Behavioral Recovery in a Primate Model of Parkinson’s Disease by Triple Transduction of Striatal Cells with Adeno-Associated Viral Vectors Expressing Dopamine-Synthesizing Enzymes*

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ABSTRACT

One potential strategy for gene therapy of Parkinson’s disease (PD) is the local production of dopamine (DA) in the striatum induced by restoring DA-synthesizing enzymes. In addition to tyrosine hydroxylase (TH) and aromatic-L-amino-acid decarboxylase (AADC), GTP cyclohydrolase I (GCH) is necessary for efficient DA production. Using adeno-associated virus (AAV) vectors, we previously demonstrated that expression of these three enzymes in the striatum resulted in long-term behavioral recovery in rat models of PD. We here extend the preclinical exploration to primate models of PD. Mixtures of three separate AAV vectors expressing TH, AADC, and GCH, respectively, were stereotaxically injected into the unilateral putamen of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-treated monkeys. Coexpression of the enzymes in the unilateral putamen resulted in remarkable improvement in manual dexterity on the contralateral to the AAV-TH/-AADC/-GCH-injected side. Behavioral recovery persisted during the observation period (four monkeys: 48 days, 65 days, 50 days, and >10 months, each). TH-immunoreactive (TH-IR), AADC-IR, and GCH-IR cells were present in a large region of the putamen. Microdialysis demonstrated that concentrations of DA in the AAV-TH/-AADC/-GCH-injected putamen were increased compared with the control side. Our results show that AAV vectors efficiently introduce DA-synthesizing enzyme genes into the striatum of primates with restoration of motor functions. This triple transduction method may offer a potential therapeutic strategy for PD.

OVERVIEW SUMMARY

Local production of dopamine (DA) in the striatum via adeno-associated viral (AAV) vector-mediated gene transfer has shown to produce long-term behavioral recovery in parkinsonian rats. To investigate whether this finding can be extrapolated to humans, a preclinical study was conducted in a primate model of Parkinson’s disease (PD). AAV vectors expressing tyrosine hydroxylase, aromatic-L-amino-acid decarboxylase, and GTP cyclohydrolase I were stereotaxically injected into the unilateral putamen of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-treated monkeys. The results showed amelioration of motor abnormalities on the contralateral side with robust transgene expression and elevated DA synthesis in the treated putamen. This gene therapy strategy is a feasible and novel treatment of PD patients.

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*This article has additional online data, accessible from this issue’s Table of Contents online at http://www.humangenetherapy.com

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INTRODUCTION

Parkinson’s disease (PD) is one of the most common neurodegenerative disorders among the elderly. Characteristic motor symptoms, which include resting tremor, muscular rigidity, and bradykinesia, are caused by a severe decrease in the dopamine (DA) content of the striatum secondary to a progressive loss of nigrostriatal DA neurons. Replacement of DA in the striatum is important for functional recovery, and oral administration of the DA precursor L-3,4-dihydroxyphenylalanine (L-DOPA) is currently used to control these symptoms. However, as the disease progresses, L-DOPA therapy eventually becomes less effective and frequent systemic administration of high-dose L-DOPA causes deleterious side effects with oscillations in motor performance (Carey et al., 1995; Jenner, 2000; Langston et al., 2000). For efficient DA synthesis, three enzymes are necessary (Elsworth and Roth, 1997): tyrosine hydroxylase (TH, EC 1.14.16.2), the rate-limiting agent, first converts tyrosine to L-DOPA. Aromatic-L-amino-acid decarboxylase (AADC, EC 4.1.1.28) then converts L-DOPA to DA. Guanosine triphosphate cyclohydrolase I (GCH, EC 3.5.4.16) is the rate-limiting enzyme for the synthesis of essential TH co-factor tetrahydrobiopterin (BH$_4$). These enzymes are antero-gradely transported from the substantia nigra to the striatum (Nagatsu et al., 1990, 1997) and a severe loss of dopaminergic nerve terminals in advanced PD is associated with profound depletion of enzyme activities (Zhong et al., 1995; Nagatsu and Ichinose, 1999a).

