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Gene Therapy for Parkinson's Disease

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Abstract

Gene therapy is a potentially powerful approach to the treatment of neurological diseases. The discovery of neurotrophic factors inhibiting neurodegenerative processes and neurotransmitter-synthesizing enzymes provides the basis for current gene therapy strategies for Parkinson's disease. Genes can be transferred by viral or nonviral vectors. Of the various possible vectors, recombinant retroviruses are the most efficient for genetic modification of cells in vitro that can thereafter be used for transplantation (ex vivo gene therapy approach). Recently, in vivo gene transfer to the brain has been developed using adenovirus vectors. One of the advantages of recombinant adenovirus is that it can transduce both quiescent and actively dividing cells, thereby allowing both direct in vivo gene transfer and ex vivo gene transfer to neural cells. Probably because the brain is partially protected from the immune system, the expression of adenoviral vectors persists for several months with little inflammation. Novel therapeutic tools, such as vectors for gene therapy have to be evaluated in terms of efficacy and safety for future clinical trials. These vectors still need to be improved to allow long-term and possibly regulatable expression of the transgene.

Index Entries: Gene therapy; recombinant adenovirus; recombinant retrovirus; glial cell line-derived neurotrophic factor; tyrosine hydroxylase; Parkinson's disease.

Introduction

Although the cause of Parkinson's disease is not known, it is associated with progressive death of dopaminergic (DA) neurons, inducing motor impairment. The characteristic symptoms of Parkinson's disease appear when up to 70% of dopaminergic nigrostriatal neurons have degenerated. There is currently no satisfactory cure for this disease. Symptomatic treatment of the disease-associated motor impairments involves oral administration of L-DOPA. L-DOPA is transported across the blood–brain barrier and converted to dopamine, partly by residual dopaminergic neurons, leading to a substantial improvement of motor function. However, after a few years, the

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degeneration of dopaminergic neurons progresses, the effects of L-DOPA are reduced, and side effects appear (Marsden and Parkes, 1976). Better therapy of Parkinson's disease is therefore necessary. Neural grafting is a possible means of substituting the lost neurons. Both rodent and nonhuman primate animal models have been developed for testing new therapeutic approaches, and the rat model of Parkinson's disease is particularly convenient (Ungerstedt and Arbuthnott, 1970). The animals are given an injection of 6-hydroxydopamine (6-OHDA) unilaterally into the ascending mesostriatal pathway, originating from the dopaminergic neurons of the substantia nigra (A9), and the mesostriatal pathway, originating from the dopaminergic neurons located near the ventral segmental area (A10). This causes denervation of the ipsilateral striatum from its dopaminergic afferents. The induced depletion of dopamine in the denervated striatum correlates with a quantitative sensorimotor asymmetry. The denervated rats respond to low doses of apomorphine by turning. Apomorphine is an agonist of the dopamine receptors that stimulates the denervation-induced supersensitive dopamine receptors. The rate of turning can be reduced by treatment that compensates the dopamine deficiency of the denervated striatum. Transplantation was studied in this animal model of Parkinson's disease as early as the late 1970s (Bjorklund and Stenevi, 1979; Perlow et al., 1979) and appeared promising (for a review see Brundin et al., 1987). Clinical trials to evaluate transplantation of human fetal dopaminergic cells as a therapy for Parkinson's disease initially gave encouraging results and suggested that beneficial treatment for patients suffering from severe "on–off" phenomena would be possible (Lindvall et al., 1990). Indeed, 3 yr after intracerebral grafting of cells originating from the ventral mesencephalon of four fetuses, patients with Parkinson's disease continued to display clear clinical improvement, manifest by a reduction of the severity of symptoms and the time spent in the "off" phase, as well as by a prolongation of the effect of a single dose of L-DOPA (Lindvall et al., 1994). However, neuronal grafts require immunosuppression and access to fetal material of a particular age. Even when successful, only a small proportion of the intracerebrally injected neurons survive transplantation.

Over the last few years gene therapy has emerged as a potentially powerful approach to the treatment of neurological diseases. Various neurotrophic factors that inhibit neurodegenerative processes or stimulate regeneration have been discovered and genes encoding neurotransmitter-synthesizing enzymes have been isolated (Mallet et al., 1987). These elements provide the basis for current developments in gene therapy for patients with neurological disease. Various vectors have been developed to transfer genes to cells for transplantation (ex vivo gene therapy) or directly into the brain (in vivo gene therapy). Adenovirus has recently been shown to be a powerful tool for in vivo gene transfer to the brain. In this article, we discuss the potential value of both ex vivo and in vivo gene therapy for Parkinson's disease.

Gene Therapy: Recombinant Retrovirus and Adenovirus as Vectors of Gene Transfer

The cloning of biologically active genes and the development of vectors for gene transfer have allowed the construction of tools to express genes in animals, suggesting that it will be possible to introduce functional genes into human cells to treat genetic disease (Friedmann and Roblin, 1972). The ideal goal of gene therapy is to replace a defective gene by the corresponding functional gene without any modification of the remaining genome. Recently, several groups have shown that mammalian cells possess the enzymatic machinery for homologous recombination, which allows targeting of genetic modifications in dividing cells (Smithies et al., 1985; Thomas et al., 1986; Nandi et al., 1988). These observations have led to methods for modify-

ing the genome of embryonic stem (ES) cells to be developed in the mouse to generate transgenic animals. This method has been used mostly to establish animal models of human genetic diseases (Kuehn et al., 1987). However, the efficiency of this method is too low to be used to correct genetic defects in whole organs. It is also not applicable to humans because human ES cell culture has not been developed. In addition, genetic modification of the human genome is currently considered as unethical.

