Published in final edited form as:

J Comp Neurol. 2009 July 1; 515(1): 15–30. doi:10.1002/cne.22037.

Impact of Levodopa Priming on Dopamine Neuron Transplant Efficacy and Induction of Abnormal Involuntary Movements in Parkinsonian Rats

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Abstract

Clinical trials of neural grafting for Parkinson's disease (PD) have produced variable, but overall, disappointing results. One particular disappointment has been the development of aberrant motor complications following dopamine (DA) neuron grafting. Despite a lack of consistent benefit, the utility of dopamine neuron replacement remains supported by clinical and basic data. In a continued effort to elucidate factors that might improve this therapy, we used a parkinsonian rat model to examine whether pre-graft chronic levodopa impacted graft efficacy and/or graft-induced dyskinesia (GID) induction. Indeed, all grafted PD patients to date have had a pre-graft history of long-term levodopa. It is well established that long-term levodopa results in a plethora of long-lasting neurochemical alterations, and genomic changes indicative of altered structural and synaptic plasticity. Thus, therapeutic dopamine terminal replacement in a striatal environment complicated by such changes could be expected to lead to abnormal or inappropriate connections between graft and host brain, and contribute to suboptimal efficacy and/or post-graft GID behaviors. To investigate the impact of pre-graft levodopa, one group of parkinsonian rats received levodopa for 4 weeks prior to grafting. A second levodopa naïve group was grafted and grafts allowed to mature for nine weeks prior to introducing chronic levodopa. We report here that in parkinsonian rats, pre-exposure to chronic levodopa significantly reduces behavioral and neurochemical efficacy of embryonic dopamine grafts. Further, dopamine terminal replacement prior to introduction of chronic levodopa is highly effective at preventing development of levodopa-induced dyskinesias, and GID-like behaviors occur regardless of pre-graft levodopa status.

Keywords

Parkinson's disease; levodopa; priming; rodent; grafting; dyskinesias

INTRODUCTION

Two landmark double-blind clinical grafting trials in individuals with Parkinson's disease (PD) have revealed that while some patients experienced clinical benefit, overall these trials failed to attain statistical significance in their primary behavioral endpoints (Freed et al., 2001; Olanow et al., 2003). Additionally, negative side-effects known as graft-induced dyskinesias (GIDs) developed in a significant number of dopamine (DA) graft recipients (Freed et al., 2001; Hagell et al., 2002; Olanow et al., 2003). While results from these most recent trials are disappointing and the utility of grafting is widely debated, the rationale of replacing cells lost to disease remains sound, and interest in therapeutic graft procedures continues. Prior to re-engaging in clinical grafting trials, resolution of issues underlying: 1) the lack of consistent clinical benefit, and 2) development of GIDs is needed. One primary focus in this regard has been to increase the survival of primary embryonic neurons as well as development of neural stem cell lines, which could serve as limitless sources of standardized cells. However, even when this primary challenge of limited cell numbers is overcome, cell replacement therapies may still face additional challenges that have yet to be identified. Indeed, there are clinical cases where large post-surgical increases in 18F-DOPA and/or large numbers of grafted tyrosine hydroxylase (TH) positive cells have been found postmortem, but without the degree of therapeutic improvement that would be predicted. Thus, while improving cell survival is vital, additional factors may still impose limitations to this therapy.

In this regard, all PD patients that have received embryonic dopamine neuron grafts to date have been treated for many years with the anti-parkinsonian medication levodopa. Despite the undisputed therapeutic benefit of levodopa, significant side effects such as the development of unwanted, involuntary movements known as dyskinesias limit its usefulness. Levodopa-induced dyskinesias (LIDs) result due to a sensitization phenomenon known as “priming”, the mechanisms of which remain elusive. In the parkinsonian striatum, where dopamine depletion itself significantly alters striatal proteins and the morphology of target neurons (e.g.: Brown et al., 2005; Day et al., 2006), exacerbation of a non-physiologic dopamine tone that accompanies standard levodopa administration results in further alterations in proteins and genes that are long-lasting, if not irreversible (Obeso et al., 2000; Olanow and Obeso, 2000; Rascol, 2000; Westin et al., 2001; Konradi et al., 2004). It is not unreasonable to propose that the array of changes that occur following long-term levodopa in the parkinsonian brain might contribute to suboptimal benefit in dopamine graft recipients and/or development of aberrant post-graft behaviors. Using the well-established 6-hydroxydopamine (6-OHDA) rat model of parkinsonism, we examined whether timing of levodopa treatment in relationship to placement of embryonic midbrain grafts had any impact on the efficacy of grafted neurons and/or development of GID-type behaviors recently described in this model (Steece-Collier et al., 2003; Maries et al., 2006; Carlsson et al., 2006; Soderstrom et al., 2008).

MATERIALS AND METHODS

An experimental timeline of all surgical procedures and treatments are shown in Fig. 1. Care and use of animals was in compliance with all applicable laws and regulations as well as principles expressed in the National Institutes of Health, United States Public Health Service *Guide for the Care and Use of Laboratory Animals*.

Nigrostriatal Dopamine Lesions

Adult male Fisher 344 rats (225-250 g at time of lesion) received a unilateral lesion of the nigrostriatal dopamine system with stereotaxic injections of 6-OHDA into both the left medial forebrain bundle and left substantia nigra. Lesions were performed at nigral

coordinates: 4.8 mm posterior to bregma, 1.7 mm lateral to midsagittal suture, and 7.5 mm deep from dura. Medial forebrain bundle coordinates were: 4.3 mm posterior to Bregma, 1.2 mm lateral to midsagittal suture, and 7.5 mm below dura. Animals were anesthetized prior to surgery with a Chloropent solution (3.0 cc/kg; chloral hydrate 42.5 mg/ml + sodium pentobarbital, 8.9 mg/ml) and placed in a stereotaxic frame. Aseptic techniques were used during all surgical procedures. The neurotoxin 6-OHDA (2.7 ug free base/ ul 0.9% saline with 0.2 mg/ml ascorbate) was injected at a rate of 0.5 ul/minute (2 ul total at each site) using a Hamilton 5 ul syringe with a 26-gauge needle. Stereotaxic coordinates were based on the Paxinos and Watson atlas (Paxinos W, Watson C. 2005. The Rat Brain in Stereotaxic Coordinates).

The presence of an adequate lesion was confirmed using amphetamine rotations. Rats were injected with amphetamine sulfate (5 mg/kg, i.p.; 0.1cc/100 g body weight) two weeks after the 6-OHDA lesion. Rotational behavior was monitored for 90 minutes using automated rotometers. Only rats rotating ipsilaterally at a rate of ≥ 6 turns per minute over 90 minutes were included in this study. We have found that rats with this rotational rate have > 95% nigral dopamine cell depletion and develop levodopa-induced dyskinesias (Steece-Collier et al., 2003).

Preparation of Tissue for Transplantation

Ventral mesencephalon (VM) from Fisher 344 rats of embryonic day 14 (E14) (crown-rump length = 10.0-11.5 mm) containing developing A8, A9 and A10 dopamine cell groups was dissected from the ventral floor of the midbrain flexure extending posteriorly to the isthmus, and laterally to the sulcus limitans (Lipton et al., 2008). After collection, VM tissue was pooled in 4°C calcium-magnesium free buffer, transferred to calcium-magnesium free buffer containing 0.1% trypsin, warmed to 37 °C for 10 minutes, rinsed in calcium-magnesium free buffer again, and triturated in 0.004% DNase using Pasteur pipettes of 1.0 mm and 0.5 mm tip diameter. The resulting suspension was pelleted by centrifugation at 500g for 10 minutes. The pellet was suspended in 1.0 ml of Neurobasal Media (Gibco), and cell number and viability was determined via trypan blue exclusion. Final suspensions were prepared at a density of 66,700 cells/ul and 100,000 cells/ul. During the transplant surgery, cells were placed on ice and used within 3-4 hours of preparation.

