protocol: 10 min of heat activation of the enzyme at 95°C, 45 cycles of denaturation at 95°C for 5 s, annealing at 60°C for 30 s, and extension at 72°C for 20 s. Fluorescence detection was performed at 72°C. Gene-specific primers were designed using Primer3 software (primer3) to amplify fragments of 150–250 bp as follows: for *D2r* (Drd2; NM 010077) forward, TCGCC ATTGTCTGGGTCCTG; reverse, TGCCCTTGAGTGGTGTCTTC; *D1r* (Drd1a; NM 010076) forward, AAGATGCCGAGGATGACAAC; reverse, CCCTC TCCAAAGCTGAGATG. The transcript amounts evaluated for *D1r* and *D2r* were normalized for the quantity and quality of each sample by division by the amount of transcript of the housekeeping gene acidic ribosomal phosphoprotein P0 (Arbp or 36B4; NM 007475) in the same sample and such relative values were presented in Figures 4A and 2S. *36B4* transcript amount was quantified using forward primer ACCCTGAAGTGCTCGACATC and reverse primer AGGAAGGCCTTGACCTTTTC.

In Situ Hybridization and Analysis of Expression Levels

In situ hybridization (ISH) was performed on 14 μm thick frozen sections with digoxigenin-labeled riboprobes synthesized from a 1680 bp *D2r* cDNA template and an enkephaline 800 bp cDNA template as described (Krezel et al., 1998). Hybridization conditions were as described previously (Krezel et al., 1998) and are available on the http://empress.har.mrc.ac.uk/ website. The amount of probe used for hybridization and signal detection conditions was adapted to avoid saturation of the chromogenic labeling (see below). Expression patterns were documented using a macroscope (Leica M420) or microscope (DM4000B), both connected to a Photometrics camera with the CoolSNAP (v. 1.2) software (Roger Scientific, Chicago, IL).

For the analysis of cell counts and expression levels of D2r, the images were transformed into gray scale and analyzed using ImageJ software (Rasband, 1997). The strongest signal observed for any of the neurons in any of the brain sections remained between 67 and 95 units in a 0 to 255 unit gray scale (0 corresponding to black), being thus 25%–30% below full saturation conditions in order to enable quantitative analysis of signal intensity. For each animal, the cell number and intensity of cellular signal was evaluated within selected regions of CPu, NAcSh and NAcCo on the same sections (for region selection see Figures 5A and 5B) at bregma 1.10 and 1.40 (Paxinos and Franklin, 2001). The mean values of cell counts or intensity for each region were calculated and compared as described in Results.

Immunohistochemistry and c-*fos* Cell Counts

Coronal sections (14 μm thick) from unfixed frozen brains of 4-month-old *Rxrγ*$^{-/-}$ mice and their WT littermates were collected on super-frost slides and stored at −80°C until analysis. Sections were postfixed in 4% paraformaldehyde and treated with 1% H_2O_2 to block endogenous peroxides. For detection of Rxrγ, we used rabbit anti-Rxrγ polyclonal antibody (SC555, batch A111, Santa Cruz, US), whereas c-*fos* was detected using rabbit anti-c-*fos* polyclonal antibody (1:1000, Chemicon). Both primary antibodies were detected using the ABC system (Vector, USA) according to the manufacturer's manual. For each animal and section, corresponding brain regions were identified according to the mouse brain atlas (Paxinos and Franklin, 2001) and c-*fos*-positive cells were counted from identical surfaces defined by region-corresponding auto-shape figures (Figures 5A and 5B) at two levels of the striatum (bregma 1.10 and 1.40; Mouse Brain Atlas; Paxinos and Franklin, 2001). The mean cell counts for each brain region were calculated and compared as described in Results.