Consequently, most gene transfer experiments addressing gene therapy are restricted to the introduction of new genes into somatic cells. Gene transfer techniques were first developed by virologists. Indeed, viruses naturally have the capacity to introduce their genome into mammalian cells. The first method was using DEAE dextran as a carrier molecule for the naked DNA (McCutchan and Pagano, 1968). The calcium-phosphate transfection method developed with the adenovirus by Graham and van der Eb (1973) was more recently modified to enhance its efficiency by Wigler et al. (1979) and Chen and Okayama (1987). This is now the most widely used method.

In 1976, pieces of exogenous DNA were propagated in mammalian cells by transfecting a defective SV40 virus bearing exogenous fragments of lambda phage together with a helper SV40 (Ganem et al., 1976; Goff and Berg, 1976; Nussbaum et al., 1976). The SV40 system was used by Mulligan et al. to express the β-globin gene, showing the potential of gene transfer to treat a gene defect disease, in this case thalassemia (Mulligan et al., 1979). However, SV40 has various limitations that precluded its further use: size limit of 2.5 kb for the inserted DNA, replication is only possible in monkey cells in the presence of a helper virus. Another group of viruses appeared as more powerful tools for gene transfer: retroviruses.

Retrovirus-Mediated Gene Transfer: A Tool for Cell Transplantation

As early as the 1970s retroviruses were known to have several advantages as tools for gene transfer. In particular, they integrate into the genome of infected cells (Baltimore, 1970; Temin and Mizutani, 1970). If a cell replicates and has receptors for the virus, one virus hitting the cell leads to the incorporation of one copy of the proviral genomic DNA into the chromosome of the cell. These viruses also propagate after transfection of their genomic DNA (Gilboa et al., 1979). Some retroviruses naturally incorporate cellular genes into their genome (Martin, 1970; Vogt, 1971) and a gene coding for thymidine kinase was inserted in vitro into the genome of the spleen necrosis virus (Shimotohono and Temin, 1981). In addition, the nucleotide sequence of the genome of the Moloney murine leukemia virus was determined (Shinnick et al., 1981), and retroviral genomes are small. There were thus numerous arguments supporting retroviruses as candidates for producing tools for gene transfer.

The steps toward the construction of tools for gene therapy include the elimination of viral genes *(gag-pol-env)* and the conservation of sequences necessary for the integration of the virus. In particular, recombinant retroviruses must possesses the two directly repeated retroviral long terminal repeats (LTR), as well as a packaging ψ-sequence (Watanabe and Temin, 1982). The 5' LTR constitutively promotes transcription of a large mRNA, whereas the 3' LTR provides sequences necessary for cleavage and polyadenylation of the mRNA. The production of recombinant retroviral particles requires introduction of the construct into a packaging transcomplementing cell line that synthesizes in *trans* the retroviral proteins and particles from a provirus containing *gag-pol-env* genes, but that is defective for its own packaging (Mann et al., 1983). Several refinements have been added to the recombinant retroviruses to increase their efficiency. To facilitate identification of infected cells, selection markers, such as the aminoglycoside 3'-phosphotransferase gene conferring resistance to neomycin, is often coexpressed with the gene to be transferred (Colbere-Garapin et al., 1981; Mulligan and Berg, 1981). This coexpression is based either

on the natural alternative splicing of retroviruses or multiple units of transcription. However, interaction between promoters can reduce expression (Emerman and Temin, 1984a,b).

It is now possible to develop a safe system by eliminating the risk of tumorigenesis and viral dissemination. Tumorigenesis may results from insertional activation of cellular oncogenes by integration of a retrovirus (Neel et al., 1981; Payne et al., 1981; Moreau-Gachelin et al., 1988) or retroposition (Heidmann et al., 1988). Oncogene activation is, however, a rare event (Teich et al., 1985), the frequency of which can be lowered further by the use of a defective LTR promoter (Yu et al., 1986; Cone et al., 1987; Dougherty and Temin, 1987). Viral dissemination may be totally excluded by using safe helper-free packaging cell lines (Danos and Mulligan, 1988; Markowitz et al., 1988; Morgenstern and Land, 1990).

Adenovirus Also Allows In Vivo Direct Gene Transfer

The recombinant adenoviruses commonly used are derived from type 5 adenovirus. This type has never been found to be associated with tumoral disease in humans, most probably because the probability of integration of the adenoviral DNA into the host cell genome is low. The effects of wild-type adenoviral infection are well documented, and more than 10 million patients have received live adenovirus tablets as an oral vaccine without major side effects (Rubin and Rorke, 1988). Finally, the natural tropism of adenovirus is the respiratory tract, and wild-type viruses are found only rarely in the human brain. However, the virus can be expressed in reactive brain microglial cells, suggesting that monocyte/microglia can mediate entry of adenovirus into the central nervous system (CNS) (Matsuse et al., 1994).