Dopamine neuron transplantation and levodopa administration

All successfully lesioned rats were divided into the following experimental groups (Figure 1): 200,000 Single-site graft; 200,000 Widespread graft ; 500,000 Widespread graft; Sham graft (as detailed below).

For both the levodopa-primed and non-primed cohorts, the following graft parameters were held constant. Rats in the 200,000 VM cell, single-site dopamine graft groups received 200,000 VM cells in a total volume of 3 ul (66,700 cells/ul) deposited at a single central striatal site (Figure 1C). Coordinates for the single injection into the striatum were anterior-posterior= +0, medial-lateral= +3.0, and dorsal-ventral= -5.5 from cranium.

Animals in the 200,000 VM cell, widespread dopamine graft groups received 200,000 VM cells dispersed into six separate sites to generate a more widespread distribution of the same number of cells as the 200,000 VM cell, single-site groups (Figure 1C). Each of these injection sites received 33,350 cells in 0.5ul volume (66,700 cells/ul) also resulting in a total volume of 3 ul. Coordinates for these six injections into the striatum were: anterior-posterior= +1.5, medial-lateral= +2.4, dorsal-ventral= -4.9 and -5.9 from cranium; anterior-posterior= +0, medial-lateral= +3.0, dorsal-ventral= -5.0 and -6.0 from cranium; and anterior-posterior -1.0, medial-lateral= +4.0, dorsal-ventral= -5.0 and -6.0 from cranium.

Animals in the 500,000 VM cell, widespread graft groups received 500,000 VM cells deposited in one rostral and one caudal striatal site. Each injection site in these animals received 250,000 VM cells in a total volume of 2.5 μ l (100,000 cells/ μ l), providing adjacent dense cell grafts. Coordinates for the two injections into the striatum were: anterior-posterior= +1.5, medial-lateral= +2.4, dorsal-ventral= -5.9 from cranium and anterior-posterior= +0, medial-lateral= +3.0, dorsal-ventral= -5.0 from cranium.

Grafted groups in this study were designed to control for transplanted cell number and for degree of graft-derived innervation as follows: 200,000 VM cell, single-site groups and 200,000 six-site groups received grafts that contained equal numbers (200,000) of VM neurons but have been shown to generate different patterns of innervation, “hot-spot” versus “widespread” (Maries et al., 2006) (Figure 1). The 200,000 VM cell, six-site and 500,000 VM cell, two-site groups received transplants that result in similar density of innervation surrounding each transplant site but are composed of different numbers of cells (200,000 vs. 500,000) (Figures 1).

In both levodopa-primed and non-primed cohorts, three groups of sham grafted animals (N= 3 – 4 each) were injected with cell-free vehicle media (Neurobasal) at one of three sets of stereotaxic coordinates; the three coordinates were the same stereotaxic coordinates as used in the three dopamine grafts paradigms (200,000 single-site, 200,000 six-site, 500,000 two-site). Thus, a third of sham animals received cell-free vehicle into a single site, a third into two sites, and a third into six-sites, as described above for DA grafted rats. Injection of identical volumes of fluid at identical coordinates in sham animals for each cohort was included to control for potential effect of tissue damage on experimental outcomes of each treatment.

For the non-primed groups (Figure 1A), unilaterally 6-OHDA lesioned, levodopa naïve rats were grafted with embryonic VM cells placed into the DA-depleted striatum four weeks after 6-OHDA lesion, but prior to any exposure to levodopa. Maturation and efficacy of grafts were tested at approximately 4, 6, and 8 weeks post-grafting by monitoring their rotational response to amphetamine sulfate (5 mg/kg, i.p.; 0.1cc/100 g body weight). One week after dopamine grafts were observed to reversed amphetamine-induced rotation, which corresponded to the ninth week after transplantation, rats began treatment with levodopa (12.5 mg/kg levodopa: 12.5 mg/kg benserazide, i.p.; 5 days/week) (Figure 1 A). While pharmacokinetics and drug responses are difficult to compare between species, the dose of levodopa for the current study was chosen because it is considered a moderately high dose for both rats and humans (e.g.: 875 mg/70 kg human=12.5 mg/kg rat). For these studies, we were interested modeling the impact of a levodopa dose equivalent that might be similar to that taken by some PD patients with advanced disease, the subset of patients thus far enrolled in clinical grafting trials. The vehicle groups received intraperitoneal injections of the vehicle sterile saline.

For the levodopa-primed groups (Figure 1B), after amphetamine confirmation of a successful unilateral 6-OHDA lesion, rats began treatment with levodopa (12.5 mg/kg levodopa: 12.5 mg/kg benserazide, i.p.; 5 days/week). This cohort of primed rats was treated for four weeks prior to grafting to induce stable, robust dyskinetic behaviors (Maries et al., 2006). After the four-week priming paradigm, embryonic dopamine neurons were grafted into the DA-depleted striatum as described above. One week of levodopa drug holiday was given per previous protocols to prevent negative interaction of newly grafted cells with levodopa (Steece-Collier et al., 1995; Lee et al., 2000; Maries et al., 2006). In levodopa primed and non-primed groups, the interaction of levodopa-induced dyskinetic behaviors and dopamine grafts was assessed for ten to twelve weeks after graft implantation, respectively (Figure 1).

Dyskinesia Behavior Rating

The word “dyskinesia” is used to describe the abnormal involuntary movements including dystonia, hyperkinesia, and/or stereotypies in experimental rats following levodopa administration. Chronic administration of levodopa to rats with unilateral striatal dopamine depletion results in abnormal involuntary muscle tone and abnormal involuntary movements involving the limbs on the side of the body contralateral to dopamine depletion as well as abnormal trunk and neck posturing toward the side of the lesion. Typical dyskinetic behaviors that are noted in sham-grafted rats, or in levodopa-treated rats prior to dopamine cell grafting, will be referred to as levodopa-induced dyskinesias (LIDs). Focal, generally stereotyped behaviors that are only noted to develop at extended post-graft time points, and fail to develop to any significant degree in sham-grafted rats will be referred to as graft-induced dyskinesias (GIDs) or GID-like behaviors.

Dyskinetic behaviors were rated three days per week (Wednesday-Friday) at approximately the same time each day, and by the same individual throughout the study. Ratings were performed by an investigator blinded to the treatment conditions and checked weekly with inter-rater reliability by a second blinded investigator. A daily dyskinesia ‘severity’ score was obtained by multiplying ‘frequency’ × ‘intensity’. ‘Intensity’ of LIDs was scored looking for specific indications of mild (score =1), moderate (score =2), or severe (score =3) dyskinesia of head, neck, forelimb and hindlimb contralateral to lesion, and trunk. Briefly, ‘intensity’ ratings included examination of specific attributes of dystonia displayed in these animals included claspings of forepaw, twisting of axial musculature, torsional movements beginning in the neck region, and twisting of limbs (details of which have been previously published, Steece-Collier et al., 2003; Maries et al., 2006). In contrast to general LIDs, ‘intensity’ of GID-like behaviors was either present or absent, and thus scores of 0= absent, 1= present. ‘Frequency’ rating for all dyskinetic behaviors was summed with the ‘intensity’ rating to allow refinement of the score by indicating time spent exhibiting this behavior; 0= absent, 1= <50% of the rating period, 2= >50% of the rating period but not constant, 3= constant. Dyskinetic behaviors were rated for two minutes at 30 minutes after the levodopa injection. Injections were timed to allow for observation of each rat at precisely 30 minutes after injection. Weekly dyskinesia severity scores were obtained by averaging the three daily severity scores for each week.