HPLC Measures of Serotonin and Its Metabolites in the Brain Tissue

The brain samples of n = 6 WT and n = 6 *Rxrγ*$^{-/-}$ male littermates (4months old) were weighed immediately after collection and frozen at −80°C until use. Before analysis, samples were thawed, and homogenized in 10 volumes (w/v) of 0.1 M $HClO_4$ containing the internal standard DHBA (125ng/ml). The homogenates were centrifuged at 12.000 rpm for 20 min at 4°C and supernatant was retained for analysis. Serotonin and its metabolite 5-hydroxyindoleacetic acid (5HIAA) were evaluated using high performance liquid chromatography (HPLC) with electrochemical detection. The chromatographic system consisted of a 25 cm × 4.6 mm Hypersyl C18 ODS column (particle size 5μm, Biochrom, France). The column was kept at a constant temperature of 30°C. The flow rate was 1.2 ml/min with a back pressure of 1500 psi (Waters Instrumentation). The system was linked to a Waters model 460 electrochemical detector with a glassy-carbon electrode. Detector potential was maintained at 0.85 V (reference, Ag/AgCl electrode). The mobile phase consisted of 0.05 M NaH_2PO_4 and 0.1 mM EDTA (pH adjusted to 4.85 with NaOH) in double-distilled water with methanol (6%). The system was calibrated by injecting various amounts (3,4 pg–34 ng) of standard solutions, containing 1.1 ng of internal standard DHBA (3–4 dihydroxybenzylamine 1 mM in $HClO_4$ 0.1 M). The supernatant of each sample was injected onto the column, and peak identification was performed by comparing retention times with the calibration solution. Results were expressed in ng/g ±SEM.

Statistical Analysis

The comparisons of behavioral performance in *Rarβ*$^{-/-}$/*Rxrγ*$^{-/-}$, *Rarβ*$^{-/-}$, and *Rxrγ*$^{-/-}$ null mutant mice were carried out using the protected least significant difference (PLSD) Fischer test. The pharmacological data for the treatments in WT and *Rxrγ*$^{-/-}$ mice were analyzed using two-way analysis of variance (ANOVA)—with treatment and genotype as two independent factors and behavioral responses as dependent variables. Comparison of the evolution of locomotor performance in the open field or actimetric cages were evaluated using ANOVA on repeated-measures. Global and post hoc statistical analyses were performed using the PLSD Fischer test and for two-group comparisons using student t test (see t values in the text). Significant differences are indicated in the corresponding figures.

SUPPLEMENTAL INFORMATION

Supplemental Information includes three figures and can be found with this article online at doi:10.1016/j.neuron.2010.05.004.

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A Phase I Study of Aromatic L-Amino Acid Decarboxylase Gene Therapy for Parkinson's Disease

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Gene transfer of dopamine-synthesizing enzymes into the striatal neurons has led to behavioral recovery in animal models of Parkinson's disease (PD). We evaluated the safety, tolerability, and potential efficacy of adeno-associated virus (AAV) vector–mediated gene delivery of aromatic L-amino acid decarboxylase (AADC) into the putamen of PD patients. Six PD patients were evaluated at baseline and at 6 months, using multiple measures, including the Unified Parkinson's Disease Rating Scale (UPDRS), motor state diaries, and positron emission tomography (PET) with 6-[^{18}F]fluoro-L-*m*-tyrosine (FMT), a tracer for AADC. The short-duration response to levodopa was measured in three patients. The procedure was well tolerated. Six months after surgery, motor functions in the OFF-medication state improved an average of 46% based on the UPDRS scores, without apparent changes in the short-duration response to levodopa. PET revealed a 56% increase in FMT activity, which persisted up to 96 weeks. Our findings provide class IV evidence regarding the safety and efficacy of AADC gene therapy and warrant further evaluation in a randomized, controlled, phase 2 setting.