One potential strategy for gene therapy of PD is the local production of DA in the striatum induced by restoring DA-synthesizing enzymes. If DA is produced efficiently in the striatum in spite of degeneration of the nigrostriatal pathway, an amelioration of motor symptoms could be induced. Moreover, in contrast to systemic L-DOPA administration, DA synthesis in the restricted target area could reduce dopaminergic stimulation in other parts of the brain, notably the mesolimbic system, thereby avoiding possible side effects, such as hallucinations (Carey et al., 1995). Among various strategies to introduce therapeutic genes directly into the brain, adenovirus-associated viral (AAV) vector is the only viral vector system that is based on a nonpathogenic and replication-defective virus. Efficient, long-term in vivo gene expression has been achieved without substantial toxicity or immune response (Kaplijt et al., 1994; Du et al., 1996; During et al., 1998; Fan et al., 1998; Mandel et al., 1999; Shen et al., 2000). We have shown that AAV vectors expressing TH, AADC, and GCH induced biochemical and behavioral improvements in parkinsonian rats, and suggested that triple transduction with these DA-synthesizing enzyme genes might be used to treat PD (Shen et al., 2000). Here, we extend the preclinical exploration to a primate model of PD, and show that this triple transduction method using AAV vectors induced a behavioral improvement with restoration of DA synthesis.

MATERIALS AND METHODS

AAV vector production

AAV vector plasmids pAAV-LacZ, pAAV-TH, pAAV-AADC, and pAAV-GCH were generated as described previously (Fan et al., 1998; Shen et al., 2000). They contain the lacZ, human TH, human AADC, and human GCH genes, respectively, with the human cytomegalovirus immediate-early promoter, human growth hormone first intron, and simian virus 40 polyadenylation signal sequence between the inverted terminal repeats of the AAV-2 genome. pHLP19 (Avigen, Alameda, CA) is a helper plasmid, containing the AAV rep and cap genes, which are required for replication and capsid formation. pladen01 (Avigen) harbors the E2A, E4, and VA RNA genes of the adeno virus genome. Transfection and purification methods were described previously (Fan et al., 1998). 293 cells were cotransfected by the calcium phosphate coprecipitation method with the vector plasmid (pAAV-LacZ, pAAV-TH, pAAV-AADC, or pAAV-GCH), pHLP19, and pl aden01. AAV vectors (AAV-LacZ, AAV-TH, AAV-AADC, and AAV-GCH) were then harvested and purified by two sequential continuous CsCl gradient ultracentrifugations. The vector titer was determined by quantitative DNA dot–blot hybridization of DNase-I-treated vector stocks, and was routinely $10^{12}$ to $10^{13}$ vector genome copies/ml. Functional titers were estimated by LacZ staining of 293 cells that were transduced with serially diluted AAV vectors. The ratio of genome copy to functional titer was about several hundred.

Animals and neurotoxin treatment

All experiments were done in full compliance with the institutional animal care and use committee of the National Institutes of Infectious Diseases. Four female cynomolgus macaques (Macaca fascicularis), M-1, M-2, M-3, and M-4, weighing 2–2.5 kg, were used for the gene therapy experiments. They were housed under standard conditions of humidity and dark–light cycles with ad libitum access to food and water. Two monkeys, M-1 and M-2, had been trained to perform a fine motor task consisting of the capture of four raisins or small pieces of apple with each of the two hands. To make bilateral striatal lesions, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP, 0.25 to 0.5 mg/kg of free base; Sigma, St. Louis, MO) in phosphate-buffered saline (PBS) was injected intravenously once per week until a stable parkinsonian syndrome was achieved. The total dose of MPTP given was 1.0 to 6.25 mg/kg for seven consecutive months. To avoid the possibility that spontaneous recovery from acute toxicity of MPTP could mimic the behavioral effect of AAV injection, monkeys were allowed to recover for 2 months after the last MPTP treatment.

Behavioral assessment

Animals were clinically evaluated twice a week, using a Primate Parkinsonism Rating Scale (PPRS) (Langston et al., 2000) and activities were recorded on digital videotapes. The PPRS was modeled on the Unified Parkinson’s Disease Rating Scale (UPDRS), but was developed specifically for nonhuman primates. It scores independently from 0 (normal) to 4 (maximal disability) parkinsonian features that consist of spatial hypokinesia (moving around cage), bradykinesia, manual dexterity (right arm/left arm), balance, and freezing, thus giving a total maximal score of 24. The disability scores given in Table 1 are average values of six evaluations done during the last 3 weeks before AAV vector injection and sacrifice.