Necropsy and Preparation of Tissue

Animals were euthanized via transcardiac perfusion approximately 10-12 weeks after graft + levodopa interactions were evaluated (Figure 1). For perfusion, animals were anesthetized with a high dose chloropent solution, perfused transcardially with room temperature 0.9% saline (150 ml) followed by cold (100 ml) 0.9% saline and 100 ml buffered, fresh 4.0% paraformaldehyde. Brains were post-fixed for 24 hours in fresh 4.0% paraformaldehyde followed by transfer to 30% sucrose solution for 48-72 hours. Coronal brain sections were sectioned on a sliding microtome at 40 um thickness and were stored in a cryoprotectant solution before processing.

Immunohistochemistry

A series composed of every sixth section through the brain was used for immunohistochemical staining of tyrosine hydroxylase (TH) (Kordower et al., 1995; Steece-Collier et al., 1995). Animals from all groups were assayed at the same time. Sections were incubated with a monoclonal TH (1:4,000) primary antibody for 24 hours at 4°C. The mouse monoclonal anti-tyrosine hydroxylase (TH) antibody (clone LNC1, Millipore-Chemicon #MAB318; lot#0509010596) was raised against purified TH protein derived from PC12 cells. This antibody recognizes an epitope on the outside of the regulatory N-terminus, and detects a unique 59-61 kDa band on western blotting using human brain tissue and is widely

used (e.g.: Croft, B.G., et al., 2005; Gutierrez-Mecinas, et al., 2005; Sanchez-Gonzalez, et al., 2005). It does not cross-react with dopamine-beta-hydroxylase, phenylalanine hydroxylase, tryptophan hydroxylase, dihydropteridine reductase, sepiapterin reductase, or phenethanolamine-N-methyl transferase. It has been shown to stain embryonic rat mesencephalic dopaminergic neurons and rat dorsal root ganglia (manufacturer's information) and stained cells with the typical morphology and distribution of TH cells in our study. Sections were then incubated for 2.5 hours in goat anti-mouse (TH) biotinylated secondary antisera (1:400; Vector Laboratories, Burlingame, CA) and developed using diaminobenzadine (0.5 mg/ml TBS). Every 12th section through the striatum was processed for immunohistochemistry for the isoforms of FosB/ Δ FosB. Sections were incubated for 1 hr in blocking buffer (10 % goat serum in TBS), followed by incubation overnight at room temperature in antibody against FosB/ Δ FosB isoforms (rabbit polyclonal anti-FosB/ Δ FosB isoforms, Santa Cruz Biotechnology, CA) at a concentration of 1:2,000 in tris-buffered saline containing 1% goat serum. The rabbit polyclonal antiserum (Santa Cruz Biotechnology, CA; # sc-48) was raised against a recombinant protein corresponding to amino acids 102-117 within an internal region of FosB of mouse origin. It uniquely recognizes a FosB protein of 45kDa by Western Blot in activated rodent neurons (manufacturer's technical information). Staining of sections through the striatum produced a pattern of FosB/ Δ FosB immunoreactivity that had identical localization and response to drug treatment as reported in previous citations (e.g. Westin et al., 2001; Cenci 2002; Maries et al., 2006). The tissue was then incubated in biotinylated goat-anti rabbit IgG (Vector Laboratories, Burlingame, CA) at a concentration of 1:300 in tris-buffered saline containing 1% goat serum for 90 min at room temperature. Sections were developed using the avidin-biotin-peroxidase complex (Vector Laboratories, Burlingame, CA) and diaminobenzadine (0.5 mg/ml TBS). Control sections were processed in an identical manner except the primary antibody was omitted. Sections were mounted on gelatin-coated slides, dehydrated, and cover slipped with Permount. The slides were visualized using light microscopy.

Stereology of Grafted Neurons

The number of grafted TH-positive cells located within the striatum was estimated using an optical dissector sampling design (Gundersen et al., 1988; West et al., 1991; Kordower et al., 2001) by an investigator blinded to the grafting conditions. Approximately thirteen equally spaced sections were sampled along the entire length of the striatum of each brain. The section sampling fraction (ssf) was 1/0.25. The distance between sections was approximately 0.14 mm. The graft was outlined using a 1.25x objective. A systematic sample of the area occupied by the graft was made from a random starting point (StereoInvestigator 2000 software; MicroBrightField, Colchester, VT). Counts of TH-positive cells were made at regular predetermined intervals ($x = 100$ μ m, $y = 100$ μ m) and a counting frame (70×70 μ m = 4900 μ m²) was superimposed on the image of the tissue sections. The area sampling fraction (asf) was 1/0.49. These sections were then analyzed using a 100x planapo oil immersion objective with a 1.4 numerical aperture. The section thickness was empirically determined. Briefly, as the top of the section was first brought into the focus, the stage was zeroed at the z-axis by software. The stage was stepped through the z-axis until the bottom of section was in focus. Section thickness averaged 16.98 ± 0.77 μ m. The optical dissector height (counting frame thickness) was 9 μ m. This method allowed for 3 μ m top, and at least 3 μ m bottom guard zones. The thickness sampling fraction (tsf) was 1/0.53 in the graft. Care was taken to ensure that the top and bottom forbidden planes were never included in the cell counting. TH-positive cells were only counted if the first recognizable labeled profiles of the cell came into focus within the counting frame (West et al., 1991). Using the optical dissector principle, TH-positive cells in each case were sampled using a uniform, systematic, and random design procedure. The total number of TH-positive neurons within the grafts was calculated using the following formula: $N = \Sigma Q^- \cdot 1/ssf \cdot 1/asf$

- 1/tsf. ΣQ was the total number of raw counts. On average, there were no more than two countable profiles per dissector. Therefore the coefficients of error (CE) were calculated according to the procedure of Schmitz and Hof as estimates precision (West and Gundersen, 1990; West et al., 1996) and the values of CE ranged from 0.05 to 0.09.

Densitometry analysis of FosB/ Δ FosB-positive cells

We performed densitometry analysis of FosB/ Δ FosB in the dorsolateral striatum of levodopa-treated parkinsonian rats for correlation with LIDs (Maries et al., 2006). Three sections through the striatum at rostral, middle, and caudal levels (anterior-posterior + 1.70, +0.70, -0.80, respectively) were analyzed for each animal by an investigator blinded to the experimental treatment. Slides were visualized at 40X using light microscopy (Olympus BX60, Olympus, USA) and were digitized using a Nikon DM1200 camera and ACT software (Nikon Microscopy, USA). At each of the three striatal levels examined, two images were obtained for both the DA-depleted and intact striatum. The first image of striatal FosB/ Δ FosB-positive cells was obtained adjacent to the lateral border of the striatum at a mid-dorsoventral level (approximately 5mm ventral to dura; anatomically still considered dorsal striatum). The second imaged area was of the same size, adjacent and just medial to the first area. Densitometry of FosB/ Δ FosB-positive cells within the digitized images was performed using Sigma Scan Pro 5.0 software (Aspire Software International, Leesburg, VA). The threshold of light intensity that defined the selected cells was set at 50% of background light intensity. The investigator blinded to the experimental conditions selected each of the FosB/ Δ FosB-positive cells in the digitized images, which were then filled in by the software. A light intensity reading was generated for each FosB-positive cell. An average of 40 to 70 cells was sampled per image (giving a total of between 80 and 140 cell intensity samples per animal). An average light intensity was calculated for all FosB/ Δ FosB-positive cells present in the two adjacent images. The background level of light intensity was determined by tracing small areas within the striatum located between the FosB/ Δ FosB-positive cells and averaging their light intensity readings. The corrected FosB/ Δ FosB light intensity was obtained by subtracting the average background light intensity from the average light intensity obtained for the FosB/ Δ FosB-positive cells. The light intensity of the FosB/ Δ FosB-positive cells present in the lateral lesioned striatum at each striatal level (rostral, middle, and caudal) was expressed as a percent increase from the value found in the unlesioned (intact) striatum at the same level.