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INTRODUCTION

Dopamine replacement has been the standard pharmacotherapy for motor impairment in Parkinson's disease (PD). Although virtually all patients benefit from levodopa at an early stage of the disease, severe loss of nigrostriatal nerve terminals in advanced PD leads to profoundly decreased activities of dopamine-synthesizing enzymes, including aromatic L-amino acid decarboxylase (AADC), an essential enzyme that converts levodopa to dopamine. Failure to respond to levodopa therapy may result from a reduction in AADC activity, decreased dopamine storage capacity in synaptic vesicles, postsynaptic changes in striatal output neurons, and abnormalities of nondopaminergic neurotransmitter systems.[1,2] Systemic administration of high-dose levodopa enhances oscillations in motor performance and complications, including hallucinations, due to dopaminergic stimulation of the mesolimbic system.

One potential treatment for advanced PD is gene therapy to restore striatum-selective dopamine production. In addition to AADC, tyrosine hydroxylase, which converts L-tyrosine to levodopa, and guanosine triphosphate cyclohydrolase I, which catalyzes biosynthesis of the essential tyrosine hydroxylase cofactor, tetrahydrobiopterine, are necessary for efficient synthesis of dopamine.[2] Viral vector–mediated gene transfer of these dopamine-synthesizing enzymes has been shown to achieve behavioral recovery in animal PD models, with efficient transduction of striatal neurons that escape degeneration.[3–6] When tyrosine hydroxylase and guanosine triphosphate cyclohydrolase I are expressed in the striatum, levodopa can be synthesized continuously. This strategy would be useful for reducing motor fluctuations associated with intermittent levodopa intake. Gene transfer of AADC alone in combination with oral levodopa administration would be a safer strategy for initial clinical trials. In the latter approach, the patients still need to take levodopa to control motor symptoms, but excess production of dopamine could be avoided by reducing the dose of levodopa. We assessed the safety, tolerability, and the potential efficacy of intraputaminal infusion of recombinant adeno-associated virus (AAV) serotype 2 vector encoding human AADC (AAV-hAADC-2) in patients with mid- to late-stage PD. We also examined whether the short-duration response to levodopa, the antiparkinsonian response that parallels the plasma levodopa levels, would change after gene therapy.[7]

RESULTS

Patient disposition and baseline characteristics

Six patients (4 men, 2 women), mean age 60 (range, 51–68) years, were enrolled (Table 1). The mean disease duration was 10 (range, 5–18) years, and time on levodopa was 9.3 (range, 5–15) years. The average baseline daily levodopa and levodopa equivalent doses were 642 and 808 mg, respectively.

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Table 1 Patients' baseline characteristics

Subject	Age (years)	Sex	Disease duration (years)	Time on levodopa (years)	Levodopa dose (mg)	Levodopa equivalents (mg)
A-1	51	M	11	9	600	900
A-2	63	M	9	9	450	650
A-3	66	F	7	7	500	700
A-4	58	M	11	11	700	700
A-5	68	F	18	15	1,000	1,100
A-6	56	M	5	5	600	800
Mean (SD)	60 (6.5)	67% M	10 (4.5)	9.3 (3.4)	642 (196)	808 (169)

Abbreviations: F, female; M, male.
Patients are listed in the order in which they received treatment. Levodopa equivalents were estimated as follows: 100 mg of levodopa with a dopa-decarboxylase inhibitor is equivalent to 0.8 mg talipexole, 1 mg pergolide, 1 mg pramipexole, and 1.5 mg cabergoline.

Primary end point

The procedure was well tolerated. All patients completed all protocol-defined visits. One patient (patient A-2) had a venous hemorrhage in the right frontal lobe just below a burr hole that was found on CT scan 3 days after infusion. The patient used his left arm less frequently than his right arm for 3 weeks; this was assumed to reflect mild frontal lobe dysfunction and resolved completely. Mild, transient headache around the burr holes was present for 2 days after surgery in all patients. There were no significant laboratory test abnormalities. All patients had mildly increased titers of anti-AAV2-neutralizing antibodies 6 months after treatment, which tended toward baseline concentrations thereafter (Table 2).

