A multitarget basal ganglia dopaminergic and GABAergic transplantation strategy enhances behavioural recovery in parkinsonian rats

K. Mukhida,1 M. Hong,1 G. B. Miles,2 T. Phillips,1 B. A. Baghbaderani,3 M. McLeod,1 N. Kobayashi,1 A. Sen,3 L.A. Behie,3 R. M. Brownstone2 and I. Mendez1

1Cell Restoration Laboratory, 2Motor Control Laboratory, Departments of Anatomy & Neurobiology and Surgery (Neurosurgery), Dalhousie University, Halifax, Nova Scotia, Canada and 3Pharmaceutical Production Research Facility, Schulich School of Engineering, University of Calgary, Calgary, Alberta, Canada

Correspondence to: Dr I. Mendez, MD PhD, Division of Neurosurgery, Queen Elizabeth II Health Sciences Centre, Room 3806, 1796 Summer Street, Halifax, Nova Scotia B3H 3A7
E-mail: mendez@dal.ca

The current transplantation paradigm for Parkinson’s disease that places foetal dopaminergic cells in the striatum neither normalizes neuronal activity in basal ganglia structures such as the substantia nigra (SN) and subthalamic nucleus (STN) nor leads to complete functional recovery. It was hypothesized that restoration of parkinsonian deficits requires inhibition of the pathological overactivity of the STN and SN in addition to restoration of dopaminergic activity in the striatum. To achieve inhibition, a multitargeted basal ganglia transplantation strategy using GABAergic cells derived from either foetal striatal primordia (FSP) cells or human neural precursor cells (hNPCs) expanded in suspension bioreactors was investigated. In hemiparkinsonian rats, transplantation of foetal rat dopaminergic cells in the striatum in conjunction with GABAergic grafts in the STN and/or SN promoted significant improvement in forelimb akinesia and motor function compared to transplantation of intrastriatal dopaminergic grafts alone or in conjunction with undifferentiated hNPCs.

In culture, FSP cells exhibited neuronal electrophysiological properties. However, recordings from GABAergic hNPCs revealed limited ionic conductances and an inability to fire action potentials. Despite this, they were almost as efficacious as FSP cells in inducing functional recovery following transplantation, suggesting that such recovery may have been mediated by secretion of GABA rather than by functional integration into the host. Thus, restoration of dopaminergic activity to the striatum in concert with inhibition of the STN and SN by GABAergic grafts may be beneficial for improving clinical outcomes in patients with Parkinson’s disease and potential clinical application of this strategy may be enhanced by the use of differentiated hNPCs.

Keywords: Parkinson’s disease; subthalamic nucleus; transplantation; GABA

Abbreviations: ChAT = choline acetyltransferase; DARPP-32 = dopamine and cyclic AMP-regulated phosphoprotein; DBS = deep brain stimulation; FSP = foetal striatal primordia; FVM = foetal ventral mesencephalic; GABA = gamma-aminobutyric acid; GFAP = glial fibrillary acidic protein; hNPCs = human neural precursor cells; MAP2 = microtubule-associated protein; SN = substantia nigra; SNr = substantia nigra pars reticulata; ST = striatum; STN = subthalamic nucleus; TUJ1 = β-III tubulin

Received November 30, 2007. Revised June 4, 2008. Accepted June 16, 2008

Introduction

Clinical trials of neural transplantation for the treatment of Parkinson’s disease (PD) have demonstrated that in only a subset of patients have modest functional outcomes been observed (Freed et al., 2001; Olanow et al., 2003). This may be related to the inability of ectopically placed dopaminergic grafts to normalize activity in other basal ganglia structures affected in Parkinson’s disease (Nakao et al., 1998), such as the subthalamic nucleus (STN) (Mitchell et al., 1989; Bergman et al., 1994; Hassani et al., 1996; Herrero et al., 1996; Vila et al., 1997; Lozano and Carella, 2002). According to the standard model of basal ganglia physiology, excessive activation of subthalamic glutamatergic efferents to basal ganglia output structures,
such as the substantia nigra pars reticulata (SNr), leads to impaired motor function by increasing inhibitory input to thalamocortical and brainstem motor systems (Alexander and Strick, 1990; DeLong, 1990; Parent and Hazrati, 1999a). Thus, subthalamotomies in animal and clinical studies (Bergman et al., 1990; Su et al., 2002; Alvarez et al., 2005), which reduce the activity of SNr neurons (Guridi et al., 1996), and deep brain stimulation (DBS) of the STN, which is thought to inhibit this structure by an as yet undefined mechanism [perhaps depolarization blockade (Burbaud et al., 1994; Benazzouz et al., 1995), depression of voltage-gated currents (Beurrier et al., 2001) or release of inhibitory neurotransmitters like gamma-aminobutyric acid (GABA, Boraud et al., 1996; Dostrovsky et al., 2000)], ameliorate parkinsonian motor symptoms (Kumar et al., 1998; Limousin et al., 1998; Martinez-Martín et al., 2002; Krack et al., 2003; Rodriguez-Oroz et al., 2005). This suggests that the efficacy of cell-based restoration of nigrostriatal dopaminergic function may be enhanced by concurrent inhibition of the STN and SNr.