Hand movements in the fine motor task were analyzed by...
counting the pixels of digital recordings, using the image subtraction method (Hashimoto et al., 1999). A video camera was situated in front of a tray on which four raisins were placed in line from back (monkey side) to front (observer side). While monkeys sequentially picked up four raisins, the number of pixels representing the hand increased when the hand was coming forward to reach each raisin. Time spent on picking up each raisin was measured before and after AAV vector injection.

To evaluate the functional asymmetry of the striatum, apomorphine (0.2 mg/kg) was administered intramuscularly, and the appearance of circling behavior was analyzed with prolonged video recordings.

**Surgery and injection of AAV**

All surgical procedures were performed in an aseptic environment with the monkeys under isoflurane (1–2%) anesthesia. The head was placed in a stereotaxic device (Kopf Instruments, Tujunga, CA). Each monkey received nine injections of AAV vectors in three tracks in the unilateral putamen. The side for injection was randomly assigned (right in M-1, and left in M-2, M-3, and M-4). Each injection was 5 µl with a 1:1:1 mixture of AAV-TH, AAV-AADC, and AAV-GCH (1 × 10¹³ copies of each vector genome per milliliter). Thus, in total, 1.5 × 10¹¹ genome copies of each vector was injected. Injections were made through a Hamilton microsyringe at a rate of 1 µl/min. The needle was left in place for an additional 5 min to prevent the loss of vectors by back flow. As a control, 15 µl of AAV-LacZ (1 × 10¹³ vector genome copies/ml in monkey M-1) or PBS (in monkeys M-2, M-3, and M-4) was injected into the contralateral putamen. The side for injection of AAV vectors in three tracks in the unilateral putamen. The side for injection was randomly assigned (right in M-1, and left in M-2, M-3, and M-4). Each injection was 5 µl with a 1:1:1 mixture of AAV-TH, AAV-AADC, and AAV-GCH (1 × 10¹³ copies of each vector genome per milliliter). Thus, in total, 1.5 × 10¹¹ genome copies of each vector was injected. Injections were made through a Hamilton microsyringe at a rate of 1 µl/min. The needle was left in place for an additional 5 min to prevent the loss of vectors by back flow. As a control, 15 µl of AAV-LacZ (1 × 10¹³ vector genome copies/ml in monkey M-1) or PBS (in monkeys M-2, M-3, and M-4) was injected into the contralateral putamen. The stereotaxic coordinates of injection sites in the putamen were as follows: for track 1: anterior, 18.1 mm; lateral, 11 mm; depth, +19, 17, and 15 mm from the midpoint of the ear bar; for track 2: anterior, 16.4 mm; lateral, 11.5; depth, +20, 18, and 16 mm; and for track 3: anterior, 13.4 mm; lateral, 12 mm; depth, +19, 17, and 15 mm.

**Microdialysis and HPLC**

Before killing the animals, microdialysis experiments were performed in monkeys M-2 and M-3. The dialysis probe used was a concentric type (0.22 mm in diameter) that had 10 mm of exposed dialysis membrane (Eicom, Kyoto, Japan). The probes were implanted in the putamen bilaterally at the following coordinates: anterior, 16.4 mm; lateral, +11.5 and −11.5 mm; depth, +18 mm. Ringer’s solution was passed through the probes at a rate of 10 µl/min for 20 min to remove DA overflow from the damaged tissue. The perfusion rate was decreased to 2 µl/min, at which it was maintained throughout the remainder of the experiment. Two hours after the insertion of probes, 30 µl of sample was collected every 15 min for 60 min (four samples). To study the effect of systemic administration of L-DOPA, L-DOPA (50 mg/kg) and carbidopa (5 mg/kg; peripheral decarboxylase inhibitor) were injected intravenously, followed by L-DOPA (50 mg/kg, d.i.v. [intravenous drip]) after the baseline samples had been obtained in monkey M-3. The levels of DA were determined by high-performance liquid chromatography with electrochemical detection, using an Eikompak MA-5ODS column (Eicom) (Warnhoff, 1984). The minimum detectable limit for DA was 1 nM.

