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Grafting Genetically Modified Cells to the Damaged Brain: Restorative Effects of NGF Expression

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Fibroblasts were genetically modified to secrete nerve growth factor (NGF) by infection with a retroviral vector and then implanted into the brains of rats that had surgical lesions of the fimbria-fornix. The grafted cells survived and produced sufficient NGF to prevent the degeneration of cholinergic neurons that would die without treatment. In addition, the protected cholinergic cells sprouted axons that projected in the direction of the cellular source of NGF. These results indicate that a combination of gene transfer and intracerebral grafting may provide an effective treatment for some disorders of the central nervous system.

CONSIDERABLE EFFORT IN RECENT years has been applied toward the development of methods for the genetic modification of mammalian cells to correct disease phenotypes in vivo. Because of their accessibility, cells of the bone marrow and skin have been studied most extensively. Because of its relative inaccessibility, a very important target organ, the brain, has received little attention. The development of methods for intracerebral neural grafting has provided new approaches towards the treatment of central nervous system (CNS) disorders. We have previously suggested that a combination of the gene transfer and neural grafting may constitute an effective approach to therapy in the CNS (*1*). Specifically, we have demonstrated that cultured cells genetically modified with retroviral vectors can survive when implanted in the mammalian brain and can continue to express foreign gene (transgene) products (*1, 2*). Before this approach can be used to treat specific CNS diseases, one prerequisite is to determine whether, under any circumstances, sufficient transgene product can be made in vivo to complement or repair an absent or previously damaged brain function.

To test the potential of this therapeutic approach, we have chosen a well-characterized model that provides the opportunity to observe a functional effect. After transection of the fimbria-fornix (the pathway connecting cholinergic neurons of the basal forebrain to their target in the hippocampus), many of the cholinergic neurons undergo retrograde degeneration, exhibit a decrease in the activities of many enzymes, and, in some cases, die (*3, 4*). This degenerative response is attributed to the loss of trophic support from β–nerve growth factor (NGF), which is normally transported retrogradely in the intact brain from the hippocampus to the septal cholinergic cell bodies (*5–10*). The importance of NGF in this response to damage is supported by experiments that demonstrate that cholinergic neurons in the medial septum can be protected from retrograde degeneration by chronic infusion of exogenous NGF (*11–14*). We report here that cultured fibroblasts, genetically modified to produce and secrete NGF and then grafted to the cavity formed in creating a fimbria-fornix lesion, will prevent retrograde cholinergic degeneration and induce axonal sprouting, thereby demonstrating a functional response to the grafted cells.

A retroviral vector, similar to one described previously (*15*), was constructed from Moloney murine leukemia virus (*16*). It contains the 777-bp Hga I–Pst I fragment of mouse NGF cDNA (*17, 18*) under control of the viral 5′ long terminal repeat. This insert corresponds to the shorter NGF transcript that predominates in mouse tissue receiving sympathetic innervation (*19*) and is believed to encode the precursor to NGF that is secreted constitutively. The vector also includes a dominant selectable marker encoding the neomycin-resistance function of transposon Tn5 (*20*) under control of an internal Rous sarcoma virus promoter. Transmissible retrovirus was produced by transfecting vector DNA into PA317 amphotropic producer cells (*21*) by the calcium phosphate co-precipitation method (*22*) and by using medium from these cells to infect Ψ2 ecotropic producer cells (*23*) in the presence of Polybrene (Sigma; 4 μg/ml). Virus from the Ψ2 clone producing the highest titer (4×10^5 colony-forming units per milliliter), was used to infect the established rat fibroblast cell line 208F (*24*) as described (*25*). Individual neomycin-resistant colonies, selected in medium containing the neomycin analog G418, were expanded and tested for NGF production and secretion by a two-site enzyme immunoassay, with commercially available reagents according to the manufacturer's protocol (Boehringer Mannheim). The clone producing the highest levels of NGF contained 1.7 ng of NGF per milligram of total cellular protein and secreted NGF into the medium at a rate of 50 pg/hour per 10^5 cells. The NGF secreted by this clone was biologically active, as determined by its ability to induce neurite outgrowth from PC12 rat pheochromocytoma cells (*26, 27*). Uninfected 208F cells, in contrast, did not produce detectable levels of NGF in either assay.