The development of a strategy that utilizes GABAergic cell transplants in concert with the current dopaminergic striatal transplantation paradigm thus represents a promising therapy that addresses both the loss of dopaminergic function in the striatum (ST) as well as the overactivity of the STN and SNr. Potential clinical application of this strategy could be achieved by the availability of a reliable source of clinical-grade GABAergic cells. Human neural precursor cells (hNPCs) are attractive candidates for such a strategy because they can be efficiently expanded in large quantities in suspension bioreactors, allowing cell expansion to take place in a standardized, reproducible manner following good manufacturing protocols (Gilbertson et al., 2006). Mammalian neural precursor cells thus expanded have been shown to maintain cell cycle kinetics and intrinsic properties characteristic to precursor cells, such as self-renewal and multipotentiality, for extended periods of time (Kallos et al., 1999; Alam et al., 2004; Mukhida et al., 2005). Moreover, bioreactor-expanded hNPCs can be reliably differentiated into a GABAergic phenotype in vitro, maintain this phenotype post-transplantation into non-neurogenic regions of the central nervous system and provide inhibitory effects that ameliorate behaviours in other animal models of central nervous system dysfunction (Mukhida et al., 2007).

In the present study, a multitarget basal ganglia dopaminergic and GABAergic transplantation strategy for Parkinson’s disease was developed to investigate whether amelioration of complex sensorimotor behavioural deficits in hemiparkinsonian rats, such as forelimb motor function and akinesia, could be achieved. It was hypothesized that these complex sensorimotor deficits that are incompletely restored by foetal striatal dopaminergic grafts alone additionally require inhibition of the STN and SNr, which could be achieved using GABAergic grafts. Moreover, utilization of bioreactor-expanded hNPCs, differentiated into a GABAergic phenotype, was investigated as a clinically practical alternative to foetal GABAergic tissue.

### Materials and Methods

#### Animals and study design

Sixty-seven female Wistar rats (Charles River) weighing 200–225 g were used. Animals were housed in pairs at a constant temperature and humidity on a 12 h light/dark cycle with ad libitum access to food and water and allowed 7 days to acclimatize before surgery or behavioural testing. The experiments were conducted in accordance with the standards and procedures of the Canadian Council on Animal Care and the University Committee on Laboratory Animals.

Hemiparkinsonism was induced by lesioning the right nigrostriatal dopaminergic pathway with two stereotactic injections of 6-hydroxydopamine (6-OHDA, Mukhida et al., 2001). The hemiparkinsonian rats were randomly assigned to one of eleven treatment groups (Table 1): animals in control groups received (i) lesions only (n = 6) or (ii) injections of 400 000 embryonic day 14 rat ventral mesencephalic (FVM) cells in the ST alone (n = 6) or in conjunction with 400 000 undifferentiated foetal telencephalon-derived hNPCs in the (iii) STN (n = 4), (iv) SN (n = 4) or (v) both (n = 5). Other groups received transplants of FVM cells in the ST in conjunction with either 400 000 embryonic day 14 rat striatal primordia in the (vi) STN, (vii) SN

<table>
<thead>
<tr>
<th>Group</th>
<th>Striatum</th>
<th>Transplantation site</th>
</tr>
</thead>
<tbody>
<tr>
<td>(i)</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>(ii)</td>
<td>400 000 FVM cells</td>
<td>400 000 Undifferentiated HNPCs</td>
</tr>
<tr>
<td>(iii)</td>
<td>400 000 FVM cells</td>
<td>400 000 Undifferentiated HNPCs</td>
</tr>
<tr>
<td>(iv)</td>
<td>400 000 FVM cells</td>
<td>400 000 Undifferentiated HNPCs</td>
</tr>
<tr>
<td>(v)</td>
<td>400 000 FVM cells</td>
<td>400 000 FSP cells</td>
</tr>
<tr>
<td>(vi)</td>
<td>400 000 FVM cells</td>
<td>400 000 FSP cells</td>
</tr>
<tr>
<td>(vii)</td>
<td>400 000 FVM cells</td>
<td>400 000 FSP cells</td>
</tr>
<tr>
<td>(viii)</td>
<td>400 000 FVM cells</td>
<td>400 000 Differentiated HNPCs</td>
</tr>
<tr>
<td>(ix)</td>
<td>400 000 FVM cells</td>
<td>400 000 Differentiated HNPCs</td>
</tr>
<tr>
<td>(x)</td>
<td>400 000 FVM cells</td>
<td>400 000 Differentiated HNPCs</td>
</tr>
<tr>
<td>(xi)</td>
<td>400 000 FVM cells</td>
<td>400 000 Differentiated HNPCs</td>
</tr>
</tbody>
</table>
or (viii) both (n = 6 for each of these groups) or 400 000 hNPCs differentiated to a GABAergic phenotype in vitro in the (ix) STN, (x) SN or (xi) both (n = 8 for each of these groups). Functional recovery was assessed by amphetamine challenge after lesioning and 4 and 8 weeks after transplantation, and by cylinder, ramp, and adjusting step tests before and after lesioning and 3, 6, and 9 weeks after transplantation. Patch-clamp electrophysiological analyses were performed on cultured foetal striatal primordia (FSP) cells and undifferentiated and differentiated GABAergic hNPCs.