**Immunohistochemistry**

Under deep anesthesia, each monkey was perfused through the ascending aorta with 0.01 M PBS, followed by 4% paraformaldehyde. The brains were removed and cut into several blocks 5 mm thick. The tissue blocks were postfixed in the same fixative, followed by a rinse for 3 days in PBS containing 15% sucrose. The blocks were cut on a cryostat into coronal sections, 30 µm thick. The sections were treated with 40% methanol and 1% H₂O₂ for 20 min to inhibit endogenous peroxidase. The sections were incubated with primary antibodies against TH (Nagatsu et al., 1979) or AADC (Nagatsu et al., 1988) diluted 1:10,000, or with GCH (Nagatsu et al., 1995) diluted 1:8000, in PBS containing 0.3% Triton X-100 at 4°C for 3 days. They were then incubated with biotinylated rabbit IgG (diluted 1:1000; Vector Laboratories, Burlingame, CA) for 2 hr at 4°C, and finally with avidin–biotin–peroxidase complex (diluted 1:1000; Vector Laboratories) for 1 hr at room temperature. Peroxidase activity was revealed in 50 mM Tris–HCl buffer (pH 7.6) containing 0.0003% H₂O₂ and 0.01% 3,3′-di-aminobenzidine-4HCl (DAB). Brain slices of a normal macaque that was killed in an unrelated experiment were used as a control. For dual immunofluorescence staining for neuronal nuclear antigen N (NeuN) and TH, sections were incubated with a mixture of mouse monoclonal anti-NeuN antibody (diluted 1:200; Table 1. Behavioral Recovery of MPTP-Treated Monkeys after AAV Vector Injections

<table>
<thead>
<tr>
<th>Monkey</th>
<th>Total MPTP (mg/kg)</th>
<th>Before AAV injection</th>
<th>After AAV injection</th>
<th>PPRS²</th>
<th>Fine motor task²</th>
<th>Apomorphine-induced turning²</th>
</tr>
</thead>
<tbody>
<tr>
<td>M-1</td>
<td>6.75</td>
<td>17.2 ± 0.17</td>
<td>6.8 ± 0.16</td>
<td>0.71 ± 0.17</td>
<td>0.20 ± 0.04</td>
<td>(+)</td>
</tr>
<tr>
<td>M-2</td>
<td>2.5</td>
<td>18.7 ± 0.21</td>
<td>6.3 ± 0.21</td>
<td>0.58 ± 0.19</td>
<td>0.23 ± 0.04</td>
<td>(+)</td>
</tr>
<tr>
<td>M-3</td>
<td>6.75</td>
<td>18.8 ± 0.17</td>
<td>6.5 ± 0.22</td>
<td>ND</td>
<td>ND</td>
<td>(+)</td>
</tr>
<tr>
<td>M-4</td>
<td>1</td>
<td>23.7 ± 0.21</td>
<td>16.5 ± 0.22</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

Definitions: MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; ND, Not done.

²Average (mean ± SEM) of six evaluation sessions.

*b*Picking up time (seconds; mean ± SEM) of eight trials after AAV-TH/-AADC/-GCH injection into the unilateral putamen. Time span of picking up each raisin decreased to the normal range (<0.3 sec/motion) on the contralateral hand.

²Turning toward the ipsilateral side.
Chemicon International, Temecula, CA) and rabbit polyclonal anti-TH antibody (diluted 1:500) followed by incubation with Alexa Fluor 488-conjugated goat anti-mouse IgG (2 μg/ml; Molecular Probes, Eugene, OR) and Alexa Fluor 594-conjugated goat anti-rabbit IgG (2 μg/ml; Molecular Probes). Immunoreactivity was assessed and viewed under a confocal laser scanning microscope (TCS NT; Leica, Bensheim, Germany).

Image analysis

The volume of transduced region was determined by TH immunohistochemistry and quantified by image analysis (KS400; Zeiss, Thornwood, NY). Every fifteenth slice in serial sections was scanned into the computer and the margins of transduced area and ipsilateral putamen were outlined. The volume of transduced region was estimated by multiplying the measured area by the distance between slices and summing across all slices within the putamen.