Our knowledge of the biology of the adenovirus is extensive. The human adenovirus genome is large (36 kb of DNA). It contains many genes, which are classified into early (E1–E4) and late (L1–L5) genes, according to whether they are expressed prior to or after viral DNA replication. The entry of human adenovirus into epithelial, endothelial, and blood mononuclear cells is a two-step process. The initial binding of the virus to specific receptors is mediated by the fiber protein. Subsequent interaction of the penton base (a 400 kDa pentavalent subunit that contains five Arg-Gly-Asp sequences) with α v integrins promotes virus internalization (Wickham et al., 1993, 1994; Goldman and Wilson, 1995; Huang et al., 1995). Thereafter, the virus is incorporated into endosomes and released into the cytoplasm by endosmolysis. The viral DNA is translocated to the nucleus, where the early regions are activated, followed by DNA replication and activation of the late regions. The viral DNA is packaged into capsid and the infected cells die while releasing the viruses. As compared to herpes simplex virus-1 (HSV-1), the transcription machinery and viral cycle of adenovirus are relatively simple, allowing straightforward obtention of purified replication-defective recombinant viruses (Revah et al., 1996).

Since the wild-type adenovirus causes a lytic infection, adenoviral vectors for gene transfer must be defective for some essential genes. The E1 region contains such genes. E1-defective viruses are complemented in *trans* in 293 cells (Graham et al., 1977). The 293 cells contain the E1 genes integrated into their genome so that after infection of this cell line the E1-deleted viruses can replicate and be amplified. These replication-deficient adenoviruses have been referred to as first-generation recombinant adenoviruses. The E3 region, which is not necessary for viral propagation in cell culture, can also be deleted. Such viruses can accommodate transgenes up to 7.5 kb in length, and can be obtained at titers as high as 10^{11}–10^{12} pfu/mL.

Recombinant adenovirus can transduce both quiescent and actively dividing cells. It is thus possible to express transgenes in neurons via direct in vivo gene transfer as well as via ex vivo gene transfer. There are several important issues for therapy by gene transfer to the brain, including

1. Extent of transgene expression;
2. Stability of transgene expression;
3. Targeting of the cells; and
4. Safety of the procedure.

The relative significance of these issues differs according to the transgene, the vector, and the host cell type.

Ex Vivo Gene Therapy for Parkinson's Disease: Intracerebral Transplantation of Genetically Modified Cells

Neurons do not proliferate and cannot be renewed in the adult human brain. The possibility of replacing lost neurons by intracerebral transplantation has therefore been investigated. Unfortunately, embryonic dopaminergic cells survive poorly and are difficult to obtain. Gene therapy can be used to facilitate this approach by allowing the generation of cells producing particular neurotransmitters or trophic factors. This method combining gene transfer with transplantation is now referred to as ex vivo gene therapy.

The first step was to modify cell lines to secrete DOPA or dopamine using recombinant retroviruses carrying the tyrosine hydroxylase (TH) gene. Such genetically modified neuronal, endocrine, and fibroblastic cell lines have been constructed and used to show the feasibility of the method in animal models of Parkinson's disease (Horellou et al., 1989, 1990a,b; Wolff et al., 1989; Uchida et al., 1990). Fibroblastic and endocrine cell lines have also been used to analyze the relative contribution of DOPA and dopamine to functional recovery after grafting. Grafted fibroblastic DOPA-secreting cells produce four to five times more extracellular dopamine than the endocrine dopamine-secreting cells, and they compensate apomorphine-induced turning behavior more efficiently (Horellou et al., 1990b).

Some primary cell types originating from the periphery have also been used. Primary fibroblasts (Fisher et al., 1991) expressing the TH gene partially compensate motor impairments in the rodent model of Parkinson's disease. The main advantage of this approach is the possibility of autotransplantation (cells from the patient himself), thereby solving the supply problem and reducing the need for immunosuppression to prevent rejection.

CNS-derived cells are of obvious potential for intracerebral transplantation. Being natural constituents of the brain, they are likely to integrate well in the long term. The use of neuronal cells may allow delivery of neurotransmitters to synapses and therefore to facilitate regulated release. We therefore studied the fate of modified rat astroglial cells and human neural progenitors after intracerebral transplantation.

Because they can be amplified in vitro, primary astrocytes are candidates for gene transfer using retrovirus associated with intracerebral transplantation. We have used recombinant retroviruses to genetically modify rat primary astrocytes such that they produce TH. The gene transduction efficiency was approx 80% (Lundberg et al., 1996). DOPA production in the transduced astrocytes was largely independent of the exogenous cofactor biopterine. Transplantation of the TH-transduced astrocytes to the striatum of unilaterally 6-OHDA-lesioned rats reduced apomorphine-induced turning by approx 50% 2 wk postgrafting. Histological analysis revealed that the transduced astrocytes survived transplantation well and that some of the grafted cells had migrated, partly along blood vessels, into the surrounding striatum. TH was detected in cells with the appearance of mature GFAP-positive astrocytes, as well as in more immature-looking cells. Primary astrocytes have also been used to produce brain-derived neurotrophic factor (Yoshimoto et al., 1995) and nerve growth factor (Cunningham et al., 1991) in the rat model of Parkinson's disease.

