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Expedited Review

Prospects for Gene Therapy in Parkinson's Disease

*†Andrew Freese, ‡Matthew Stern, §Michael G. Kaplitt, †William M. O'Connor, †Maureen V. Abbey, *‖Michael J. O'Connor, and ¶Matthew J. During

**Division of Neurosurgery, University of Pennsylvania Medical Center, †Laboratory of Molecular Neurosurgery, and ‡Department of Neurology, Graduate Hospital, Philadelphia, Pennsylvania; §Department of Neurosurgery, Cornell Medical School/New York Hospital, New York, New York; ‖Division of Neurosurgery, Graduate Hospital, Philadelphia, Pennsylvania; and ¶Section of Neurosurgery, Yale University School of Medicine, New Haven, Connecticut, U.S.A.*

Summary: Numerous advances in in vivo and ex vivo gene-therapy approaches to Parkinson's disease offer promise for direct clinical trials in patients in the next several years. These systems are predicated on introducing genes that encode enzymes responsible for dopamine biosynthesis or neurotrophic factors that may delay nigrostriatal degeneration or facilitate regeneration. We review the current status of experimental approaches to gene therapy for Parkinson's disease. Comparative advantages and disadvantages of each system are enumerated, and preclinical trials of some of the systems are evaluated. Although the specific in vivo or ex vivo methods used for gene transfer into the brain are likely to be supplanted by newer technology over the next decade, the principles and approaches developed in current studies likely will remain the same. **Key Words:** Parkinson's disease—Gene therapy—Transgenes—Viral vector—Transplant.

Over the past several years, a number of advances in molecular biology have made it possible that gene therapy for patients with Parkinson's disease (PD) will occur within a decade. Newly developed approaches are based on either in vivo gene-transfer systems, predicated on the introduction of therapeutic foreign genes (transgenes) directly into the host brain or ex vivo gene-transfer systems, which use cell lines transfected with transgenes; these transfected cell lines are in turn grafted into the host brain. Significant advances in each of these approaches have resulted in testing in animal models of PD, setting the stage for the consideration of clinical trials in patients. The prospect of gene therapy may offer an alternative to tissue-transplantation methods based on fetal mesencephalic sources and may provide an adjunct to or replacement for conventional pharmacotherapy.

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Address correspondence and reprint requests to Dr. A. Freese at Division of Neurosurgery, Silverstein 5, Hospital of the University of Pennsylvania, 3400 Spruce St., Philadelphia, PA 19104, U.S.A.

IN VIVO APPROACHES

A number of techniques have been developed for the introduction of genes into mammalian cells, including viruses, plasmids, and direct DNA transfer (1). Of these, vectors based on DNA viruses hold particular promise for application to PD, because they can efficiently transfect the postmitotic central nervous system (CNS), contain large enough genomes to carry one or more therapeutically relevant genes, and can be sufficiently attenuated to prevent a destructive infection in the brain. Vectors based on three types of DNA viruses (1–11) have been used for application to PD: (a) herpes simplex virus-1 (HSV-1), (b) adeno-associated virus (AAV), and (c) adenovirus. Each virus vector type has advantages and disadvantages, and it is difficult to predict which will ultimately prevail. Preclinical

studies have been performed, with promising results for each virus vector type.

HSV-1 Vectors

HSV-1 has a number of attractive features as a CNS gene-transfer vector, including observations that (a) it has a wide host range—it can infect a variety of cells in a number of organisms, including humans, primates, and rodents; (b) it can infect postmitotic cells, such as neurons, in adult animals or in culture; (c) it can be maintained indefinitely in a latent state, during which it is quiescent (i.e., DNA replication does not occur, but expression of certain viral genes can proceed, and no progeny virus are produced, preventing a virulent infection); (d) electrophysiologic properties of latently infected neurons are unaltered; (e) its gene expression occurs in a regulated cascade, and its genes are transcribed by the host cell RNA polymerase II; and (f) it has a large genome (150 kb) that can accommodate large transgenes or even multiple transgenes (1,2,5–11).

Two categories of HSV-1 vectors have been developed for gene transfer into the brain; these have been termed *recombinant vectors* and *defective viral vectors*. In a recombinant vector, or replicon, one or more essential genes are removed from the HSV-1 genome. Removal of a gene, such as that encoding HSV-1 thymidine kinase (TK), may significantly decrease viral replication and subsequent cytoxicity in nondividing cells of the brain, while permitting growth in actively dividing cells in tissue culture (12,13) or in vivo in experimental models of brain tumors (14,15). Alternatively, another essential gene in the HSV-1 life cycle, such as the ICP4 gene, can be removed, and this completely abolishes viral replication in both dividing and nondividing cells. In turn, viral stocks of the deleted HSV-1 virus to be used for gene-therapy studies can then be produced only by using cell lines that have been engineered constitutively to express the missing gene product (16,17).

After the removal of such an essential gene, which then limits viral replication, a new transgene can be inserted into the viral chromosome by homologous recombination. The resulting recombinant vectors can be and have been used to transfer a number of functional genes into the rodent brain. They have also been successfully used in gene-therapy approaches to malignant brain tumors by selectively killing dividing tumor cells, with limited damage to the nondividing, healthy brain (14,15,18).

Recombinant vectors with a single gene deletion retain all but one of the ~70 HSV-1 genes. Several of these remaining genes are still capable of directing expression of viral proteins, despite the single gene deletion. Although viral replication is certainly one determinant of vector pathogenicity, expression of viral proteins in the absence of replication may still result in either direct cytotoxicity to the recipient cell or the stimulation of an immune response directed against the target cells. Thus further to debilitate these viruses, vectors containing deletions in several genes have been created, and these are not only replication deficient, but they also result in significantly less toxicity, because fewer viral genes are expressed in the target cell (19; J. Glorioso, personal communication). Such multiple deleted vectors may prove promising; however, there is still concern regarding continued viral gene expression. Furthermore, there is the possibility that all HSV-1 vectors could recombine with herpes viruses naturally present in the majority of the adult population in this country, resulting in an encephalitis.

An alternative, second HSV-1 vector type is the defective viral vector. This vector type is based on the concept of *defective interfering HSV-1 particles*, which occur in nature when HSV-1 is grown at high multiplicities of infection (moi; the ratio of the number of viral particles to the number of cells in a culture dish or animal). Defective interfering particles contain only a limited portion of the entire 150-kb HSV-1 genome, which is repeated until ~150 kb of DNA is generated; then these repeated sequences are packaged into an HSV-1 virion particle. Defective interfering viruses require help from wild-type HSV-1, because they do not themselves contain all of the genes necessary for viral replication and packaging; however, they all contain an HSV-1 origin of DNA replication and a cleavage/packaging signal, necessary for packaging the DNA into an HSV-1 coat (5,20,21). A prototype plasmid, initially developed by Spaete and Frenkel (5), contains these two recognition sequences and a foreign gene, but all the genes encoding HSV-1 proteins were eliminated. In the presence of proteins provided by a helper virus, this plasmid was replicated, cleaved into 150-kb units, and packaged into an HSV-1 coat. The plasmid was termed an *amplicon*, because packaging multiple repeats of the plasmid allowed delivery of several copies of the gene of interest to a single cell, amplifying the signal. The resulting packaged viral vector, in turn, was termed

a defective viral vector. Thus a defective HSV-1 vector contains a gene of interest packaged into an HSV-1 coat, but the vector cannot replicate autonomously and contains no or few genes that encode immunogenic or cytotoxic HSV-1 proteins. Unfortunately, to provide missing proteins needed to generate a virus stock for gene-therapy application, these defective vectors require the presence of a helper virus, which can either be a temperature-sensitive mutant or a deletion mutant in essential HSV-1 life-cycle genes.

These defective HSV-1 vectors are versatile gene-transfer systems and have been used in a variety of experimental systems for application to the CNS. We and others showed that defective HSV-1 vectors can transfect dividing cells and neurons (1,2,9,22–25) in tissue culture, resulting in the expression of a number of transgenes. As a prototype for application of HSV vectors to the CNS, we and others (1,2,9) developed the vector pHSVlac (Fig. 1A), which places the *Escherichia coli* Lac Z marker gene under the control of the HSV-1 Immediate Early (IE) 4/5 promoter, a constitutive promoter active in most mammalian cells, including neurons and glia. The Lac Z gene encodes *E. coli* β-galactosidase, which is normally absent from mammalian cells, and whose expression in these cells could be readily verified. This vector directed the expression of β-galactosidase in a variety of neurons in vitro. Subsequently we demonstrated that defective HSV-1 vectors, such as pHSVlac, could successfully transfer genes into the adult rat brain (26–28). Since then, a number of other research groups have developed a variety of defective HSV-1 vectors that contain many transgenes of interest for studies focusing on CNS function and disease. These defective HSV-1 systems have been used for a wide variety of purposes in vivo, including expression of growth factors to promote peripheral nerve regeneration (29), expression of glucose transporters to provide neuroprotection (30), and analysis of neuronal promoters (31).

Application of HSV-1 Vectors to PD

Perhaps the most relevant to PD is a defective HSV vector containing a gene encoding a human tyrosine hydroxylase (TH; EC 1.14.16.2), the rate-limiting enzyme in dopamine biosynthesis. In this vector, pHSVth (Fig. 1B), the type II TH complementary DNA (cDNA) replaces the Lac Z gene in

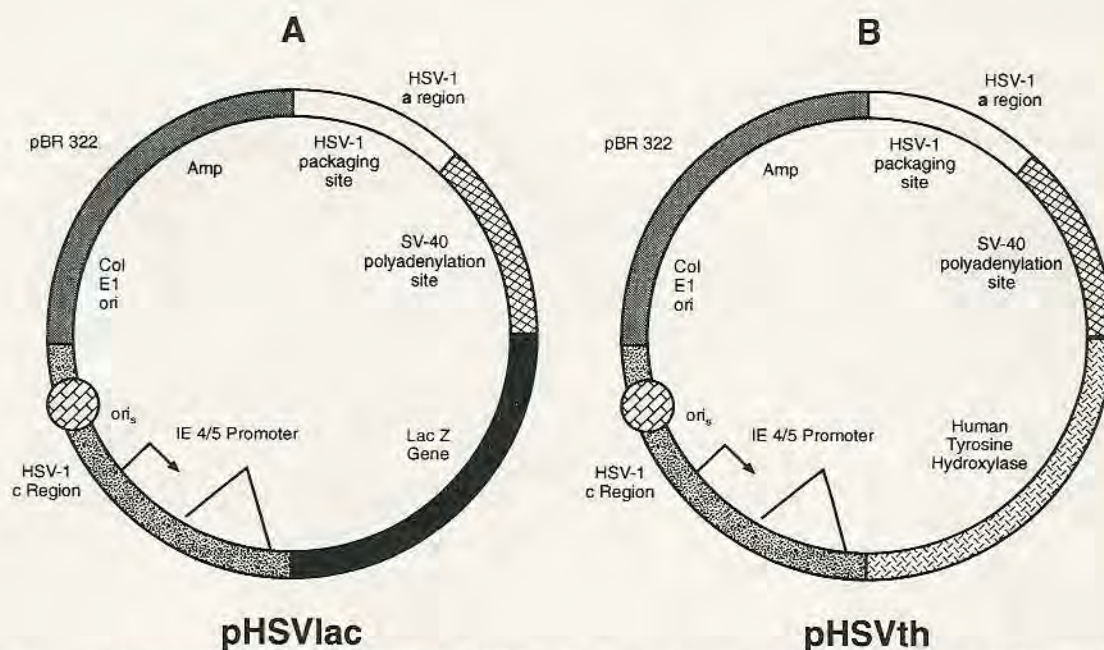

FIG. 1. Schematic diagram of pHSVlac and pHSVth. **A:** pHSVlac: The transcription unit in pHSVth contains the HSV-1 IE 4/5 promoter (arrow), the intervening sequence after the promoter (triangle), the *Escherichia coli Lac Z* gene encoding the marker enzyme, β-galactosidase (black segment), and the SV-40 polyadenylation site (checkerboard). Other sequences include an HSV-1 origin of DNA replication (ori_s; circle with brick pattern), the HSV-1 packaging site (a region; clear segment) to support packaging into HSV-1 particles, and pBR322 sequences (lightly shaded segment). **B:** pHSVth: The lac Z gene in pHSVlac has been replaced with the human TH-II cDNA. pHSVlac was digested completely with *Hin*dIII and partially with *Eco*RI to remove the 3.3-kb *lac Z* fragment, which was replaced with the 1.7-kb TH cDNA (form II) fragment (stippled segment), by using the identical restriction endonuclease sites.

the prototype vector (pHSVlac, described previously), thus placing the TH gene under control of the HSV-1 IE (IE 4/5) promoter, which permits constitutive and sustained expression of a variety of genes in CNS cells in vitro and in vivo (24,26).