RESULTS

Behavioral recovery in parkinsonian monkeys

After chronic administration of MPTP, monkeys developed severe bilateral parkinsonism manifested by a loss of spontaneous motor activity, bradykinesia, impairment of manual dexterity, tremor, and freezing. Monkey M-4 was the most sus-

FIG. 1. Examples of a video-analyzed pattern representing hand movement of a monkey performing a fine motor task. Monkey M-2 picked up four raisins sequentially with each of the two hands 1 month after AAV-TH/-AADC/-GCH injection into the left putamen. The horizontal black bar above each major peak represents hand movement to pick up each raisin and corresponds to picking up time shown in Table 1. With the left (ipsilateral to the vector injection) hand, the parkinsonian monkey took longer to pick up raisins, and this action was often disturbed by tremor expressed as multiple small peaks on major peaks. In contrast, the time spent and pattern of grabbing the four raisins improved with the right (contralateral side) hand after vector injection.
ceptible and became highly symptomatic at the minimum MPTP dosage (1 mg/kg; Table 1). Parkinsonian features were stable for 2 months from the last MPTP treatment. One week after a mixture of AAV-TH, AAV-AADC, and AAV-GCH was injected into the putamen unilaterally (referred to as the ipsilateral side), all four monkeys showed a marked behavioral improvement in the contralateral limbs. Monkeys began to use contralateral limbs, in which tremor and bradykinesia were ameliorated, more frequently and deftly. Maximal recovery was observed 2 weeks after vector injection and remained stable during the observation period (M-1, 48 days; M-2, 65 days; M-3, 50 days; M-4, >10 months; after the AAV vector injection). Monkey M-4 remained in a condition of severe motor impairment requiring assisted feeding, but showed distinct recovery on the side contralateral to the vector injection. A reduction in parkinsonian features on the contralateral side was demonstrated by both PPRS score and systematic analysis of digital videotapes. Before MPTP treatment, all monkeys scored 0 on the PPRS. After MPTP, but before AAV injection, the score ranged from 17.2 to 23.7 on the PPRS. After AAV treatment, the score was reduced by up to 64% (Table 1), most noticeable as an improvement in dexterity of the contralateral hand. Two monkeys showed an improvement in a fine motor task consisting of picking up four raisins with the contralateral hand. Before MPTP, monkeys picked up raisins quickly, using each of the two hands at approximately the same speed (<0.3 sec/motion). After MPTP, monkeys took longer (>0.5 sec/motion) to pick up all four raisins, and this action was frequently disturbed by tremor. After AAV treatment, the time spent and pattern of grabbing the four raisins improved remarkably on the contralateral side (Fig. 1).

Before AAV vector injection, apomorphine induced stereotyped movements in both arms but did not induce circling behavior or any behavioral signs of asymmetric striatal dopamine function. Two weeks after AAV vector injection, animals showed apomorphine-induced dystonic postures, in which the body and face turned toward the ipsilateral side. Video analysis revealed a circling tendency toward the ipsilateral side after systemic administration of apomorphine, indicating reduced DA receptor supersensitivity in the AAV-TH/-AADC/-GCH-injected putamen (Table 1) (see videos 1–7 online at http://www.humangenetherapy.com).

Transduction of the striatum

Histological assessment of the brains was performed in three monkeys (48, 65, and 50 days after AAV vector injection for M-1, M-2, and M-3, respectively; M-4 was kept alive to evaluate long-term effects). Immunostaining for TH showed bilateral depletion of DA cells in the substantia nigra (>90%) in all three animals (data not shown). TH immunoreactivity (TH-IR), AADC-IR, and GCH-IR in the striatum on the AAV-LacZ- or PBS-injected side were profoundly reduced. In contrast, monkeys undergoing gene therapy showed TH-IR, AADC-IR, and GCH-IR at similar or greater levels relative to non-MPTP-treated animals in the putamen and external globus pallidus on the AAV-TH/-AADC/-GCH-injected side (Fig. 2). TH-IR, AADC-IR, and GCH-IR remained at a reduced level in the ipsilateral caudate. The transduced region occupied >90% of the putamen (Table 2). The majority of transduced cells (>90%) appeared to have a morphology typical of medium spiny neurons (Fig. 3A) and were also positive for the neuronal marker NeuN (Fig. 3B). Hematoxylin–eosin staining showed no signs of cytotoxicity in the AAV vector-injected putamen. A slight infiltration of mononuclear cells and residual hemosiderin was observed only around the needle track (Fig. 4).