**Cell culture—foetal rat cells**

Ventral mesencephalic (E14) and the medial and lateral eminences of striatal primordia (E15) tissues were harvested from Wistar rat foetuses. Cell suspensions for each of these tissue types were prepared using mechanical dissociation as previously described (Mukhida et al., 2001). A final concentration of 200 000 cells/μl in 0.05% deoxyribonuclease (DNase, Sigma)/DMEM was prepared for each of the tissue types with viability exceeding 98% using the trypan blue dye exclusion method.

A sample of the FSP cells prepared for transplantation was plated on 13 mm poly-I-lysine-coated glass coverslips in 24-well plates at a density of 100 000 cells/ml in a medium containing 2% B27 (Stem Cell Technologies), 1% N2 (Gibco), 1% penicillin–streptomycin (Hyclone) and 1% foetal bovine serum (Hyclone) in DMEM. The cells were fed every 3 days by replacing 50% of the medium to differentiate the hNPCs also contained BDNF and valproic acid. After 7 days, the cells were fixed with 4% paraformaldehyde in 0.1 M phosphate buffer (PB) in preparation for immunocytochemistry.

**Cell culture—human neural precursor cells**

Telencephalon-derived hNPCs were harvested from a foetal brain (10 weeks gestational age) using protocols developed in our centre as part of our clinical programme of foetal tissue transplantation for Parkinson’s disease (Mendez et al., 2002, 2005). The tissue was prepared as a single cell suspension and transferred to suspension bioreactors for expansion as previously described (Mukhida et al., 2007, 2008). Using the suspension bioreactors, the overall cell-fold expansion was approximately 190 000 over 61 days in culture from passage level 2–6 (Mukhida et al., 2008).

For transplantation, cryopreserved bioreactor-expanded hNPCs were thawed and passaged two additional times in stationary tissue culture flasks. The resulting neurospheres were rinsed four times in 0.05% DNase/DMEM and incubated for 20 min in 0.25% trypsin at 37°C. The neurospheres were rinsed in 0.05% DNase/DMEM four more times before being mechanically triturated into a single cell suspension using a 1 ml Eppendorf pipette. The undifferentiated cells were centrifuged at 1000 rotations per minute for 5 min and the pellet was suspended in 0.05% DNase/DMEM to make a cell suspension for transplantation with a concentration of 200 000 cells/μl and viability exceeding 99%.

Bioreactor-expanded hNPCs were differentiated into a GABAergic phenotype using the methods outlined by Laeng and colleagues (2004). Neurospheres were dissociated into a single cell suspension as described and then cultured in the presence of brain-derived neurotrophic factor (BDNF; Regeneron; 50 nM) and valproic acid (Sigma; 5 mM). After one week of differentiation, the cells were collected and centrifuged at 1000 rotations per minute for 5 min. A single cell suspension with a final concentration of 200 000 cells/μl was prepared with viability exceeding 98%.

Undifferentiated and differentiated hNPCs were plated on 13 mm poly-I-lysine-coated glass coverslips at a density of 100 000 cells/ml for 7 days in a medium consisting of 2% B27, 1% N2 and 1% penicillin–streptomycin in DMEM as previously described (Mukhida et al., 2007). The medium to differentiate the hNPCs also contained BDNF and valproic acid. After 7 days, the cells were fixed with 4% paraformaldehyde in 0.1 M PB in preparation for immunocytochemistry.

