Multi-site partitioned delivery of human tyrosine hydroxylase gene with phenotypic recovery in Parkinsonian rats

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Parkinson's disease (PD) is a leading candidate for neurological gene therapy, given our increasing knowledge of the functional anatomy of the striatonigral system and the localized nature of the affected cell populations. Here we report that stereotactic introduction of a human tyrosine hydroxylase (TH-2) gene using multi-site partitioned doses resulted in behavioral recovery in 6-OHDA-lesioned rats, with transient 100% recovery observed in some animals. We also show correlation between numbers of TH-immunoreactive cells and loss of apomorphine induced rotation, with a near-linear relationship between TH expression and phenotypic recovery. Furthermore, the data suggest that only a fraction of striatal cells need to be transduced in order to exert phenotypic effects, and therefore TH partitioned gene transfer may have clinical potential in PD. *NeuroReport* 11:1145–1151 © 2000 Lippincott Williams & Wilkins.

Key words: Adenovirus; Apomorphine; Behavioural recovery; Gene transfer; Human; 6-OHDA; Parkinsonian; Partitioned delivery; Striatum; Tyrosine hydroxylase

INTRODUCTION

In vivo gene transfer presents an alternative to current treatments for Parkinson's disease (PD) such as dopamimetic drugs, ablative surgery, and cell transplantation. Following stereotactic injection of viral vector, transduced cells can produce continuous, physiological expression of a therapeutic transgene. *In vivo* microdialysis studies have demonstrated that expression of the human TH gene, which catalyzes the rate-limiting step in dopamine biosynthesis, increases local production of both L-dopa and dopamine in animal models of Parkinsonism, as long as sufficient cofactor is available [1,2]. A number of vectors (HSV, AAV, adenoviral, and liposomal) have been introduced to Parkinsonian animals to increase striatal L-dopa and dopamine, leading to 20–60% behavioral recovery, as measured by reductions in the rate of apomorphine-induced asymmetrical rotations [1,3–5].

In this study a high titer replication-defective (with deletions in E1A, E1B, and E3) adenovirus was used to drive human TH expression in the 6-OHDA-lesioned rat model of PD. Previous studies have not addressed the quantitative relationship between numbers of virally transduced TH-immunoreactive (TH-IR) cells and phenotypic recovery, and our primary goal was to define this relationship, determining both the maximum recovery possible using TH and the minimum number of transduced cells required to obtain a significant phenotypic effect.

Betz *et al.* [6] reported that altering parameters such as infusion rate and volume (with a constant viral dose) did not have a significant effect on the quantity of transduced tissue, but that the dose of virus is an important parameter. We experimented with different numbers and locations of injection sites, using the same total dose but partitioning the volume for multiple injections, in order to maximize the amount of tissue transduced by the adenovirus. The effective dose delivered to the striatum was found to

depend on the number of sites injected, even with the same total volume.

MATERIALS AND METHODS

An adenoviral vector was constructed (AdTH) expressing human TH-2 driven by a Rous sarcoma virus (RSV) promoter with the Ad5 backbone dl309. Control vectors expressed β-galactosidase (AdLac), as described previously [7]. Both vector systems were rendered replication defective and were propagated and purified by the University of Iowa Gene Transfer Vector Core with particle to infectious unit (i.u.) ratios 1:100, assessed by infecting permissive 293 cells with different multiplicities of infection (MOI).