Neural progenitors are another attractive tool for intracerebral transplantation. A rat neural progenitor cell line transformed by a temperature-sensitive SV40-T antigen (HiB5) has been isolated and transplanted into rat neonatal hippocampus and cerebellum where it

acquired morphological characteristics of the neurons and glial cells (Renfranz et al., 1991). Other immortalized rodent neural progenitor cell lines with similar properties have been isolated (Snyder et al., 1992; Onifer et al., 1993). Interestingly, an immortalized rodent neural progenitor isolated from substantia nigra has been modified with a TH expression vector (Anton et al., 1994), which improved the efficacy of conditionally immortalized nigral neural cells in rodent and nonhuman primate models of Parkinson's disease following neural transplantation. This improvement was accompanied by evidence of increased L-DOPA production and immunocytochemical evidence of higher TH activity in the transfected cells than in the parental line (Anton et al., 1994). For human applications, it seems important to avoid the transformation of cells by conditional oncogenes, and human primary cells stimulated to grow with mitogens that would retain properties of neural progenitors may be the ideal cell type for intracerebral transplantation. Recently, we developed a strategy based on the use of such cells. In vivo, neuroepithelial progenitor cells have three relevant properties: They are dividing cells, they are the multipotential precursors of all neuronal and glial cells present in the CNS, and they show plasticity in new environments. We explanted human neural progenitors from germinative zones of the CNS of 6–10 wk-old fetuses, obtained after legal abortions. Immediately after explantation, the phenotype of more than 95% of the cells was that of neuroepithelial and/or neuroblastic cells. We established conditions allowing their differentiation into neuronal and glial derivatives, attesting the multipotentiality of the initial cell population. In serum-free culture medium containing basic fibroblast growth factor, these cells could be propagated quasi-indefinitely as dividing progenitors (Buc-Caron, 1995). We used this amplified population of human neural progenitors as a vehicle for recombinant adenovirus. First, we established conditions allowing the majority (about 65%) of the cells to express the β-galactosidase gene in vitro without toxicity. Large numbers of human neuroblasts expressed β-galactosidase activity in three of four immunosuppressed rats that had each received an intrastriatal injection of 10^6 infected neural progenitors (Sabaté et al., 1995).

Human neural progenitors amplified in vitro and adenoviruses could be used to treat Parkinson's disease. The number of cells expressing a potentially therapeutic gene (encoding a trophic factor or TH enzyme, for example) that could be obtained from a single fetus would be sufficient to graft several patients. In addition, the in vitro step of amplifying the cells allows testing for the absence of any contaminating agent from the fetal tissue and thereby improved safety.

In Vivo Gene Therapy for Parkinson's Disease: Direct Intracerebral Gene Transfer

In vivo gene therapy for the CNS consists of the direct intracerebral injection of genetic material using appropriate vectors. Three types of vector have been constructed. HSV-1, adeno-associated virus (AAV), and adenovirus vectors were all recently shown to mediate gene expression after intracerebral injection. Each has been used to express the TH gene in the rat model of Parkinson's disease (During et al., 1994; Horellou et al., 1994; Kaplitt et al., 1994).

Various defective HSV-1 vectors have been developed to deliver heterologous genes into the rat brain by stereotaxic injection (Palella et al., 1989; Fink et al., 1992; Wolfe et al., 1992; During et al., 1994; Bloom et al., 1995). In these in vivo experiments, the transgene was expressed in neurons of different brain areas surrounding the injection site and in neurons whose axons project to the injection site. Long-term expression has been described with HSV1 vectors (Bloom et al., 1995) and in particular with TH in denervated striatum (During et al., 1994). However, only a small number of cells express the transgene, probably because HSV-1 enters a latent phase during which the genes it carries are silent (Wolfe et al., 1992; During et al., 1994).

A TH recombinant AAV has also been recently shown to produce TH in neurons (Kaplitt et al., 1994). However, the risk of a contamination of both the HSV-1 and the AAV vectors by competent viruses is a major problem. Further safety improvements are required before these tools can be applied clinically.

Recombinant adenovirus has already been used in clinical trials because it can be purified to be completely free of any contaminating agent. Recently, a system for direct gene transfer to the brain has been developed with a recombinant human adenovirus carrying the *E. coli lacZ* reporter gene (Akli et al., 1993; Bajocchi et al., 1993; Davidson et al., 1993; Le Gal La Salle et al., 1993). Stereotaxic intracerebral injection of the recombinant adenovirus results in evident and a robust labeling of a large number of cells of various types around the inoculation site. The labeled cells (i.e., those infected with the recombinant human adenovirus 5 and expressing β-galactosidase) include neurons, astrocytes, oligodendrocytes, and microglial and ependymal cells. The efficiency of the in vivo infection is relatively high: About one cell appeared to express β-galactosidase for 100 injected recombinant adenovirus (Akli et al., 1993). The neuropathological consequences of low inocula were minimal in rats, and neuronal death with gliosis and vascular inflammatory response was observed only when high titers of virus were injected (Akli et al., 1993; Le Gal La Salle et al., 1993). Like HSV-1 vectors, recombinant adenovirus can be transported in a retrograde manner to nerve cell bodies from axonal terminals (Ridoux et al., 1994).

