Vesicular Monoamine Transporter-2 and Aromatic L-Amino Acid Decarboxylase Enhance Dopamine Delivery after L-3,4-Dihydroxyphenylalanine Administration in Parkinsonian Rats

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Medical therapy in Parkinson's disease (PD) is limited by the short-duration response and development of dyskinesia that result from chronic L-3,4-dihydroxyphenylalanine (L-DOPA) therapy. These problems occur partly because the loss of dopamine storage sites leads to erratic dopamine delivery. Vesicular monoamine transporter-2 (VMAT-2) plays a critical role in dopamine storage by packaging dopamine into synaptic vesicles and regulating sustained release of dopamine. To restore the capacity to produce and store dopamine in parkinsonian rats, primary skin fibroblast cells (PF) were genetically modified with aromatic L-amino acid decarboxylase (AADC) and VMAT-2 genes. After incubation with L-DOPA in culture, the doubly transduced fibroblast cells (PFVMAA) produced and stored dopamine at a much higher level than the cells with either gene alone. PFVMAA cells in culture released dopamine gradually in a constitutive manner. Genetically modified fibroblast cells were grafted in parkinsonian rat striata, and L-DOPA was systemically administered. Higher dopamine levels were sustained for a longer duration in rats grafted with PFVMAA cells than in those grafted with either control cells or cells with AADC alone. These findings underscore the importance of dopamine storage capacity in determining the efficacy of L-DOPA therapy and illustrate a novel method of gene therapy combined with precursor administration to overcome the major obstacles of PD treatment.

Key words: short duration response; wearing-off; gene therapy; Parkinson's disease; dopamine; L-DOPA; AADC; VMAT

The dopamine precursor L-3,4-dihydroxyphenylalanine (L-DOPA) is the mainstay of treatment for Parkinson's disease (PD) (Birkmayer and Hornykiewicz, 1961; Cotzias et al., 1967). Chronic L-DOPA treatment is limited by the short duration of behavioral improvement and fluctuating responses to the drug, resulting in "wearing-off" phenomena and dyskinesia (Marsden and Parkes, 1977; Peppe et al., 1993; Marsden, 1994). These complications originate partly from the loss of dopamine terminals that can store dopamine, buffer its sudden surge, and sustain its gradual release (Fabbrini et al., 1987; Papa et al., 1994; Nutt and Holford, 1996). Long-term intermittent L-DOPA delivery, inherent in oral pharmacological therapy, worsens fluctuations and is also associated with changes downstream from the dopaminergic synapses in the striatum, including the imbalance of indirect and direct efferent pathways (Engber et al., 1992; Nutt et al., 1992; Gnanalingham and Robertson, 1993). Continuous delivery of dopamine can reverse fluctuating responses in PD patients (Shoulson et al., 1975) and imbalance of the striatal efferents in animal models of PD (Gerfen et al., 1990; Mouradian et al., 1990). Therefore, increasing the efficiency of conversion of L-DOPA to dopamine and achieving sustained levels of dopamine within the striatum may provide an effective tool to treat the motor symptoms of PD with minimal untoward effects.

L-DOPA is decarboxylated to dopamine by aromatic L-amino acid decarboxylase (AADC), whose major striatal source is the very dopaminergic neurons that degenerate in PD. However, the source and site of the residual AADC in animal models and patients with PD are controversial (Melamed, 1988; Hadjiconstantinou et al., 1995; Zhu and Juorio, 1995). The capacity to decarboxylate L-DOPA to dopamine appears to be limited; thus, augmenting available AADC may increase the efficiency of conversion of L-DOPA to dopamine in the denervated striatum (Kang et al., 1993). Endogenous dopamine is sequestered into storage organelles by a vesicular monoamine transporter (VMAT) (Erickson et al., 1992; Liu et al., 1992). VMAT is a transporter that resides in the membrane of secretory vesicles and partitions monoamines from cytoplasm into vesicles by the vesicular proton gradient (Erickson et al., 1992; Liu et al., 1992; Erickson and Eiden, 1993; Henry et al., 1994). Two homologous but distinct VMAT genes have recently been cloned; VMAT-2 is primarily expressed in the brain (Mahata et al., 1993; Peter et al., 1995) and has higher affinity for catecholamines than VMAT-1 (Erickson et al., 1996). VMAT-2 regulates stimulated quantal release of monoamines (Amara, 1995; Cooper et al., 1996). Even when VMAT-2 is expressed in non-neuronal cells, such as fibroblast cells, it can still function as a monoamine transporter, presumably sequestering dopamine into acidic endosomal compartments (Gasnier et al., 1994; Merickel et al., 1995). However,

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whether dopamine can be released from such preparations is not known.

Here, we genetically modified primary fibroblast cells with AADC and VMAT-2 genes to address the efficacy of the double-gene transduction in L-DOPA therapy both *in vitro* and *in vivo* in parkinsonian rats. We specifically tested whether VMAT-2 can enhance dopamine storage and prolong the duration of dopamine release after exogenous L-DOPA administration.

MATERIALS AND METHODS

Retroviral vector construction and transfection. A full-length bovine AADC cDNA was cloned into a retroviral vector (LDcSHL; with selection marker hygromycin-B-phosphotransferase under the control of an internal simian virus 40 early promoter) as described previously (Kang et al., 1993). The 1.6 kilobase fragment containing the full coding region and the part of the 3′ untranslated region of rat VMAT-2 cDNA [a gift from R. Edwards, University of California at San Francisco, San Francisco, CA (Merickel et al., 1995)] was removed by *Eco*RI, blunted, and ligated into LINX vector [a gift from F. H. Gage, Salk Biological Institute, La Jolla, CA (Hoshimaru et al., 1996)] downstream from the $P_{hCMV^{*}\text{-}1}$ promoter. Both were used to generate amphotropic retroviral producer lines from PA317 cells (PAAADC and PAVMAT). The isolation and culture of primary skin fibroblasts (PF) from inbred Fischer 344 rats have been described previously (Kang et al., 1993; Bencsics et al., 1996; Wachtel et al., 1997). PFs were infected with either PAAADC or PAVMAT and selected with hygromycin (150 μg/ml) or G418 (400 μg/ml) to establish PFAADC or PFVMAT cells, respectively. PFAADC was secondarily infected with PAVMAT and underwent additional selection in media containing G418 and hygromycin to generate a bulk population of primary fibroblast cells expressing both AADC and VMAT-2 (PFVMAA).