Male Fischer 344 rats (280–300 g) were handled according to Animal Care and Use Guidelines of the NIH and Yale University. Animals were anaesthetized with ketamine and xylazine (67 and 6.7 mg kg^{-1}) and lesioned with 12 μg of the neurotoxin 6-OHDA-HBr (Research Biochemicals, Inc.) injected stereotaxically into the right substantia nigra, at a free base concentration of 4 μg/μl (0.9% saline/0.2% ascorbic acid). Stereotactic coordinates, measured in millimeters from lambda, were +3.5 antero-posterior (AP), +2.1 medio-lateral (NIL) and −7.1 dorso-ventral (DV) [8]. A Harvard Apparatus microdialysis pump was used to deliver the 6-OHDA at a rate of 0.5 μl/min with a 10 μl Hamilton syringe connected to a 30-gauge stainless steel cannula with polyethylene tubing. Three weeks after lesioning, animals were screened for complete lesions with apomorphine (1 mg/kg in 0.9% saline, 0.2% ascorbic acid) using a hemispheric rotometer. The number of rotations (contralateral minus ipsilateral) were recorded over a 5 min interval (15–20 min) after apomorphine administration [9]. A baseline rotation rate was previously established with three tests conducted 1 week apart. Only animals with a consistent rotation rate >5 rotations/min (with less than ~25% intraindividual variation between tests) were included in the experimental groups.

The viral concentration was optimized for minimal tissue damage and maximal gene expression. The AdTH stock solution was measured at 1.4×10^{12} physical particles/ml. AdTH and AdLac were diluted with sterile PBS to an optimal concentration of 1.4×10^{8} i.u./μl. Animals were then randomly assigned into one of five groups. Gene delivery was performed 1 month after animals were lesioned. All animals received a 4 μl infusion of PBS, AdTH or AdLac (5.6×10^{8} i.u./animal). Injections were performed using the stereotaxic injection system described above with a slow infusion rate (0.1 μl/min). One group of animals ($n=4$) received a single 4 μl injection of AdTH to a centrally located site in the striatum (Group A). The second group ($n=5$) received 4 μl AdTH injected into four separate striatal sites at 1 μl/site (Group B). A last tripartite group, consisting of AdTH ($n=5$), PBS ($n=5$), and AdLac ($n=4$) received eight separate striatal injections at 0.5 μl/site (Groups C_1, C_2, C_3). Thus the three groups (A, B, C) differed in the number of injection sites and the location of viral vector dispersion (Table 1).

Animals were tested for apomorphine induced rotational behavior 2, 3, 4 and 6 weeks post-surgery. At 8 weeks post-surgery, two animals from each group (those showing the maximum and minimum behavioral recovery) were chosen for histochemical analyses. These animals were euthanized with chloral hydrate and perfused transcardially with 60 μl ice-cold 1 M PBS followed by 180 ml ice-cold 4% paraformaldehyde. The brains were removed and post-fixed with 4% paraformaldehyde for 24 h at 4°C, equilibrated in a solution of 30% sucrose in PBS, and 30 μm sections of selected brains from each group were sliced for immunohistochemistry. Every third section was tested for TH-IR cells, using incubation overnight with a polyclonal primary TH-antibody (Pel Freez, 1:750 dilution) followed by incubation of free-floating sections using the VectaStain biotinavidin system (Vector Laboratories). Antibodies were visualized with a DAB (3'-3'diaminobenzidine) solution containing hydrogen peroxide with nickel ammonium sulfate and cobalt chloride intensifying reagents. The AdLac group, which served as a control for transduction efficiency and non-specific effects, was reacted with X-Gal substrate to demonstrate β-galactosidase transgene activity. Those sections were incubated overnight with β-Gal substrate (5-bromo-4-chloro-3-indolyl-β-galactoside) and counterstained with hematoxylin/eosin. Free-floating sections were mounted on poly-L-lysine-coated slides, dehydrated in serial washes of methanol and xylene, and coverslipped. The technique we used eliminates background staining by endogenous β-galactosidase [3], and accordingly we did not detect X-gal staining in the TH or PBS injected animals. Remaining animals from each group were used to assay long-term presence of the tyrosine hydroxylase transcript using RT-PCR analysis (Fig. 5).