TH is the rate-limiting enzyme in dopamine biosynthesis, as shown in Fig. 2, using tyrosine as a substrate and tetrahydrobiopterin as a cofactor. This enzyme has been thoroughly characterized; its expression is highly regulated by numerous transcriptional and translational mechanisms, and its activity responds to a variety of physiologic and metabolic controls (24,26,27,32). O'Malley et al. and Kobayashi et al. (27,33,34) identified four forms of human TH (1–4), which are based on alternative RNA splicing, resulting in structural diversity in the N terminal, but with only slight differences in kinetic parameters. A number of studies have demonstrated that augmenting the activity of TH will increase L-dopa and, subsequently, dopamine production and its consequent release into the extracellular fluid compartment. In a similar vein, increasing the amount of TH synthesized causes elevated levels of L-dopa (and dopamine) in most systems studied (24,26,27,35).

Because PD is characterized largely by a specific loss of dopaminergic neurons projecting from the substantia nigra pars compacta to the neostriatum, with a concomitant reduction in striatal dopamine levels, augmenting dopaminergic function in the neostriatum has been the cornerstone of current pharmacologic therapy for PD. The success of L-dopa (with a peripheral decarboxylase inhibitor) and dopamine agonists in ameliorating the symptoms of this disease is well known. However, the disadvantages of current pharmacologic therapies are now equally well established, including motor fluctuations and dyskinesias (36–43). Other strategies, such as sustained-release formulations, monoamine oxidase inhibitors, and combinations of levodopa with dopamine agonists have been used to achieve adequate symptomatic benefit without adverse effects (38–43). However, PD continues to progress, and current drug therapy remains imprecise and imperfect. This may in part be due to erratic or nonphysiologic drug delivery to target neurons in the striatum. Continuous intravenous infusions of levodopa can reduce the variance between on and off states in patients with advanced PD with levodopa-related fluctuations, suggesting that intermittent oral dosing may play a role in motor fluctuations, that dopamine receptors can develop reversible functional alterations, and that more "physiologic" dopamine delivery may offer a

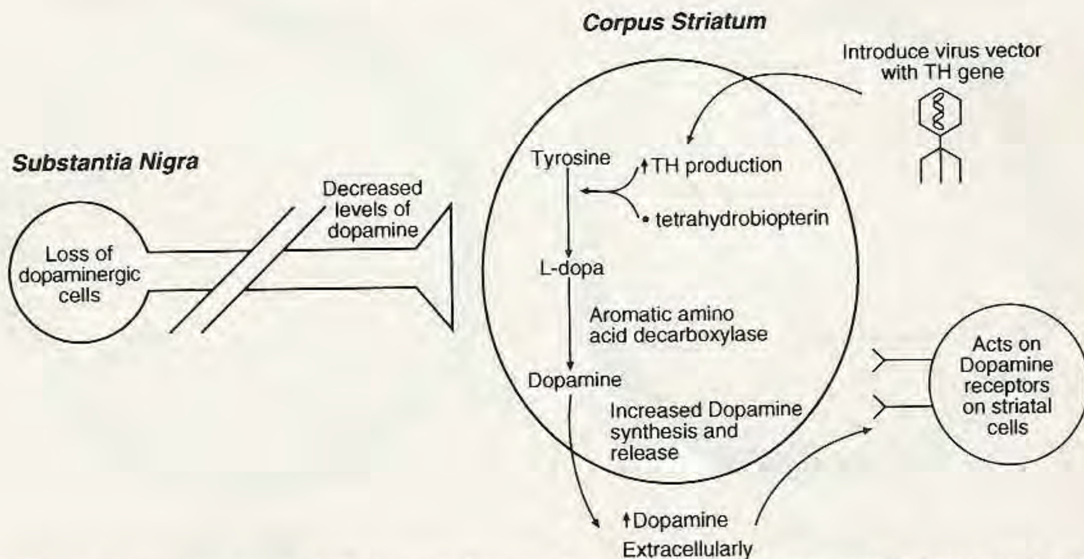

FIG. 2. Schematic of in vivo gene therapy for Parkinson's disease with viral vectors expressing tyrosine hydroxylase (TH). In PD, there is a loss of dopaminergic nigrostriatal fibers, resulting in decreased levels of dopamine in the striatum. By introducing the TH gene directly into the striatum, increased TH production results. In turn, this results in increased endogenous dopamine biosynthesis and release extracellularly, which then restores dopaminergic tone in the striatum. TH requires the presence of the cofactor, tetrahydrobiopterin, as well as the substrate, tyrosine, for synthesis of L-dopa. Aromatic amino acid decarboxylase decarboxylates L-dopa into dopamine. Under certain conditions, in which excess L-dopa is present or TH activity is sufficiently increased, aromatic amino acid decarboxylase can become rate limiting.

significantly improved, long-term approach to PD management (37,39,43–47).

We have therefore initiated a series of studies using the vector, pHSVth, which may provide a novel approach to therapy in PD, and which may address some of the fundamental flaws of current pharmacologic agents. By introducing the TH transgene directly into the neostriatum, the resulting increased amount of TH might permit endogenous dopamine biosynthesis within the striatum, bypassing the dopaminergic nigral afferent input (Fig. 2). Although TH converts only tyrosine into L-dopa, endogenous decarboxylases have been found in brain that can in turn convert L-dopa into dopamine. Lesioning studies have shown a significant amount of amino acid decarboxylase activity endogenous to the striatum, including that found in a population of medium spiny neurons, astrocytes, and vascular endothelial cells (48–50). Such cells could contribute to the conversion of secreted L-dopa to dopamine.

Initially we evaluated the ability of pHSVth to direct the expression of human TH RNA and protein in striatal cells in dissociated cell cultures derived from neonatal rat (24,51). Several days after transfection, cellular RNA was extracted and, by using reverse transcriptase polymerase chain reaction (rtPCR), the presence of human TH mRNA was confirmed in the rat striatal cells transfected with pHSVth, which was not present in controls (treated with either buffer or HSV-1 vectors that did not contain the TH transgene).

To determine whether the vector pHSVth could direct expression of TH in striatal cells, immunocytochemical analysis was performed by using a mouse monoclonal antibody against human TH (24,51). Of the cells demonstrating positive immunostaining, ~90% had neuronal structure; we previously confirmed the identification of such cells as neurons by using histologic, ultrastructural, immunohistochemical, and electrophysiologic techniques (52–54). TH immunoreactive cells were observed 1 day and 1 week after transfection, suggesting the potential for long-term expression. Cells in transfected cultures maintained normal structure, with no evidence of direct cytopathic effects.

L-Dopa and dopamine release from striatal cultures transfected with pHSVth or controls was analyzed. pHSVth, but not controls, induced significant L-dopa release into the extracellular medium, and this release could be stimulated by physiologically depolarizing conditions. Similarly, amplification of extracellular dopamine levels was observed, which was further enhanced by the addition of the biosynthetic precursor, tyrosine, and the TH cofactor, tetrahydrobiopterin, to the extracellular medium. Both L-dopa and dopamine release were found to be dependent on extracellular calcium, results consistent with normal neurotransmitter release mediated by exocytosis (24).

In cultures transfected with pHSVth, the amount of TH enzyme produced was comparable to that found in native catecholaminergic neurons (24). Furthermore, the rate of L-dopa production in transfected striatal cells was comparable to that reported in genetically engineered cell lines that express TH and have been used successfully for ex vivo gene-therapy approaches to animal models of PD (see the following).

In Vivo Studies with pHSVth

Once we had established that the pHSVth vector could in fact direct the expression of a functional TH in neostriatal neurons, we initiated a study to evaluate its in vivo effects. A well-established rat model for PD is based on inducing unilateral lesions of the substantia nigra with the toxin, 6-hydroxydopamine (6-OHDA). Resulting asymmetry in postsynaptic receptor sensitivities between intact and denervated striatum is responsible for the abnormal rotational behavior observed after administration of dopaminergic agonists, such as apomorphine. In turn, the severity of striatal dopamine depletion correlates closely with the rate of rotational behavior (32,55,56). However, only those animals with ≥90% loss of dopamine levels result in predictable and reproducible rat rotational behavior with apomorphine introduction, and thus prescreening of adequate rotation is required in 6-OHDA-treated rats, before their use (56). Established drugs used in PD therapy reduce the abnormal rotation, and this behavioral restoration is frequently used to screen novel candidate pharmaceuticals for potential efficacy in PD.

Phosphate-buffered saline (PBS), pHSVlac, or pHSVth was stereotactically introduced into the ipsilateral denervated striatum of 6-OHDA-lesioned rats (26). Two weeks later, apomorphine-induced rotation was monitored in all the rats. In those rats receiving the experimental vector, pHSVth, compared with controls (pHSVlac or PBS treated), there was a 64 ± 6% reduction in rotational behavior, which remained stable for 1 year (Fig. 3). Several months after pHSVth or control injection,

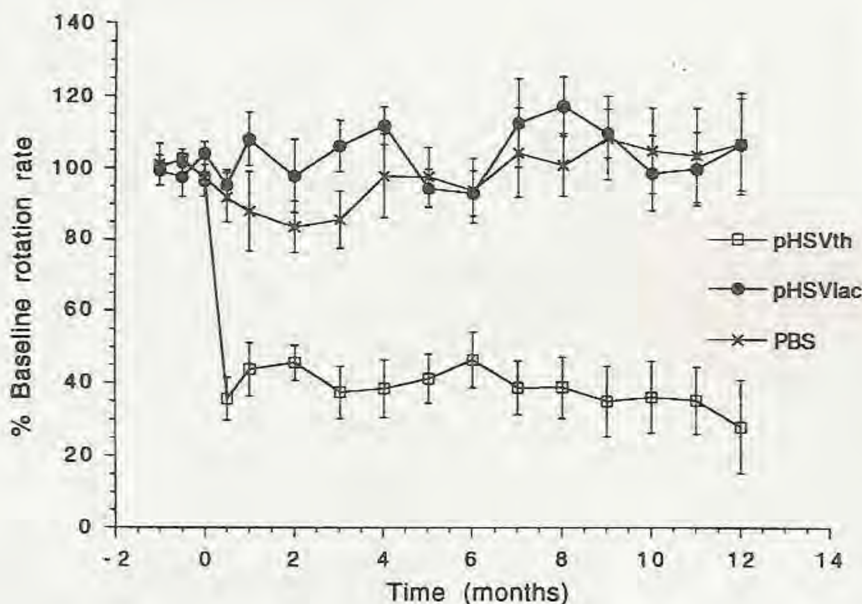

FIG. 3. Behavioral recovery in the 6-OHDA rat rotational model after intrastriatal stereotactic introduction of pHSVth. Apomorphine-induced rotational behavior in rats treated with 6-OHDA was established for ≥6 weeks before introduction of the pHSVth or pHSVlac vectors or phosphate-buffered saline (PBS). Thereafter rats were randomly placed into the three groups that received by stereotactic injection 2 μl of PBS (n = 6 rats), pHSVlac (n = 5 rats), or pHSVth (n = 9 rats) into the right striatum at each of three sites. Two weeks after injection, the rotational rate was evaluated every 2 weeks for 3 months and thereafter at monthly intervals. Statistical analysis of the rotational rate was performed by using repeated analysis of variance with post hoc tests; error bars, standard error of the mean. A significant reduction in rotational behavior was observed in the first measurement after introduction of the TH vector compared with controls (either PBS or pHSVth), which was sustained for 12 months (see ref. 26 for further details). (Reprinted with permission from *Science* 266:1399–1403. Copyright 1994 American Association for the Advancement of Science.)

