-specific proteins. Immunostaining with antibodies directed against GFAP demonstrates astrocytic migration into the grafts and, therefore, integration within the host brain. The minor macrophage infiltration observed with OX-42 immunostaining also indicates the integration as well as the viability of FF/BDNF grafts at the longest time point depicted in this study. These data, in combination with previous reports from our laboratory demonstrating long-term graft viability using ultrastructural analysis (Kawaja et al., 1991), suggest that the structural integrity of FF/BDNF grafts at 6 weeks was sufficient to pursue further grafting experiments.

**BDNF effects on the adult dopaminergic system in vivo**

BDNF promotes the survival of fetal dopaminergic neurons in vitro (Hyman et al., 1991; this study), but the role that BDNF plays in the adult dopaminergic system is
unclear. We noted increased turn-over of dopamine as evidenced by a small but significant difference in HVA/dopamine ratios ipsilateral to FF/BDNF grafts placed near the substantia nigra. We did not, however, observe differences in amphetamine-induced rotation behavior following grafting into the midbrain. When rhBDNF protein is administered in vivo at 12.5 μg/day, several effects have been reported. BDNF infusion into the midbrain produces slight amphetamine-induced rotation behavior in the normal rat and enhances dopamine turnover in the ipsilateral striatum, as noted by increased HVA/dopamine ratios (Altar et al., 1992; Martin-Iverson et al., 1994). We believe that the differences between our data and the infusion data of Altar et al. (1992) are due to lesser amounts of BDNF introduced to the brain by somatic cell delivery. We expect that FF/BDNF grafts deliver approximately nanogram or lesser amounts of BDNF, as they consist originally of 2–3 × 10^6 cells/graft (with rough estimates of tens of nanograms produced by 10^6 cells in vitro). This fact, in and of itself, could explain the modest effect from our grafts, although our results parallel those of (Obryan and high dose infusion. Because HVA levels reflect both intra- and extraneuronal dopamine metabolism, they may be one of the more sensitive parameters describing dopamine metabolism. The significance of augmented rotation behavior is ambiguous, however. Although BDNF apparently has a biochemical effect, behavioral effects observed following very high infusion concentrations are likely to be nonphysiological.

The most striking effect we observed was the sprouting of dopaminergic neurons of the substantia nigra into FF/BDNF grafts. TH-IR and NF-IR profiles are only evident when grafts are placed directly adjacent to, or partially in, the SNC. We did not observe sprouting of dopaminergic, neuronal profiles when grafts were stereotaxically placed in the striatum; neither does intraventricular infusion of BDNF induce sprouting of dopaminergic profiles (Knusel et al., 1992). Dopaminergic ingrowth is observed only when grafts directly interrupt the cell bodies of nigral neurons, suggesting that levels of BDNF are highest directly surrounding the graft. In situ hybridization studies show BDNF mRNA in the striatum, the target region of nigral neurons (Hofer et al., 1990), but BDNF mRNA is also expressed in the cell bodies of nigral neurons themselves (Friedman et al., 1991; Gall et al., 1992). This finding greatly complicates the classical view of neurotrophic factors but is interesting in light of our results. BDNF may function in an autocrine fashion as a locally derived, rather than target-derived, neurotrophic factor in vivo (Schecterson and Bothwell, 1992). Although retrograde transport of 125I-BDNF to the substantia nigra following infusion into the striatum has been reported (Wiegang et al., 1991), this may not be the normal mechanism by which BDNF exerts its effects on dopaminergic cell bodies.

BDNF also protects fetal dopaminergic neurons from MPP+ and 6-OHDA toxicity (Hyman et al., 1991; Spina et al., 1992). Protection may be mediated via the glutathione system, reducing the toxicity of free radicals generated from these toxins. Implications for the pathogenesis of and possible therapeutic approaches to PD are significant. We saw no sparing of TH-IR neurons or axons or evidence of dopaminergic sprouting into the grafts. The lack of sprouting probably reflects the fact that our lesions were complete and the toxicity too potent to overcome. FF/BDNF grafts do not protect dopaminergic neurons from 6-OHDA toxicity in vivo. This is consistent with reports that BDNF is not able to support dopaminergic neurons following transection of the MFB (Knusel et al., 1992). Protective effects of BDNF for dopaminergic neurons in vitro may not be an adequate model for the in vivo situation; however, higher doses of BDNF, other methods of administration, or BDNF’s introduction in combination with other trophic factors may be effective.