Table 2 Changes in neutralizing AAV2 antibody titers in sera following gene therapy

Subject	Pre	2 weeks	6 months	1 year
A-1	1:2	1:4	1:4	1:4
A-2	<1	1:32	1:4	1:2
A-3	1:32	1:64	1:64	1:32
A-4	1:32	1:32	1:256	1:64
A-5	1:4	1:32	1:32	1:32
A-6	<1	1:16	1:32	1:32

Abbreviations: AAV, adeno-associated virus.
Titers are determined by *in vitro* assay and represented as "1:" dilutions.

Clinical evaluations

The clinical results are summarized in Table 3. Intraputaminal AAV-hAADC-2 infusion significantly improved both total and motor scores of the unified Parkinson's disease rating scale (UPDRS) in the OFF state. Five of six patients showed substantial improvement in UPDRS motor ratings in the OFF state (Figure 1). Changes in the UPDRS ON state and the percent of ON state hours in a day were not significant. One patient with relatively mild motor symptoms at baseline did not improve on UPDRS (A-3 in Figure 1). However, this patient showed a remarkable increase in mobile time as measured by the diaries (28% at baseline to 58% at 6 months after gene transfer; Figure 2). The daily dose of levodopa was unchanged in two patients (A-2 and A-5) and reduced in three patients (A-1, A-3, and A-5) at 6 months. Patient A-6, who had daytime sleepiness, preferred to reduce pramipexole instead of levodopa after gene therapy.

The last three patients underwent the levodopa test after our institutional review board confirmed the safety of AADC gene transfer in the first three patients. The short-duration response to levodopa did not change significantly after gene therapy in these three patients, though UPDRS motor scores at 6 months showed slight improvement at 30 minutes in patient 5 and at 120 minutes in patient 4 after levodopa intake (Figure 3). Significantly higher peak plasma levodopa concentrations were observed in these two patients after gene therapy.

The mini-mental state examination (MMSE) and geriatric depression scale (GDS) scores did not change significantly.

Table 3 Clinical outcomes of six patients

	Baseline	6 months	*P* value
UPDRS Total OFF	53 (12.4)	38 (10.1)	0.049*
UPDRS Total ON	15 (7.2)	10.7 (2.9)	0.262
UPDRS Part III (Motor) OFF	25.3 (9.4)	13.7 (6.0)	0.024*
UPDRS Part III (Motor) ON	5.2 (4.6)	1.8 (1.5)	0.120
Percent day spent in mobile state	48.8 (12.9)	55.4 (14.8)	0.348
Daily levodopa equivalents dose, mg	808 (169)	707 (233)	0.097

Abbreviations: OFF, off-medication state; ON, on-medication state; UPDRS, Unified Parkinson's Disease Rating Scale.
Data are presented as means (SD). The UPDRS scores in each patient did not change during the 2 months of the screening period.
*$P < 0.05$.

PET analysis

PET imaging revealed increased 6-[^{18}F]fluoro-L-*m*-tyrosine (FMT), a tracer for AADC, activity 4 weeks postoperatively, which persisted at 6-month evaluation (Figure 4). The mean increase in FMT uptake from baseline in the combined (right and left) putamen at 24 weeks was 56%. Two patients (A-1 and A-2) who had PET scans 96 weeks after surgery showed persistently increased FMT uptake. In these two patients, motor performance in the OFF state also maintained its improvement at 96 weeks.

DISCUSSION

Extensive preclinical studies on both rodent and nonhuman primate models of PD have shown that AAV vectors can express exogenous genes for a long time in the brain target areas without

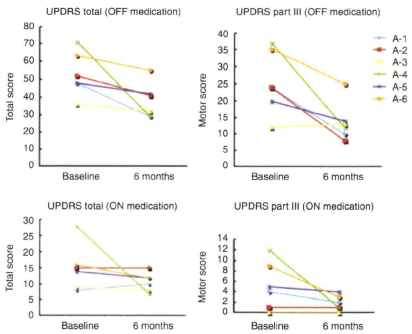

Figure 1 Changes in UPDRS scores. Absolute changes in scores from baseline to 6 months for individual patients. OFF, off-medication state; ON, on-medication state; UPDRS, Unified Parkinson's Disease Rating Scale.