some rats were subjected to intrastriatal microdialysis to evaluate extracellular catecholamine levels. Dopamine levels were increased by 120% in the pHSVth-treated rats, compared with controls, and depolarizing conditions (56 m*M* potassium in the microdialysis perfusate) resulted in even further dopamine elevations (of 310%), compared with controls, indicating the anticipated effect specific to the particular transgene introduced (TH). As expected, levels of other striatal neurotransmitters [γ-aminobutyric acid (GABA) and acetylcholine] were unaltered by the presence of pHSVth compared with controls. In the presence of an inhibitor of amino acid decarboxylase (AADC, the enzyme that converts L-dopa to dopamine), a 60% increase in striatal dialysate L-dopa levels was observed in those lesioned animals that had received pHSVth, compared with controls (26).

Immunocytochemical evaluation for TH staining in the brains of rats treated with pHSVth or controls revealed the presence of numerous immunoreactive neurons in pHSVth-treated rats, which were not observed in controls, although there was significant variability in the number of cells demonstrating staining among different rats. In addition, by using PCR and rt-PCR methods, human TH RNA and DNA were detected in pHSVth-treated rats; these were not present in controls (26).

These combined results indicated that the vector, pHSVth, directed introduction of the human TH transgene into the denervated rat striatum, with subsequent transcription and translation, resulting in the production of a functional protein that had a significant and sustained behavioral effect. However, in this experimental series, a small but significant percentage (<10%) of rats died, with evidence of HSV-1-mediated cytopathic effects, presumably due to the reversion to wild type of the helper virus or the production of cytotoxic or immunogenic viral proteins. In addition, a diminution of TH expression was observed with time; this may be due to downregulation of the IE 4/5 promoter or other properties associated with the HSV-1 amplicon system. Finally, because the IE 4/5 promoter is constitutively expressed in all cells, excess synthesis of TH and subsequent production in situ of dopamine is possible, which may prove toxic to the striatum; application of cell-specific and controllable promoters may obviate this potential problem. Thus although they are encouraging, these combined findings indicate some obstacles with this vector-system approach (26).

HSV-1 Vectors: Alternative Therapeutic Transgenes

A complementary approach to transmitter synthetic enzyme replacement as a target for gene therapy in PD is the strategy to alter the function of the surviving dopaminergic neurons, thereby facilitating or amplifying their activity and improving dopaminergic transmission. Dopamine neurons, like other CNS cells, have a multitude of extracellular stimuli that ultimately may influence neuronal membrane potential, firing mode and frequency, and transmitter release. The key mediators in these pathways, coupling extracellular signals to transmitter release, are signal-transduction proteins. We showed that defective HSV vectors can direct the stable expression of enzymes involved in a number of signal-transduction pathways in neurons. Of specific interest are those enzymes that have a domain structure; a recombinant encoding the catalytic fragment alone can be constructed such that transduced cells will express an unregulated, fully active catalytic domain of a signal-transduction enzyme. We performed such experiments in vitro, by using HSV vectors containing genetic sequences encoding catalytic domains of adenylate cyclase, protein kinase C (PKC), and calcium–calmodulin protein kinase II (CaCmKII; 23,57,58).

Recently we used these constructs in vivo (59,60). Of interest is the finding that transduction of neurons with these genes alters their physiology, with adenylate cyclase influencing both basal and stimulated neurotransmitter release. In contrast, PKC and CaCmKII have little effect on basal catecholamine release, but they markedly potentiate release stimulated by depolarizing conditions.

The relevance to PD of this approach is that we can specifically target the dopaminergic nigrostriatal cells by using a combination of stereotactic surgery directed at the substantia nigra with further cell specificity obtained by using the TH promoter driving the expression of a signal-transduction enzyme. By using an HSV-1 vector with the catalytic domain of PKC driven by the rat TH promoter, we have been able to demonstrate increased dopaminergic transmission and induction of rotational behavior in nonlesioned, otherwise normal rats (59,60). This facilitated dopamine release suggests that a similar strategy could be used in PD, particularly in early clinical stages in which there remains a significant population of dopaminergic neurons. Alternatively, such a strategy could be coupled to a neuroprotective or neuroregenerative approach to facilitate the function of surviving dopamine neurons.

Considerations for Application of HSV Vectors

Although HSV-1-based vectors were the first successfully to introduce genes into postmitotic neurons, as suggested previously, there are significant disadvantages of HSV-1 vectors. These include the finite but real possibility of reversion of mutants to wild-type HSV-1, resulting in a lytic encephalitis (1,2,5,20,21). Of concern, the combined morbidity and mortality of HSV-1 encephalitis in humans is >50% in most studies. Because the majority of the American population has latent HSV-1 residing in the trigeminal ganglion, the theoretic possibility exists for a recombination event between this latent HSV-1 and the engineered vector system to develop a lytic infection or the reactivation of latent HSV-1 in the CNS. A number of studies also revealed evidence of cytotoxicity associated with several HSV-1 vectors (26,61,62).

Recent approaches have attempted to reduce the potential for cytopathic effects associated with HSV-1 vectors. One group (63) has taken advantage of a cell type–specific factor, Oct-2, which is expressed in B-lymphocyte cells and the developing CNS. Isoforms Oct 2.4 and 2.5 are believed to interfere with HSV lytic infections. An amplicon vector containing the Oct 2.4 and 2.5 genes under the control of a tetracycline (tet) sensitive operon has been developed. This system directs Oct 2.4 and 2.5 expression in transfected cells, which reduces the chance of a lytic infection. As mentioned earlier, Glorioso et al. (19) recently developed novel recombinant HSV-1 vectors with multiple deletions that appear to reduce the risk of neurovirulence and cytotoxicity. Most recently, Geller et al. (64) reported the development of HSV-1 amplicon vector stocks that are entirely free of helper virus, which would likely markedly reduce or eliminate cytotoxic effects.

In addition, novel HSV-1 replicon vectors, containing a variety of directly and indirectly acting cytopathic genes, were developed for application to malignant brain tumors. Planned clinical trials in patients with these fatal tumors will provide significant experience with HSV-1 vectors in human patients (N. Fraser, personal communication), which may pave the way for application to other, nonmalignant CNS diseases, such as PD.

Adeno-associated Virus Vectors

In part motivated by the potential and established deficiencies of HSV-1 vectors, and because of rapid advances in the development of alternative DNA

virus vector systems, we initiated a series of investigations (4,65) focusing on a novel virus vector, based on AAV (66). Wild-type AAV is a nonpathogenic DNA parvovirus, which has two cardinal features: (a) the virus is incapable of autonomous replication and spread; productive infections require non-AAV helper virus coinfection to provide necessary functions for AAV replication; and (b) wild-type AAV can integrate into the host's chromosomal DNA, a property unique to AAV among DNA viruses that permits increased DNA stability (4,66, 67).

During the generation of AAV vectors, >90% of the wild-type genome is deleted, and only the terminal-repeat sequences, which contain DNA replication and packaging signals, remain (4,66). In turn, by cotransfecting with a helper plasmid that contains the missing AAV genes but lacks replication and packaging signals, the missing AAV structural proteins are provided in trans. After infection with adenovirus, which is required as a helper virus for a productive infection to obtain progeny AAV vector, two types of particles are generated: progeny AAV vector with the transgene(s) and progeny helper adenovirus. No helper AAV plasmid is generated because it lacks the packaging sequences required. Progeny AAV vector can be separated from progeny helper adenovirus based on physicochemical distinctions in viral coat proteins.

Thus AAV vectors can be obtained that are entirely free of helper viruses and that do not encode or express any endogenous viral proteins. This is a significant advantage over other DNA virus vector systems that retain the ability to synthesize endogenous viral proteins. The resulting immunogenicity of these viral proteins may be responsible for some of the cytopathic effects associated with these other viral vector systems. Although wild-type AAV can integrate into the host chromosome, the fate of AAV vectors remains less well defined. In human cultured cells, AAV vectors have been shown to integrate, but episomal copies of AAV vector genomes, located outside the host chromosome, also have been observed (4).

A further advantage of AAV vectors is the apparent absence of significant cytotoxicity. In all published in vitro or in vivo experiments using AAV vectors in CNS cells, no evidence of cytopathic effects was observed, in contrast to the established potential for toxicity observed in some studies with HSV and adenovirus vectors. Furthermore, we recently observed that expression or extracellular secretion of protein markers for subtle neuronal injury, such as heat shock protein-72 (hsp-72) or lactate dehydrogenase (LDH), are stimulated by certain HSV-1 and adenovirus vectors but not by AAV vectors (68). Thus it appears that AAV vectors may be particularly suited for gene-transfer approaches to the CNS.

However, disadvantages also exist for AAV. Viral titers are significantly lower than those that can obtained with adenovirus vectors (see the following), but recent technical improvements permit titers of $\leq 10^9$ particles/ml (R. J. Samulski, personal communication). Furthermore, because of the relatively small AAV genome, AAV vectors can accommodate only ~5-kb transgene sequences, unlike HSV, which has a 150-kb genome and can accommodate much larger genetic sequences, although most genes of therapeutic significance for PD are small enough to fit within AAV. Finally, although likely in the near future, Food and Drug Administration (FDA) and National Institutes of Health (NIH) RAC approval is not yet available for the human application of AAV vectors.

Preliminary Studies with AAV Vectors

Based on the significant promise of CNS applications of AAV, we developed several AAV vectors relevant to PD. Initially, a prototype vector, AAV-lac (Fig. 4A) was generated (4), placing the lacZ gene encoding *E. coli* β-galactosidase under control of the human cytomegalovirus (CMV) IE promoter, which permits rapid gene expression. AAVlac introduction into primary dissociated striatal cultures derived from neonatal rat resulted in sustained functional β-galactosidase expression, as monitored by immunocytochemical and histochemical staining (unpublished results). AAVlac was also stereotactically microinjected into the corpus striatum of rats. Several days or several months later, these rats were killed. Histochemical analysis for β-galactosidase activity revealed numerous positively staining cells in the striatum. In addition, PCR permitted the amplification and visualization of viral vector DNA in situ, several months after transfection. Of note, no evidence of cytopathologic conditions was observed other than the actual injection tract from the needle (4). We also demonstrated recently that AAVlac can successfully transfect human brain tissue slices obtained from patients undergoing temporal lobe resections for intractable seizures, resulting in the specific expression of *E. coli* β-galactosidase in human neurons (69).