TH-IR cells within the target area of the striatum were counted using light microscopy for Group A, B and C_1, using the method of Abercrombie as modified by McLachlan and Janig [10]. Neurons, oligodendrocytes, astrocytes and microglia were included. Counting was restricted to separate cell bodies, and isolated TH-IR cell fibers were disregarded. To quantify the percentage area of caudate stained per animal, a projection microscope produced a ×10 magnified image of sections onto a transparent grid for the caudate to be outlined. The number of grid units containing TH-positive cell components, including cell bodies and solitary fibers, were counted. The entire area of the caudate was calculated by integrating the number of squares the projected image covered. In this way all sections from AdTH treatment groups were counted and

Table 1. Injection sites by group, in mm from bregma (AP, ML) or from dura mater (DV).

Group	AP	ML	DV	General position
A	0.5	3.0	−5.6	Rostral
B	1.4	3.2	−5.4	Rostral
	1.4	1.8	−5.4	Rostral
	0.2	3.2	−5.6	Mid-rostral
	0.2	2.2	−5.6	Mid-rostral
C	1.8	2.2	−5.2	Rostral
	1.1	2.0	−5.6	Rostral
	1.0	3.2	−5.6	Rostral
	0.4	2.4	−5.6	Mid-rostral
	0.3	4.0	−5.8	Mid-rostral
	0.2	3.3	−5.4	Mid-rostral
	−0.8	4.3	−5.8	Caudal
	−1.8	4.4	−6.0	Caudal

an overall percentage of area stained in the caudate was measured. Cell count and percentage area caudate studies were analyzed using linear regression.

RESULTS

In vivo injection of AdTH into the striatum of 6-OHDA lesioned rats resulted in behavioral improvements for up to 6 weeks, with immunohistochemical evidence of TH expression. Reductions from baseline values of apomorphine-induced turns were used as an index of behavioral recovery (Fig. 1). Parametric tests (*t*-tests for dependent samples) showed a statistically significant difference in mean pre- *vs* post-treatment rotational behavior within the 1-site ($p < 0.01$), 4-site and 8-site ($p < 0.008$) AdTH-treated groups of animals. Control animals showed no statistically significant change in rotational behavior from baseline values by the same tests. In addition a repeated measures ANOVA with selected *post hoc* tests was performed using the complete rotational values for each group, which demonstrated a significant difference in mean values among treatment groups and compared to controls for 1-site ($p < 0.025$), 4-site ($p < 0.001$) and 8-site ($p < 0.0005$) animals.