To test the functional efficacy of adenovirus-mediated gene transfer, we expressed the TH gene in the rat model of Parkinson's disease. A defective human adenovirus carrying the human TH-1 cDNA (Ad-RSVhTH) was constructed. The entire coding sequence of TH was placed under the transcriptional control of the LTR RSV promoter (in place of the E1 gene of the adenovirus). We improved both the expression level and the dispersion of the transgene in the striatum by optimizing the conditions of intracerebral injection. A dose of 15×10^7 was found to be optimum for maximal expression of the transgene and minimal tissue necrosis as assessed by microscopic analysis of striatum stained with neutral red. We improved the dispersion of transgene expression by increasing the number of injection sites to nine and by decreasing the rate of injection from 1 μL/min to 0.5 μL/min. The increase of the number of injection sites was achieved by releasing the virus at three dorso-ventral positions (equidistant at 0.4 mm) while removing the cannula from each of the three antero-postero-lateral coordinates. This modification of previous methodology resulted in distinctly more dispersed expression through the dorso-ventral axis. The lower rate of injection (0.5 μL/min) increased the diffusion of the virus as indicated by the greater dispersion of the cells expressing β-galactosidase.

To test the capacity of the Ad-RSVhTH to express the TH enzyme in vivo, the purified Ad-RSVhTH was injected into the striatum of 6-OHDA denervated rats as described above. Control animals received a defective adenovirus encoding β-galactosidase (Ad-RSVβGal). At least 10 d after the 6-OHDA lesion and 1–2 wk before intracerebral injection, rats were tested for apomorphine-induced turning.

The injected animals were tested again for apomorphine-induced turning 7 and 14 d after intracerebral injection. One week post injection the mean decrease in turning was approx 30% and after 2 wk it was 22%. These reductions were both statistically significant. One week post injection, the control rats injected with the Ad- RSVβGal virus showed no modification of apomorphine-induced turning behavior and 2 wk post injection none of the control rats showed a significant decrease. Indeed, the group showed mean increase of 19%. The difference between the Ad-RSVhTH and Ad-RSVβGal groups was significant as determined using one-way analysis of variance (ANOVA).

Two weeks after the intrastriatal injections of adenovirus, the efficiency of gene transfer to the striatum was determined in the Ad-RSVhTH group by immunohistological

staining with an anti-TH antibody, and in the Acl-RSVβGal group by reaction with X- Gal. In most animals, a large number of cells throughout the entire denervated striatum expressed the transgene. Expression was confined to the striatum, from its rostral to caudal regions, and to the anterior part of the external capsule. This wide distribution was presumably the result of the optimized injection protocol described above. Double GFAP/TH immunostaining, and the morphological aspect, indicated that most infected cells were reactive astrocytes. Some cells (a minority) had a clear neuronal morphology. To evaluate the extent of neural tissue destruction, the inflammatory response, and gliosis, adjacent sections were analyzed by acetylcholinesterase histochemistry and GFAP immunohistochemistry. At the site of injection, discrete zones of tissue destruction were observed equally in the Ad-RSVhTH and the Ad-RSVβGal-injected rats. A similar pattern of GFAP reactive astrocytes was found around the injection site in both groups of animals (Horellou et al., 1994).

Our work with E1/E3 deleted adenovirus, and that with HSV-1 and AAV vectors show that virus-mediated TH gene transfer decreases the apomorphine-induced turning behavior in 6-OHDA-lesioned rats. Thus, it appears that dopamine depletion in the striatum of denervated rats can be compensated using in vivo gene transfer.

In Vivo Gene Transfer of GDNF Using Adenovirus

Glial cell line-derived neurotrophic factor (GDNF) is structurally related to members of the transforming growth factor-β superfamily. In vitro, GDNF promotes survival, high-affinity dopamine uptake, and neurite outgrowth of embryonic DA-neurons (Lin et al., 1993). GDNF also stimulates recovery of developing DA-neurons after damage by 1-methyl-4-pyridinium (Hou et al., 1996). Intracerebral administration of GDNF to the substantia nigra (SN) completely prevents nigral cell death and atrophy in a rat model of Parkinson's disease (Sauer et al., 1995). Axotomy results in loss of TH-containing neurons in the SN, but this is largely prevented by repeated injections of GDNF adjacent to the SN (Beck et al., 1995). In mice, GDNF injected into the SN or the striatum before 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine strongly protects the DA-system and with both modes of administration, motor activity is increased above normal levels (Tomac et al., 1995). Recently, intracerebral injection of the factor into rhesus monkeys was shown to protect the nigrostriatal DA pathway in this species closely related to humans (Gash et al., 1996). For all these reasons, this trophic factor is a potential candidate for the treatment of Parkinson's disease.

An appropriate mode of administration of this factor is essential for its clinical efficacy. Intracerebral injection is necessary because this type of trophic factor cannot cross the blood–brain barrier. However, the direct delivery of purified proteins into the brain would necessitate repeated injections. A gene transfer method may therefore be a good alternative to obtain continuous activity after a single injection. In addition, because GDNF is a disulfide-bonded homodimer protein that is heterogeneously glycosylated (Lin et al., 1994), its constant delivery in its biologically correct form may best be obtained through exogenous expression after gene transfer.