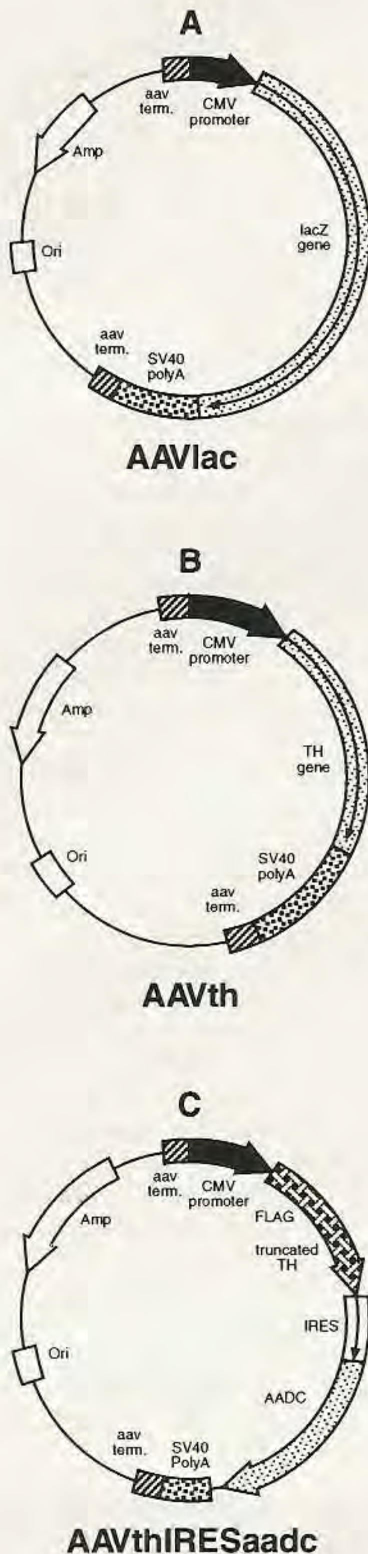

FIG. 4. Schematic diagrams of the adeno-associated virus vectors: AAVlac, AAVth, and AAVthIRESaadc. A: AAVlac: To generate AAVlac (4), plasmid pSub201 was digested with *Xba*I to remove nearly the entire AAV genome, leaving only the terminal repeats. A cytomegalovirus (CMV) promoter-*lac Z* gene-SV40 polyadenylation signal cassette was isolated from plasmid pHCL by digestion with *Spe*I and *Xba*I, and this was inserted into *Xba*I-digested pSub101 to create AAVlac. B: AAVth: AAVlac was digested with *Hin*dIII and *Xba*I to remove the *Lac Z* gene and polyadenylation signal. Then a *Hin*dIII-*Xba*I fragment from pREP4 (Invitrogen), containing an SV40 polyadenylation signal and a polylinker, was inserted. This construct was then digested with *Hin*dIII and *Bam*HI, followed by insertion of the human TH form II cDNA. C: AAVthIRESaadc: To generate this "bicistronic" construct, cDNA of human TH (form II) was truncated to 1.1 kb by deleting its N-terminal regulatory region. To permit assessment of transgene expression within cells already containing TH, an FLAG epitope was added to this truncated TH cDNA to form a novel recombinant domain, FLAG-TH. FLAG encodes a nonfunctional peptide that can be readily assayed immunocytochemically, permitting evaluation of transgene expression in transfected cells that endogenously synthesize the same gene. Human AADC cDNA was added to FLAG-TH, and the entire resulting construct (FLAG-TH-AADC) was packaged into an AAV vector by using the emc virus IRES sequence, resulting in the final bicistronic vector.

Application of AAV Vectors to PD

Based on this prototype AAV vector (AAVlac) we developed AAV vectors containing therapeutically relevant genes. In one construct, we replaced the lacZ gene with the human TH (form II) cDNA (4). The resulting construct, AAVth (Fig. 4B), caused TH expression in cultured striatal neurons, as determined by immunocytochemical staining; cultures transfected with AAVlac or treated with PBS showed no such staining (unpublished results). AAVth also was placed into the partially denervated striatum of rats with unilateral 6-OHDA lesions (4). Subsequent expression of TH was verified at various times, extending up to 4 months, by using immunocytochemical staining. Controls, which included 6-OHDA-lesioned rats treated with buffer or AAVlac, had no endogenous TH immunocytochemistry. The majority of immunoreactive cells after AAVth injection were neurons, as monitored by double-labeling experiments using antibodies directed against the neuron-specific antigen, neurofilament, or the glia-specific antigen, glial fibrillary acidic protein (GFAP) (4). Although present for the duration of the experiment, TH expression did appear to diminish with time.

Similar to the observed behavioral effect of the HSV vector, pHSVth, on rotation, injection of AAVth into 6-OHDA-lesioned rats resulted in a significant and sustained recovery of apomorphine-induced asymmetrical rotation for 2 months (Fig. 5). In contrast, animals treated with intrastriatal

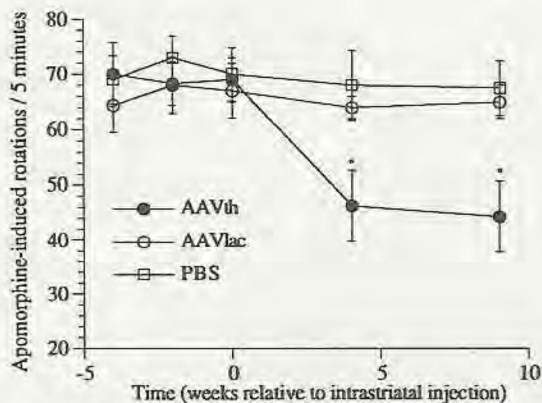

FIG. 5. Behavioral effect of AAVth on 6-OHDA-lesioned rat rotational behavior. After establishing a baseline rotational behavior for ≥6 weeks after 6-OHDA lesioning, rats were injected unilaterally and intrastriatally with AAVth ($n = 4$), AAVlac ($n = 3$), or phosphate-buffered saline (PBS; $n = 4$). Only those animals injected with the TH vector demonstrated a significant and sustained reduction in rotational behavior compared with baseline (and PBS control). Asterisks, significant results ($p < 0.05$) with post hoc analysis. Error bars, standard error of the mean (4). (Reprinted with permission from *Nature Genet* 1994;8:148–154. Copyright 1994 Nature America, Inc.)

AAVlac and PBS injections showed no alteration from baseline (4). AAVth directed synthesis only of TH, which is responsible for the conversion of tyrosine into L-dopa; as mentioned earlier, decarboxylases endogenous to the striatum presumably mediated the subsequent conversion of L-dopa into dopamine.

Given the uncertainty of the origin of the decarboxylase activity responsible for this conversion, we recently developed a "bicistronic" construct, AAVthIRESaadc (Fig. 4C), which contains both the human TH and AADC genes (70,71). As discussed earlier, AADC is responsible for the conversion of L-dopa to dopamine in dopaminergic neurons, and under certain conditions (in which TH activity is markedly enhanced), the biochemical step mediated by AADC can become rate limiting. To generate this bicistronic construct, cDNA of human TH (form II) was truncated to 1.1 kb by deleting its N-terminal regulatory region. To permit assessment of transgene expression within cells already containing TH, a FLAG epitope was added to this truncated TH cDNA to form a novel recombinant domain, FLAG-TH. FLAG encodes a nonfunctional peptide that can be readily assayed immunocytochemically and thus allows evaluation of transgene expression in transfected cells that endogenously synthesize the same gene. In turn, human AADC cDNA was added to FLAG-TH, and the entire resulting construct (FLAG-TH-AADC) was packaged into an AAV vector by using the emc virus IRES sequence, resulting in the final bicistronic vector, which we termed AAVthIRESaadc. This vector, which contains the TH and AADC genes, was used to transduce HEK 293T cells, which in turn released high levels of dopamine into the medium, compared with controls (72). Levels of dopamine were also higher than in cells transfected with the TH vector alone (unpublished results). The bicistronic vector also resulted in both FLAG and human TH immunoreactivity in primary rat striatal cultures (unpublished results).

Primate Studies with AAV Vectors

Introduction of the synthetic toxin, MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine), into monkeys results in a striking neurochemical and behavioral paradigm for PD and is used frequently for the assessment of novel therapeutic approaches to PD (32). Thus in a recent multicenter study done in conjunction with D. E. Redmond, Jr. and J. Sladek, Jr., (70,71) experimental vector (AAVthIRESaadc) or control vector (AAVlac) were evaluated in 12 MPTP-treated African green monkeys. Four monkeys received intrastriatal injections of the experimental vector unilaterally and six bilaterally; two monkeys received intrastriatal injections of the control vector, AAVlac, bilaterally. One week to 4 months later, the monkeys were killed. PCR and rtPCR confirmed the presence of both TH and AADC DNA and RNA, which were not found in the controls, in the AAVthIRESaadc-treated monkeys. Histologic evaluation showed no evidence of cytotoxicity, and within a few millimeters of the injection site, TH-immunoreactive cells were observed. Consistent with increased dopamine neurotransmission, an increase in dopamine concentrations and a decrease in the homovanillic acid/dopamine ratio, which were not seen in the controls, were observed in the brains of monkeys treated with the experimental vector. Preliminary behavioral evaluation suggested a positive effect from the experimental vector, but further analysis of the data is under way. These results indicate the significant potential of this AAV-based system in a primate model of PD (70–72).

AAV Vectors with Neuronotrophic Genes

More recently, in another collaborative study with Redmond and R. Roth at Yale (73), we and others developed an AAV vector (AAVgdnf) that

contains the cDNA for glia-derived neuronotrophic factor (GDNF). GDNF, first characterized several years ago, is a potent and specific neurotrophic factor that promotes midbrain dopaminergic neuron survival (74–76). In embryonic midbrain cultures, GDNF has been shown to induce differentiation, enhance survival, and increase high-affinity dopamine uptake in dopaminergic neurons. This effect on dopamine uptake is specific and is not seen for other transmitters, such as GABA or serotonin (76–78). Although GDNF is trophic for cortical and brainstem motor neurons, as well as hippocampal neurons (77,79), the relative specificity to dopaminergic neurons, compared with GABAergic and serotoninergic neurons, in midbrain is unique to GDNF, because other growth factors, including fibroblast growth factors, insulin-like growth factors, and epidermal growth factor have generalized effects on all three of these transmitter systems. In addition, these other growth factors are mitogens, and their 50% effective concentration (EC_{50}) is one to three orders of magnitude greater than that for GDNF. Recent studies have indicated that GDNF may provide protection for and induce regeneration in nigrostriatal dopaminergic neurons in PD animal models, with significant implications for developing a protective or regenerative strategy in patients with PD (74–79).

In embryonic mesencephalic rat cells in culture, the AAVgdnf vector increased the survival of dopaminergic neurons. In turn, two in vivo animal models of nigral degeneration were used. In one, rats had nigrostriatal neurons prelabeled with fluorogold, and thereafter bilateral 6-OHDA lesioning took place. AAVgdnf introduced unilaterally before 6-OHDA administration resulted in neuroprotection, as monitored by increased striatal dialysate levels of dopamine ipsilaterally and rotational behavioral improvement over controls (73). In two MPTP-treated African green monkeys, stereotactic introduction of AAVgdnf unilaterally resulted in an increase in single-photon emission–computed tomography (SPECT) imaging of dopamine transporter ipsilaterally. Further analysis of biochemical, histologic, and behavioral correlates are under way (73).

Perhaps an ideal approach to patients with PD would be to both enhance dopaminergic function and provide protection from further nigrostriatal degeneration. Thus we could envisage a combined approach that uses restorative vectors, such as AAVthIRESaadc, and protective vectors, such as AAVgdnf. In addition, because TH is dependent on its cofactor, tetrahydrobiopterin, for maximal activity, we can also imagine developing a vector system that contains the gene encoding the rate-limiting enzyme in this cofactor's biosynthesis, guanosine triphosphate (GTP) cyclohydrolase I (80).