We defined the percentage behavioral recovery for a

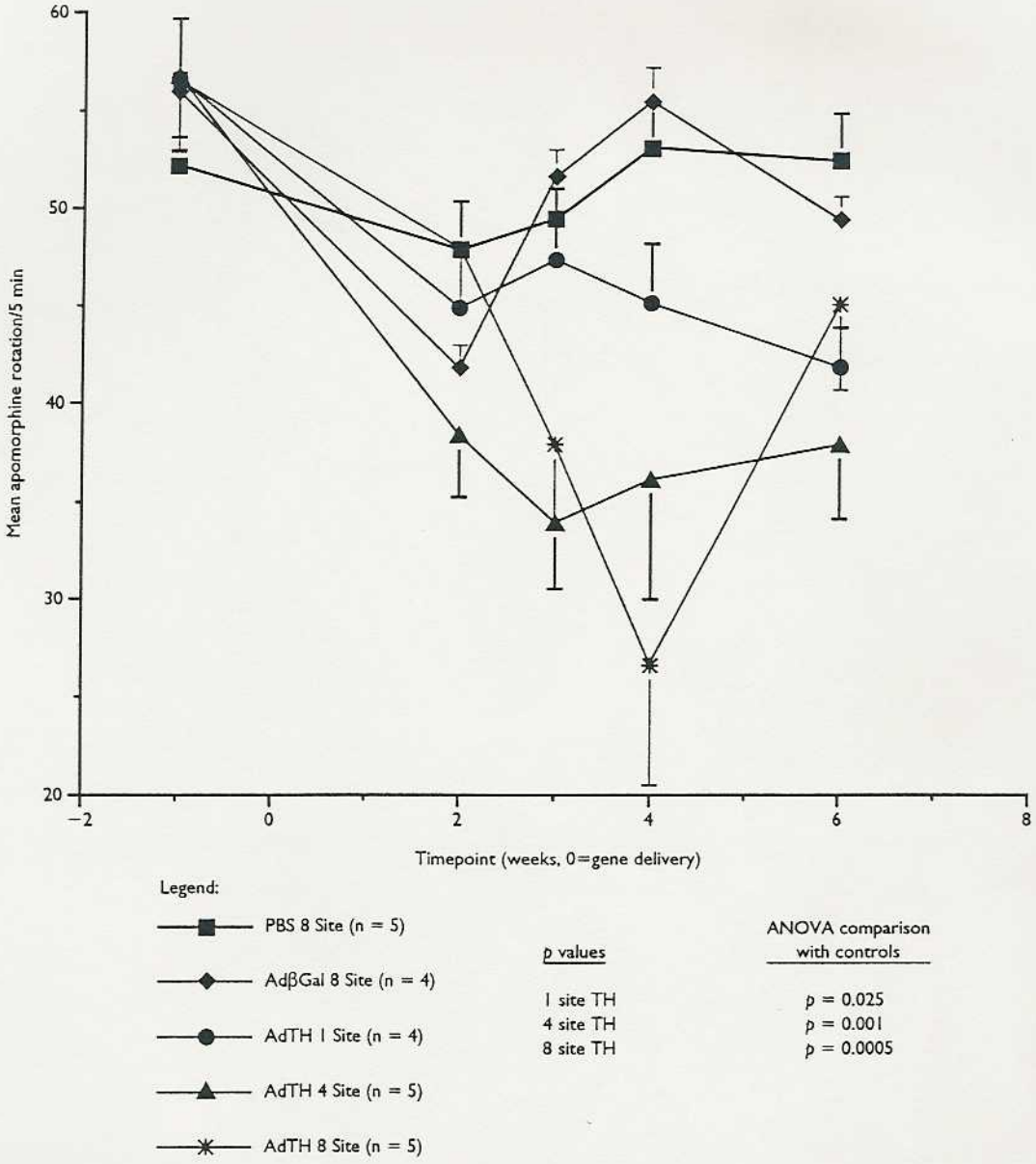

Fig. 1. Apomorphine-induced asymmetrical rotational behavior. The symbols represent mean values with connecting lines for three basal measurements and post-surgical data points at 2, 3, 4, and 6 weeks (error bars = s.d.).

given animal at any time point as $100 \times [1-(A/M)]$, where A/M is the post-treatment number of net contralateral apomorphine-induced rotations (A) for that animal, divided by the mean baseline net contralateral rotation (M) for that animal. For each animal the percentage recovery was calculated at the at 2, 3, 4, and 6-week timepoints. These data were used to plot the mean % behavioral recovery *vs* cell counts for selected animals. In addition, data were pooled within each treatment group and between-group differences in mean values were compared using a *t*-test for independent samples. There was no statistically significant difference in mean percentage behavioral recovery between the two control groups (PBS *vs* β-gal injected animals). The three treatment (AdTH) groups were each compared with the PBS-injected control group which showed a statistically significant difference in mean percentage behavioral recovery for the 1-site ($p < 0.04$), 4-site ($p < 0.035$), and 8-site ($p < 0.02$) animals, consistent with the ANOVA analysis comparing mean rotational values within and among groups.

Two weeks following surgery all groups showed some reduction from baseline in apomorphine-induced turns, due to non-specific effects of the surgery. However, the treatment groups (A, B, C_1) maintained behavioral improvements at 3, 4, and 6 weeks following surgery, whereas control groups (C_2, C_3) returned to baseline. Behavioral recovery for the 1-site TH animals appeared stable at 3 and 6 weeks, with recoveries at any given timepoint ranging from 12 to 45%. Behavioral recovery at any given timepoint among the 4-site animals ranged from 18 to 100%, with transient 100% recovery in one animal at 4 weeks. Behavioral recovery at any given timepoint among the 8-site animals ranged from 15 to 100%, with transient 100% recovery in one animal at 3 weeks (Fig. 2). By 6 weeks mean rotational recoveries had declined in both the 4 and 8-site groups to ~37%, while PBS and AdLac controls remained close to baseline.