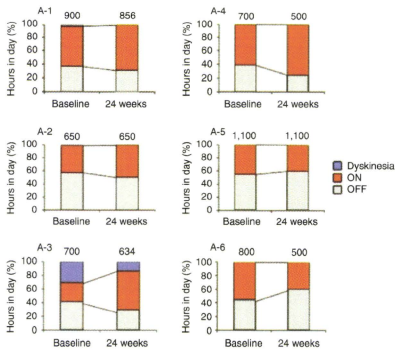

Figure 2 Evaluation of patients' diaries and daily doses of levodopa equivalents. For each 30-minute interval throughout the day, the patients recorded whether they were mobile (ON), immobile (OFF), or asleep. They also recorded the time with troublesome dyskinesias (Dyskinesia). The graph shows the percentage of hours in a day spent in each condition at baseline and at 6 months. The numbers on the bars indicate the mean daily doses of levodopa equivalents (mg). OFF, off-medication state; ON, on-medication state.

significant toxicity.[3,4,6,8,9] Recently, three phase I clinical trials of gene therapy for advanced PD demonstrated that AAV vector–mediated gene delivery into the subthalamic nucleus or putamen was safe and tolerable.[10–13] In this study, the safety of the AAV vectors for clinical use in the human brain was confirmed. Although one patient developed a venous hemorrhage in the subcortical white matter along the trajectory, it is well known that cerebral bleeding occasionally occurs in association with surgical procedures for deep brain stimulation in which electrodes are inserted into the basal ganglia through the frontal lobe white matter.[14,15] PET imaging in this patient showed that putaminal AADC expression was not affected by the subcortical venous hemorrhage and persisted up to 96 weeks. Thus, the venous hemorrhage was probably due to the surgical procedure and not gene transduction.

Although the present trial was a small, open-label study, and the nonblinded, uncontrolled analysis limits the interpretation, the initial efficacy outcomes are encouraging. Our patients showed improved motor performance in the OFF state. Levodopa has a relatively short plasma half-life (60–90 minutes), and antiparkinsonian effects observed after levodopa administration have generally been recognized as short- and long-duration responses. The short-duration response roughly parallels the plasma levodopa concentrations and is thought to be closely linked to dyskinesia, whereas the long-duration response builds up over weeks and improves trough (worst) motor performance in the OFF state.[7] Because the pattern of the short-duration response to levodopa did not change after gene therapy in our patients, the beneficial effect on the OFF state appears to be attributed to augmentation of the long-term response to levodopa.[16] In the preclinical studies with animal models of PD, AAV vectors mainly transduced medium spiny neurons that have dopamine receptors, and extracellular dopamine was increased in the striatum after administration of levodopa.[5,17] The mechanism underlying the long-duration response is not sufficiently understood, and future study is necessary to determine how nonphysiologic production of dopamine in the striatal neurons could enhance the response. It has been reported that the sustained long-duration response to levodopa is greater in patients treated with higher single doses of levodopa.[18] Thus, it is likely that increased dopamine in the putamen after gene transfer may enhance the stable long-duration response. Motor fluctuations in PD are associated with increased response to levodopa with a deeper trough in motor performance, rather than shortening of the response. Improving trough or OFF state motor function by augmenting the long-term response would likely reduce motor fluctuation.[16] Two of three patients in whom the short-duration response to levodopa was studied showed increased peak plasma levodopa concentrations after gene therapy. This finding may simply reflect variable absorbance of levodopa, and it remains to be elucidated whether changes in gastrointestinal absorption could be related to better motor performance in the OFF state.[19]

Activities and levels of AADC mRNA and protein are profoundly reduced in advanced PD,[2] but there are still several types of AADC-containing cells in the striatum, such as serotonin neurons, intrinsic dopamine neurons, AADC-containing "D" neurons, and glial cells.[20] These cells may act as a local source of dopamine. However, dopamine produced in nondopamine cells may not be taken up into dopamine cells and stored in synaptic vesicles, as dopamine transporter and vesicular monoamine transporter 2 are also reduced in advanced PD. The functional efficacy of dopamine produced from exogenous levodopa in these cells may be limited, at least in primates.[2,3] Striatal output neurons, main targets in AADC gene therapy, play a principal role in dopamine modulation of motor function in the basal ganglia. Dopamine synthesized in the striatal neurons themselves may more easily stimulate both synaptic and extrasynaptic receptors.