Adenovirus Approaches to PD

In 1993, several reports were published on the use of recombinant adenoviruses to deliver genes into the mammalian brain (3,81–83). These articles demonstrated that after intracerebral injection of adenovirus containing the marker gene, lac Z, significant staining surrounded the injection site. Moreover, the adenoviral vectors were relatively efficient (with ~1 transduced cell per 100 infectious particles). The transduced cells appeared to include neurons, astrocytes, oligodendrocytes, microglia, and ependymal cells. Relatively low titers ($<2 \times 10^7$ pfu) of the recombinant adenoviral vectors resulted in low toxicity; however, at higher titers ($>5 \times 10^7$ pfu), significant toxicity occurred with both gliosis and vascular inflammatory responses. Despite the large numbers of cells transduced in the short term, at 1 month, the vast majority of staining had disappeared, and by 2 months, there was little or no expression in the majority of brain regions studied (B. Davidson and J. Mallet, personal communication). These results parallel those found with first-generation recombinant adenoviral vectors used in other tissues in which the inherent immunogenicity of the adenovirus vectors resulted in tissue inflammation and contributed to the loss of expression.

Despite these limitations, Horrelou et al. (84) reported the use of a recombinant adenoviral vector expressing TH in the 6-OHDA rodent model of PD. By using a vector that contains the human TH form I, they showed a significant (mean, 30%) reduction in rotational behavior at 1 week and a 22% reduction at 2 weeks, compared with no significant change in the apomorphine-induced rotations in controls (rats treated with the adenovirus vector expressing the Lac Z gene). Moreover, they were able to show striatal neurons that were immunoreactive for TH at 15 days after the vector administration. This study was restricted to just 2 weeks, partly because of the known short-lived expression of the adenoviral vector.

We recently conducted similar studies in collaboration with B. Davidson (unpublished results). By using a vector that contains the human TH form II driven by a Rous sarcoma virus (RSV) promoter,

we were able to demonstrate significant immunoreactivity for TH in primary rat striatal cultures (Fig. 6). When this vector was introduced into 6-OHDA lesioned rats, a mean rotational recovery of 40% was observed at 2 weeks, which persisted in two of four rats for >2 months (unpublished results). Thus although we cannot be confident that this recovery was due to persistent transgene expression, the results suggest that, in addition to HSV and AAV, adenoviral vectors also may be useful for gene therapy in PD, particularly with newer generations of adenovirus vectors being developed with reduced immunogenicity and toxicity.

FIG. 6. Immunocytochemical staining of striatal cells after transfection with an adenovirus vector containing the TH cDNA. Primary striatal cultures derived from neonatal rat (22) were transfected with either AdenoRSVlac or AdenoRSVth (moi, 10^3). Nine days later, these cultures were fixed in 4% paraformaldehyde, and immunocytochemistry was performed as described previously (4,24) for expression of TH. **Top panel:** cells treated with AdenoRSVlac demonstrated no significant TH staining, whereas 10–30% of neurons demonstrated significant positive staining in cultures treated with AdenoRSVth (**bottom panel**). These results indicate successful transfer and sustained expression of the TH transgene in primary striatal cultures by using an adenovirus vector (Lowen et al., unpublished results).

Another recent study (85) developed a defective adenovirus vector (Ad-huGDNF) containing the GDNF gene under control of an RSV promoter. In vitro, this vector increased the number of TH immunoreactive neurons and extent of neurite outgrowth in primary cultures of dissociated embryonic rat ventral mesencephalon. In vivo studies with this vector are planned.

Of concern for application of adenovirus vectors to human neurologic diseases is a recent report by Woo et al. (86) demonstrating significant morbidity (one of four animals died, and another showed progressive clinical deterioration, in the presence of ganciclovir) and histopathology (extensive coagulative necrosis, significant cerebral edema with mass effect, and acute inflammation at the site of injection) associated with injection of high titers of defective adenovirus vectors containing the TK gene into the brain of primates. Lower titers resulted in less evidence of cytotoxicity, but nonetheless, some inflammatory response and necrosis were observed.

Delivery Issues

PD is predominantly a focal neurodegenerative disease, with the brunt of damage occurring within the nigrostriatal system. A strategy based on stereotactic delivery of vector systems to either the striatum or the substantia nigra is relatively straightforward. Multiple inoculations in different locations will permit a larger area of tissue delivery and may be needed for sufficient numbers of cells to be transfected to produce adequate enzyme or neurotrophic factor. If clinical application of the in vivo gene-therapy systems were to occur, existing methods for ablative or tissue-transplant surgery in PD could be modified to accommodate the viral vectors.

However, PD can have more diffuse neurologic manifestations that can influence cognition and affect, with altered function of the mesolimbic dopamine pathways, as well as other dopamine systems, including retinal and olfactory. Thus global delivery of viral vectors may prove to be desirable, particu-

larly for vectors that contain genes encoding neuroprotective proteins. This issue is being addressed by using two broad approaches. The first is based on osmotic disruption of the blood–brain barrier by using intravenous introduction of high-molecular-weight compounds. Reports from Neuwelt's laboratory (87–90) have indicated that particles the size of viruses can be induced to cross the blood–brain barrier by using this approach. Recently, effective delivery and transgene expression throughout the brain of cats was achieved by Neuwelt et al. (89, unpublished results) by using an adenovirus vector containing a gene encoding a lysosomal enzyme.

As an alternative, second approach, Oldfield's group, (91), developed a convection-enhanced delivery system in the brain based on pressure gradients inducing bulk flow of interstitial fluid carrying macromolecules. With this approach, large particles that were infused into white matter were redistributed over the next 24-h period throughout the grey matter in the cortex. However, the precise mechanism responsible for this diffusion remains controversial (90). Intraventricular delivery has also been tried, resulting in preferential expression in ependymal and circumventricular tissues (83). Based on these and other innovative delivery systems, it is possible that global CNS delivery of viral gene-therapy vectors will be feasible, with implications for therapy in PD as well.

EX VIVO GENE-THERAPY APPROACHES

A number of studies have explored the possibility of developing cell lines that contain the TH gene or other transgenes or both for application as grafts in PD. Such approaches have been termed ex vivo because they do not use direct in vivo gene transfer into the host brain. Much of this work was pioneered by Gage et al. (92), who initially demonstrated that grafted nerve growth factor (NGF)-secreting fibroblasts could prevent the degeneration of cholinergic neurons and promote sprouting of axons in animal models of fimbria–fornix lesions. Subsequently numerous investigators developed a variety of cell lines, based on cultured neuroblastoma, glioma, and neuroendocrine cells, immortalized and primary fibroblast cells, myoblast and myotube cultures, Schwann cells, and other cell types, that have been transfected with either rat or human TH cDNA. Most of these cell lines express TH, direct L-dopa or dopamine biosynthesis and secretion or both, and have a positive behavioral effect in the 6-OHDA-lesioned rat rotational model of PD. However, in many cases, the immortalized cell lines formed solid, expanding tumors and thus have limited clinical application. In addition, transgene expression in grafts is frequently limited to a few weeks or months, although recent modifications suggest that long-term expression can be achieved under certain circumstances.

Techniques for Introduction of Transgenes into Cell Lines

Gage et al. (93) and other groups established that a variety of techniques can be used to transfer a transgene into fibroblast cell lines, including calcium phosphate precipitation, lipofection, electroporation, and application of retroviral vectors. Variability of subsequent transgene expression was in part due to the particular method used, but could also be attributed to the type of promoter used and the host cell chromatin structure at or near the site of integration of the transfected DNA. Palmer et al. (94) demonstrated that fibroblasts genetically modified with retrovirus vectors persisted after grafting, but gradual inactivation of the transgene typically took place. In this study, the human adenosine deaminase and neomycin phosphotransferase genes were introduced into skin fibroblasts by using a retrovirus. Although the fibroblasts survived transplantation for >8 months, transgene expression decreased by >1,500-fold after 1 month. Neither cellular nor humoral-mediated immune responses were observed in the transplanted animals. Furthermore, transgene expression could not be restored after recultivation of the fibroblasts from the grafts, indicating that genetic regulatory elements, rather than immune responses, were responsible for the diminution of gene expression with time.

In another study, Scharfmann et al. (95) demonstrated that long-term transgene expression could be achieved with a "housekeeping" gene promoter, such as the dihydrofolate reductase (DHFR) gene promoter, instead of viral promoters, such as the CMV IE promoter, frequently used in gene-therapy experiments. Recent studies used adenovirus vectors to deliver the TH gene into cell lines; evaluation of these systems is ongoing (96,97).

Initial Studies with Immortalized Cell Lines

Gage et al., and others (98,99) initially focused their attention on immortalized cell lines of fibroblasts as donors for genetic modification. Such cells

grow easily and can be readily manipulated genetically in vitro. The rat 208F fibroblast cell line was infected with a retrovirus vector containing rat TH cDNA. In vitro, in the presence of tetrahydrobiopterin, these cells synthesized L-dopa and secreted it into the extracellular medium. These genetically modified cells were then implanted into the brain of rats with unilateral 6-OHDA lesions, and rotational behavior was assessed. A significant reduction in aberrant rotational behavior induced by apomorphine administration was observed for 2 weeks after grafting in those animals receiving rostral caudate grafts, which was not seen in rats receiving caudal caudate grafts. Immunocytochemical analysis could not identify TH staining, and suboptimal survival of the grafts occurred, presumably because of immune rejection due to a genetic mismatch between graft and host. Thus as a next step (99), an immortalized, syngeneic Fischer fibroblast cell line transfected with the rat TH cDNA was used in Fischer rats. Although behavioral data were supportive of a physiologic recovery, invasive growth of the grafted cells to develop tumors precluded long-term survival of the animals.

In similar studies, Uchida et al. (100,101) transfected both a C6 glioma cell line and an immortalized rat kidney fibroblast cell line (NRK-49F) with TH cDNA. In vitro TH activity was subsequently detected, and L-dopa was found within the cells and the extracellular medium. The transfected C6 cells were able to synthesize L-dopa without introduction of the TH cofactor, tetrahydrobiopterin, presumably because these cells can synthesize this cofactor independently. In contrast, the transfected NRK cells required the addition of tetrahydrobiopterin to the culture medium before L-dopa production occurred (100). These findings confirm the need for a source of the active cofactor for TH. When transfected C6 glioma cells were introduced into the rat brain, staining for TH was observed in the grafted cells 10 days later, but large tumors had formed in situ (101).

Studies by Horellou and Mallet et al. (102,103) evaluated the potential of a number of immortalized cell lines to serve as the donor for genetic modification. The neuroblastoma NS20Y cell line and the neuroendocrine AtT20 cell line were transfected with rat TH cDNA and human type I TH cDNA, respectively. Cells were then further selected in tyrosine-free medium, yielding high levels of TH activity. Both cell types were implanted into rats with unilateral 6-OHDA lesions and successfully reduced the apomorphine-induced rotational asymmetry for ≤2 weeks. However, after this period, it was noted that some of the animals had seizures, and histologic evaluation revealed that the grafts had become large tumors.