Fimbria-fornix lesions were made in 16 rats; 8 rats received grafts of infected cells and the remaining 8 received uninfected control cells (*28*). After 2 weeks, all animals were killed and processed for immunohistochemistry (*28*). Staining for fibronectin, a fibroblast-specific marker, revealed robust graft survival in all 16 animals that was comparable in both groups (Fig. 1, A and B). Sections stained for choline acetyltransferase (ChAT) to evaluate the survival of cholinergic cell bodies indicated a greater number of remaining neurons on the lesioned side of the medial septum in all 8 animals that had received grafts of infected

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cells than in the 8 animals that had received uninfected control grafts (Fig. 1, C to F). Neuronal survival in all animals was quantitated and expressed as the mean number of cells counted on each side of the septum (Fig. 2). The number of cells surviving in the lesioned side of animals that had received NGF-secreting grafts ranged from 158 to 173, whereas the range with control 208F grafts was 49 to 121. When expressed as a percentage of the remaining cholinergic cells in the septum ipsilateral to the lesion relative to the intact contralateral septum, survival was shown to be 92% in animals grafted with NGF-secreting cells but only 49% in animals grafted with uninfected control cells. The results from the control group are comparable to previous observations in lesioned animals that had received no grafts (*4, 11–14*).

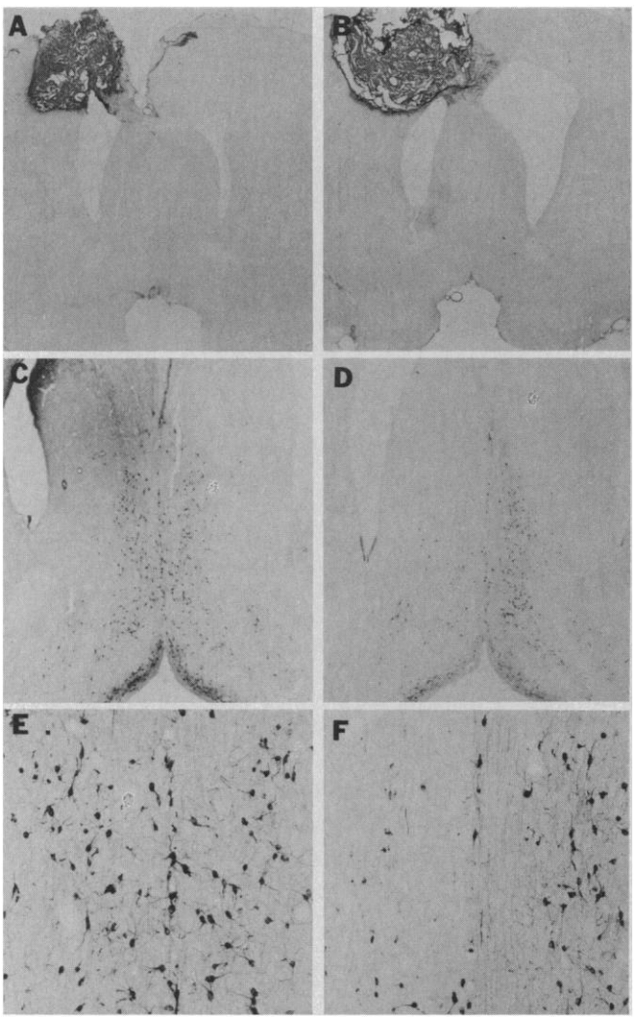

Fig. 1. Photomicrographs of immunohistochemical staining for fibronectin and ChAT. (**A** and **B**) Fibronectin staining in fibroblasts grafted into the fimbria-fornix cavity. (**C** to **F**) Coronal section through medial septum of tissue stained for ChAT. (A, C, E) Animal with graft of retrovirus-infected cells. (B, D, F) Animal with graft of control cells. Magnification: A and B, ×20; C and D, ×70; E and F, ×220.