We tested the effect of injection of an Ad-GDNF into rat striatum to allow expression in both DA-axon terminals and DA-cell bodies via retrograde transport (Bilang-Bleuel et al., 1997). We used the rat model of Parkinson's disease developed by Sauer and Oertel (1994) in which 6-OHDA injected into the striatum causes progressive degeneration of DA-cells. GDNF was delivered to both DAterminals and DA-cell bodies by injecting the virus unilaterally into the striatum to obtain expression at the site of the injection as well as in the SN via retrograde transport of the virus. After 6 d, the rats received 6-OHDA in their previously injected striatum. Three weeks after the unilateral 6-OHDA lesion, the animals were

sacrificed. Immunohistochemical analysis using specific anti-*Escherichia coli*-βGal antibodies showed numerous infected cells in the injected striatum and in the ipsilateral SN. Substantial transgenic βGal expression was detected for at least 4 wk following adenoviral delivery. This suggests that Ad-GDNF drove a high level of production of transgenic GDNF. The survival of DA neurons was analyzed throughout the SN between the coordinates AP –4.3 and –6.4 mm from bregma (Fig. 1 and Table 1). The animals treated with 6-OHDA alone or with Ad-βGal 6 d before the lesion showed a similar degree of DA-neuron degeneration, with only about 30% of DA cells surviving throughout the SN. The value for the Ad-GDNF group was 60–62%, showing more significant protection by GDNF against 6-OHDA than with no treatment (p = 0.0003), or AdβGal (p = 0.0009).

The Ad vector injected into the brain induces inflammation. We therefore investigated the toxicity of the virus by histological analysis. The injected striatum of animals treated with Ad vectors were more inflammatory and atrophied than those treated with 6-OHDA alone (about 13 vs 2%, Table 1). The inflammation and atrophy induced by Ad-βGal and AdGDNF were not significantly different (Table 1). The overall protective action of Ad-GDNF was not only apparent from better survival of DA-neurons, but also from more TH innervation in the striatum and SN following 6-OHDA administration than in Ad-βGal-treated animals (Table 1, Fig. 2). Therefore, it appears that the Ad-GDNF injection in the striatum protected DA-cell bodies as well as DA terminals in the striatum from the toxicity of 6-OHDA.

To evaluate the behavioral consequence of the DA-neuron degeneration, we monitored amphetamine-induced turning 1, 2, and 3 wk following the lesion (Fig. 3). Control animals that received 6-OHDA had a mean rotation score of 1020 ± 160 net ipsilateral turns per 90 min 1 wk after the lesion. This turning behavior was stable for at least 3 wk after the lesion. Injection of Ad-βGal 6 d prior to the lesion

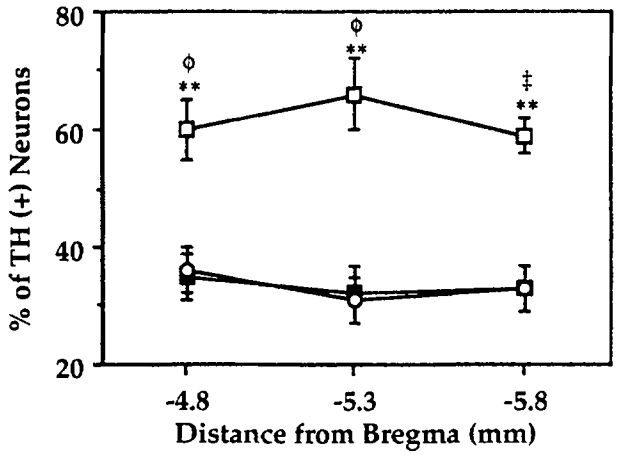

Fig. 1. Survival of DA neurons in the SN of 6-OHDA-lesioned rats. The animals received intrastriatal Ad injections followed by 6-OHDA 6 d later. Three weeks after 6-OHDA injection, animals were sacrificed for TH-immunohistochemistry. The number of TH (+) cell bodies present in the SN at the coordinates AP 4.8, 5.3, and –5.8 mm from bregma (3–4 sections per region of each animal) was determined. The values reported are means for 6–11 rats per group ± SEM and are expressed as percentages of TH (+) cell counts in the contralateral nonlesioned SN. The survival of DA neurons is significantly higher in animals injected with AdGDNF (□) than with Ad-βGal (■) or than in animals that received 6-OHDA alone (○). $^{**}p < 0.01$ vs 6-OHDA alone, $^{\phi}p < 0.01$, and $^{\ddagger}p<0.001$ vs Ad-βGal.

slightly decreased the rotation score to 810 ± 150 at 1 wk post lesion. The score decreased, but not significantly, thereafter. The rotation score of the animals that received Ad-βGal was not significantly different from that of the animals that received 6-OHDA alone 1 wk postlesion (p = 0.38). A statistical difference was observed 2 wk post lesion (p = 0.03) but did not persist to the third week post lesion (p = 0.06) (Fig. 3). Injection of Ad-GDNF 6 d prior to the lesion reduced the rotation score to 200 ± 30, 1 wk post lesion. The rotation score decreased further to 70 ± 25 after 2 wk and 47 ± 17 after 3 wk. The difference in rotation score between animals injected with Ad-GDNF and animals that received Ad-βGal (p = 0.004, 0.002, and 0.03 at 1, 2, and 3 wk, respectively) or 6-OHDA alone (p = 0.0008, 0.0002, and 0.0001) was highly significant (Fig. 3).