Horrelou et al. (103) also evaluated the fibroblast 3T3 cell line and the pancreatic β-cell derived RIN cell line as graft donors. These cell lines were transfected with a retrovirus containing the cDNA-encoding form I of human TH. When grafted into unilaterally denervated striatum of 6-OHDA-lesioned rats, both cell types were able to survive implantation, express TH, and synthesize L-dopa or dopamine, as monitored by microdialysis techniques, for 8–9 days after implantation. The 3T3 cells secreted L-dopa, which was in turn decarboxylated to dopamine by the host striatum. No significant storage of dopamine was observed, and depolarizing solutions in the microdialysis perfusate did not result in significant increases of dopamine release. In contrast, the RIN cells, which possess endogenous AADC activity, synthesized dopamine directly, and this dopamine appeared to be stored and released in a regulated manner. In these cells, the catecholamine biosynthetic rate closely paralleled that seen in the rat pheochromocytoma cell line, PC12. After hyperkalemic depolarizing conditions in vitro, a significant increase in dopamine release was observed. Similarly, increasing extracellular calcium induced dopamine secretion, suggesting normal vesicular exocytosis. In vivo, depolarizing solutions in the dialysis perfusate induced a 13-fold increase in dopamine release in the RIN grafts in rats, consistent with regulated storage and release. Tissue levels of dopamine in RIN grafts were 57% of control striatum, compared with depleted levels in the lesioned side, also suggesting restoration of dopamine storage.

Surprisingly, although the genetically modified RIN cells had regulated storage and release of dopamine, the modified 3T3 cell grafts resulted in higher striatal dialysate levels of dopamine and superior behavioral recovery (103). Graft tissue levels of catecholamines in the 3T3 cells were only 2.6% of the control striatum, indicating minimal storage of catecholamines by these cells and preferential extracellular secretion. These findings suggest that cell lines that synthesize only L-dopa may prove superior to those containing AADC, which can convert L-dopa to dopamine, because L-dopa may preferentially be secreted by these cells and not undergo regulated vesicular release.

Primary Cultures

Given the problem of in situ tumor formation with immortalized cell lines, investigators more recently turned their attention to primary cell cultures, which are more likely to remain dormant within the brain.

In one study, Kawaja and Gage (99) evaluated the morphologic and neurochemical features of striatal implantation of cultured primary skin fibroblasts of Fischer 344 rats. Isogeneic primary fibroblasts survived for >6 months. Although these cells demonstrate robust growth in culture, once they form a confluent monolayer, contact inhibition arrests further cell division. After striatal implantation, an intense astrocytic response, as monitored by GFAP immunocytochemistry was seen for ≤8 weeks throughout the striatum. Although this response diminished among striatal astrocytes distant to the transplant, it persisted in the cells adjacent to the border of the graft. Capillary formation within the graft was demonstrated, and by using intravenous infusion of peroxidase, a rudimentary endothelial blood–graft barrier could be demonstrated, similar to that seen in the capillaries of the host striatum. On ultrastructural examination, the grafts contained many fibroblasts and extracellular matrix with collagen. Reactive astrocytic processes with intermediate filaments were found dispersed throughout the graft. Hypertrophied astrocytes could be demonstrated to form a border between the graft and the host brain.

In another study, Fisher et al. (104) then used primary fibroblasts derived from the skin of inbred Fischer rats as recipient cells for the TH transgene. When these were subsequently grafted in 6-OHDA-lesioned Fischer rats, the grafts survived for ≥10 weeks and expressed the transgene throughout this period. Behavioral recovery was observed, confirming synthesis of L-dopa; this was seen in in vitro studies as well.

In another study (105), Kang et al. transduced primary skin fibroblasts from Fischer 344 rats with either the TH or the AADC gene. Those cells with the AADC gene were able efficiently to convert L-dopa into dopamine. Co-cultures of the two cell types, containing either TH or AADC genes in various proportions, permitted control of the amount of dopamine produced. Application of these co-cultures to animal models of PD is under way.

More recent studies have evaluated primary fibroblast cells transfected with the GTP cyclohydrolase 1 (GTPCH1) and TH genes. Primary fibroblast cultures contain all the enzymes needed to synthesize the TH cofactor, tetrahydrobiopterin, except for this enzyme (GTPCH1). Fibroblasts were engineered to contain both genes encoding TH and GTPCH1. An enzyme assay revealed that these cells were able to synthesize L-dopa in the absence of exogenously provided tetrahydrobiopterin. In vivo these cells survived well in the rat striatum, and analysis in the rat rotational model is under way (80).

Jiao et al. (106–108) pioneered the use of autologous muscle grafts in the brain that take advantage of the postmitotic state of myofibers and the stem-cell characteristics of myoblasts. By mincing muscle grafts, long-term survival was observed in rat cortex in vivo. Co-transplantation of cultured myoblasts and myotubes permitted long-term expression of reporter genes. In one study, primary rat muscle cells were lipofected with a TH expression vector; several days later in vitro, ~20% of the myoblasts and 50% of myotubes demonstrated TH immunoreactivity. These genetically engineered myoblasts and myotubes were transplanted together into unilaterally 6-OHDA-lesioned rats. TH immunoreactivity was observed in the muscle grafts, which persisted for ≤6 months. A 75% reduction in apomorphine-induced rotation was observed for this same period. At the end of various periods in this study, striatal sections were excised and assayed for catecholamines. Dopamine levels were restored to nearly normal levels for ≥4 months with the muscle grafts, whereas lesioned rats with no graft had virtually no dopamine. Similar results were seen for L-dopa levels. In this study, nonspecific neurotrophic effects due to the muscle graft alone could not be fully excluded.

Another cell type that has been investigated as a candidate for ex vivo gene therapy is the Schwann cell, the glial cell type that myelinates axons in the peripheral nervous system. Pure populations of primary Schwann cells can be isolated from either neonatal or embryonic rats, and several studies have suggested that these cells can survive implantation in the CNS (27). For application to humans, Schwann cells could be readily obtained from peripheral nerve biopsies, which would thus provide a source of isogeneic cells. By using a retrovirus vector, O'Malley and Geller (27) expressed rat TH cDNA in rodent Schwann cells. In initial studies using immortalized Schwann cell lines, L-dopa secretion was more than twice that seen with fibro-

blast cell lines. As a next step, primary Schwann cells were developed that contained rat TH. Variable expression was seen, such that ~10% of the cells expressed high levels of TH. Thus these cells could serve as an alternate tissue graft in an ex vivo approach to PD.

Alternative Ex Vivo Approaches

Other approaches are focusing on the application of tumor cell lines that, under appropriate conditions, can differentiate into neurons. Morassutti et al. (109) reported transfection of the rat TH gene into P19 murine embryonal carcinoma cells, which can terminally differentiate into neurons or glia in the presence of retinoic acid. Under control of a murine phosphoglycerate kinase (PGK) promoter, rat TH gene expression was observed in transfected embryonal carcinoma cells. Dopamine synthesis depended on exogenous availability of tetrahydrobiopterin in vitro in those cells not exposed to retinoic acid, whereas in the presence of retinoic acid, differentiation of cells occurred, and dopamine production became independent of exogenous tetrahydrobiopterin. These differentiated, transfected cells were then grafted into 6-OHDA-lesioned rats, and long-term graft survival was observed. However, TH immunostaining did not differ from that in controls, and no behavioral improvement was observed. By using tyrosine-free media, clonal selection of this transfected cell line may improve the degree of transgene expression. Because this approach depends on the retinoic acid–based induction and subsequent maintenance of the differentiated state of otherwise undifferentiated tumor cells, there is concern about the potential dedifferentiation and resulting formation of malignant tumors, and thus application to human patients is uncertain.

Other recent studies evaluated the potential application of primary fibroblasts engineered to produce trophic factors. In one study, Frim, Isacson, and their colleagues (110) developed a fibroblast cell line that contains the brain-derived neurotrophic factor (BDNF). In adult rats infused with MPP^+ intrastriatally, implantation of these engineered fibroblasts dorsal to the substantia nigra attenuated the loss of nigral dopaminergic neurons. Similar studies are being pursued with cell lines that express GDNF and other growth factors (111).

Recent studies based on developing genetically altered pig CNS tissue as a xenograft may also provide a readily available source of tissue (O. Isacson, personal communication), but further details are pending. Clearly suppressing immune system responses to such tissue will play an important role in the application of any strategy involving xenografts and may limit its success.

New advances in the development of "biohybrid" implants that contain living cells supported in a hydrogel matrix and enveloped by a semipermeable membrane may be of value for the ex vivo approach to PD (112). Genetically engineered cells that secrete dopamine or neurotrophic factors may be placed in such a device, permitting long-term survival and containing the cells within the physical contraints of the device. Further evaluation of these systems in animal models of PD must first be performed.

Advantages and Disadvantages of Ex Vivo Approaches

Ex vivo approaches have a number of attractive features. Included among them is a clear identification of the genetic constitution of the grafted cells, unlike in vivo approaches that use viral vectors with poorly defined intracellular genetic events. In many studies of cultured cells, long-term transgene expression can be observed, with appropriate control of expression by selected promoters. Although some grafts can form tumors, others have been developed that apparently are dormant within the CNS. Because the genetic engineering of these cells occurs entirely outside the patient, before implantation, risks associated with some viral vector systems caused by helper viruses or viral protein synthesis can be obviated.

However, there are problems with the ex vivo approaches as well. Once they are implanted in situ, gene expression often is transient, and its control is less well characterized. Grafts are often immunogenic, and evidence of long-term survival beyond a few months is scant. Depending on their source, grafts can form tumors, which continue to divide until the animal dies of the tumor. To obtain sufficient protein expression, grafts must be large enough, and thus may be a space-occupying lesion within the limited space of the brain. Limited diffusion within the CNS of the transgene-directed transmitter or trophic factor from the graft may restrict its efficacy, although this may prove to be a constraint for in vivo gene-therapy approaches as well. Finally, the mechanism of cell death responsible for PD in the first place may also affect longevity of transplanted cells, although there is no evidence available to address this issue.

SUMMARY

Rapid advances in molecular biology and tissue-transplant techniques have permitted the development of a number of gene-therapy approaches to neurologic diseases, including PD. Several in vivo and ex vivo–based systems have been developed, with demonstrated efficacy in animal models of PD, which make it possible that clinical trials will occur within the next several years. Similar to the rapid evolution of the personal computer industry over the past decade, novel permutations and improvements in existing gene transfer technology are likely and may well permit the development of long-term and effective gene therapy for PD, obviating the need for systemic pharmacologic therapy. Perhaps the most exciting aspect of these approaches is the potential to develop a dual restorative and protective/regenerative approach to PD, based on the long-term restoration of dopaminergic function and the prevention of further deterioration or the induction of regeneration in the nigrostriatal system.

Problems with gene therapy for PD are significant but surmountable. Long-term expression (exceeding several months) has not been demonstrated. In most in vivo and ex vivo systems, cytotoxicity and immune responses are problematic. Techniques for delivery of the vector or cells into the appropriate location have not been perfected. Current gene-transfer systems have used promoters that do not allow precise control over gene expression; however, newer generation vectors are being developed and allow cell-specific and controlled expression—features likely to improve the efficacy of any gene-therapy approach to PD. Before implementation of clinical trials, such problems must be solved. Nonetheless, the rapid pace of new discoveries in this field offers exciting hope for patients burdened by PD; it is possible that clinical trials will begin before the end of the "decade of the brain."