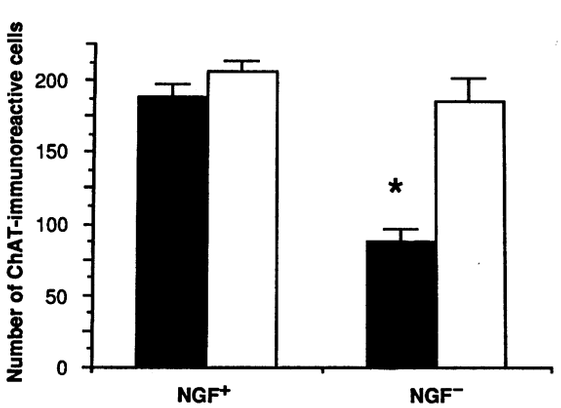

Fig. 2. Effect of grafted NGF-producing (NGF^+) fibroblasts; survival of ChAT-immunoreactive cells in the septum. Two sections, 200 μm apart, from all 16 animals were stained for ChAT through the septum and used to evaluate the extent of cholinergic cell survival. All the ChAT-positive cells in the ipsilateral septum and in the contralateral septum were counted separately and sized for planar area with an Olympus Que-2 image analysis system. The values reported are the mean number of cells per side per animal in each group. The error bars represent standard error of the mean; the asterisk indicates significance at the $P < 0.001$ level by unpaired t test. Solid bars, lesion side; open bars, control side.

In addition to the increase in the percentage of ChAT-positive cells in the NGF group, these animals also showed an increase in acetylcholinesterase (AChE)-positive fiber and cell staining (Fig. 3). Most striking was the observation of a robust sprouting response in the dorsal lateral quadrant of the septum, with the most intense staining abutting the cavity containing the graft. This intense increase in AChE staining was not observed in the group receiving control grafts (Fig. 3). We have previously observed this enhanced sprouting in response to chronic NGF infusion (*14*), but not to the extent seen in the present study. In those studies, NGF was administered at a rate of approximately 1 ng of active NGF per hour. The cells used in the present study secreted NGF at 50 pg/hour per 10^5 cells in culture before grafting. Assuming a similar initial rate in vivo, each graft secreted a total of 0.2 ng of NGF per hour, a rate substantially lower than that of the infusion study. Therefore, the magnitude of the cholinergic response in the present study may not have been due to NGF alone. It is possible that the grafts also secreted additional factors that augmented the action of NGF. For example, basic fibroblast growth factor has been shown to prevent the death of lesioned cholinergic neurons (*29*). Such putative factors alone apparently do not produce a response, as control grafts did not affect cholinergic survival. It is also possible that the brain itself normally produces a factor that enhances NGF production by the graft. For example, the Moloney murine leukemia virus long terminal repeat used in the retroviral vector has been shown to contain an enhancer-like element that responds to epidermal growth factor and phorbol esters (*30*).

Several groups have recently reported continued transgene expression by cells grafted to the periphery (*31–34*). The present study not only extends the feasibility of such an approach to the CNS but also demonstrates a functional restoration in whole animals brought about by grafted, genetically modified cells.