**Electrophysiology**

Whole-cell patch–clamp recordings were made from FSP cells (n = 30), differentiated hNPCs (n = 12) and undifferentiated hNPCs (n = 7) cultured for 7 days. Experiments were performed in a recording chamber that was perfused continuously with oxygenated artificial cerebrospinal fluid (aCSF) at room temperature using solutions previously described (Miles et al., 2004). Signals were recorded from cells visualized with differential interference contrast microscopy using patch electrodes (resistance 4.0–5.0 MΩ) pulled on a Sutter P-87 puller (Sutter Instrument Company) from 1.5 mm outer diameter filamented borosilicate glass (World Precision Instruments). Signals recorded were amplified using a Multi-clamp 700B amplifier (Molecular Devices) and acquired at ≥10 kHz using a Digidata 1322A digitizer and pClamp software (Molecular Devices). Series resistance, whole-cell capacitance (Cm) and input resistance (Rm) values were calculated using pClamp 9 software. An on-line P4 leak subtraction protocol (Heineman, 1995) was used for all recordings of voltage-activated currents. All voltage- and current-clamp protocols are described in Results. Signals were analysed off-line using Clampfit software (Molecular Devices). Alexa Fluor 594 (Invitrogen) was added to all pipette solutions before the experiments with a final concentration of 0.2%. For experiments investigating FSP post-synaptic currents (n = 16), bicuculline (10 μM) was added to the perfusate.

**Transplantation**

Three weeks post-lesioning, rats were stereotactically transplanted ipsilaterally to the 6-OHDA lesion using a glass microcapillary with an outer opening diameter of between 50 and 70 μm attached to a 2 μl Hamilton microsyringe at a rate of 1 μl of cell suspension per minute as previously described (Nikkhah et al., 1994; Mukhida et al., 2001). All rats, except those that received lesions only, received a total of 400 000 FVM cells in the ST equally divided between the following coordinates (in millimetres and with reference to bregma and the surface of the brain): (i) AP +1.3, ML −2.1, DV −5.5 and −4.3; (ii) AP +0.6, ML −2.9, DV −5.5 and −4.3 and (iii) AP +0.3, ML −3.7, DV −5.5 and −4.3, with the incisor bar set at 3.3 mm below the interaural line. For animals that also received intranigral grafts of either FSP cells, differentiated hNPCs, or undifferentiated hNPCs, a total of 400 000 cells were transplanted in the SN, equally divided between the following coordinates (in millimetres and with reference to bregma and the surface of the brain): (i) AP −4.8, ML −2.0 and DV −8.3 and −8.1; (ii) AP −5.0, ML −2.3 and DV −8.2 and −8.0; and (iii) AP −5.3, ML −2.6 and DV −8.1 and −7.9. Animals that received STN grafts received a total of 400 000 FSP cells, differentiated hNPCs, or undifferentiated hNPCs in this structure, equally divided between the following two sites (in millimetres and with reference to bregma and the surface of the brain): (i) AP −3.8, ML −2.6 and DV −8.0 and (ii) AP −3.8, ML −2.2 and DV −8.2. All animals that were transplanted with hNPCs received cyclosporine (Novartis, 10 mg/kg, intraperitonial) daily beginning two days prior to transplantation and until they were sacrificed.
Behavioural assessment

Rotational behaviour
Rats were challenged with amphetamine (3 mg/kg, intraperitoneal) 3 weeks after being lesioned and 4 and 8 weeks after transplantation. Rotational behaviour was monitored for 70 min using a computerized video activity monitoring system (Columbus Instruments).

Sensorimotor testing
All behavioural tests were performed blinded by the same investigator between 0700 and 1200 h pre- and post-6-OHDA lesioning and 3, 6 and 9 weeks post-transplantation. A second investigator recorded data, ensured that tests were technically performed in a consistent manner, and verified results.

(i) The adjusting step and initiation time components of the stepping tests were used to assess forelimb function and the motivational component of akinesia, respectively (Olsson et al., 1995; Chang et al., 1999; Deumens et al., 2002). The animals were trained once per day for 2 weeks prior to baseline testing. The adjusting step test involved immobilizing the hind limbs and one forepaw as the rats were moved across a 0.9 m wooden plank at a consistent speed over 5 s in each of the backhand and forehand directions. The total number of adjusting steps made with the free forepaw to maintain balance was recorded. The initiation time component of the test involved attaching the wooden ramp to the animals’ home cage, holding the animals in a similar manner as in the adjusting step test and determining the time, for each forelimb, required to initiate movement up the ramp to the cage. The first three scores obtained for each forelimb during each adjusting step and initiation time test session were used for analysis;

(ii) Forelimb akinesia was assessed using the cylinder test (Lundblad et al., 2002). The number of wall contacts made with full appositions of the left and right forepaws was counted up to a total of 50 contacts. The test evaluates voluntary preference for forelimb use, since rats demonstrate a lack of motivation to use the impaired forelimb if given that option (Ewenden and Robbins, 1984; Barne´oud et al., 2000), and therefore provides information distinct to that provided by the stepping test in which rats are not permitted to choose which forelimb to use.