Selected animals underwent histochemical analysis (Fig. 4). All six AdTH animals stained positive for TH, while the PBS and AdLac control groups were essentially negative for TH-IR in the striatum. In the AdLac group, one set of sections was reserved for reaction with X-gal as an indicator of gene expression. Strong and near-equivalent β-galactosidase activity was observed in sections tested from all animals. Microscopic analysis revealed adenoviral infection of a range of cell types: neurons (medium spiny, medium smooth, large spiny), oligodendrocytes, astrocytes, and microglia (fibrous and protoplasmic) were all identified on the basis of morphology [11,12].Three-dimensional reconstruction of the transduced areas based on the full extent of TH-IR sections resulted in volumes that appeared oblong in shape in coronal sections. TH-IR cells were particularly dense around the points of injection. Group A (1-site AdTH animals) had a maximal dispersion range of ~1.0 mm medio-laterally and ~1.6 mm in the anteriorposterior direction. Group B (4-site AdTH animals) had a wider dispersion, ~1.4 mm medio-laterally and ~3.3 mm in the anterior-posterior plane. Group C_1 (8-site AdTH animals) had the widest dispersion, with TH-positive cells extending medio-laterally over ~2.5 mm, nearly the entire width of the central caudate, and ~4.5 mm distance in the anterior-posterior axis.

To define the relationship between behavioral recovery and number of transfected cells, TH-IR cells were counted for each of the six sets of AdTH brain sections. Linear regression was used to correlate total cell counts and mean percent recovery for a given animal (Fig. 3a). This relationship was significant for the line of best fit, with Pearson's $r = 0.97$. In order to verify cell counts using another method, the percentage of caudate volume showing TH-

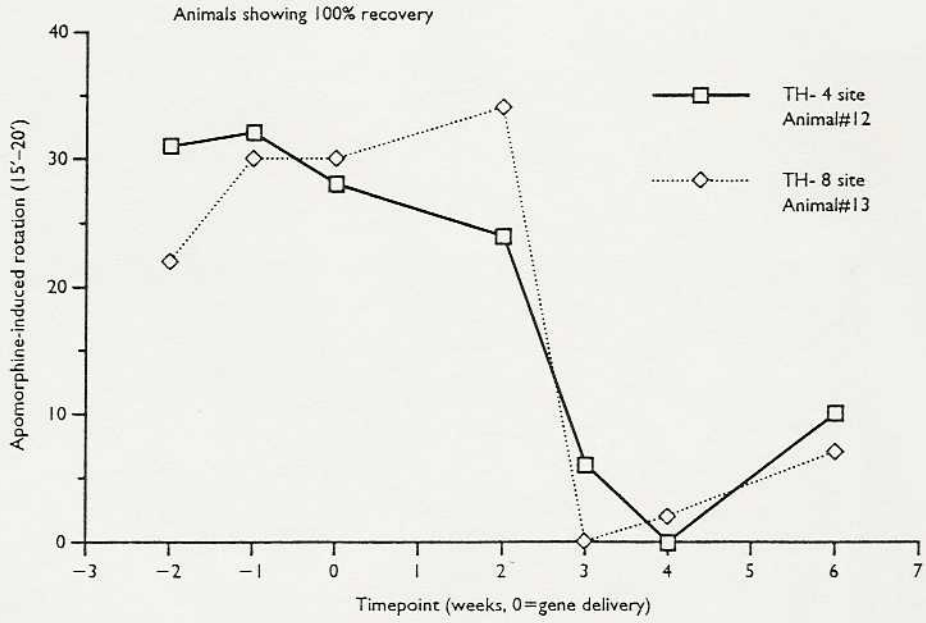

Fig. 2. Apomorphine-induced rotation rates for two individuals that showed decreases in rotation rates to zero net turns following gene delivery.