Immunohistochemical staining. For staining cultured cells, control and transduced fibroblasts were grown on chamber slides (Lab-Tek, San Francisco, CA), fixed with 4% phosphate-buffered paraformaldehyde, and permeabilized with 0.2% Triton X-100. Cells were immunostained with a rabbit polyclonal antibody against a synthetic peptide corresponding to the C terminus of VMAT-2 (Phoenix Pharmaceuticals Inc., Mountain View, CA) at a dilution of 1:5000 or a polyclonal antibody against bovine AADC (Protos Biotech Corp., New York, NY) at a dilution of 1:1000, followed by incubation with a biotinylated goat anti-rabbit IgG secondary antibody. The signal was amplified by avidin and biotinylated horseradish peroxidase using the Elite ABC Vectastain kit (Vector Laboratories, Burlingame, CA). VMAT-2 and AADC immunoreactivity were visualized using 3,3′-diaminobenzidine tetrachloride dihydrate (Aldrich, Milwaukee, WI) as a chromogen, enhanced with cobalt chloride–nickel ammonium.

For *in vivo* studies, rats were anesthetized 1 d after microdialysis and transcardially perfused with 125 ml of normal saline, followed by 250 ml of ice-cold 4% paraformaldehyde. Brains were removed, post-fixed overnight, and transferred to 30% sucrose until equilibrated. Forty micrometer sections were cut from the frozen brains and stained for Nissl substance or immunostained with AADC and VMAT-2 antibodies as described above.

AADC and transporter assay. AADC activity was assayed by a modification of a CO_2 trapping as described previously (Lamprecht and Coyle, 1972) but without using radioactivity. Supernatant from homogenized cells was added to a reaction solution containing 30 mM potassium phosphate buffer, 0.3 mM EDTA, 20 μM pyridoxal 5′-phosphate, and 200 μM L-DOPA. Dopamine levels after reaction were measured by reverse-phase HPLC using a Velosep RP-18 column (100 × 3.2 mm; Applied Biosystems, Foster City, CA) and an ESA Coulochem II electrochemical detector equipped with a 5014 analytical cell. For the transporter assay, cells were washed and homogenized. Cell debris was removed by centrifugation in a microcentrifuge at 4000 × *g* for 5 min. A Bradford assay (Bio-Rad, Hercules, CA) was performed to measure the protein concentration, and supernatant was diluted in sucrose HEPES (SH) buffer to a final concentration of 10 mg/ml. Twenty microliters of the supernatant were added to 200 μl of SH buffer containing 4 mM KCl, 2.5 mM $MgSO_4$, 2 mM ATP (potassium salt), and 50 nM [^{3}H]serotonin (DuPont NEN, Boston, MA) at 29°C for 5 min. The termination of reaction, filtration, and radioactivity measurement were performed as described previously (Gasnier et al., 1994; Merickel et al., 1995). Experiments were performed in triplicate, and background uptake at 4°C at 0 min was subtracted.

In vitro *cell culture and catecholamine measurement.* For all culture experiments, fibroblast cells (2.5×10^5) were plated in six well plates in DMEM media with 10% fetal calf serum with appropriate selection markers as described previously (Kang et al., 1993; Bencsics et al., 1996; Wachtel et al., 1997). Only fibroblasts of passages 5–18 were used. Catecholamine measurements were made in DMEM media (1 ml in each well), except for the experiments on the effect of calcium, which were done in a physiological incubation medium consisting of 135 mM NaCl, 3 mM KCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$, 10 mM glucose, 200 μM ascorbic acid, and 10 mM HEPES, pH 7.3. After incubation, cell pellets and media were precipitated with perchloric acid and EDTA solution. The supernatant was collected, and catecholamines in cell pellets and media were isolated by alumina extraction. The levels of dopamine, L-DOPA, and dihydroxyphenyl acetic acid (DOPAC) in the media and cell pellets were determined by HPLC analysis as described above.

Medial forebrain bundle lesion. The *in vivo* protocols were approved by the Institutional Animal Care and Use Committee of the University of Chicago. For dopamine depletion, female Fischer 344 rats (150–200 gm) were anesthetized with a mixture of ketamine (75 mg/kg), acepromazine (0.75 mg/kg), and xylazine (3.8 mg/kg). As described previously for the medial forebrain bundle (MFB) lesion (Chang et al., 1999), 8 μg (free base weight) of 6-hydroxydopamine was infused unilaterally in 2 μl of normal saline at a rate of 0.5 μl/min at the following coordinates: anteroposterior (AP), −4.4 mm; mediolateral (ML), 1.2 mm relative to bregma and −7.5 mm from the dura. Animals that show >400 rotations/hr after D-amphetamine (5 mg/kg, i.p.) administration, indicating near complete lesions, were used for the microdialysis study.

Grafting and in vivo *microdialysis.* PF, PFAADC, and PFVMAA cells were washed, trypsinized, and suspended in Dulbecco's PBS. Two microliters of the cell suspension (75,000 cells/μl) were infused at each of four ventral sites [AP, 1.2 and −0.3; ML, 2.3 and 3.0; dorsoventral (DV), −4.0] and 1 μl each at four dorsal sites within the same needle tracts (DV, −3.5) for a total of 900,000 cells per animal. The cell suspensions were injected at the rate of 0.5 μl/min using a 10 μl syringe and an infusion pump. Grafting used only cells below passage 15. Microdialysis probes were implanted at the center of the four tracks of grafts (AP, 0.45; ML, 2.65; DV, 5.2) under anesthesia 3 d after grafting. The microdialysis probes were of a vertical concentric design as described previously (Wachtel and Abercrombie, 1994). Before implantation, the microdialysis probes (2 mm active area) were calibrated *in vitro* for relative recovery rates to ensure consistency, but the data were not corrected for the recovery rate. Artificial CSF (147 mM NaCl, 2.5 mM KCl, 1.3 mM $CaCl_2$, and 0.9 mM $MgCl_2$, pH ~7.4) was infused continuously through the probe at a rate of 1.5 μl/min. The day after probe placement, dialysates were collected at 20 min intervals for 340 min before and after L-DOPA administration in awake, freely moving animals. Twenty microliters of each dialysate sample were analyzed by HPLC for L-DOPA, dopamine, and DOPAC concentrations.