REFERENCES

1. Freese A, Neve R, Geller AI. HSV-1 vector mediated neuronal gene delivery: strategies for neuroscience and neurology. *Biochem Pharm* 1990;40:2189–2199.
2. Geller AI, Freese A. Transfection of cultured central nervous system neurons with a defective HSV-1 vector which expresses β-galactosidase. *Proc Natl Acad Sci USA* 1990; 87:1149–1153.
3. Davidson BL, Allen ED, Kozarsky KF, Wilson JM, Roessler BJ. A model system for *in vivo* gene transfer into the central nervous system using an adenoviral vector. *Nature Genet* 1993;3:219–223.
4. Kaplitt MG, Leone P, Samulski RJ, et al. Long-term gene expression and phenotypic correction using adeno-associated virus vectors in the mammalian brain. *Nature Genet* 1994;8:148–154.
5. Spaete RR, Frenkel N. The herpes simplex virus amplicon: a new eucaryotic defective virus cloning-amplifying vector. *Cell* 1982;30:295–304.
6. Palella TD, Hidaka Y, Silverman LJ, Levine M, Glorioso J, Kelley WN. Expression of human HPRT mRNA in brains of mice infected with a recombinant herpes simplex virus-1 vector. *Gene* 1989;80:137–144.
7. Baringer JR, Sworeland P. Recovery of herpes-simplex virus from human trigeminal ganglions. *N Engl J Med* 1973; 228:9593–9596.
8. Cook ML, Bastone UB, Stevens JG. Evidence that neurons harbor latent herpes simplex virus. *Infect Immun* 1974;9: 946–951.
9. Geller AI, Breakfield XO. A defective HSV-1 vector expresses *Escherichia coli* β-galactosidase in cultured peripheral neurons. *Science* 1988;241:1667–1669.
10. Martuza RL, Malick A, Markert JM, Ruffner KL, Coen DM. Experimental therapy of human glioma by means of a genetically engineered virus mutant. *Science* 1991;252:854–856.
11. Geller AI, During MJ, Neve RL. Molecular analysis of neuronal physiology by gene transfer into neurons with herpes simplex virus vectors. *Trends Neurosci* 1991;14:428–432.
12. Chiocca EA, Choi BB, Cai W, DeLuca NA, et al. Transfer and expression of the *Lac Z* gene in rat brain neurons mediated by herpes simplex virus insertion mutants. *New Biol* 1990;2:739–746.
13. Mellerick DM, Fraser NW. Physical state of the latent herpes simplex virus genome in a mouse model system: evidence suggesting an episomal state. *Virology* 1987;158: 265–275.
14. Markert JM, Coen DM, Malick A, Mineta T, Martuza RL. Expanded spectrum of viral therapy in the treatment of nervous system tumors. *J Neurosurg* 1992;77:590–594.
15. Kaplitt MG, Tjuvajev JG, Leib DA, et al. Mutant herpes simplex virus induced regression of tumors in immunocompetent rats. *J Neurooncol* 1994;19:137–147.
16. Dobson AT, Margolis TP, Sedarati F, Stevens JG, Feldman LT. A latent, nonpathogenic HSV-1-derived vector stably expresses β-galactosidase in mouse neurons. *Neuron* 1990; 5:353–360.
17. Andersen JK, Garber DA, Meaney CA, Breakefield XO. Gene transfer into mammalian central nervous system using herpes virus vectors: extended expression of bacterial *lacZ* in neurons using the neuron-specific enolase promoter. *Hum Gene Ther* 1992;3:487–499.
18. Randazzo BP, Freese A, Kesari S, Brown SM, Maclean AR, Fraser NW. Treatment of cerebral metastases with herpes simplex virus one mutant 1716: culture and animal studies. *Proc Am Assoc Neurol Surg* 1995;63:1395.
19. Glorioso JC, Goins WF, Meaney CA, Fink DJ, DeLuca NA. Gene transfer to brain using herpes simplex virus vectors. *Ann Neurol* 1994;35:28–34.
20. Vlazny DA, Frenkel N. Replication of herpes simplex virus DNA: localization of replication recognition signals within defective virus genomes. *Proc Natl Acad Sci USA* 1981; 78:742–746.
21. Vlazny DA, Kwong A, Frenkel N. Site-specific cleavage/packaging of herpes simplex virus DNA and the selective maturation of nucleocapsids containing full-length viral DNA. *Proc Natl Acad Sci USA* 1979;79:1423–1427.
22. Freese A, Geller AI. Infection of cultured striatal neurons with a defective HSV-1 vector: implications for gene therapy. *Nucl Acids Res* 1991;19:7219–7223.
23. Geller AI, During MJ, Haycock JW, Freese A, Neve R. Long term increases in neurotransmitter release from neuronal cells expressing a constitutively active adenylate cy-

clase from a herpes simplex virus type 1 vector. *Proc Natl Acad Sci USA* 1993;90:7603–7607.
24. Geller AI, During MJ, Oh YJ, Freese A, O'Malley K. A HSV-1 vector expressing tyrosine hydroxylase causes production and release of L-dopa and dopamine from cultured striatal neurons. *J Neurochem* 1995;64:487–496.
25. Kwong D, Frenkel N. The herpes simplex virus virion host shutoff function. *J Virol* 1989;63:4834–4839.
26. During MJ, Naegele JR, O'Malley KL, Geller AI. Long-term behavioral recovery in parkinsonian rats by an HSV vector expressing tyrosine hydroxylase. *Science* 1994;266: 1399–1403.
27. O'Malley K, Geller AI. Gene therapy in neurology: potential application to Parkinson's Disease. In: Vecsei L, Freese A, Swartz KJ, Beal MF, eds. *Neurological disorders: novel experimental and therapeutic strategies*. West Sussex: Ellis Horwood Limited, 1992:223–248.
28. Kaplitt MG, Pfaus JG, Kleopoulos SP, Hanlon BA, Rabkin SD, Pfaff DW. Expression of a functional foreign gene in adult mammalian brain following *in vivo* transfer via a herpes simplex virus type 1 defective viral vector. *Mol Cell Neurosci* 1991;2:320–330.
29. Federoff HJ, Geschwind MD, Geller AI, Kessler JA. Expression of nerve growth factor *in vivo* from a defective herpes simplex virus 1 vector prevents effects of axotomy on sympathetic ganglia. *Proc Natl Acad Sci USA* 1992;89: 1636–1640.
30. Ho DY, Mocarski ES, Sapolsky RM. Altering central nervous system physiology with a defective herpes simplex virus vector expressing the glucose transporter gene. *Proc Natl Acad Sci USA* 1993;90:3655–3659.
31. Kaplitt MG, Kwong AD, Kleopoulos SP, Mobbs CV, Rabkin SD, Pfaff DW. Preproenkephalin promoter yields region-specific and long-term expression in adult brain after direct *in vivo* gene transfer via a defective herpes simplex viral vector. *Proc Natl Acad Sci USA* 1994;19:8979–8983.
32. Yurek DM, Sladek JR Jr. Dopamine cell replacement: Parkinson's disease. *Annu Rev Neurosci* 1990;13:415–440.
33. O'Malley KL, Anhalt MJ, Martin BM, Kelsoe JR, Winfield SL, Ginns EI. Isolation and characterization of the human tyrosine hydroxylase gene: identification of 5' alternative splice sites responsible for multiple mRNAs. *Biochemistry* 1987;26:6910–6914.
34. Kobayashi K, Kiuchi K, Ishii A, et al. Expression of four types of human tyrosine hydroxylase in COS cells. *FEBS Lett* 1988;238:431–434.
35. Cooper JR, Bloom FE, Roth RH. *The biochemical basis of neuropharmacology*. New York: Oxford University Press, 1986.
36. Sabel BA, Dominiak P, Hauser W, During MJ, Freese A. Levodopa delivery from a controlled release polymer matrix: delivery of more than 600 days and 225 days of elevated plasma levels after subcutaneous implantation in rats. *J Pharmacol Exp Ther* 1990;255:914–922.
37. Birkmayer W. Experimentalle ergebnise uber die Kombinationsbehandlung des Parkinson-syndroms mit L-dopa und einem decarboxylasehemmer. *Wiener Klin Wochenschr* 1969;81:677–679.
38. Fahn S. "On-off" phenomenon with levodopa therapy in parkinsonism. *Neurol* 1974;24:431–441.
39. Freese A, Sabel BA, Saltzman WM, During MJ, Langer R. Controlled release of dopamine from a polymeric brain implant: *in vitro* characterization. *Exp Neurol* 1989;103:234–238.
40. Mouradian MM, Juncos JL, Fabbrini G, Chase TN. Motor fluctuations in Parkinson's disease: pathogenetic and therapeutic studies. *Ann Neurol* 1987;22:475–479.
41. Barbeau A. The clinical physiology of side effects in long term L-dopa therapy. *Adv Neurol* 1974;5:347–356.
42. Chase TN, Juncos J, Serrati C, Fabbrini G, Bruno G. Fluctuations in response to chronic levodopa therapy: pathogenetic and therapeutic considerations. In: Yahr MD, Bergmann KJ, eds. *Advances in neurology: Parkinson's disease*. New York: Raven Press, 1986:477–480.
43. Hardie RJ, Lees AJ, Stern GM. On–off fluctuations in Parkinson's disease: a clinical and neuropharmacologic study. *Brain* 1984;107:487–506.
44. Bittkau S, Przuntek H. Chronic sc lisuride in Parkinson's disease—motor performance and avoidance of psychiatric side effects. *J Neural Transm Suppl* 1988;27:35–54.
45. During MJ, Freese A, Sabel BA, et al. Controlled release of dopamine from a polymeric brain implant: *in vivo* characterization. *Ann Neurol* 1989;25:351–356.
46. Sabel BA, Dominiak P, Hauser W, During MJ, Freese A. Extended levodopa release from a subcutaneously implanted polymer matrix in rats. *Ann Neurol* 1990;28:714–717.
47. Cedarbaum JH, Breck L, Kuff H, McDowell FH. Controlled-release levodopa/carbidopa. I. Sinemet CR3 treatment of response fluctuations in Parkinson's disease. *Neurology* 1987;37:233–241.
48. Horrelou P, Brundin P, Kalen P, Mallet J, Bjorklund A. *In vivo* release of dopa and dopamine from genetically engineered cells grafted to the denervated rat striatum. *Neuron* 1990;5:393–402.
49. Hefti F, Melamed E, Wurtman RJ. The decarboxylation of dopa in the Parkinsonian brain: *in vivo* studies on an animal model. *J Neural Transm* 1980;16:95–101.
50. Melamed E, Hefti F, Wurtman RJ. Non-aminergic striatal neurons convert exogenous L-dopa to dopamine in parkinsonism. *Ann Neurol* 1980;8:558–563.
51. Freese A, During MJ, O'Malley K, Geller AI. Expression of tyrosine hydroxylase in striatal cells with a HSV-1 vector causes stable production and regulated release of dopa and dopamine: potential gene therapy for Parkinson's disease. *Abstr Soc Neurosci* 1991;17:424.15.
52. Freese A, DiFiglia M, Beal MF, Martin JB. Characterization and mechanism of glutamate toxicity in primary striatal cultures. *Brain Res* 1990;521:254–264.
53. Koroshetz WJ, Freese A, DiFiglia M. Correlation between excitatory amino acid induced current responses and excitotoxicity in striatal cultures. *Brain Res* 1990;521:265–272.
54. Freese A, Finklestein S, DiFiglia M. Basic fibroblast growth factor protects striatal neurons *in vitro* from NMDA receptor mediated excitotoxicity. *Brain Res* 1992;575:351–355.
55. Hefti F, Melamed BJ, Sahakian BJ, Wurtman RJ. Circling behavior in rats with partial, unilateral nigrostriatal lesions: effects of amphetamine, apomorphine and DOPA. *Pharmacol Biochem Behav* 1980;12:185–188.
56. During MJ, Freese A, Deutch AY, et al. Biochemical and behavioral recovery in a rodent model of Parkinson's disease following stereotactic implantation of dopamine-containing liposomes. *Exp Neurol* 1992;115:193–199.
57. During MJ, Geller AI, Freese A, Neve R. Neuronal expression of parvalbumin and calcium/calmodulin dependent protein kinase II from HSV-1 vectors. *Abstr Soc Neurosci* 1990;16:216.4.
58. During MJ. Genetic manipulation of signal transduction pathways in the adult mammalian brain. *Abstr Soc Neurosci* 1992;18:453.3.
59. Leone P, Geller AI, During MJ. A HSV-1 vector expressing unregulated PKC from the TH promoter improves spatial navigation performance following intrahippocampal injection in young adult rats. *J Neurochem* 1993;61:135–137.
60. Song S, Leone P, Wang Y, et al. Rotational behavior in adult rats is induced by selective activation of the protein kinase C pathway in substantia nigra pars compacta neu-

rons with a HSV-1 vector. *Abstr Soc Neurosci* 1993;19: 328.2.