Immunocyto- and immunohistochemistry

Ten weeks post-transplantation, the rats received an anaesthetic overdose and were transcardially perfused with 300 ml of ice-cold 0.1 M PB followed by 300 ml of ice-cold 4% paraformaldehyde in 0.1 M PB. The brains were extracted, post-fixed in 4% paraformaldehyde in 0.1 M PB for 24 h and then cryoprotected in 30% sucrose in 0.1 M PB at 4°C for 24 h. Coronal or sagittal sections (40 μm) were cut serially on a freezing microtome.

Immunocyto- and immunohistochemistry was performed on cultured cells and brain sections, respectively, as previously described (Mukhida et al., 2007). Primary antibodies used included rabbit anti-GABA (1:1000, Sigma), rabbit anti-glutamic acid decarboxylase 65/67 (GAD 65/67, 1:1000, Chemicon), rabbit anti-glial fibrillary acidic protein (GFAP, 1:2000, Sigma), mouse anti-human nuclei (HuN, 1:1000, Chemicon), rabbit anti-microtubule-associated protein (MAP2, 1:1000, Chemicon), rabbit or chicken anti-BII tubulin (TUJ1, 1:1000, Chemicon), rabbit anti-nestin (1:1000, Chemicon), rabbit anti-serotonin (1:1000, Chemicon), rabbit anti-Ki67 (1:1000, Chemicon), rabbit anti-choline acetyltransferase (ChAT, 1:1000, Chemicon), rabbit anti-CD3 (1:100, Biocare) or rabbit anti-Iba1 (1:1000, Wako). Secondary antibodies used included goat anti-rabbit Alexa 488 (1:300, Molecular Probes), goat anti-mouse Alexa 555 (1:300, Molecular Probes) or goat anti-chicken Alexa 633 (1:300, Molecular Probes). Standard ABC methodology was used to process cells for tyrosine hydroxylase (TH) and dopamine and cyclic AMP-regulated phosphoprotein (DARPP-32) immunohistochemistry using rabbit anti-TH (1:2500, Pel-Freeze Biologicals) and rabbit anti-DARPP-32 (1:1000, Chemicon) (Mukhida et al., 2001).

The proportion of transplanted undifferentiated and differentiated hNPCs that demonstrated immunoreactivity for GFAP, GABA or TUJ1 were determined using methods modified from those of Karimi-Abdolrezaee and colleagues (2006). Two brain sections that contained hNPC grafts from each of three randomly selected animals that were transplanted with undifferentiated hNPCs and from each of three randomly selected animals that were transplanted with differentiated hNPCs were selected. For each grafted section, confocal microscopy using the 40× objective was used to obtain a Z-stack from each of the medial, central and lateral aspects of each of the grafts. The proportion of HuN-immunoreactive cells within each Z-stack that expressed GFAP, GABA or TUJ1 was quantified.

The total number of surviving transplanted undifferentiated and differentiated HuN-immunoreactive cells transplanted in the SN and/or STN, TH-immunoreactive cells in the ST and DARPP-32-immunoreactive cells transplanted in the SN and/or STN were estimated in an unbiased manner stereologically using the optical fractionator probe (Sterio, 1984; West et al., 1991) and stereological software (Stereo Investigator 6.01) by two blinded investigators. The optical fractionator probe was used on every fourth immunostained section throughout the grafted areas and consisted of a 100 × 100 μm counting frame with a height of 11.5 μm for the HuN-immunoreactive cells, a 110 × 110 μm counting frame with a height of 11.5 μm for the TH-immunoreactive cells and a 120 × 120 μm counting frame with a height of 18 μm for the DARPP-32-immunoreactive cells. The sampling grid (300 × 300 μm for the HuN grafts, 185 × 185 μm for the TH grafts and 120 × 120 μm for the DARPP-32 grafts) and counting grids were randomly placed in the grafted areas. The section thickness was estimated every fifth dissector measurement and then averaged for each section.

The volumes of the TH-immunoreactive FVM grafts in the ST and DARPP-32-immunoreactive FSP grafts in the STN and/or SN were assessed using the Cavalieri method on every fourth section throughout the grafted area.

TH-immunoreactive reinnervation of the ST by FVM grafts was assessed by optical density using a densitometry software program (Scion Image Beta 4.03, National Institutes of Health). The relative
optical density of TH-immunoreactivity in the transplanted ST compared to that in the unlesioned ST was calculated [in order to correct for non-specific background staining (Kirik et al., 2001)]. The graft deposits were excluded from optical density measurements. The TH-immunoreactive fibre outgrowth in the transplanted ST was expressed as the optical density measurement for the transplanted ST as a percentage of the score for the contralateral ST. The sections used for analysis included one rostral to the graft, two through the grafted area and one caudal to the graft.