Statistical analysis. Statistical analyses were performed using the GB-STAT statistical package (Dynamic Microsystems, Silver Spring, MD). SEs were calculated for each mean, and statistical differences among groups were determined by two-way repeated ANOVA for time course data in Figure 2, *A* and *B*, and Figure 4, and one-way randomized ANOVA was used for the rest of the data, unless otherwise indicated. A Newman–Keuls test was used for *post hoc* comparison, and $p < 0.05$ was used as the minimum criteria for statistical significance.

RESULTS

Transgene expression in modified fibroblast cells

First, transgene expression in these genetically modified fibroblast cells was confirmed by immunohistochemistry against AADC and VMAT-2. AADC immunoreactivity was detected in primary skin fibroblast cells modified to express AADC (PFAADC) and those modified to express both AADC and VMAT-2 (PFVMAA). Similarly, VMAT-2 immunoreactivity was only noted in cells with VMAT-2 (PFVMAT) and in the doubly transduced PFVMAA cells (Fig. 1).

The functions of transgene products were then examined by biochemical assays. AADC activities were significantly higher in groups with AADC transgenes than in control PF cells ($F_{(2,6)} = 122.8$; $p < 0.0001$). VMAT activities were likewise significantly

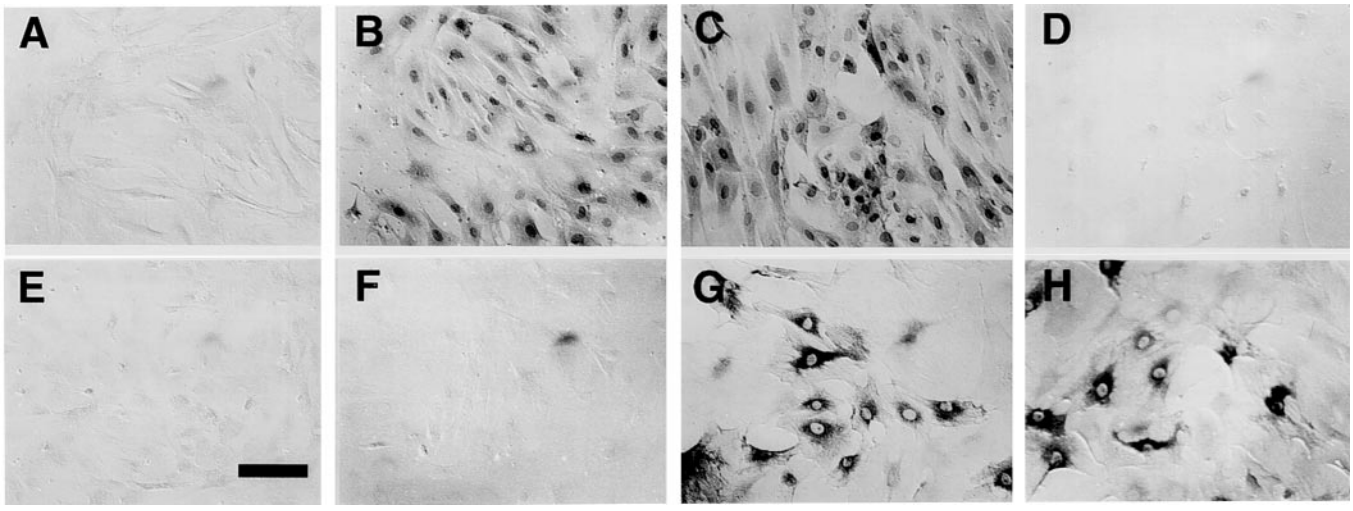

Figure 1. Transgene expression in genetically modified fibroblasts. The *top row* (*A–D*) shows AADC immunohistochemistry. The *bottom row* (*E–H*) shows VMAT-2 immunohistochemistry. PFVMAA (*C*, *G*) shows immunoreactivity to both AADC and VMAT-2. PFAADC (*B*, *F*) shows immunoreactivity to only AADC antibody and PFVMAT (*D*, *H*) to only VMAT antibody. PF (*A*, *E*) does not show any immunoreactivity to AADC or VMAT-2 antibody. Scale bar, 100 μm.

Table 1. Activities of recombinant enzymes in genetically modified fibroblasts

	PF	PFAADC	PFVMAA	PFVMAT
AADC activity (pmol mg/min dopamine)	0.0 ± 0.0	316.4 ± 8.8*	293.5 ± 26.1*	—
VMAT activity (pmol mg/min serotonin)	0.0 ± 0.0	0.0 ± 0.0	2.3 ± 0.2*	3.3 ± 0.2*

Data represent the mean ± SEM ($n = 3$). *$p < 0.01$ from PF control groups by Newman–Keuls test.

higher in those with VMAT transgenes than in control cells ($F_{(2,8)} = 109.4$; $p < 0.0001$) (Table 1).