Analysis of FosB/ Δ FosB immunohistochemical staining intensity in the ventral striatum, to correlate with GIDs (Maries et al., 2006), was obtained from a striatal section just anterior to that which included the decussation of the anterior commissure (approximately AP -0.26) (Maries et al., 2006). This anterior-posterior level was chosen as it provided the most unequivocal anatomical landmarks for obtaining matching levels between subjects. This level also excluded nucleus accumbens and pallidum. The striatal section for each animal was visualized at 40X using light microscopy (Olympus BX60, Olympus, USA). Density measurements for the ventral striatum were obtained from two adjacent images. The location of the first image was adjacent to the lateral striatal border at the intersection with the anterior commissure; the second neighboring image was immediately medial (Maries et al., 2006). Images were obtained for both the lesioned and unlesioned (intact) striatum and were digitized using a Nikon DM1200 camera and ACT software (Nikon Microscopy, USA). Densitometry of FosB/ Δ FosB-positive cells within the digitized images was performed using Sigma Scan Pro 5.0 software (Aspire Software International, Leesburg, VA) by an investigator blinded to the treatment conditions as described above. The light intensity of the FosB/ Δ FosB-positive cells present in the ventrolateral striatum on the denervated hemisphere was expressed as a percent increase from the value found in the unlesioned (intact) striatum.

Striatal TH-positive Reinnervation

The distance in the rostral-caudal, dorsal-ventral, and medial-lateral plane occupied by TH-positive grafted neurites was calculated using an Olympus BX60 light microscope with a 10X objective. The investigator was blinded to the type of graft. A striatal region was considered positive for graft-derived fibers if at least 10 distinct and separate, darkly stained TH-positive fibers were evident in the striatum. None of striatal sections from sham animals fulfilled this criterion. Lesion parameters used here resulted in a greater than 99% reduction of TH-positive neurons in the lesioned substantia nigra ($99.65\% \pm 0.13\%$). To determine the rostral-caudal extent of TH-positive fibers, the entire series of brain sections containing striatum was examined. For the dorsal-ventral measurements a single site, which corresponded to the central graft site (anterior-posterior = +0, medial-lateral = +3.0) in the 200,000 single-site animals was examined. The extent of graft-derived innervation in each plane was computed for all animals using a micrometer and expressed in millimeters.

Statistical Analysis

Two-way repeated measures analyses of variance (RM-ANOVA) was performed for both primed and non-primed groups to determine significant effects of treatment, time or treatment $\times$ time interactions for dyskinetic behaviors. Significant differences of main effects were conducted using Bonferroni post-hoc analyses. To determine significant differences between primed and non-primed groups RM-ANOVAs were performed followed by Bonferroni post-hoc analyses for dystonic and graft-induced behaviors. On significant time-points of interest, one-way ANOVAs were performed followed by Fisher LSD post-hoc analyses to determine any effect of group for each behavior analyzed.

Differences in TH-positive cell counts, FosB/ Δ FosB immunohistochemistry intensity and extent of graft-derived reinnervation were analyzed using one-way ANOVAs, followed by Tukey's post-hoc analyses. Student t-tests were used to compare the extent of graft-derived reinnervation and FosB/ Δ FosB intensity between levodopa-primed and non-primed rats receiving the same type of transplant. Correlation between FosB intensity and LIDs was performed using a Spearman's correlation test. Sigma Stat 3.1 software (Aspire Software International, Leesburg, VA) was employed for all statistical analyses with a level of statistical significance set at 0.05.

RESULTS

Levodopa-induced dyskinesia

The components and characteristics of levodopa-induced dyskinesia in parkinsonian rats have been detailed in previous studies from our lab (Steece-Collier et al., 2003; Maries et al., 2006). Briefly, levodopa-induced behavioral abnormalities in parkinsonian rats consist of forelimb hyperkinesia, manifest as increased frequency of random and/or stereotypic movements, and dystonia. Dystonia was expressed in these rats as sustained muscle contractions resulting in twisting and abnormal postures or positions of various body parts including forelimb, hindlimb, trunk and neck. Hyperkinesia and/or dystonia always involved the limb and/or were oriented toward the side contralateral to DA-depletion and dopamine graft. In this study, all sham grafted animals displayed high levels of dyskinetic behaviors through the duration of the study (Figure 2). In contrast, grafting of embryonic VM cells into the DA-depleted striatum of rats resulted in a significant dampening of LIDs expression, the level of which was dependent on number of grafted cells and timing of levodopa administration as detailed below.

Reversing Levodopa-induced Dyskinesias: Dopamine Grafts in Levodopa-Primed Rats

In primed rats that received 4 weeks of chronic levodopa injections prior to grafting, all dopamine-grafted rats showed significant reduction in the levels of LIDs by 4 weeks post-grafting when compared to sham-grafted rats (week 4 post-grafting: $F^{3,27} = 7.73$, $p < 0.001$; Fig 2A). However, in levodopa-primed parkinsonian rats, neither the number of grafted cells (200,000 or 500,000) nor the number of injections sites (1, 2 or 6) affected the final antidyskinetic graft efficacy as there were no significant differences between grafted groups in the final weeks post-grafting ($p > 0.05$; Fig 2A). However, at 2 weeks post-grafting, rats receiving the largest number of cells (500,000) already showed a significant decrease in LIDs when compared with sham-grafted rats ($p = 0.028$). This early, significant post-graft reduction in LIDs was not seen in rats receiving 200,000 cells, regardless of number of injection sites ($p > 0.05$), demonstrating that while larger grafts in this model can result in a faster amelioration of levodopa-induced dyskinesias, the final impact was the same regardless of whether 200,000 or 500,000 VM cells were grafted.

Preventing Levodopa-induced Dyskinesias: Dopamine grafts in Non-Primed Rats

The cohort of parkinsonian rats designated as non-primed were levodopa naïve at the time of grafting. These rats received embryonic VM cells placed into the DA-depleted striatum, and grafts were allowed to mature for nine weeks prior to the introduction of chronic levodopa. In these rats, the presence of grafted dopamine neurons significantly *prevented* the development of LIDs. Specifically, the moderately high dose of 12.5 mg/kg levodopa when administered to parkinsonian rats with mature dopamine grafts, failed to elicit the same high level of LIDs noted in sham grafted rats (sham versus all dopamine grafted groups, week 4 through 12 post-levodopa: $F^{3,48} = 9.48$, $p < 0.001$; Fig 2B). In contrast to levodopa-primed rats, the impact of dopamine grafts in non-primed rats was influenced by the number of VM cells grafted (200,000 vs. 500,000 cells). Specifically, rats grafted with 500,000 VM cells showed generally less severe LIDs compared with their 200,000 cell grafted counterparts (week 4 and 12 ($p < 0.05$) post-levodopa administration) (Fig 2B). Behavioral efficacy in this model was not dependent on the number of injections sites ($p > 0.05$).