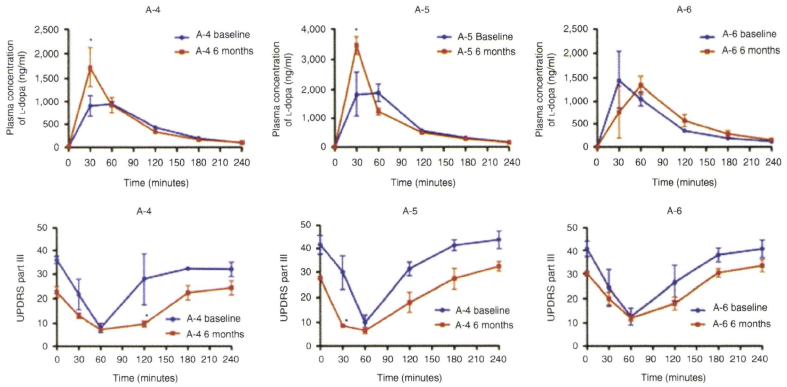

Figure 3 Short-duration response to levodopa. Comparison of short-duration response to levodopa before (blue) and after gene therapy (brown) in three patients (A-4, A-5, and A-6). Patients took 100 mg of levodopa with 25 mg benserazide orally after 20 hours without dopaminergic medication. Values represent means and SE of three trials. Upper panels: plasma levodopa levels; lower panels: Unified Parkinson's Disease Rating Scale motor scores. *$P < 0.05$.

Results of a similar phase I protocol were reported recently for the 10 patients treated with AAV-hAADC-2 (ref. 10). That study used the same vector preparations as this study. The subjects were divided into two groups that received the same or one-third dose of the vector used in this study, respectively. Although the present patients had slightly milder initial symptoms, the patients treated with the same dose of vector in the two studies showed similar improvement in the OFF state and putaminal FMT uptake on PET. These findings provide independent confirmation of the safety, tolerability, and potential efficacy of AADC gene therapy. Future studies focusing on optimal vector dosing and defining the relationship between vector dose and clinical effects are necessary.[21]

In conclusion, these data indicate that AAV vector–mediated gene transfer of AADC is safe and may benefit advanced PD patients.

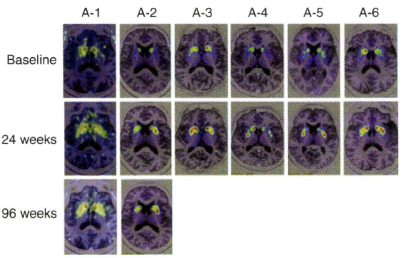

Figure 4 FMT-PET images. Axial images at the level of the putamen are shown before and 24 weeks after gene therapy for all six patients. Increased FMT uptake persisted until 96 weeks in two patients. The 4-week images are not shown because they are similar to the 24-week images. FMT, 6-[^{18}F]fluoro-L-*m*-tyrosine; PET, positron emission tomography.

MATERIALS AND METHODS

Study design. The protocol and consent forms were approved by the institutional review board. The protocol was also reviewed by the committee of the Ministry of Health, Labour and Welfare of Japan. A data safety monitoring board reviewed the ongoing study. All subjects reviewed the consent form and provided their written, informed consent.