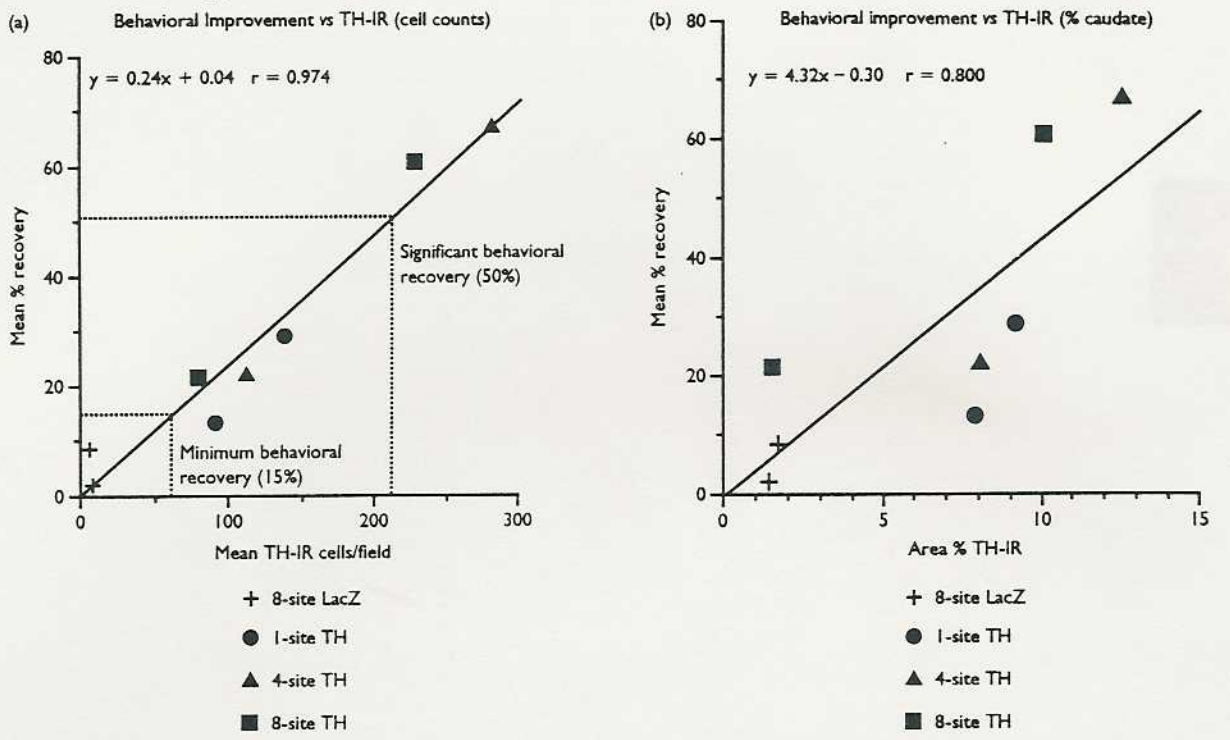

Fig. 3. (a) Total number of TH-IR cell bodies in the striatum *vs* behavioral recovery. The abscissa is the total number of TH-IR cells per section, and the ordinate is the percentage recovery from baseline for apomorphine-induced asymmetrical rotation. (b) The area of the caudate stained positive for TH *vs* behavioral recovery. The abscissa is the percentage of the total caudate area which was TH-IR, and the ordinate is the percentage recovery from baseline for apomorphine-induced asymmetrical rotation.

immunoreactivity was investigated for each animal using the projection method outlined above. Linear regression was used to calculate the association between the percentage of area stained and behavioral recovery (Fig. 3b). This relationship was significant for the line of best fit, with Pearson's $r = 0.80$.

To estimate the minimum number of cells necessary to produce a trend in behavioral recovery, the number of TH-positive cells (per tissue section) was calculated from the regression line between TH-positive cells and percentage behavioral recovery. The threshold for a trend toward behavioral recovery was arbitrarily chosen to be 15%. Taking the estimate of TH-positive cells per section to be 65 and multiplying by the mean longitudinal extent of TH-immunoreactivity gave a figure of ~7350 cells.