Dopamine assays in the putamen

To assess the biochemical outcome of AAV vector-mediated gene therapy, DA was measured in the bilateral putamen of two monkeys (M-2 and M-3), using in vivo dialysis. Measurable DA levels were observed only on the AAV-TH/-AADC/-GCH-injected side (Figs. 5 and 6). Levels of DA in the putamen were elevated further on the AAV-TH/-AADC/-GCH-injected side after intravenous administration of L-DOPA with peripheral decarboxylase inhibitor in monkey M-3 (Fig. 6).

| Table 2. Volumes of Transduced Region in the AAV Vector-Injected Putamen |
|-----------------|-----------------|-----------------|
| Transduced region | Total volume (µL) | Volume (µL) | Percent |
| M-1 | 601 | 559 | 93 |
| M-2 | 627 | 577 | 92 |
| M-3 | 588 | 552 | 94 |

DISCUSSION

In our previous study using a rat model of PD, local production of DA in the striatum via AAV vector-mediated gene transfer produced long-term behavioral recovery. However, before the introduction of this strategy to the clinical arena, studies of a primate model are necessary to evaluate motor functions more properly and to determine whether the striatum of primates, which is much larger than that of rodents, can be efficiently transduced. By using AAV vectors to transduce unilateral putamen of parkinsonian monkeys, the present study showed extensive expression of DA-synthesizing enzymes TH, AADC, and GCH, resulting in behavioral recovery with restoration of DA levels in the ipsilateral putamens. Amelioration of parkinsonism was remarkable in the contralateral limb, and was characterized by rapid dexterous movement and reduced tremor. Given that we made bilateral striatal lesions by systemic and chronic administration of MPTP, we could evaluate behavioral recovery by comparing motor abilities between bilateral limbs. Contralateral putamen served as an internal control, and the ipsilateral limb remained disabled, suggesting that behavioral recovery of the contralateral limb cannot be explained by spontaneous recovery that may be expected to occur over time (Elsworth et al., 2000). Unilateral transduction would affect mainly contralateral limb movement, although 20% of thalamic projections from basal ganglia are crossed in monkeys (Parent and Hazrati, 1995). Further amelioration of global parkinsonism, including enhanced spontaneous activity and improved balance, would be expected with bilateral transduction.
FIG. 2. Dense (a) TH-, (b) AADC-, and (c) GCH immunoreactivity in the unilateral putamen of monkey M-2, 65 days after AAV-TH/-AADC/-GCH injection. There was a comprehensive loss of immunoreactivity in the striatum on the control (AAV-LacZ- or PBS-injected) side. (d) AADC immunostaining in a control monkey. Scale bars: 0.5 cm.

FIG. 3. Morphology of TH-immunoreactive cells in the putamen. (A) TH-IR cells in a ventral portion of the putamen. Almost all of the transduced cells after AAV vector injection displayed a morphology typical of medium spiny neurons. Scale bar: 50 μm. (B) Confocal view of a neuron double positive for TH-IR (green) and NeuN-IR (red). Scale bar: 20 μm.
The AAV vector is one of the most attractive gene delivery vehicles for direct introduction of therapeutic genes into the brain in the treatment of neurological diseases. Its unique characteristics include the lack of any disease associated with the wild-type virus, its ability to infect non-dividing cells, long-term transgene expression without a substantial immune response, and the physical stability of viral particles (Monahan and Samulski, 2000). AAV in combination with lentivirus is an efficient and persistent vector system for use in the nervous system (Kordower et al., 2000; Trono, 2000), and one that might be practical clinically with respect to safety. We and other researchers have demonstrated that direct introduction of DA-synthesizing enzyme genes into the brain, using AAV vectors, successfully restored activities of the enzymes in rat models of PD (Fan et al., 1998; Mandel et al., 1998; Leff et al., 1999; Shen et al., 2000). However, there is no report showing significant behavioral recovery in primate models of PD using AAV vectors. In a study in which a bicistronic AAV vector expressing both TH and AADC was used to transduce the caudate of MPTP models of St. Kitts green monkeys, behavioral changes were not significantly correlated with the vector treatment, although levels of DA near injection tracks were elevated after vector injection (During et al., 1998). One possible explanation for the unsatisfactory result was the failure to deliver AAV vector in sufficient quantities to the brain, likely because of a relatively low titer of AAV vectors ($1 \times 10^7$ transducing units/ml). In