In vitro dopamine production and storage in genetically modified fibroblast cells

To determine whether the doubly transduced PFVMAA cells can convert and store dopamine from exogenous L-DOPA, fibroblast cells were incubated with 1 μM L-DOPA, and the dopamine levels in both the cells and media were measured. We have shown previously that control fibroblasts do not produce any detectable dopamine from L-DOPA (Kang et al., 1993). Intracellular dopamine levels were significantly higher in PFVMAA than in PFAADC ($F_{(1,4)} = 179.6$; $p = 0.0002$ for group effect) (Fig. 2*A*). The intracellular storage of dopamine in PFAADC cells was negligible. Intracellular dopamine levels in PFVMAA significantly increased with time ($F_{(4,16)} = 28.0$; $p < 0.0001$ for time effect; and $F_{(4,16)} = 28.8$; $p < 0.0001$ for the interaction of group and time) and plateaued after 2 hr, indicating saturation of the storage capacity (Fig. 2*A*). Levels of dopamine in the media incubating PFVMAA cells increased with time ($F_{(4,16)} = 59.6$; $p < 0.0001$ for time effect) and were significantly higher than those corresponding to PFAADC cells ($F_{(1,4)} = 182.6$; $p = 0.0002$ for group effect; and $F_{(1,16)} = 35.4$; $p < 0.0001$ for the interaction of group and time) (Fig. 2*B*).

When fibroblast cells with only VMAT expression were directly incubated with 1 μM dopamine in the medium for 1 hr, intracellular dopamine levels were 22.5 ± 2.5 pmol/10^6 cells (mean ± SEM; $n = 3$) compared with 122.1 ± 6.8 at the same 1 hr time point in PFVMAA cells ($t = 12.3$; $p = 0.0002$; t test) (Fig. 2*A*), despite a slightly higher VMAT activity of PFVMAT than that of PFVMAA ($p < 0.05$) (Table 1).

To further demonstrate that the increased dopamine production and storage were caused by VMAT-2 expression, cells were incubated with L-DOPA (1 μM) plus the VMAT inhibitor reserpine (3 μM) for 2 hr, and dopamine levels were measured. There were significant differences among the groups for both intracellular ($F_{(3,8)} = 882.42$; $p < 0.0001$) and extracellular ($F_{(3,8)} = 121.7$; $p < 0.0001$) dopamine levels (Fig. 2*C*). Reserpine had no effect on PFAADC but depleted intracellular dopamine from PFVMAA completely and decreased extracellular dopamine level of PFVMAA to the level comparable with that of PFAADC cells. Total dopamine production in PFVMAA cells was higher than that in the same cells treated with reserpine or in PFAADC cells (Fig. 2*C*), despite comparable AADC activities (Table 1). Intracellular DOPAC levels were below detection limits in all of the groups, and extracellular levels were significantly different among the groups ($F_{(3,8)} = 119.2$; $p < 0.0001$) (Fig. 2*D*). DOPAC was not detected in PFVMAA cells, and reserpine treatment of PFVMAA cells increased DOPAC levels to that of PFAADC, suggesting that dopamine was protected from metabolism after its presumed sequestration into endosomes by VMAT-2.

Dopamine release from PFVMAA

To investigate whether stored dopamine can be released spontaneously from PFVMAA, we preincubated cells with 1 μM L-DOPA for 2 hr to store dopamine, replaced with media lacking L-DOPA every hour, and measured dopamine levels in the media recovered. Dopamine stored in PFVMAA cells was released spontaneously and gradually over a few hours in the absence of a continuing supply of the precursor L-DOPA (Fig. 3*A*). The time course of dopamine release was not analyzed in PFAADC cells because there had been no detectable intracellular dopamine accumulation in PFAADC cells, even after prolonged incubation with L-DOPA (Fig. 2*A*). To investigate the mechanism of dopamine release in the PFVMAA cells, intracellular calcium level was manipulated by using either calcium-free media or the cal-