Levodopa-induced Dystonia: Primed versus non-primed

To examine more directly the impact of grafts between primed and non-primed rats, we compared the effect on total body dystonia scores between these two cohorts of rats (Fig 3). Total body dystonia involves dystonic posturing and/or movements of the forelimb, hindlimb, trunk and neck, but excludes forelimb hyperkinesias and orolingual behaviors because forelimb and orolingual behaviors are exacerbated by certain grafting paradigms (e.g.: GIDs; detailed below).

Comparison of total body dystonia between levodopa primed and non-primed rats revealed the following. First, the levodopa-primed cohort showed a significant reduction of dystonic behaviors that acclimated to the same level found in non-primed rats by three weeks post-grafting. Indeed, total body dystonia in the non-primed animals grafted with 200,000 VM cells (both single-site and six-site) was significantly lower than total dystonia in levodopa-primed animals with the same grafts only during the first weeks after grafting (Fig 3 A&B; $p < 0.05$). Thus, with a small-to-moderate number of VM cells, i.e.: 200,000, there is a similar overall anti-dyskinetic efficacy of dopamine grafts regardless of pre-graft levodopa exposure, or distribution of cells within the striatum of parkinsonian rats.

Second, levodopa-naïve, parkinsonian rats that received 500,000 VM cells prior to the introduction of levodopa showed an overall lesser degree of total body dystonia compared to levodopa-primed rats grafted with the same 500,000 cells (rating session 3: $t^{1,18} = 3.437$; rating session 6: $t^{1,18} = 2.543$ when compared to their primed counterparts; Fig 3C). Thus, it

appears that increasing the number of grafted cells provides for additional behavioral benefit in this model only when graft recipients have no prior history of chronic levodopa treatment.

Amphetamine-induced Rotational Behavior

To determine lesion success and an additional measure of post-graft behavioral recovery, primed and non-primed rats from all groups were challenged with amphetamine (5mg/kg), once following 6-OHDA and again on weeks 3-4 and 6-8 post-grafting (Fig 4 A-B). Two weeks post-lesion, but prior to graft implantation, primed rats, ($F^{3,27} = 0.01$, $p = 0.96$) and non-primed rats ($F^{3,48} = 0.12$, $p = 0.95$) in all groups showed a similar severity in amphetamine-induced contralateral turns per 90 minutes. By week 4 post-grafting, all dopamine-grafted groups showed significant improvements compared to sham groups (primed: $F^{3,27} = 14.63$, $p < 0.001$; non-primed: $F^{3,48} = 9.99$, $p < 0.001$) that were sustained through the last time points examined post-grafting (primed: $F^{3,27} = 43.64$, $p < 0.001$; nonprimed: $F^{3,45} = 17.70$, $p < 0.001$; Fig 4 A-B). This behavioral endpoint, which is relatively easy to impact with even small numbers of grafted dopamine neurons, showed no significant effect of priming at any of the time-points analyzed between primed and non-primed groups ($p < 0.05$).

FosB/ Δ FosB Immunohistochemistry in the Dorsolateral Striatum: Correlation with levodopa-induced dyskinesias

Increased expression of FosB/ Δ FosB transcription factors within the lateral striatum has been shown to be a reliable marker of dyskinetic behavior in rats treated with levodopa (Andersson et al., 1999; Winkler et al., 2002). Similar to these previous studies, we have found a positive correlation between the level of cellular FosB/ Δ FosB staining intensity in the dorsolateral dopamine-depleted striatum and levodopa-induced dyskinesia severity ($r = 0.459$, $p < 0.001$; Fig 5A). Dopamine grafts placed into levodopa-primed and non-primed subjects were capable of reversing (primed) or preventing (non-primed) the increase in FosB/ Δ FosB staining intensity in the dorsolateral striatum that is normally observed in levodopa-treated, sham-grafted animals ($F^{3,27} = 65.66$, $p < 0.001$) and non-primed ($F^{3,24} = 17.06$, $p < 0.001$) rats respectively (Fig 5B). However, dopamine grafts were significantly more effective at preventing this neurochemical change than reversing it once it was altered (200,000 cells to 1 site, primed versus non-primed: $t^{1,12} = 2.401$, $p = 0.03$; 200,000 cells to 6 sites, primed versus non-primed: $p = 0.616$; 500,000 cells to 2 sites, primed versus non-primed: $t^{1,13} = 2.16$, $p = 0.50$).

Graft-induced Dyskinesia: Primed versus non-primed

Similar to our previous findings (Maries et al., 2006), delivery of dopamine grafts to the dopamine-depleted striatum resulted in the development of novel (not seen prior to grafting) dyskinetic behaviors. While both primed and non-primed parkinsonian rats showed an emergence of novel, aberrant behaviors following implantation of embryonic dopamine neuron grafts, some characteristics of these GID-like behaviors varied.

Both levodopa-primed and non-primed rats displayed facial forelimb dyskinesia (FFD), which we have described in detail previously (Maries et al., 2006). Briefly, FFD is a goal-directed, repetitive, stereotypic grabbing and gnawing of cage litter involving the forepaw contralateral to the graft. However, a second graft-induced abnormal behavior was also noted in non-primed rats receiving dopamine grafts, which we will refer to in this text as tapping dyskinesia (TPD). This behavior also involved the forelimb contralateral to the graft but was manifest more simply as repetitive, stereotypic pushing or tapping of cage litter by rats with the forepaw contralateral to the graft and is described in detail elsewhere (Soderstrom et al., 2008). Again, as we noted previously, these GID-like behaviors were significant following levodopa administration only in dopamine-grafted animals, and were

not seen to any significant degree in non-grafted sham animals (Fig 6). To evaluate the effect of levodopa priming on GID-like behaviors, we have compared the expression of total graft-induced dyskinesias (FFD+TPD) between grafted rats receiving levodopa-priming versus non-primed rats (Fig 6).

A second difference was temporal in nature. Specifically, GID-like behaviors in levodopa-primed rats were more gradual, appearing as the grafted cells matured and other parkinsonian behavioral profiles improved (first significant occurrence at 4 weeks post-grafting; pre-transplant week -1 vs post-transplant $p < 0.005$; Maries et al, 2006; Fig 6B). In the non-primed rats, because the grafts were allowed to mature prior to introducing levodopa, GID-like behaviors were observed in the first week of levodopa treatment in dopamine-grafted rats (Fig 6A). A third difference was noted, with the larger, 500,000 cell grafts, where non-primed rats showed a small but significantly higher level of GIDs expression at later rating sessions compared to the primed rats ($p > 0.05$; Fig 6C).