**FIG. 3.** Continued.

**FIG. 4.** Hematoxylin and eosin staining of the M-3 brain. (A) Low-power magnification showing a needle track. Scale bar: 0.5 cm. (B) High-power view of the injection site [arrowhead in (A)]. Residual hemosiderin and slight infiltration of mononuclear cells were observed along the needle track. Scale bar: 100 μm.
contrast, we were able to transduce extensive areas of putamen by a simple injection method using high-titer AAV vectors (each $1.5 \times 10^{11}$ genome copies). Although one proposed convection-enhanced delivery method is reportedly efficient (Bankiewicz et al., 2000), no complicated approach is necessary if high-titer AAV vectors are available. The minimal volume of transduced region that is necessary for functional recovery remains to be elucidated for human brains, in which the putamen is approximately 10 times larger than in monkey brain (Lange et al., 1976; Lieberman et al., 1995). However, our promising results suggest that it will also be feasible to transduce a broad region of the human putamen by increasing the number of injection sites reasonably. The second major difference from the previous study is the inclusion of the GCH gene in addition to TH and AADC genes to enhance DA synthesis. In addition to the fact that BH$_4$ availability tightly regulates TH enzyme activity in vivo (Nichol et al., 1985; Nagatsu and Ichinose, 1999b), GCH may play a role in stabilization of the TH protein expressed as a transgene (Ichinose et al., 1994; Wu and Cepko, 1994; Leff et al., 1999). In a rat model of PD, expression of TH and GCH enhanced L-DOPA production (Bencsics et al., 1996; Mandel et al., 1998) and expression of GCH in addition to TH and AADC augmented the biochemical and behavioral improvements (Shen et al., 2000). Another difference in the strategy is that we chose putamen instead of caudate as a target for transduction. Cortical inputs in the striatum are segregated anatomically and functionally in primates (Smith et al., 1998). Motor and premotor cortices project to the putamen,

![FIG. 5. In vivo synthesis of dopamine (DA) after striatal AAV transductions. DA in the bilateral putamen was measured via microdialysis in monkey M-2. The level of DA was remarkably higher in the AAV-TH/-AADC/-GCH-treated side than in the control side. Levels were averaged (mean ± SEM) over four consecutive 15-min samples.](image)

![FIG. 6. Enhancement of in vivo dopamine DA synthesis after systemic administration of L-DOPA with peripheral decarboxylase inhibitor. The level of DA was remarkably elevated in the AAV-TH/-AADC/-GCH-treated putamen of monkey M-3. Levels were averaged (mean ± SEM) over four consecutive 15-min samples.](image)
whereas the associative cortical areas project to the caudate. For more complex motor tasks that require higher levels of neural plasticity in associative cortices, transduction of both caudate and putamen appears to be necessary to modulate motor behavior in a subtle manner.

In our microdialysis study, we detected efficient baseline and L-DOPA-induced DA synthesis in the AAV-TH/-AADC/-GCH-injected putamen. Replacement of DA in the striatum is important for functional recovery regardless of damage sustained by the nigrostriatal DA pathway. Cell transplantation studies have demonstrated that grafted dopaminergic cells, which lack normal afferents, are effective in restoring motor functions in PD patients and in animal models (Bjorklund and Lindvall, 2000). Baseline striatal dopaminergic neurotransmission in normal striatum is maintained by tonic synaptic and non-synaptic DA release, which is largely independent of changes in neuronal impulse flow in the nigrostriatal pathway. As in previous studies (Leff et al., 1999; Bankiewicz et al., 2000; Kirik et al., 2000; Shen et al., 2000), the majority of transduced cells were neurons. Because most of the striatal neurons express DA receptors that are internalized in response to alterations in dopaminergic tone (Dumartin et al., 1998; Muriel et al., 1999), DA produced in striatal neurons might bind to cytoplasmic DA receptors in addition to being released by a non-synaptic mechanism.

Monkeys did not show any complications related to AAV vector injection, including dyskinesia. It has been shown clinically that short-acting pulsatile DA agonists are more likely to induce dyskinesia in PD patients than drugs that have a long duration of effect, and to produce tonic receptor stimulation (Jenner, 2000). Continuous DA production in the striatum may account for the reduced likelihood of dyskinesia in our experiment. In conclusion, unilateral intraputaminal introduction of DA-synthesizing enzyme genes, using AAV vectors, induced functional recovery in MPTP monkeys with robust transgene expression and efficient local production of DA. Although further studies, such as those developing vector constructs that allow regulation of gene expression, may be necessary, this approach represents a clinically feasible protocol for gene therapy for PD.

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