Linear regression was also used to estimate the minimum number of transduced cells necessary to produce a significant behavioral recovery. The average number of apomorphine-induced turns <2 s.d. below the mean was calculated for all the AdTH groups combined. This value (~30 turns) is ~50% of the average baseline pre-treatment value for those groups. The estimate of cells per section necessary to produce a 50% rotational recovery was 210, which was multiplied by the mean longitudunal extent of TH-immunoreactivity to give a total value of 24 000 cells.

DISCUSSION

Parkinsonian symptoms usually present in human patients when the dopamine content in the putamen and caudate nucleus is decreased by 70–80%, to levels 20–30% of normal [13,14]. This high threshold of dopamine loss (up to 80%) before the appearance of symptoms is due to a large functional reserve of nigrostriatal dopaminergic neurons that increase dopamine synthesis and release as cell loss progresses [15–17], which suggests that even a moderate increase in dopamine production may be sufficient to completely ameliorate symptoms in the early stages of PD. Our results, showing behavioral recovery in 6-OHDA Parkinsonian rats, provide an animal model for this clinical finding. The estimate we provide for the minimum number of transduced cells necessary (7350) to produce a trend in behavioral improvement is small, relative to the massive neurodegeneration of the nigrostriatal pathway, and suggests that only a fraction of striatal cells needs to be converted into DOPAergic cells to exert phenotypic effects. This finding has implications for the practicality of gene transfer for the treatment of PD in humans. Moreover, based on our counting techniques and the morphological homogeneity of medium spiny neurons which constitute the majority of neurons in the rat striatum [18], it is likely that our approximation is an over-estimate rather than an

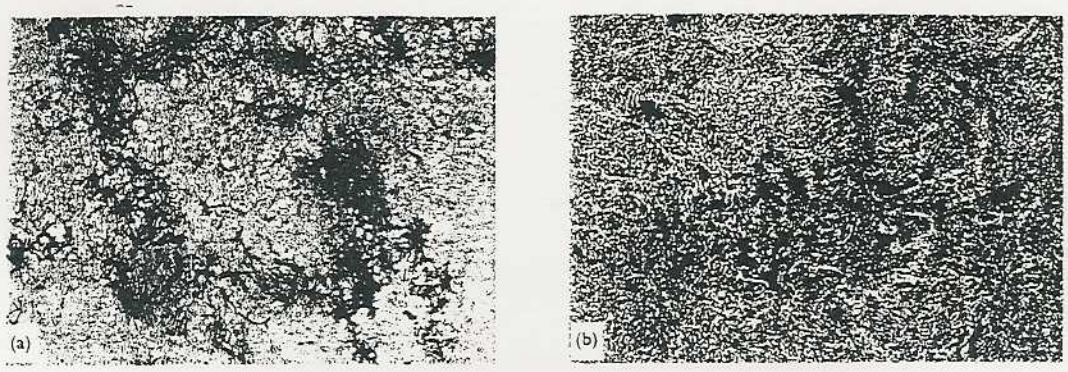

Fig. 4. TH-IR cells in the caudate-putamen of representative animals. (**a**) ×10 AdTH 1-site, (**b**) ×10 AdLac control (Xgal/H and E).

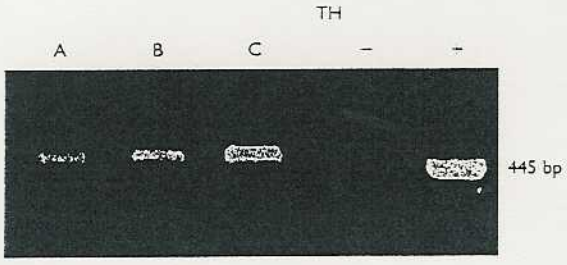

Fig. 5. Representative samples from the caudates of 1-site A, 4-site B and 8-site C animals 2 months after gene delivery, demonstrating TH-positive RT-PCR to a 445 bp vector-specific probe.

under-estimate, and it is therefore possible that a smaller number of cells would suffice for behavioral recovery.