Grafted Cell Number and Reinnervation: Primed versus non-primed

A detailed description of graft phenotype using the current grafting paradigms has been previously published by our group (Maries et al., 2006). Similar to our previous findings, when 200,000 VM cells were grafted into a single striatal site, there was a localized cylindrically-shaped cluster of densely distributed TH+ cells located primarily at the periphery of the graft (data not shown). Grafts containing the same number of grafted cells but delivered to 6 sites (3 rostral-caudal coordinates with two dorsal-ventral drops) appeared as 3 to 6 distinct deposits of TH+ neurons. Embryonic VM grafts containing 500,000 cells implanted at 2 separate rostral-caudal sites appeared as two distinct grafts within the dopamine-depleted striatum. Despite the differences in deposition of grafted cells, there was no significant difference between primed and non-primed groups in the total percentage of surviving cells regardless of the number of cells grafted or injection sites used (200,000 cells to 1 site: $t^{1,18} = 0.66$, $p = 0.52$; 200,000 cells to 6 sites: $t^{1,19} = -0.35$, $p = 0.73$; 500,000 cells to 2 sites: $t^{1,19} = 0.63$, $p = 0.53$; Fig 7). Further, there was no significant impact on TH-positive cell number between primed and non-primed rats with identical graft parameters (e.g.: single site 200,000 primed versus single site 200,000 non-primed) (Fig 7; stereological TH-positive cell numbers: 200,000 cells to 1 site: primed = 2887 ± 471 , non-primed = 2429 ± 437 ; 200,000 cells to 6 sites: primed = 2495 ± 447 , non-primed = 2873 ± 790 , 500,000 cells to 2 sites: primed = 9680 ± 2181 , non-primed = 7377 ± 1000).

While priming status had no impact on the overall survival of grafted TH-positive cells or general appearance of the grafts, it did impact the area of TH+ reinnervation surrounding the grafted cells (Fig 8). In rats receiving 200,000 cells to a single injection site there was no significant difference between the area of reinnervation between primed and non-primed rats in the rostral-caudal axis (RC: $p = 0.10$), or medial-lateral axis (ML: $p = 0.052$), however, there was a significant increase in reinnervation area in the dorsal-ventral axis (DV: $t^{1,17} = 5.348$, $p < 0.001$) with significantly greater innervation for non-primed rats. This differential increase in reinnervation area for non-primed rats was additionally seen in rats receiving 200,000 cells to 6 sites at all levels of analysis (RC: $t^{1,18} = 5.951$, $p < 0.001$; ML: $t^{1,18} = 5.964$, $p < 0.001$; DV: $t^{1,18} = 11.981$, $p < 0.001$).

Discussion

The primary findings of the current study are that in parkinsonian rats: 1) pre-exposure to chronic levodopa significantly reduced behavioral and neurochemical efficacy of embryonic dopamine neuron grafts, 2) dopamine terminal replacement prior to the introduction of chronic levodopa was highly effective at preventing development of LIDs, and 3) GID-like behaviors occurred regardless of pre-graft levodopa status.

Levodopa Priming and Graft Efficacy

Previous studies in parkinsonian rats have demonstrated that embryonic dopamine grafts placed into parkinsonian striatum after the induction of LIDs results in a significant decrease in the level of these preexisting behaviors over time (Lee et al., 2000; Steece-Collier et al., 2003; Maries et al., 2006; Carlsson et al., 2006; Lane et al., 2006). The current study extends our understanding of the interaction between dopamine grafts and dyskinetic behaviors by demonstrating that the ability of grafted dopamine neurons to reduce these previously established LIDs is apparently limited (i.e.: ceiling effect), and not necessarily dose dependent. Specifically, we report here that although the antidyskinetic efficacy of larger grafts had a more rapid time-course, increasing VM graft cell number from 200,000 to 500,000 had no additional overall benefit if the graft recipients were exposed to chronic levodopa prior to grafting. This loss of dose-dependent efficacy contrasts with what we observed when VM cells were grafted into levodopa-naïve rats. Specifically, in levodopa-naïve rats when more than twice as many VM cells (200,000 versus 500,000) were grafted, as might be anticipated, there was an approximately two-fold increase in anti-dyskinetic efficacy.

We have previously shown that levodopa exposure prior to and immediately following transplantation of embryonic dopamine neurons decreased viability of TH-positive cells (Steece-Collier et al., 1990). However, the current paradigm, which involved a one-week post-graft drug-holiday, had no deleterious affect on the number of surviving TH-positive cells. Thus, the same number of surviving TH-positive cells resulted in twice the behavioral efficacy when cells were grafted prior to the introduction of chronic levodopa. In spite of levodopa priming having no impact on the number of grafted TH-positive cells, the levodopa-primed group did show a small, but statistically significant decrease in the degree of TH-immunoreactive striatal innervation compared to the non-primed group. While this modest impact of levodopa priming on neurite outgrowth of grafted cells cannot be discounted, it is useful to note that while rats that received 200,000 VM cell grafted into 6 distinct sights showed up to a 50% greater (i.e. dorsal-ventral dimension) neurite outgrowth than when this same number of VM cells was grafted into a single site, the behavioral impact of these two graft paradigms on dyskinesias was identical. As such, these data suggest that mechanism(s) other than differential graft-derived neurite outgrowth are involved in decreasing the antidyskinetic efficacy of grafted neurons following levodopa priming in rats.

Preventing LIDs with Dopamine Terminal Replacement

While downstream mechanisms of dyskinetic movements remain uncertain, it is clear that presynaptic dopamine denervation together with intermittent drug delivery plays an important role in the development of abnormal involuntary movements following repeated administration of dopaminergic drugs such as levodopa (Mones et al., 1971; Langston and Ballard, 1984; Caligiuri and Lohr, 1993; Fahn 2000; DiMonte et al., 2000). The current study is the first to show that replacing dopamine terminals in the parkinsonian brain through grafting of embryonic midbrain neurons *prior* to the introduction of levodopa can provide long-term, stable and significant protection against dyskinesia development in a rodent model of parkinsonism. This protection against development of LIDs was realized despite 10-week chronic levodopa administration of a relatively high dose of levodopa (12.5 mg/kg). Indeed, in sham-grafted parkinsonian rats, chronic administration of this dose resulted in robust expression of dyskinetic behaviors within just one to four days. Further, as would be predicted from PD patients (Langston and Ballard, 1984; Caligiuri and Lohr, 1993) and parkinsonian non-human primates (DiMonte et al., 2000), the magnitude of antidyskinetic effect of dopamine terminal replacement in this rodent model of pre-levodopa grafting was dependent on the density of graft-derived dopamine terminals.

Practical Aspects of Preventing versus Reversing LIDs

In the rat and non-human primate models of PD, Δ FosB-like transcription factors that have been recognized to be critically involved in development of LIDs (Andersson et al., 1999; Cenci et al., 1999; Calon et al., 2000; Andersson et al., 2003). Specifically, the appearance of LIDs behaviors are accompanied by an upregulation Δ FosB-like immunoreactivity in the dorsolateral striatum, and anti-sense to this transcription factor can prevent their development (Cenci, 2002). Furthermore, dopamine grafts that down-regulate this factor can normalize (Maries et al., 2006) these abnormal behaviors. Similarly, postmortem histopathological analyses have demonstrated an increase in the level of FosB immunostaining in the putamen of dyskinetic PD patients compared to healthy control subjects (Cenci et al. 2002).