61. Johnson PA, Miyanohara A, Levine F, Cahill T, Friedmann T. Cytotoxicity of a replication-defective mutant of herpes simplex virus type 1. *J Virol* 1992;66:2952–2965.
62. Huang Q, Vonsattel J-P, Schaffer PA, Martuza RL, Breakefield XO, DiFiglia M. Introduction of a foreign gene (*Escherichia coli lacZ*) into rat neostriatal neurons using herpes simplex virus mutants: a light and electron microscopic study. *Exp Neurol* 1992;115:303–316.
63. Jacoby DR, Breakefield XO, Latchman DS. Construction of an HSV-1 amplicon with an inducible Oct-2 operon to modulate cytopathic effect. *Proceedings of the International Conference on Gene Therapy for CNS Disorders* 1995;1.31.
64. Geller A, Song S, Lim F, et al. A helper virus-free packaging system for HSV-1 plasmid vectors that supports efficient gene transfer in the rat brain. *Abstr Soc Neurosci* 1995;21:330.1.
65. Freese A, Kaplitt MG, O'Connor MJ, O'Connor WM, During MJ. Gene transfer into human hippocampal slices with an adeno-associated virus vector. *Abstr Soc Neurosci* 1994; 20:592.15.
66. Samulski RJ, Chang L-S, Shenk T. Helper-free stocks of adeno-associated viruses: normal integration does not require viral gene expression. *J Virol* 1989;63:3822–3828.
67. Muzyczka N. Use of adeno-associated virus as a general transduction vector for mammalian cells. *Curr Top Microbiol Immunol* 1992;158:97–129.
68. Lippman C, Welsh F, Kaplitt MG, O'Connor WM, During MJ, O'Connor MJ, Freese A. Transfer of the human heat shock protein-72 gene into cultured cells using an adeno-associated virus vector. *Proceedings of the International Conference on Gene Therapy for CNS Disorders* 1995;4.11.
69. Freese A, O'Connor MJ, Kaplitt MG, O'Connor WM, During MJ. Adeno-associated virus vector mediated gene transfer into human hippocampal slices. *Epilepsia* 1994;35: 62.
70. Redmond DE Jr, O'Malley KL, Kaplitt MG, et al. AAV vectored gene therapy in primate parkinsons model. *Abstr Soc Neurosci* 1995;21:224.22.
71. Redmond DE, O'Malley KL, Kaplitt MG, et al. AAV vector gene therapy in a primate Parkinson's model. *Proceedings of the International Conference on Gene Therapy for CNS Disorders* 1995:3.10.
72. During MJ, Kaplitt MG, Leone P, et al. AAV-mediated transfer of tyrosine hydroxylase and aromatic amino acid decarboxylase into the primate brain: a direct gene therapy approach to Parkinson's disease. *Abstr Soc Neurosci* 1994; 20:602.10.
73. During MJ, Choi-Lundberg D, Leone P, et al. Neuroprotection and neuroregeneration in rodent and primate parkinsonian models using an AAV vector expressing GDNF. *Abstr Soc Neurosci* 1995;21:225.9.
74. Lin L-FH, Doherty DH, Lile JD, Bektesh S, Collins F. GDNF: a glial cell line-derived neurotrophic factor for midbrain dopaminergic neurons. *Science* 1993;260:1130–1132.
75. Lindsay RM. Neuron saving schemes. *Nature* 1995;273: 289–290.
76. Tomac A, Lindqvist E, Lin L-FH, et al. Protection and repair of the nigrostriatal dopaminergic system by GDNF *in vivo*. *Nature* 1995;373:335–339.
77. Yan Q, Matheson C, Lopez OT. *In vivo* neurotrophic effects of GDNF on neonatal and adult facial motor neurons. *Nature* 1995;373:341–344.
78. Beck K, Valverde J, Alexi T, et al. Mesencephalic dopaminergic neurons protected by GDNF from axotomy-induced degeneration in the adult brain. *Nature* 1995;373: 339–341.
79. Oppenheim RW, Houenou LJ, Johnson JE, et al. Developing motor neurons rescued from programmed and axotomy-induced cell death by GDNF. *Nature* 1995;373:344–346.
80. Kang UJ, Bencsics C, Cahill AL, Hatakeyama K, Milstein S. Regulation of L-dopa production by 5,6,7,8 tetrahydro-L-biopterin in genetically modified cells. *Proceedings of the International Conference on Gene Therapy for CNS Disorders* 1995:3.11.
81. Le Gal La Salle G, Robert JJ, et al. An adenovirus vector for gene transfer into neurons and glia in the brain. *Science* 1993;259:988–990.
82. Akli S, Caillaud C, Vigne E, et al. Transfer of a foreign gene into the brain using adenovirus vectors. *Nature Genet* 1993;3:221–228.
83. Bajocchi G, Feldman SH, Crystal RG, Mastrangeli A. Direct *in vivo* gene transfer to ependymal cells in the central nervous system using recombinant adenovirus vectors. *Nature Genet* 1993;3:229–234.
84. Horrelou P, Vigne E, Castel M-N, et al. Direct intracerebral gene transfer of an adenoviral vector expressing tyrosine hydroxylase in a rat model of Parkinson's disease. *Neuroreport* 1994;6:49–53.
85. Lin O, Choi-Lundberg DL, Ravenell J, et al. Adenoviral (Ad)-mediated gene transfer of GDNF and β-galactosidase (lac Z) to the rat nigrostriatal system *in vitro* and *in vivo*. *Abstr Soc Neurosci* 1995;21:224.12.
86. Goodman JC, Trask TW, Chen S-H, et al. Toxicity of adenoviral mediated brain tumor gene therapy in primates. *Proceedings of the International Conference on Gene Therapy for CNS Disorders* 1995:2.10.
87. Neuwelt EA, Barranger JA, Brady RO. Delivery of hexosaminidase A to the cerebrum after osmotic modification of the blood-brain barrier. *Proc Natl Acad Sci USA* 1981; 78:5838–5841.
88. Neuwelt EA, Pagel MA, Dix RD. Delivery of ultraviolet-inactivated ^{35}S-herpes virus across an osmotically modified blood-brain barrier. *J Neurosurg* 1991;74:475–479.
89. Doran SE, Ren XO, Betz AL, et al. Gene expression from recombinant viral vectors in the central nervous system after blood-brain barrier disruption. *Neurosurgery* 1995;36: 965–970.
90. Neuwelt EA, Weissleder R, Milaver G, et al. Delivery of virus-sized iron oxide particles to rodent CNS neurons. *Neurosurgery* 1994;34:777–784.
91. Bobo RH, Laske DW, Akbasak A, Morrison PF, Dedrick RL, Oldfield EH. Convection-enhanced delivery of macromolecules in the brain. *Proc Natl Acad Sci USA* 1994;90: 6165–6169.
92. Rosenberg MB, Friedmann T, Robertson RL, et al. Grafting genetically modified cells to the damaged brain: restorating effects of NGF expression. *Science* 1988;242: 1575–1578.
93. Ray J, Gage FH. Gene transfer into established and primary fibroblast cell lines: comparison of transfection methods and promoters. *Biotechniques* 1992;13:598–603.
94. Palmer TD, Rosman GJ, Osborne WRA, Miller AD. Genetically modified skin fibroblasts persist long after transplantation but gradually inactivate introduced genes. *Proc Natl Acad Sci USA* 1991;88:1330–1334.
95. Scharfmann R, Axelrod JH, Verma IM. Long-term *in vivo* expression of retrovirus-mediated gene transfer in mouse fibroblast implants. *Proc Natl Acad Sci USA* 1991;88:4626–4630.
96. Mallet J, Horrelou P, LeGal LaSalle G, et al. Gene therapy approaches to neurodegenerative diseases. *Proceedings of the International Conference on Gene Therapy for CNS Disorders* 1995:3.01.
97. Horrelou P, Sabate O, Buc-Caron M-H, et al. *In vivo* and *ex vivo* gene therapy in a rat model of Parkinson's disease.

Proceedings of the International Conference on Gene Therapy for CNS Disorders 1995:3.15.
98. Wolff JA, Fisher LJ, Xu L, et al. Grafting fibroblasts genetically modified to produce L-dopa in a rat model of Parkinson disease. *Proc Natl Acad Sci USA* 1989;86:9011–9014.
99. Kawaja MD, Gage FH. Morphological and neurochemical features of cultured primary skin fibroblasts of Fischer 344 rats following striatal implantation. *J Comp Neurol* 1992;317:102–116.
100. Uchida K, Ishii A, Kaneda N, Toya S, Nagatsu T, Kohsaka S. Tetrahydrobiopterin-dependent production of L-dopa in NRK fibroblasts transfected with tyrosine hydroxylase cDNA: future use for intracerebral grafting. *Neurosci Lett* 1990;109:282–286.
101. Uchida K, Takamatsu K, Kaneda N, et al. Synthesis of L-3,4-dihydroxyphenylalanine by tyrosine hydroxylase cDNA-transfected C6 cells: application for intracerebral grafting. *J Neurochem* 1989;53:728–732.
102. Horellou P, Marlier L, Privat A, Mallet J. Behavioral effect of engineered cells that synthesize L-dopa or dopamine after grafting into the rat neostriatum. *Eur J Neurosci* 1990;2:116–119.
103. Horellou P, Brundin P, Kalen P, Mallet J, Bjorklund A. *In vivo* release of dopa and dopamine from genetically engineered cells grafted to the denervated rat striatum. *Neuron* 1990;5:393–402.
104. Fisher LJ, Jinnah HA, Kale LC, Higgins GA, Gage FH. Survival and function of intrastriatally grafted primary fibroblasts genetically modified to produce L-dopa. *Neuron* 1991;6:371–380.
105. Kang UJ, Fisher LJ, Joh TH, O'Malley KL, Gage FH. Regulation of dopamine production by genetically modified fibroblasts. *J Neurosci* 1993;13:5203–5211.
106. Jiao S, Wolff JA. Long-term survival of autologous muscle grafts in rat brain. *Neurosci Lett* 1992;137:207–210.
107. Jiao S, Gurevich V, Wolff JA. Long-term correction of rat model of Parkinson's disease by gene therapy. *Nature* 1993;362:450–453.
108. Jiao S, Schultz E, Wolff JA. Intracerebral transplants of primary muscle cells: a potential "platform" for transgene expression in the brain. *Brain Res* 1992;575:143–147.
109. Morassutti DJ, Atasoy E, Staines WA, McBurney MW. Characterization of murine P19 EC-derived neurons expressing foreign rat TH cDNA-*in vitro* and *in vivo*. *Proceedings of the International Conference on Gene Therapy for CNS Disorders* 1995:3.12.
110. Frim DM, Uhler TA, Galpern WR, Beal MF, Breakefield XO, Isacson O. Implanted fibroblasts genetically, engineered to produce brain-derived neurotrophic factor prevent 1-methyl-4-phenylpyridium toxicity to dopamine neurons in the rat. *Proc Natl Acad Sci USA* 1994;91:5104–5108.
111. Hofer MM, Barde Y-A. Brain-derived neurotrophic factor prevents neuronal death *in vivo*. *Nature* 1988;331:261–262.
112. Lysaght MJ, Frydel B, Gentile F, Emerich D, Winn S. Recent progress in immunoisolated cell therapy. *J Cell Biochem* 1994;56:196–203.