**BDNF effects on cholinergic neurons in vivo**

Some reports indicate that intraventricular and intraparenchymal infusions of microgram amounts of BDNF rescue cholinergic neurons following lesions of the fimbria-fornix (Knusel et al., 1992; Morse et al., 1993). However, more recent data suggest that the protective effect of BDNF is limited to p75NGFR induction (Koliatsos et al., 1994). Grafts of FF/BDNF did not protect ChAT-immunoreactive septal cells following fimbria-fornix lesions. Discrepancies between our results and those of others may be due to differences of several orders of magnitude (nanograms/day vs. micrograms/day) of delivery of BDNF to the septal cholinergic population. Despite the lack of protection of cholinergic cells by FF/BDNF grafts, as indicated by quantification of ChAT-immunoreactive neurons, we did observe a slight increase in the proportion of neurons (ipsilateral/contralateral) expressing p75NGFR. Widmer et al. (1993) recently showed more complete protection of septal neurons expressing p75NGFR than ChAT with BDNF infusion, and data from Koliatsos et al. (1994) suggest that the effect of BDNF on lesioned cholinergic neurons is primarily that of p75NGFR induction. The slight trend towards increased p75NGFR expression we observed is consistent with these findings. Up-regulation of p75NGFR, which occurs with neurotrophin administration in vivo (Cavicchioli et al., 1989; Higgins et al., 1989), may indicate small differences brought about by expression of the BDNF transgene.

**In vivo delivery of BDNF by genetically modified fibroblasts**

On the surface, there appear to be some discrepancies between our data and findings from infusion studies. Three explanations may account for these disparities: 1) FF/BDNF grafts do not secrete biologically active protein in vivo or do so only very transiently; 2) FF/BDNF are not able to produce enough of the correctly processed protein to produce the expected result in vivo; and 3) given that the amount of protein is sufficient to produce the expected effect, it is not able to reach the cell population of interest.

It is likely for several reasons that FF/BDNF grafts secrete BDNF protein in vivo. BDNF mRNA and protein are produced in vitro, and expression of BDNF mRNA is seen in vivo for up to 2 weeks. NGF, which is similar in signal sequence, size, and homology, is secreted from FF/NGF grafts in vivo, as indicated by cholinergic sprouting into the grafts and a sparing of septal cholinergic neurons following fimbria-fornix transection. Additionally, grafts of FF/BDNF induce dopaminergic sprouting and increase HVA/dopamine ratios when grafted into the midbrain. FF/BDNF grafts may also increase expression of the p75NGFR in the fimbria-fornix lesioned brain. We acknowledge, however, that limits to the amount of transgene produced exist, and stable, long-term gene expression in fibroblasts, as well as graft survival and integration into host tissue, are additional factors that must be considered.
The more likely explanation for differences between our in vivo findings and those described by others is that the level of BDNF produced by genetically modified fibroblasts is not comparable to amounts of BDNF infused. The small but significant differences we see in the HVA/dopamine ratios in FF/BDNF-grafted rats are consistent with this interpretation. Likewise, the trend towards increased expression of p75NGFR may indicate small differences brought about by expression of the BDNF transgene.

Another major factor concerning the bioavailability of BDNF in both our studies and infusion studies is that diffusion of BDNF is limited in vivo. Truncated forms of trkB lacking the catalytic kinase domain (Klein et al., 1990; Middlemas et al., 1991) may be responsible for sequestering the protein. Experiments involving the infusion of BDNF following fimbria-fornix lesions illustrate the importance of this phenomenon. Cholinergic neurons are rescued after axotomy following daily infusion of microgram amounts of BDNF (Kendall et al., 1992; Mercer et al., 1993). However, the preventive effect of BDNF on degeneration of cholinergic cells after axotomy is significantly more pronounced when the trophic molecule is infused intraparenchymally rather than intraventricularly (Morse et al., 1993), presumably because intraparenchymal infusion circumvents the crucial determinant. In support of this, sprouting of TH-, NF-IR processes is only observed in grafts directly adjacent to the cell bodies of nigral neurons.

Our results suggest that genetically modified fibroblasts may provide an effective method of delivering trophic molecules in vivo. FF/BDNF grafts afford the possibility of discrete, localized delivery, which is especially important in light of truncated forms of trkB receptors that limit diffusion of trophic molecules in vivo. Moreover, fibroblast grafts allow us to study uniquely the neuritie-promoting properties of trophic molecules in vivo. Despite limits in the amount and length of time of trophic factor expression by fibroblasts in vivo, we observe dopaminergic sprouting into FF/BDNF grafts implanted into the SNC as well as increased HVA/dopamine levels. Additionally, there is a trend towards increased expression of the p75NGFR in septal cholinergic neurons following fimbria-fornix transaction. It remains unclear whether or not BDNF has a protective effect on adult dopaminergic neurons in vivo and is potentially therapeutic for PD. When extrapolating these data to clinical situations, however, it should be emphasized that PD is a chronic process involving cell death over a long time period, whereas 6-OHDA toxicity causes acute degeneration of dopaminergic neurons. We are yet in need of better models of neurodegenerative processes to test the effects of substances like BDNF.

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LITERATURE CITED