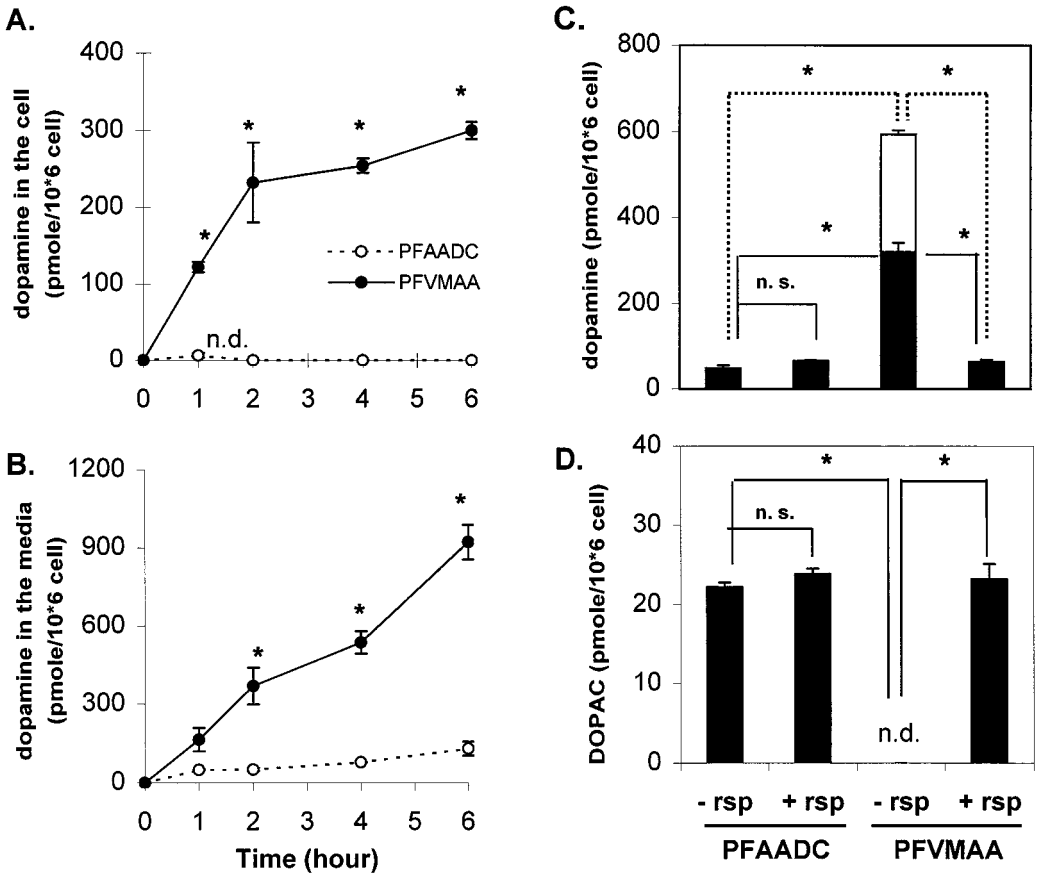

Figure 2. Dopamine production from exogenous L-DOPA and its storage in genetically modified fibroblast cells. Time course of intracellular (*A*) and extracellular (*B*) dopamine levels in PFAADC cells (*open circles* with *dotted line*) and PFVMAA cells (*filled circles* with *solid line*) after 1 μM L-DOPA incubation for various durations (0–6 hr). $^*p < 0.01$ compared with the PFAADC group and also with time 0 by Newman–Keuls test. Dopamine (*C*) and DOPAC (*D*) levels in PFAADC cells and PFVMAA cells after a 2 hr incubation with 1 μM L-DOPA and with 3 μM reserpine (+ *rsp*) or without reserpine (− *rsp*). The catecholamine levels were measured both in the media (*filled bars*) and in the cells (*open bars*). Data represent the mean ± SEM ($n = 3$) from a representative set of several experiments. *n.d.*, Not detectable; *n.s.*, not significant at $p > 0.05$ level. $^*p < 0.01$ by Newman–Keuls test. *Solid lines* show comparison among extracellular values, and *dotted lines* compare intracellular levels.

cium ionophore A23187. These treatments did not significantly influence the dopamine levels in the cells ($F_{(2,6)} = 2.13$; $p = 0.2$) but significantly changed the media levels ($F_{(2,6)} = 10.3$; $p = 0.01$) (Fig. 3*B*). There was a minor, but significant, decrease in dopamine release by calcium depletion, suggesting that some of the release is calcium-dependent. Treatment with the calcium ionophore produced no statistically significant increase in the release of dopamine (Fig. 3*B*), although there was a slight trend for increase that became more discernible with longer duration of preincubation with L-DOPA (data not shown). Therefore, the major portion of release was calcium-independent. Incubation with high-potassium media (40 mM KCl) for 3 min did not increase dopamine release (data not shown).

Biochemical changes in parkinsonian rats after grafting

We next examined whether PFVMAA cells would increase dopamine release and prolong the duration of release that occurs after systemic administration of L-DOPA *in vivo*. We made unilateral near complete lesions of the nigrostriatal system by injecting 6-hydroxydopamine into the MFB in rats. Grafts were placed in the central area of the striatum along four vertical tracks. Three days later, a microdialysis probe was placed at the center of these four tracks, and the next day, catecholamine levels were monitored after intraperitoneal administration of L-DOPA and the peripheral AADC inhibitor benserazide. Consistency and completeness of the lesions were ensured by selecting only those rats that showed >400 rotations/hr after amphetamine administration and by confirming the lack of detectable dopamine and DOPAC by microdialysis before L-DOPA infusion. There was significant effect of group ($F_{(4,19)} = 32.9$; $p < 0.0001$), time course ($F_{(17,323)} = 89.4$; $p < 0.0001$), and interaction of group and time course ($F_{(68,323)} = 20.9$; $p < 0.0001$) on intrastriatal L-DOPA levels after exogenous L-DOPA treatment. These effects were primarily attributable to the significant difference between the group that received a higher dose of L-DOPA (25 mg/kg group) and the other groups (Fig. 4*A*). Similar L-DOPA levels were achieved in the denervated striatum in all other groups that received 6 mg/kg of L-DOPA (Fig. 4*A*). There was significant effect of group ($F_{(4,19)} = 12.9$; $p < 0.0001$), time course ($F_{(17,323)} = 33.3$; $p < 0.0001$), and interaction of group and time course ($F_{(68,323)} = 7.3$; $p < 0.0001$) on intrastriatal dopamine levels. For the same L-DOPA treatment, dopamine levels were significantly higher and remained high for longer periods of time in PFVMAA grafted striata compared with control and PFAADC grafted groups (Fig. 4*B*). Additional exogenous AADC provided by the PFAADC cells was not sufficient to increase conversion