**Statistical analyses**

Behavioural test scores and stereological results are presented as mean ± standard error of the mean throughout. Between and within group differences for behavioural tests were assessed using a mixed design two-way analysis of variance (ANOVA) followed by Bonferroni’s post hoc test when appropriate (only animals for which complete data sets were obtained were included for analysis). Between group differences for transplanted cell survival (TH-, DARPP-32 and HuN-immunoreactive cell survival), graft volume (TH- and DARPP-32 immunoreactive graft volumes) and TH-immunoreactive fibre density were assessed for between group differences using a one-way ANOVA followed by Bonferroni’s post hoc test when appropriate.

**Results**

**Behavioural studies**

**Rotation test**

A mixed design two-way ANOVA used to analyse the rotational behaviour of the groups over time revealed a statistically significant mean effect of time \( [F(2, 70) = 178, P<0.0001] \) and group \( [F(10, 70) = 6.121, P<0.0001] \) and a significant interaction \( [F(20, 70) = 2.207, P = 0.0080] \). Prior to transplantation, there was no significant difference between groups in the number of amphetamine-induced rotations \( (P>0.05, \text{Fig. 1}) \). By 4 weeks after transplantation, all rats that received intrastriatal FVM grafts demonstrated significant attenuation of this behaviour \( (P<0.05 \text{ for within-group differences}) \), and there was no significant difference between these groups in the number of rotations made at either 4 or 8 weeks post-transplantation \( (P>0.05 \text{ for between group-differences}) \).

**Cylinder test**

A mixed design two-way ANOVA used to analyse spontaneous forepaw motor function in the cylinder test demonstrated a statistically significant mean effect of time \( [F(4, 204) = 131.2, P<0.0001] \) and group \( [F(10, 204) = 5.382, P<0.0001] \) and a significant interaction \( [F(40, 204) = 2.880, P<0.0001] \). There was no significant bias in spontaneous forepaw use during the cylinder test prior to 6-OHDA lesioning (between-group differences \( P>0.05, \text{Fig. 2} \)). After lesioning, the left forepaw was held flexed and the right forepaw was preferentially used to explore the cylinder \( (P<0.05 \text{ for within-group differences}, P>0.05 \text{ for between group differences}) \). Analysis of within-group differences revealed that a significant increase in the proportion of left forepaw touches compared to post-lesion scores was exhibited as early as 6 weeks post-transplantation by animals that received intrastriatal FVM grafts in conjunction with FSP grafts in the STN (0.38 ± 0.09, 6 weeks post-transplantation versus 0.14 ± 0.04, post-lesion), FSP grafts in both the STN and SN (0.49 ± 0.04 versus 0.14 ± 0.03), differentiated hNPC grafts in the SN (0.31 ± 0.06 versus 0.13 ± 0.02) or differentiated hNPC grafts in the STN and SN (0.35 ± 0.07 versus 0.23 ± 0.03). By 9 weeks post-transplantation, animals that received intrastriatal FVM grafts in conjunction with either foetal-derived GABAergic grafts in the STN or both the STN and SN or hNPC-derived GABAergic grafts in the STN, SN or both demonstrated significant improvement compared to post-lesion scores \( (P<0.05 \text{ for within-group differences}, \text{9 weeks post-transplantation scores versus post-lesion scores 0.36 ± 0.05 versus 0.14 ± 0.04, 0.54 ± 0.05 versus 0.14 ± 0.03, 0.34 ± 0.06 versus 0.17 ± 0.01, 0.30 ± 0.06}) \).

![Fig. 1 Amphetamine-induced rotational behaviour of rats after 6-OHDA lesions and 4 and 8 weeks after transplantation. Animals that received FVM grafts in the ST demonstrated significant attenuation of rotational asymmetry by 4 weeks post-transplantation. * represents a significant difference compared to post-lesion scores (within-group differences, P<0.05) as well as compared to scores for the lesion-only group (between-group differences, P<0.05).](https://academic.oup.com/brain/article-abstract/131/8/2106/268174)
Fig. 2 Spontaneous motor forelimb function was assessed using the cylinder test pre-lesion, post-lesion and 3, 6 and 9 weeks post-transplantation. All animals demonstrated a significant decrease in the proportion of left forepaw touches post-lesion. By 6 weeks post-transplantation, only rats that received FVM grafts in the ST as well as FSP grafts in the STN or STN and SN, or that received differentiated hNPC grafts in the STN, SN or both demonstrated significant improvement of left forepaw motor use. At 9 weeks post-transplantation, animals that received either FSP or differentiated hNPC basal ganglia grafts as well as FVM grafts in the ST demonstrated significantly improved cylinder test scores compared to animals that received FVM grafts in the ST alone. [0] represents a statistically significant difference at $P<0.05$ compared to group pre-lesion scores (within-group differences); ” represents a statistically significant difference at $P<0.05$ compared to scores for the lesion only group (between-group differences); # represents a statistically significant difference at $P<0.05$ compared to scores for animals that received FVM grafts in the ST only (between-group differences); # represents no statistically significant difference at $P>0.05$ compared to scores pre-lesion (within-group differences).