Table 1
Survival of DA Neurons and Degree of TH Innervation in 6-OHDA-Lesioned Rats Subjected to Different Treatments[a]

Group	DA-cells[b]	TH-SN[c]	Striatal size[d]	TH-striatum[e]
6-OHDA (n = 10)	31 ± 4	+	− 2.1 ± 0.7	+
Ad-βGal (n = 8)	31 ± 3[ns]	+	−13.6 ± 1.8***	+
Ad-GDNF (n = 7)	62 ± 5***‡	+++	−13.1 ± 2.3***	+++

[a]Animals were injected with 6-OHDA 6 d after treatment (n: number rats per group). Coronal sections of SN and striatum were processed for TH-immunohistochemistry. Values given in [b] and [c] correspond to the analysis of 5–6 brain sections for each animal, where TH (+) cell bodies were counted only in the SN and restricted to the coordinate AP −5.3 mm from bregma. Values given in [d] and [e] correspond to the analysis of 10–12 brain sections per animal (between the coordinates AP = 1.7 and +0.2 mm from bregma). DA cell bodies and DA neurites were more protected from 6-OHDA toxicity by Ad-GDNF than by Ad-βGal. ***$p < 0.001$, ns: not significant vs 6-OHDA alone. ‡$p < 0.001$ vs Ad-βGal.

[b]TH (+) cells in SN (% of contralateral ± SEM).

[c]Estimation of TH-neurite density in SN (++++: 100%,+++: 75%, ++: 50%, and +: 25% of contralateral).

[d]Decrease in striatum size (% of contralateral ± SEM).

[e]Estimation of TH-neurite density in the striatum (scale as in [c]).

The correlation between the extent of DA-neuron survival in the SN and the rate of amphetamine-induced rotation 3 wk after 6-OHDA injection was analyzed by plotting the two variables against each other. A significant correlation was found between amphetamine rotation (Y) and the percentage of surviving DA-cells (X): ($Y = 1652 - 23.8X$; $r^2 = 0.447$; $p = 0.0003$; $n = 25$). Since the groups of rats that received 6-OHDA alone or Ad-βGal before 6-OHDA had similar DA-cell survival rates (Fig. 1) and similar amphetamine-induced rotation rates (Fig. 3), they were pooled for this regression analysis ($Y = 1811 - 27.5X$; $r^2 = 0.259$; $p = 0.03$; $n = 18$). Interestingly, the animals that received Ad-GDNF 6 d before 6-OHDA gave a regression curve with a much lower slope ($Y = 187 - 2.2X$; $r^2 = 0.597$; $p = 0.04$; $n = 7$) than the other groups. This difference in the linear regression curves illustrates the better motor functional score (lower amphetamine-induced rotation response) for animals that received the AdGDNF than predicted by their higher DA-cell survival rate in comparison to animals receiving 6-OHDA alone or 6-OHDA and Ad-βGal. Histological analysis showed a higher protection and/or sprouting of axons in the striatum and of dendrites in the SN of animals that received Ad-GDNF (Table 1 and Fig. 2). These observations suggest that the reduced turning behavior results from both the improved DA-cell survival and the protection of DA neurites in the striatum and/or in SN. It can be concluded that Ad-GDNF protected DA cells in the SN and protected or stimulated DA-neurite arborization, resulting in better motor function.

In our study, the availability of the neurotrophic factor to both the DA-cell bodies and to the DA-nerve terminals prevented not only DA-cell death but also striatal denervation. This most probably allowed the functional recovery that was observed following Ad-GDNF administration. Interestingly, in a similar lesion model, Winkler et al. (1996) injected GDNF protein into the SN and reported no reduction of rotational behavior, although DA-cell survival improved. Recently, Choi-Lundberg et al. (1997) reported a similar study using the same lesion model as we did with injection of GDNF- recombinant Ad near the SN. However, they show a protective effect of Ad-GDNF only on DA survival, and they report no behavioral data. Therefore, the value of Ad-GDNF injection near the SN for therapy appears small. Our study shows that adenoviral GDNF expression in both striatum and SN not only allows protection of striatal DA-innervating fibers and of DA-cell bodies but also limits motor impairment, suggesting a possible therapeutic value of this method. Our findings

experiments with recombinant SV40, to compare the various tools available today and to improve their performance in terms of efficacy and safety. With powerful tools, such as expression vectors and biologically active genes, we can hope to treat some neurological disorders by delivering genes into the brain either through transplantation of genetically modified cells or by direct injection of genes. Major efforts are being made to develop different types of vectors to obtain a sustained, regulated, and long-term expression of transgenes in the brain.

Recombinant adenoviruses are of particular interest as vehicles to transfer genes encoding neurotransmitter-synthesizing enzymes or trophic factors into somatic cells. They allow efficient expression in brain cells after intracerebral injection of purified recombinant viruses. In addition, it should be possible to develop a safer system by further inactivating viral genes. Such tools offer the possibility of transferring biologically active genes *in situ* by direct intracerebral injection. They allow modification of cells that do not divide and can therefore be used to direct the expression of genes in neurons. The use of tissue-specific promoters would also be valuable for cell-specific expression.