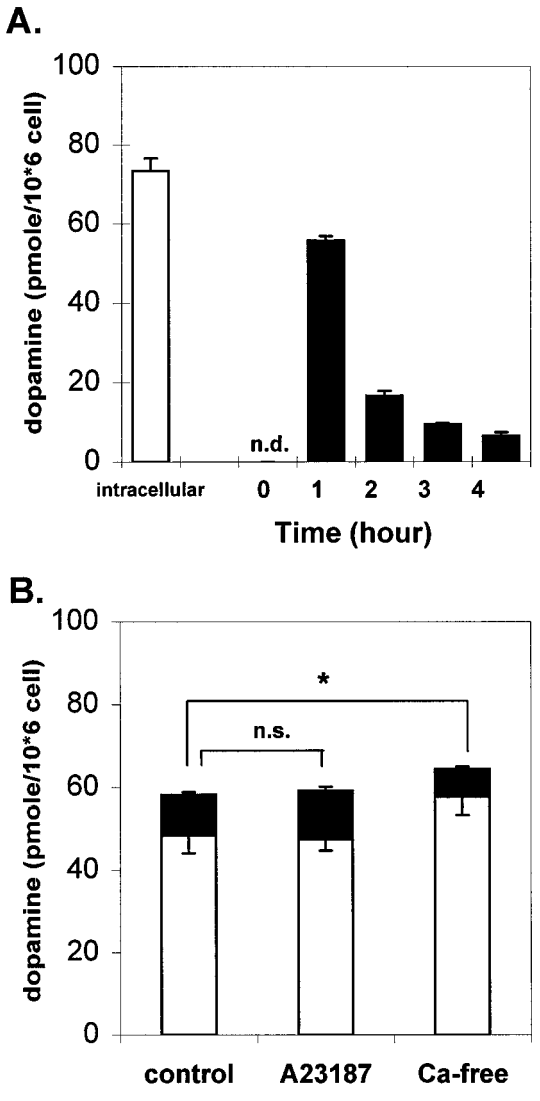

Figure 3. Release of stored dopamine from PFVMAA. *A*, Time course of dopamine release from PFVMAA cells that were preincubated with 1 μM L-DOPA for 2 hr to store dopamine (intracellular, *open bar*). After preincubation, the media were replaced with fresh media without L-DOPA every hour, and dopamine levels in the recovered media (*filled bars*) were measured at 1 hr intervals. *B*, The dopamine levels in the media (*filled bars*) and in the cell pellets (*open bars*) of PFVMAA cells in different calcium conditions. Cells were preincubated with 1 μM L-DOPA in the media for 2 hr, which were replaced with fresh physiological media (*control*), physiological media with 20 μM the calcium-ionophore A23187, or calcium-free media with 10 mM EGTA for 20 min of further incubation. Data represent the mean ± SEM ($n = 3$). *n.d.*, Not detectable; *n.s.*, not significant. $^*p < 0.05$ by Newman–Keuls test for media levels. Dopamine levels in the cells were not significantly different among three groups.

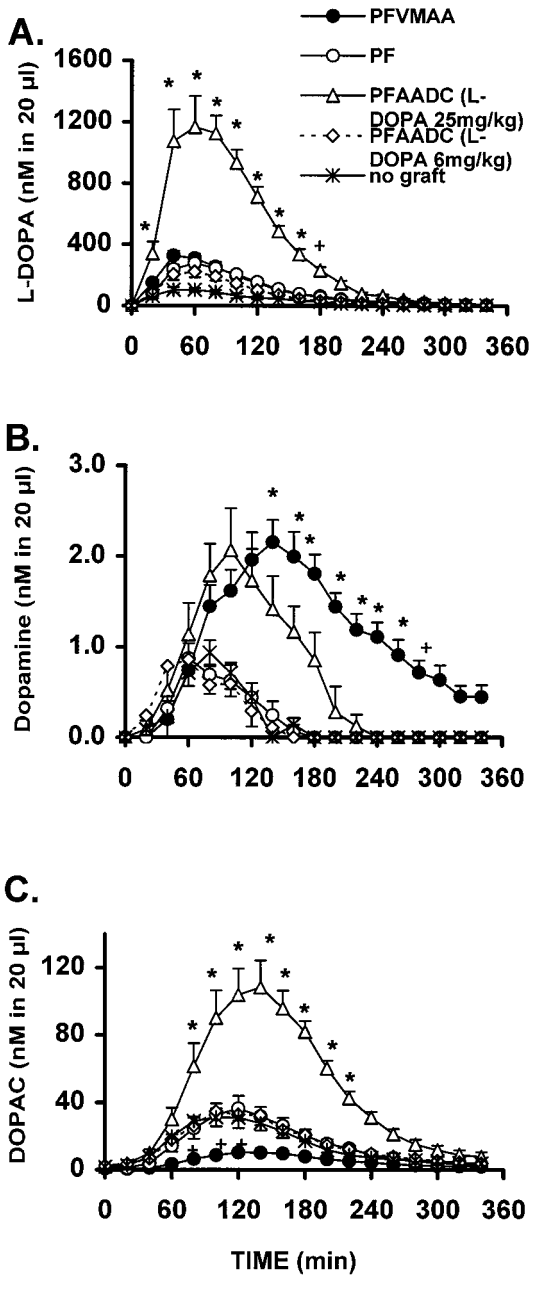

Figure 4. Time course of biochemical changes after L-DOPA injection. Microdialysate concentrations of L-DOPA (*A*), dopamine (*B*), and DOPAC (*C*) were measured in 6-OHDA-denervated striata containing genetically modified grafts at 20 min intervals after L-DOPA intraperitoneal injection (6 mg/kg, except for 1 PFAADC group, which received 25 mg/kg). Benserazide was given at 25 mg/kg intraperitoneally in all groups. Data represent the mean ± SEM ($n = 3$ for no graft control; $n = 6$ for PF; $n = 4$ for PFAADC with 6 mg/kg; $n = 5$ for PFAADC with 25 mg/kg; $n = 6$ for PFVMAA). $\dagger p < 0.05$; $^*p < 0.01$, relative to all the other unmarked groups in *A* and *C*, and relative to AADC 25 mg/kg group in *B*, by Newman–Keuls test.

from the exogenous L-DOPA to intrastriatal dopamine beyond the levels achieved by endogenous AADC in the denervated striatum in control groups with no graft or control fibroblast cells (Fig. 4*B*). To exclude the possibility that the longer duration of dopamine elevation seen with PFVMAA may be simply attributable to a higher peak level, we compared the duration of dopamine elevation in the PFAADC group treated with a higher dose of L-DOPA (25 mg/kg) that attained the same peak dopamine levels as the PFVMAA group given 6 mg/kg of L-DOPA. The duration of dopamine elevation was significantly longer in the PFVMAA group than in the PFAADC group with higher dose of precursor administration, despite the same peak levels (Fig. 4*B*). These data are consistent with prolonged dopamine release by PFVMAA cells, in addition to increased total dopamine level. DOPAC levels were also significantly different among groups ($F_{(4,19)} = 22.3; p < 0.0001$), with a significant effect of time ($F_{(17,323)} = 76.6; p < 0.0001$) and the interaction of group and