versus $0.13\pm0.02$ and $0.39\pm0.08$ versus $0.23\pm0.03$, respectively). Animals that received FSP grafts in the STN and SN demonstrated normalization of this behaviour by 6 and 9 weeks post-transplantation, respectively ($P>0.05$ for within-group differences for 6 and 9 week post-transplantation scores compared to pre-lesion scores). Analysis of between-group differences revealed that although by 6 weeks post-transplantation animals that received intrastriatal FVM grafts in conjunction with either FSP grafts in the STN or differentiated hNPC grafts in the SN demonstrated significantly greater recovery of forelimb motor function compared to animals that were not transplanted or received intrastriatal FVM grafts alone ($P<0.05$), these improvements did not persist to 9 weeks post-transplantation ($P>0.05$). In contrast, animals that received intrastriatal FVM grafts as well as either FSP or differentiated hNPC grafts in the STN and SN demonstrated significantly improved scores in the cylinder test compared to animals that were not transplanted or received intrastriatal FVM grafts alone ($P<0.05$) that persisted for the duration of the experiment ($P<0.05$).

**Adjusting step test**

In the adjusting step test, the total number of steps that the rats made in the forehand and backhand directions was recorded. Rats made $24.2\pm0.18$ steps with the left forepaw prior to 6-OHDA lesions, which was not significantly different than the right forepaw scores ($24.7\pm0.17$ steps, unpaired two-tailed t-test $P>0.05$, Fig. 3). A mixed design two-way ANOVA used to analyse the number of adjusting steps made by the right forepaw revealed a statistically significant mean effect of time [$F(4, 640)=16.50$, $P<0.0001$] and group [$F(10, 640)=3.92$, $P<0.0001$] and a significant interaction [$F(40, 640)=2.34$, $P<0.0001$]. Overall, the number of adjusting steps made with the right forepaw was consistent within the groups at all time points, but there were differences in the number of steps made by animals that received intrastriatal FVM grafts in conjunction with FSP STN grafts and undifferentiated hNPC grafts in the STN or both the SN and STN compared to animals that were not transplanted at 3 weeks post-transplantation, post-lesion and 9 weeks post-transplantation and post-lesion and 9 weeks post-transplantation, respectively ($P<0.05$). A mixed design two-way ANOVA used to analyse the number of adjusting steps made by the left forepaw revealed a statistically significant mean effect of time [$F(4, 640)=1803$, $P<0.0001$] and group [$F(10, 640)=64.96$, $P<0.0001$] and a significant interaction [$F(40, 640)=49.98$, $P<0.0001$]. Lesions impaired the ability to make adjusting steps with the left forepaw such that the limb was dragged with lack of coordination in the backhand and forehand directions; the mean number of left forepaw steps for all treatment groups significantly decreased to between $1.33\pm0.40$ steps and $4.0\pm0.72$ steps ($P<0.05$). By 3 weeks post-transplantation, only animals that received either FSP- or hNPC-derived GABAergic grafts in the STN, SN or both in conjunction with intrastriatal FVM grafts demonstrated significantly improved forelimb motor function ($P<0.05$ for within group differences), with adjusting step scores ranging from $13.7\pm1.1$ to $17.4\pm1.2$ steps. Adjusting step scores for the left forepaw
continued to improve over the duration of the experiment. The improvement that these animals that received either FSP- or hNPC-derived GABAergic grafts demonstrated at 3, 6 and 9 weeks post-transplantation also was significantly different than the scores for rats that were not transplanted or received intrastriatal FVM grafts only ($P < 0.05$ for between-group differences).

**Initiation time test**

After training, animals were able to initiate movement up the ramp with either forelimb immediately after positioning (Fig. 4). There was no significant difference in these times between the forepaws, which ranged from mean $0.61 \pm 0.05$ and $0.93 \pm 0.07$ s for the right forepaw and mean $0.61 \pm 0.03$ and $0.96 \pm 0.08$ s for the left forepaw (unpaired two-tailed $t$-test, $P > 0.05$). There was no significant difference in right forepaw initiation times between groups as determined by a mixed design two-way ANOVA [statistically insignificant mean effect of time $[F(4, 520) = 0.7286, P = 0.5727]$ and group $[F(10, 520) = 1.341, P = 0.2153]$ but a significant interaction $[F(40, 520) = 1.782, P = 0.0028]$]. Lesions induced an apparent increase in initiation times for the left forelimb, which increased from mean $3.89 \pm 0.48$ to $14.9 \pm 7$ s, but this did not reach statistical significance due to the large variability in initiation times ($P > 0.05$). There was no difference in post-transplantation scores between groups that received intrastriatal FVM grafts alone or in conjunction with other basal ganglia grafts ($P > 0.05$).