Although the percentage behavioral recovery for all three AdTH groups was significantly different from that of controls, the level of statistical significance varied between the injection protocols, with the 4-site and 8-site groups having a smaller *p*-value than 1-site animals. The fact that multiple-site animals showed highly statistically significant behavioral recoveries compared with controls suggests that injections spread over a greater area of the caudate may lead to more pronounced improvements. In support of this hypothesis, we found that the multi-site and single-site groups had the highest and lowest overall volumes of caudate staining, respectively. Counts of TH-IR cells in sections also demonstrated the most TH-positive cells in the 8-site and 4-site animals.

Different functional subsets of striatal neurons are known to co-exist even within very circumscribed portions of the nigrostriatal pathway [19]. For instance, it has been shown that different profiles of recovery are obtained in the 6-OHDA model of PD depending on the location within the caudate (rostral or caudal) in which fetal nigral tissue is placed [20]. Our results show that viral transduction of the striatum can lead to near-complete reversal of phenotype in the rat, and that the volume of TH– cells was proportional to the recovery obtained. In a larger model it may be desirable to specifically target certain regional subsets of striatal cells for maximum effect, given the much greater volume that needs to be infiltrated, and to use even more injection tracts.

Despite multiple fine needle insertions in the multiple-site injection protocol, which might be expected to increase local tissue damage, lower regional injection volumes may actually reduce the local injury and inflammation, while a larger volume injection may cause an inflammatory reaction that may interfere with viral transduction efficiency. Previous work with stereotactic injections of virus or saline solution to the rodent brain followed by immunologic analyses (OX-18 or OX-32 staining) suggested that larger individual injection volumes could elicit a non-specific immune response [33]. Present results also suggest that multiple low volume injections of adenovirus vector can augment transduction efficiency and allow for maximum dispersion.

Whether the transfer of other genes, in addition to TH, enhances dopamine synthesis is a question that has aroused some debate. Potential genes include GTP cyclohydrolase-1, the rate-limiting enzyme in the biosynthesis of tetrahydrobiopterin (BH4), and aromatic amino acid decarboxylase (AADC) which converts L-dopa to dopamine [21–27]. While administration of these genes may augment dopamine production under certain conditions, our data suggest that replacement of a single gene (TH) is sufficient to change behavior in the 6-OHDA rodent model. The relevance of this finding to human disease may depend on whether sufficient levels of other enzymes and cofactors such as AADC and BH4 persist in end-stage PD.

There has been some debate over the mechanism by which viral vectors encoding TH result in behavioral recovery in the lesioned rodent model. Alternative explanations such as inflammatory responses, release of cytokines or other non-specific vector effects have been suggested [28,29]. In support of our interpretation, that locally produced TH is responsible for the behavioral changes, others have demonstrated a relationship between the number of functional nigral cells remaining after partial lesions and behavioral recovery as assessed by apomorphine- or amphetamine-induced turns [15,30] or between surviving transplant TH positive neurons and apomorphine induced rotations [31,32]. The latter studies also showed that the injection method of fetal dopaminergic

cells was an important determinant of graft function, with multiple injection sites providing superior results.

CONCLUSION

Our data support a direct relation between transgene expression and phenotypic effects. We found a local dose–response effect of TH gene delivery in the rat model of PD, with 8-site AdTH injections providing superior gene expression to 4-site and 1-site injections. We conclude that multi-site stereotactic injections are superior to single-site injections for maximizing TH expression, and moreover that subregional effects of TH expression within the striatonigral pathway may be important for behavioral recovery. Of note, the animals in which we observed transient 100% recovery originally had the lowest baseline level of apomorphine-induced rotation within their respective groups, suggesting that they had a slightly less extensive lesion. This point is relevant to human disease, because clinical improvement may depend in part on the total amount of surviving dopaminergic afferents at the time of gene transfer.

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