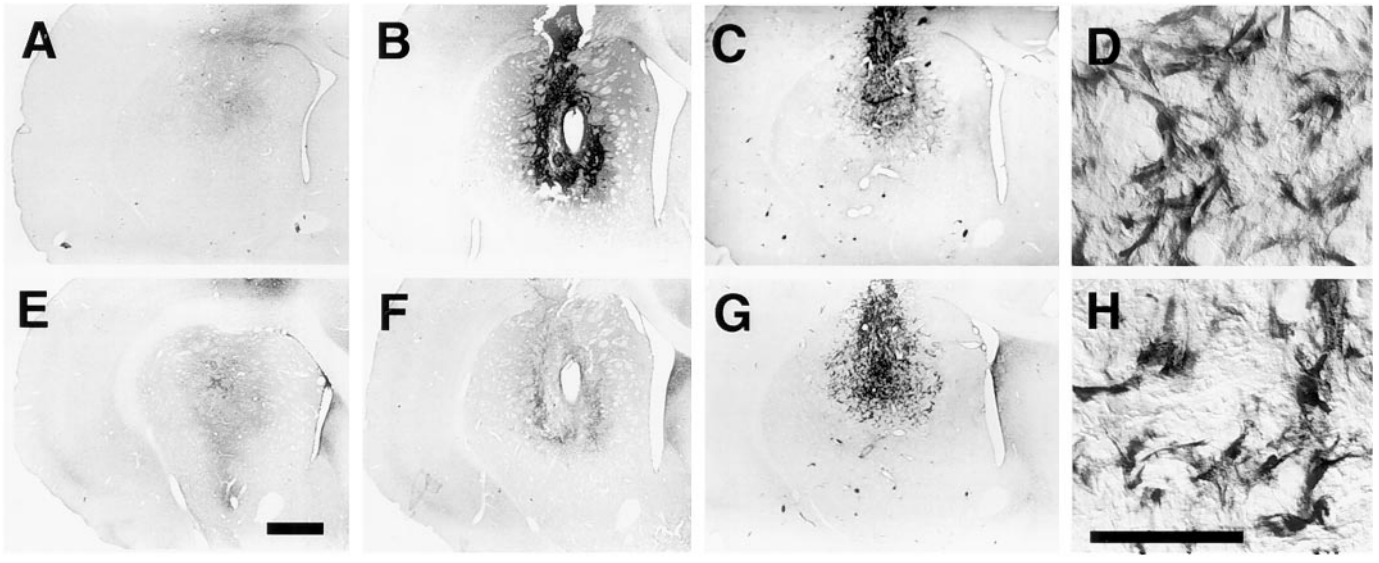

Figure 5. Genetically modified grafts in dopamine-denervated striatum. The grafts contained PF (*A*, *E*), PFAADC (*B*, *F*), or PFVMAA (*C*, *D*, *G*, *H*) cells. The void area shows the site of microdialysis probe placement near the grafts (visible in *B* and *F*). The *top row* (*A–D*) shows AADC immunohistochemistry, and the *bottom row* (*E–H*) shows VMAT-2 immunohistochemistry. *D* and *H* are high-power views of *C* and *G*, respectively. Scale bars: *E*, 1 mm; *H*, 100 μm.

time ($F_{(68,323)} = 13.0$; $p < 0.0001$). The DOPAC levels were significantly lower in the PFVMAA grafted striatum than in all the other groups, despite the higher dopamine levels in PFVMAA group (Fig. 4*C*), most likely because VMAT protects dopamine from monoamine oxidase (MAO)-mediated metabolism by sequestering cytoplasmic dopamine into endosomes.

The genetically modified grafts survived well, and graft size and locations were comparable among groups. The extent of dopaminergic lesions were noted by the absence of dopaminergic fibers in the striatum, and *in vivo* expression of transgenes were also confirmed by immunostaining for AADC and VMAT-2 (Fig. 5).

DISCUSSION

Gene therapy experiments in animal models of PD have focused primarily on providing a source of L-DOPA within the striatum by TH (Fisher et al., 1991; Mattioli et al., 1992; During et al., 1994) and GTP cyclohydrolase 1 gene transfer (Bencsics et al., 1996; Leff et al., 1998; Mandel et al., 1998). However, the major limitation of current pharmacological therapy with L-DOPA lies in the erratic nature of the dopamine delivery rather than a total lack of L-DOPA delivery into the CNS. Therefore, as an alternative approach, we tested the hypothesis that increasing the capacity to decarboxylate the L-DOPA to dopamine and to store dopamine within the striatum is sufficient to restore dopamine in parkinsonian rats after L-DOPA therapy. The strategy of precursor L-DOPA administration and gene therapy with AADC and VMAT were combined. We demonstrated the beneficial effects of such double-gene transduction in the production, storage, and gradual release of dopamine by genetically engineered fibroblast cells both *in vitro* and *in vivo*.

Increased dopamine storage and production by PFVMAA

We demonstrated that VMAT-2 is functionally active when expressed in fibroblast cells. In addition, it confers the capacity to store dopamine intracellularly in fibroblast cells. Intracellular levels of dopamine are negligible in PFAADC cells, and almost all of the dopamine produced is released into the medium. However, a significant intracellular accumulation of dopamine was noted in PFVMAA cells. When VMAT-2 is expressed in fibroblast cells, dopamine is presumably sequestered into acidic endosomal compartments, because there are no synaptic vesicles in these non-neuronal cells (Gasnier et al., 1994; Merickel et al., 1995).

Cotransduction of VMAT-2 with AADC in fibroblast cells was essential because dopamine does not enter cells efficiently nor cross the blood–brain barrier. Despite the fact that PFVMAA cells had slightly lower transporter activities than PFVMAT cells, intracellular dopamine levels were higher in PFVMAA cells than in PFVMAT cells when they were incubated with comparable levels of L-DOPA or dopamine, respectively. This difference in intracellular dopamine levels indicates that L-DOPA enters cells much more efficiently than dopamine. Using L-DOPA as a precursor and providing AADC in the cells for intracellular conversion into dopamine resulted in higher levels of dopamine storage than using dopamine itself.

In addition to having increased dopamine storage capacity, PFVMAA cells compared with PFAADC cells produced significantly increased total dopamine levels and decreased DOPAC levels for the same doses of L-DOPA both *in vivo* and *in vitro*. These findings are consistent with the view that dopamine is protected from intracytoplasmic oxidation by MAO (Cubells et al., 1994), presumably because it is sequestered in the endosomal compartments. Although the lack of dopamine metabolism contributes to higher total dopamine in PFVMAA than in PFAADC cells with equivalent decarboxylation capacity, it did not account for the entire difference in dopamine levels between these two cell types. Sequestration of the dopamine away from the site of synthesis in the cytoplasm into other inaccessible compartments may also facilitate total dopamine production by relieving feedback inhibition. In neurons, VMAT-2 also promotes uptake of dopamine at the plasma membrane because transporting dopamine into vesicles lowers its cytoplasmic concentrations. Specificity of VMAT-2 in producing these effects was confirmed by

reserpine treatment, which reverted the catecholamine levels of PFVMAA to those of PFAADC. In summary, VMAT-2 not only increases the intracellular storage capacity and reduces the metabolism of dopamine, but it also increases overall production of dopamine in fibroblasts genetically modified with AADC.