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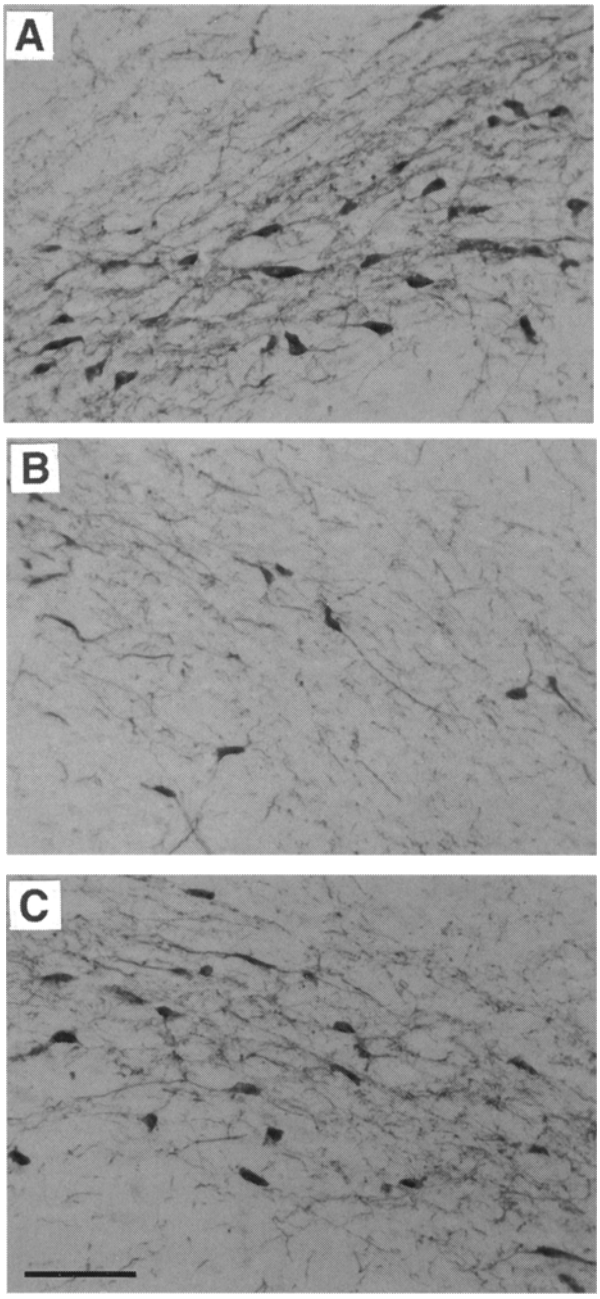

Fig. 2. Histological analysis of SN TH-neurons of treated rats. Representative pictures of 14 μm-thick coronal sections through the SN processed for TH-immunohistochemistry are shown. **(A)** Section contralateral to the lesion; **(B, C)** sections ipsilateral to the lesion of animals injected with Ad-βGal (B) or Ad-GDNF (C). The aspect of the ipsilateral SN from rats that received only 6-OHDA is comparable to those of rats that received 6-OHDA + Ad-βGal (B). Scale bar corresponds to 100 μm. The number of TH (+) cell bodies and density of TH-stained fibers were both higher in rats that received Ad-GDNF than Ad-βGal (C vs B).

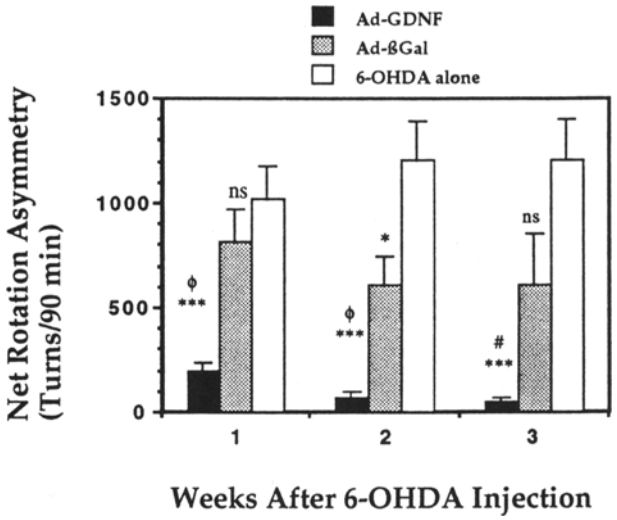

Fig. 3. Effect of Ad-GDNF on amphetamine-induced rotational behavior in 6-OHDA-lesioned rats. Ad-GDNF (n = 7) or Ad-βGal (n = 8) were delivered into the left striatum of animals by stereotaxic injection. Six days thereafter, 20 μg of 6-OHDA-hydrochloride was injected into the left striatum of both groups of animals. A third group of animals received no preinjection before 6-OHDA lesion (6-OHDA only, n = 10). The ability of the different treatments to counteract the neurotoxin action was assessed by following asymmetric rotational behavior induced by amphetamine administration 1, 2, and 3 wk after 6-OHDA injection. The values reported are means ± SEM (bars) of net ipsilateral turns over 90 min (turns contralateral to the lesion subtracted). $^{*}p < 0.05$, $^{***}p < 0.001$, and ns: not significant Ad-GDNF vs 6OHDA alone. $^{\#}p < 0.05$ and $^{\phi}p < 0.01$ Ad-GDNF vs Ad-βGal.

also suggest that to obtain a neuroprotection with motor functional efficacy, the expression of GDNF in the striatum is important, as recently shown with intrastriatal injection of the GDNF protein (Shults et al., 1996).

Conclusions and Future Work

It appears necessary, as illustrated by the history of gene transfer methods since the initial

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