Numerous issues need to be addressed before this approach can be considered for

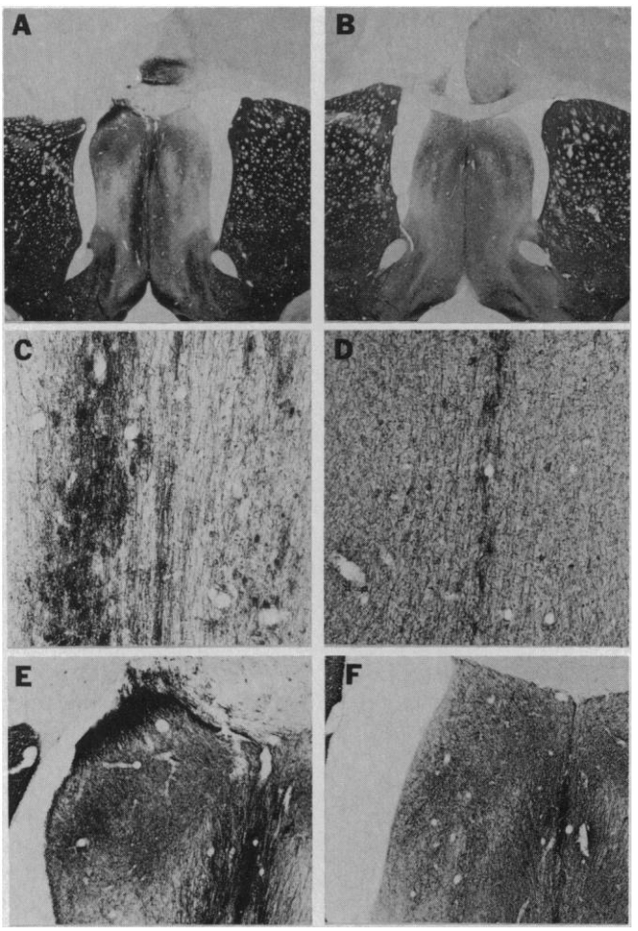

Fig. 3. Photomicrographs of AChE histochemistry. (**A**) Low-power magnification of animal grafted with NGF-infected cells. (**C**) Higher power magnification of (A) through the medial septum, which shows increased AChE staining on the lesioned side that received the NGF-infected cells. (**E**) High-power magnification of (A) through the dorsal lateral quadrant of the septum showing the increased fiber staining with a gradient of intensity highest in proximity of the NGF-producing cells. (**B**, **D**, and **F**) Animal grafted with control cells, as described for A, C, and E. Magnification: A and B, ×20; C to F, ×220. Sections were stained for AChE activity as described (*35*).

therapeutic applications. First, we used an established fibroblast cell line because of its ease of manipulation in culture and the ability to identify clones that express the transgene at high levels. This does not imply that these cells are the best suited for this approach, and we are currently investigating other cell types. We are particularly interested in primary skin fibroblasts because of their potential use in autologous grafting. Second, the interval between the lesion and histological evaluation was 2 weeks, because this was the time course we used previously in chronic NGF infusion studies. Because the present study examined only short-term effects, subsequent studies should address issues of long-term graft survival and transgene stability. Third, it is important to understand the possible immune responses of the host animal to the grafted cells or to the transgene products. Finally, it should be determined whether or not the blood-brain barrier is reestablished in these grafts, because lack of a blood-brain barrier could have adverse effects on graft or host brain function.

Although significant questions remain concerning the potential applications for grafting genetically modified cells to the brain, the present results indicate that this approach, combining the approaches of retroviral gene transfer and neural grafting, can have a reparative influence in the CNS. The cholinergic neurons described here are similar to those known to become depleted in human Alzheimer's disease patients, and it is, of course, important to determine if sources of NGF such as that described here could affect the neuronal loss in this severe CNS disorder.

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Evidence That the M2 Membrane-Spanning Region Lines the Ion Channel Pore of the Nicotinic Receptor

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Site-directed mutagenesis and expression in *Xenopus* oocytes were used to study acetylcholine receptors in which serine residues (i) were replaced by alanines (α, δ subunits) or (ii) replaced a phenylalanine (β subunit) at a postulated polar site within the M2 transmembrane helix. As the number of serines decreased, there were decreases in the residence time and consequently the equilibrium binding affinity of QX-222, a quaternary ammonium anesthetic derivative thought to bind within the open channel. Receptors with three serine-to-alanine mutations also displayed a selective decrease in outward single-channel currents. Both the direction of this rectification and the voltage dependence of QX-222 blockade suggest that the residues mutated are within the aqueous pore of the receptor and near its cytoplasmic (inner) surface.

THE NICOTINIC ACETYLCHOLINE RECEPTOR (AChR) contains both binding sites for agonists and an aqueous cation-selective channel or pore that opens for times on the order of milliseconds as a result of agonist binding. The receptor from skeletal muscle is an integral membrane protein comprised of four homologous polypeptide subunits, in the stoichiometry $\alpha_2\beta\gamma\delta$. Hydrophobicity analysis of the translated cDNA sequences indicates that each subunit probably contains at least four membrane-spanning regions (MSRs) denoted M1 through M4. There are many important questions about the relation of primary sequence to (i) the three-dimensional structure of the receptor and (ii) gating and permeation through the channel. The MSRs are likely to be crucial for these functions; the present study uses a combination of site-directed mutagenesis and electrophysiological measurements to locate a site on the M2 MSR that forms part of the lining of the pore.