This 24-week, phase I, open-label study was primarily designed to evaluate the safety and tolerability of intraputaminal AAV-hAADC-2 infusion in idiopathic PD. Patients were evaluated preoperatively and monthly postoperatively for 6 months, using multiple measures, including the UPDRS, motor state diaries, the MMSE, the short form of the GDS, and laboratory tests. The UPDRS was done in the practically defined OFF state 12 hours after withdrawal of all antiparkinsonian medications, and in the ON state 1 hour after administration of the usual morning dose of medication. Motor scores for the UPDRS can range from 0 to 56, with higher scores indicating poorer function. Using diaries that separated the day into half-hour segments, the patients recorded their mobility during the 4 days before admission and for another 4 days at 6 months after admission. They were trained to rate their condition as sleeping, immobile, mobile without troublesome dyskinesias, or mobile with troublesome dyskinesias. The total number of hours spent in each of these categories was calculated, and the differences between the baseline and the 6-month scores were compared between the groups.

The short-duration response to levodopa was evaluated in three patients (patients 4–6) at baseline and 6 months after gene transfer; they took 100 mg of levodopa orally with 25 mg benserazide after 20 hours without dopaminergic medication. Motor symptoms based on UPDRS motor (part III) and plasma levodopa concentrations were assessed at baseline and 30 minutes, 1, 2, 3, and 4 hours after levodopa intake.

Patients. The main entry criteria were: age 45–75 years; diagnosis of moderate to advanced PD, defined as Hoehn and Yahr Stage IV and UPDRS in the practically defined OFF condition of at least 20; at least

5 years of levodopa therapy; a minimum 8-point improvement in the UPDRS motor score after levodopa intake; and motor complications not satisfactorily controlled with medical therapy. The main exclusion criteria were atypical parkinsonism, dementia (MMSE score <20), and previous neurosurgical treatment for PD.

Vector and stereotaxic infusion. The vector used in this trial was a recombinant AAV2 with an expression cassette consisting of a human cytomegalovirus immediate-early promoter, followed by the human growth hormone first intron, complementary DNA of human AADC, and simian virus 40 polyadenylation signal sequence.[3–6] Clinical grade AAV-hAADC-2 was manufactured by Avigen (Alameda, CA) and provided by Genzyme (Boston, MA). The patients received AAV-hAADC-2 *via* bilateral intraputaminal infusions. Two target points were determined in the putamen that were sufficiently separated from each other in dorsolateral directions and identified on a magnetic resonance image. One burr hole was trepanned in each side of the cranial bone, through which the vector was injected into the two target points *via* the two-track insertion route. The vector-containing solution was prepared to a concentration of 1.5×10^{12} vector genome/ml, and 50 µl per point of the solution were injected at 1 µl/min; each patient received 3×10^{11} vector genome of AAV-hAADC-2.

Neutralizing antibody titers against AAV2 were determined by measuring β-galactosidase activities in HEK293 cells transduced with 5×10^3 vector genome/cell of AAV2 vectors expressing β-galactosidase in various dilutions of sera.[22]

PET. The AADC expression level in the putamen was assessed on PET imaging with FMT 6 days before surgery and 1 and 6 months after gene transfer. All patients stopped dopaminergic medications 18 hours before PET and took 2.5 mg/kg of carbidopa orally 1 hour before FMT injection. Subsequently, 0.12 mCi/kg of FMT in saline were infused into an antecubital vein, and a 90-minute dynamic acquisition sequence was obtained. The PET and magnetic resonance imaging data were co-registered with a fusion processing program (Syntegra; Philips, Amsterdam, The Netherlands) to produce the fusion images. Radioactivities within volumes of interest drawn in the putamen and occipital lobe were calculated between 80 and 90 minutes after tracer injection. A change in putaminal FMT uptake from baseline to 24 weeks was assessed using the putaminal–occipital ratio of radioactivities.

Statistical analysis. Values at baseline and 6 months after gene transfer were compared using Student's *t*-test (paired analyses). A two-sided *P* value <0.05 was taken to indicate significant differences. Two-way analysis of variance with Bonferroni correction of *P* values was used for the short-duration response to levodopa.

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