In the current study, in both primed and non-primed rats, levels of LIDs behaviors were correlated with FosB/ Δ FosB-immunoreactive optical density in the dorsolateral striatum. It is noteworthy that similar to dyskinesia behavioral indices, the largest impact of grafted dopamine cells on FosB/ Δ FosB-immunoreactive optical density was seen in the non-primed cohort of rats. Specifically, both LIDs and FosB/ Δ FosB-immunoreactive optical density were significantly lower in non-primed rats compared to levodopa-primed rats receiving the same single site 200,000 and dual site 500,000 VM cell grafts. These data could be taken to suggest that once pathological alteration of this factor occurs, grafted dopamine cells are limited in their ability to reverse the change. Taken together, the current data demonstrate that in the rat PD model, dopamine terminal replacement is more effective at preventing the development of dyskinesia behavior and alterations in the FosB/ Δ FosB transcription factor than it is at reversing them once they have occurred. Such data is in keeping with an abundant literature that has demonstrated that many pathophysiological alterations in genes and proteins in the dyskinetic brain are long lasting, if not reversible (e.g.: Obeso et al., 2000a, b; Rascol, 2000; Gerfen, 2000a, b; Westin et al., 2001). Thus, from a practical standpoint the best approach to treating levodopa-induced dyskinesias may be to prevent pulsatile dopamine stimulation (Fahn 2000) and consequently the occurrence of the priming phenomenon that generates them.

Historically, neural grafting has been approached as means to reversing symptoms of advanced PD, a task that has been met with limited success. An alternative view could be to envision terminal replacement, whether through grafting or dopamine neurotrophic factors, as a therapeutic approach to prevent development of dyskinesias. Benefits in a levodopa-naïve, early stage PD patient population would be anticipated to include positive symptomatic effects via replacement of dopamine in the environment of modest dopamine depletion, thereby delaying the requirement for levodopa therapy and initiating medication at lower dosages. This delay in levodopa treatment, decreased magnitude of dosing and potential decreased total duration of levodopa treatment may prevent the priming of levodopa dyskinesias for the effective functional lifetime of these patients. However, prior to considering such a use for grafting, understanding why grafting embryonic neurons into parkinsonian subjects, both clinically and in animal models, results in abnormal GID-type behaviors is critically needed.

Levodopa-priming and Graft-induced Dyskinesias

While differences have been noted between GID-like behaviors in individuals with PD and in parkinsonian rats, it has become clear that grafting embryonic dopamine neurons into the dopamine-depleted striatum results in emergence of focal, aberrant motor behaviors that were not present prior to grafting (Freed et al., 2001; Hagell et al., 2002; Olanow et al., 2003; Steece-Collier et al., 2003; Maries et al., 2006; Lane et al., 2006; Carlsson et al., 2006; Soderstrom et al., 2008). While the etiology of GIDs are uncertain, it is well established that

neuroleptic-induced dyskinesias (Meredith et al., 2000) and psychomotor stimulant sensitization (Robinson and Kolb 1997) produce structural changes of target neurons. Specifically, these classes of drugs, whose mechanisms of action are through alterations of dopamine neurotransmission, cause structural changes in synapses and dendrites indicative of drug-induced synaptic plasticity (for review see Harrison 1999). Recent data suggests that levodopa-induced sensitization and dyskinesia development also results in alterations of structural and synaptic plasticity (Konradi et al., 2004). Although future studies are needed to identify the nature of structural changes associated with LIDs, such structural abnormalities of target neurons would be anticipated to complicate therapeutic treatments for PD, including cell replacement strategies.

All PD patients that have received a dopamine graft to date have had a history of long-term levodopa treatment, and thus the role of levodopa priming in development of GID has been debated. Indeed, placement of a dopamine graft in a striatal environment with significantly altered neurochemistry or neuronal structural could be hypothesized to lead to establishment of abnormal or inappropriate connections between graft and host brain that could give rise to post-graft GID-type behaviors. In support of this idea, we have recently demonstrated that increases in specific aberrant synaptic features within the grafted striatum correlate statistically with the severity of GID-like behaviors in dopamine grafted, parkinsonian rats (Soderstrom et al. 2008). Such data strongly supports the idea that altered synaptic plasticity is linked to GID-like behaviors, however, the current study suggests that while pathological alterations that accompany levodopa priming can preclude some behavioral benefits of grafting, they do not appear to be necessary for development of post-graft behavioral abnormalities. Specifically, in dopamine grafted animals, either with or without pre-graft history of levodopa, a robust, novel (not seen prior to grafting) forelimb-facial stereotypic GID-like behavior emerged.

While the current study could be taken to indicate that dopamine terminal replacement into a severely DA-depleted striatum might always give rise to aberrant post-graft behavior (e.g.: regardless of levodopa history), additional issues warrant consideration prior to accepting this assumption. In the normal striatum, dopamine terminals from the substantia nigra predominantly synapse onto the neck of numerous dendritic spines found on target striatal medium spiny neurons (Freund et al., 1984; Groves et al., 1980). Dopamine depletion leads to a significant loss of these dendritic spines (McNeill et al., 1988; Ingham et al., 1998; Zaja-Milatovic et al., 2005; Stephens et al., 2005; Day et al., 2006). Thus, it is not surprising that grafted dopaminergic neurons form new synapses with dendritic shafts more often than spines (Freund et al., 1985; Mahalik et al., 1985; Bolam et al., 1988; Clarke et al., 1988, Triarhou et al., 1990; Stromberg et al., 1990; Soderstrom et al. 2008). In fact we recently found that this change in target is associated with an atypical post-synaptic phenotype between graft-derived innervation and the parkinsonian striatum, and statistically correlates with graft-induced motor abnormalities (Soderstrom et al., 2008). Thus, regardless of the approach to increase striatal dopamine terminals in the parkinsonian brain (e.g.: primary embryonic dopamine neurons, stem cell derived dopamine neurons, viral vector-induced trophic factors) it is possible that synaptic connectivity of new terminals with structurally altered target medium spiny neurons within the striatum may lead not only to aberrant behavior (i.e.: GID), but also preclude optimal therapeutic benefit. Taken together, the fact that dendritic spines are lost with increasing severity of DA-depletion, and that levodopa priming appears to induce pathological changes that reduce both behavioral and neurochemical indices of dopamine graft efficacy, it will be important to consider novel approaches to dopamine terminal replacement that may include early stage disease intervention.

Summary

While the mechanisms of the levodopa sensitization phenomenon that results in development of abnormal involuntary movements remain elusive, the current findings are consistent with the view that chronic exposure to levodopa induces stable changes in the biology of the basal ganglia that may not be conducive to amelioration by dopamine terminal replacement therapies. Indeed, the levodopa-primed, parkinsonian rat is more resistant to behavioral improvements mediated by grafted cells, less responsive to graft-mediated regulation of FosB transcription proteins in striatum, and presents a less permissive environment for graft-derived reinnervation. Chronic levodopa pre-treatment does not however appear to be a necessary factor in the development of GID-type behaviors in this model of PD. Continued investigation into the mechanisms of factors that generate aberrant interactions between grafted dopamine neurons and the host striatum to induce focal, aberrant behaviors in both clinical trials and animal models of PD are needed to allow continued consideration of dopamine terminal replacement strategies as part of the arsenal of therapeutics for PD.

Acknowledgments

The authors would like to acknowledge the outstanding technical assistance of Nathan Levine, Jennifer Stancati and Brian Daley. This work was supported by the National Institutes Neurological Disorders and Stroke (RO1NS045132, KSC).