Low-resolution structural data on the AChR, deduced by electron microscopy, reveal that the five individual subunits are arranged with radial pseudosymmetry in a roughly cylindrical structure; the transmembrane pore is at the axis (*1*). In most structural models, homologous MSRs from each subunit associate to form the lining of the pore. The available structural data do not yet reveal the identities of the particular MSRs involved. An atomic resolution structure by x-ray diffraction is not yet available but will eventually provide a detailed structure and will aid in interpreting changes in the function of mutants with altered amino acid sequence. However, at present, for the AChR as for many other proteins, site-directed mutagenesis is being combined with functional measurements to identify the roles of various domains and of individual amino acids (*2, 3*). For membrane channels in particular, electrophysiological measurements are an appropriate way to obtain high-resolution data on properties that define the function of the protein: gating and blockade on the millisecond time scale, single-channel conductance, and voltage sensitivity.

We have therefore constructed and studied receptors with substitutions at a postulated site (*4*), containing polar but uncharged amino acids on the M2 regions of three subunits. For both the α and δ subunits, the existing serine was changed to an alanine; in the β subunit, the existing phenylalanine was changed to serine. Electrophysiological measurements of two types on these mutated receptors strongly indicate that the M2 MSRs of these subunits form part of the lining of the pore. (i) As serines are removed, there are consistent effects on the kinetics and equilibria of receptor blockade by the cationic open-channel blocker, QX-222, which is thought to bind within the pore near its cytoplasmic (inner) end. (ii) For receptors with three fewer serines than normal, outward currents through the channel are selectively decreased; this rectification could be caused by an increased energy barrier for ion flux within the pore and near its cytoplasmic (inner) end. These results thus identify specific serine residues in the M2 helices that interact both with permeant cations and with an open-channel blocker. Because most structural models of the AChR also place these residues near the cytoplasmic (inner) end of the M2 MSR, we refer to them as the inner polar site (IPS).

The hypothesis that the M2 MSRs, which are amphiphilic helices, form part of the lining of the ion pore was suggested by reports that noncompetitive inhibitors attach covalently to serines at the IPS in at least α, β, and δ *Torpedo* AChR subunits (*4*). There is a 1:1 stoichiometry of label to receptor, but serines are labeled in the M2 region of several subunits. [However, labeling was reported within the M1 region of the α subunit with the use of a different compound (*5*).] Also, experiments with chimeric calf-*Torpedo* δ subunits suggest that a region containing the M2 helix and the adjacent bend between M2 and M3 is involved in determining the rate of ion transport in the absence of external divalent cations (*3*).

Mouse AChRs from BC3H-1 cells contain serine residues at the postulated IPS on only the α and δ subunits (*6*). Residues Ser^{248} in the α subunit and Ser^{262} in the δ subunit were therefore replaced (*7*) by alanine (subunits bearing the mutation are denoted by the subscript A). In addition, residue Phe^{259}, which occupies an equiva-

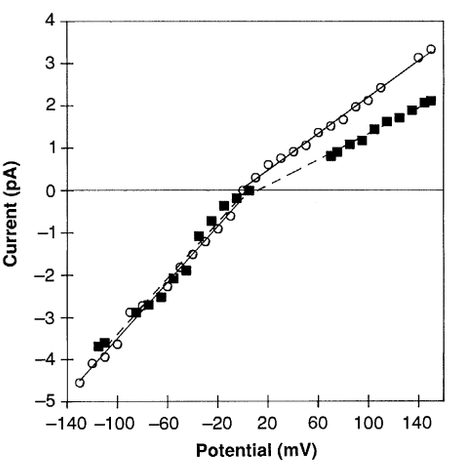

Fig. 1. Single-channel current-voltage relations for representative patches containing $\alpha\beta\gamma\delta$ (○) or $\alpha_A\beta\gamma\delta_A$ (■) AChRs. Data were obtained and analyzed as described in Table 1.

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