Increased dopamine release by fibroblast cells expressing both AADC and VMAT-2 genes

Despite the lack of neurotransmitter secretory pathways in fibroblast cells, dopamine stored within PFVMAA cells was released spontaneously and gradually into the medium. The release was primarily calcium-independent and unaffected by potassium-induced depolarization. The mechanism of dopamine release from PFVMAA is most likely to be constitutive exocytosis of endosomal vesicles. Calcium-independent release of other classic neurotransmitters and peptides from genetically modified fibroblast cells without added storage capacity has been noted previously (Ruppert et al., 1993; Misawa et al., 1994). VMAT-2 has been found in tubulovesicular organelles of cell bodies and dendrites of dopaminergic neurons (Hattori et al., 1979; Mercer et al., 1979; Nirenberg et al., 1996). Somatodendritic release of dopamine from dopaminergic neurons may occur from this pool of dopamine in a manner similar to our proposed mechanism of dopamine release from PFVMAA cells. Likewise, calcium-independent constitutive release of catecholamine has been noted in PC-12 cells and chromaffin cells (von Grafenstein et al., 1992; Sulzer et al., 1996).

Increased levels and prolonged duration of dopamine elevation *in vivo* by PFVMAA grafting

PFAADC grafts in the denervated striatum did not enhance dopamine levels after L-DOPA administration compared with PF grafts or ungrafted control animals. This demonstrates the presence of sufficient endogenous capacity for L-DOPA decarboxylation, even in the face of severe denervation of dopaminergic afferents. In addition, AADC contributed by PFAADC grafts does not add significantly to this endogenous capacity for dopamine production. However, when the same capacity to decarboxylate L-DOPA was accompanied by the ability to store dopamine, there was a significant enhancement of dopamine delivery by PFVMAA compared with PFAADC. In addition, PFVMAA grafts also prolonged duration of intrastriatal dopamine elevation *in vivo* compared with PFAADC grafts given the same dose of L-DOPA. Studying the duration of improvement in dopamine elevation rather than just focusing on the level of dopamine is more relevant to these common clinical problems of wearing-off phenomena and short-duration response.

There are clinical examples that underscore the importance of subsequent processing steps of L-DOPA, namely L-DOPA decarboxylation and dopamine storage. For instance, DOPA-responsive dystonia is associated with mutations in the GTP cyclohydrolase 1 gene that result in an absence of the cofactor tetrahydrobiopterin and a consequent lack of dopamine production (Ichinose et al., 1994; Furukawa et al., 1996). The dramatic and smooth response to L-DOPA seen in these patients (Nygaard et al., 1992) is most likely attributable to their intact L-DOPA decarboxylation and dopamine storage. In addition, the major effect of fetal transplantation in PD has been to enhance the response of patients to L-DOPA, not to alleviate the need for the drug. Again, this is likely because of an added capacity to decarboxylate L-DOPA and to store the formed dopamine, as has been demonstrated by fluoro-DOPA positron emission tomography scans (Melamed, 1988; Sawle et al., 1992; Lindvall et al., 1994).

Significance of dopamine storage in the therapy for Parkinson's disease

The use of similar grafts expressing both AADC and VMAT in humans would allow a lower dose of L-DOPA to achieve the same level of striatal dopamine as a higher dose of L-DOPA in a patient without grafts. This would decrease the diffusion of L-DOPA to other parts of the brain, notably the limbic system, and minimize side effects such as hallucinations and confusions. A sustained dopamine release will ameliorate the wearing-off in moderate and advanced stages of PD. In addition to prolonging the effect of L-DOPA, use of these grafts along with L-DOPA therapy may prevent the development of fluctuations that result from the loss of storage capacity as the PD advances. Furthermore, by varying the amount of L-DOPA given in conjunction with grafting, one could regulate the final dopamine delivery, hence alleviating concerns about unregulated gene expression and increasing flexibility to adjust the therapy in an individual patient. Such an approach may permit realistic clinical application, even with the limited gene therapy vehicles that are currently available.

In addition, genes that are essential for L-DOPA production, such as tyrosine hydroxylase and GTP cyclohydrolase 1 (Bencsics et al., 1996), could be combined with AADC and VMAT-2 to maximize the production and delivery of dopamine by gene therapy. When only AADC is introduced to cells that produce L-DOPA in an attempt to facilitate conversion to dopamine, this resulted in less dopamine delivery because of feedback inhibition by the end product, dopamine (Wachtel et al., 1997). Therefore, introduction of both AADC and VMAT will be essential to sequester dopamine into storage sites away from the site of synthesis in this combined approach. Such multiple gene transduction could also be applied to other donor cell types for transplantation, including neuronal stem cells, as well as to direct *in vivo* transfer methods using viral vectors, such as herpes virus, adenovirus, adeno-associated virus, and lentivirus.

In addition to its effect on dopamine delivery, gene therapy with VMAT may also reduce the cytoplasmic concentration of potential neurotoxins that are substrates for vesicular transport such as 1-methyl-4-phenylpyridinium by sequestering them into the endosome and reducing the exposure of other organelles to such toxins (Erickson et al., 1992; Liu et al., 1992, 1994; Del Zompo et al., 1993; Takahashi et al., 1997). Such a strategy for neuroprotection may involve direct gene transfer of VMAT-2 into the vulnerable neurons *in vivo*.

In conclusion, the data presented here underline the importance of dopamine storage in the pathogenesis and treatment of PD. The enhancement of key processing steps of L-DOPA by VMAT-2, as outlined, may lead to successful amelioration of symptoms of PD by overcoming the major limitations of the current L-DOPA therapy.

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