Table of abbreviations used in manuscript text

PD	Parkinson's disease
GID(s)	Graft-induced dyskinesia
LID(s)	Levodopa-induced dyskinesia
VM	Ventral mesencephalon
TH	Tyrosine hydroxylase
6-OHDA	6-hydroxydopamine
FFD	Facial-forelimb dyskinesia
TPD	Tapping dyskinesia
RM-ANOVA	repeated measures - analysis of variance

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Figure 1. Experimental timeline

Animals were given a unilateral 6-OHDA-induced lesion of the nigrostriatal dopamine system, and lesion success confirmed with amphetamine rotations. Parkinsonian rats were grafted with either a localized single-site graft of 200,000 total VM cells, or widespread graft consisting of either 200,000 total VM cells deposited into 6 sites, or 500,000 total VM cells grafted into 2 sites. Sham grafted rats received cell-free media using all three implantation paradigms. (A) Grafts in the non-primed groups were allowed to mature prior to introduction of levodopa. Graft maturation and efficacy were tested in these animals with amphetamine rotations performed at 4, 6 and 8 weeks post-grafting. Nine weeks after grafting, this group began chronic levodopa (*12.5 mg/kg levodopa: 12.5 mg/kg benserazide, i.p.). (B) Primed groups received levodopa (*) beginning two weeks after 6-OHDA, and for four weeks prior to grafting. All animals were euthanized approximately 10 weeks after evaluation of graft + levodopa interaction. Abbreviations: LD= levodopa; Amph(et)= amphetamine; 200K= 200,000; 500K= 500,000. Stippled bar above timeline indicates levodopa administration.

Figure 2. Levodopa-induced Dyskinesia in Levodopa Primed (A) and Non-primed (B) rats
 (A) In rats primed with levodopa there was a significant groups effect ($F(3,185)=p<0.05$) with all dopamine grafted groups showing a significant decrease in dyskinesia severity from sham grafted rats by week 4 post-grafting ($*p<0.05$). It is notable that parkinsonian rats grafted with the largest VM cell number (500,000) showed a more rapid decrease, with significant difference from sham-grafted rats by 2 weeks post-grafting ($**p<0.05$). There was, however, no significant difference in any of the dopamine graft recipients, regardless of the graft cell number or distribution, after 4 weeks post-grafting ($p>0.05$). (B) In non-primed rats that began chronic levodopa 9 weeks post-grafting, again, all dopamine grafted groups showed significantly less LIDs compared with sham-grafted rats (weeks 12, 16, 20 post-grafting, $##p<0.05$). However, in contrast to primed rat, non-primed rats receiving 500,000 cells showed an additional significantly lower level of LIDs than rats receiving 200,000 cell grafts (weeks 12, $#p<0.05$; and 20 ($#p<0.05$) post-grafting).

Figure 3. Total Body Dystonia in Levodopa Primed versus Non-Primed Grafted Rats
Rats receiving 200,000 VM cell grafts to 1 site (A) or 200,000 cells to 6 sites (B) both showed a significant difference between primed and non-primed rats only at rating session 1 (the first rating session following grafting; * $p<0.001$, + $p=0.03$). For rats receiving 500,000 VM cell grafts (C) non-primed rats showed significantly less dystonia on rating sessions 1,3 and 6 (# $p<0.05$) compared to their levodopa primed counterpart.

Figure 4. Amphetamine-induced Rotations in Levodopa Primed (A) and Non-primed (B) Rats
 (A) Primed rats showed a significant decrease in amphetamine-induced rotational behavior weeks 4 through 8 post-grafting (* $p < 0.05$, all 200,000 VM grafts versus sham; ** $p < 0.01$, 500,000 VM grafts: 4 wks versus 8 weeks). (B) Non-primed rats showed a significant decrease in amphetamine-induced rotations at 4, 6 and 8 weeks post-grafting (+ $p < 0.05$, all VM grafts versus sham), with complete reversal in all three dopamine graft groups at week 8 post-grafting. Comparative analyses between primed and non-primed groups showed no significant differences at any of the time-points analyzed ($p < 0.05$).

Figure 5. Dorsolateral FosB/ Δ FosB Staining Intensity: Correlation with Levodopa-induced Dyskinesias

(A) The percent increase in FosB/ Δ FosB staining intensity in the dopamine-depleted dorsolateral striatum over that of the intact hemisphere correlated significantly with the severity of levodopa-induced dyskinesia in all rats ($p < 0.001$). (B) The implantation of dopamine cells to the dopamine-depleted striatum ameliorated the increase in FosB/ Δ FosB staining intensity in the dorsolateral striatum in all grafted groups in both primed (+ $p < 0.001$) and non-primed (++ $p < 0.001$) rats. In rats receiving 200,000 cells to 1 site (# $p = 0.03$) or 500,000 cells to 6 sites (## $p = 0.05$), non-primed rats showed a significantly greater decrease in FosB/ Δ FosB staining intensity compared to their levodopa primed counterpart.

Figure 6. Graft-induced Dyskinesia Profiles for Levodopa Primed (A) and Non-Primed (B) Grafted Rats

Rats receiving 200,000 VM cell grafts to 1 site or 200,000 VM cells to 6 sites showed no significant difference between primed and non-primed rats at all rating sessions ($p \geq 0.105$). (A) Primed rats grafted with 200,000 VM cells into a single site, but not grafted into 6 sites, had significantly elevated level of GIDs at rating sessions 8 and 12 compared to sham grafted rats ($p < 0.025$). Primed rats grafted with 500,000 VM cell showed significantly less graft-induced dyskinesia on rating session 3 ($p = 0.02$). (B) Non-primed rats grafted with 200,000 VM cells, either into 1 or 6 sites, had significantly elevated level of GIDs at rating sessions 4, 8 and 12 compared to sham grafted rats ($p < 0.018$). Rating Sessions: (A) For the primed cohort, the rating sessions corresponded to: '1' = -3 weeks (pre-graft), '2' = 2 weeks post-graft, '3' = 6 weeks post-graft, '4' = 10 weeks post-graft. (B) For the non-primed cohort, the rating sessions corresponded to: '1' = 9 wks post-graft (1 wk post-levodopa); '2' = 12 weeks post-graft, '3' = 16 weeks post-graft, '4' = 20 weeks post-graft.

Figure 7. TH- positive Graft Cell Number

The percentage of surviving TH-positive grafted cells (total TH-positive cells counted/total VM cells injected) did not differ significantly between any dopamine grafted primed and non-primed groups. (Raw cell counts: 200,000 cells to 1 site: primed= 2887 ± 471 , non-primed= 2429 ± 437 ; 200,000 cells to 6 sites: primed= 2495 ± 447 , non-primed= 2873 ± 790 , 500,000 cells to 2 sites: primed= 9680 ± 2181 , non-primed= 7377 ± 1000).

Figure 8. Area of TH-positive Innervation in Levodopa Primed and Non-primed dopamine Grafted Rats for the Rostro-caudal Axis (A), Medio-lateral Axis (B) and Dorso-ventral Axis (C) (A) TH-positive fiber outgrowth in the rostral-caudal axis, revealed a significant increase for non-primed rats receiving 200,000 cells delivered to 6 sites compared to their primed counterpart group (* $p < 0.001$). There was no significant difference between primed and non-primed rats in graft-derived reinnervation for rats receiving 200,000 cells to 1 injections site ($p = 0.10$) or 500,000 cells ($p > 0.05$). (B) In the medio-lateral axis, there was a significant increase in TH-positive outgrowth in the non-primed rats receiving 200,000 cells grafts to 6 sites (# $p < 0.001$), and 500,000 cells ($p < 0.01$). No significant effect of priming was noted in this dimension in rats receiving 200,000 cell grafts delivered to a single site ($p = 0.052$). (C)

In the dorso-ventral axis, there was a significant increase in TH-positive outgrowth in non-primed compared to primed groups for rats receiving 200,000 cell grafts to 1 site ($+p<0.001$) and 6 sites ($++p<0.001$), but not in rats receiving 500,000 cells ($p>0.05$).