**Histological analyses**

Cultured FSP cells were immunoreactive for DARPP-32 and the majority of these cells were immunoreactive for GABA (Fig. 5A), GAD 65/67 (Fig. 5B), TUJ1 and MAP2 (Fig. 5C). Undifferentiated hNPCs demonstrated multipotentiality in vitro and differentiated into cells immunoreactive for nestin, GFAP, TUJ1 and MAP2. Culturing hNPCs in the presence of BDNF and valproic acid significantly increased the proportion of cells immunoreactive for GABA (Fig. 5D).
and TUJ1 (Fig. 5E) and decreased the proportion of cells immunoreactive for GFAP (Fig. 5F) or nestin (Fig. 5G). We have previously reported the results of stereological analyses of cultured hNPCs and found that in BDNF- and valproic acid-supplemented cultures, 93.09 ± 3.70% and 74.59 ± 6.34% demonstrated immunoreactivity for GABA and TUJ1, respectively (Mukhida et al., 2007). In contrast, fewer hNPCs cultured in non-supplemented media demonstrated GABAergic (13.14 ± 2.57%) or neuronal phenotypes (30.74 ± 1.40%) (Mukhida et al., 2007).

All animals that received transplants of FVM cell suspensions demonstrated viable grafts at 10 weeks post-transplantation that were composed of numerous TH-immunoreactive cell bodies and fibres that restored some of the TH-immunoreactive neurophil lost due to the 6-OHDA lesions (Fig. 6). There were no significant differences [F(9, 50) = 0.7586, P = 0.6543] in the mean number of surviving transplanted TH-immunoreactive cells within the striatal grafts between any of the groups (FVM in ST only = 3901 ± 776 cells; FVM in ST and FSP in STN = 3396 ± 277 cells; FVM in ST and FSP in SN = 4269 ± 1173 cells; FVM in ST and FSP in STN and SN = 3936 ± 601 cells; FVM in ST and differentiated hNPCs in STN = 3850 ± 556 cells; FVM in ST and differentiated hNPCs in SN = 2443 ± 2487 cells; FVM in ST and differentiated hNPCs in STN and SN = 2904 ± 584 cells; FVM in ST and undifferentiated hNPCs in STN = 2870 ± 604 cells; FVM in ST and undifferentiated hNPCs in SN = 2842 ± 455 cells and FVM in ST and undifferentiated hNPCs in STN and SN = 3183 ± 778 cells). Also, there were no statistically significant differences in TH-immunoreactive graft volumes between groups of animals that received FVM grafts in the ST [F(9, 49) = 1.313, P = 0.2548]; FVM in ST only = 0.759 ± 0.18 mm³; FVM in ST and FSP in STN = 1.12 ± 0.28 mm³; FVM in ST and FSP in SN = 0.762 ± 0.20 mm³; FVM in ST and FSP in STN and SN = 1.03 ± 0.16 mm³; FVM in ST and differentiated hNPCs in STN = 0.667 ± 0.087 mm³; FVM in ST and differentiated hNPCs in SN = 0.571 ± 0.15 mm³; FVM in ST and differentiated hNPCs in STN and SN = 0.724 ± 0.14 mm³; FVM in ST and undifferentiated hNPCs in STN = 1.35 ± 0.45 mm³; FVM in ST and undifferentiated hNPCs in SN = 0.919 ± 0.36 mm³ and FVM in ST and undifferentiated hNPCs in STN and SN = 0.679 ± 0.10 mm³). Optical densitometry was used to assess TH-immunoreactive fibre density in the ST of animals that received FVM grafts (expressed as the percentage of TH-immunoreactivity present in the grafted ST compared to that in the contralateral unlesioned.

Fig. 4 Forelimb akinesia was assessed using the ramp test. The time for rats to initiate movement with the right forepaw was unaffected by ipsilateral 6-OHDA lesions (A) but apparently increased in the left forepaw (B), although this did not reach statistical significance (P > 0.05). Consequently, no statistically significant decrease in initiation times observed compared to post-lesion scores.

Downloaded from https://academic.oup.com/brain/article-abstract/131/8/2106/268174 by guest on 30 April 2018
ST: FVM in ST only = 46 \pm 2.2; FVM in ST and FSP in STN = 50 \pm 2.4; FVM in ST and FSP in SN = 50 \pm 1.8; FVM in ST and FSP in STN and SN = 46 \pm 2.5; FVM in ST and differentiated hNPCs in STN = 44 \pm 2.2; FVM in ST and differentiated hNPCs in SN = 38 \pm 2.8; FVM in ST and differentiated hNPCs in STN and SN = 38 \pm 3.1; FVM in ST and undifferentiated hNPCs in STN = 51 \pm 3.5; FVM in ST and undifferentiated hNPCs in SN = 45 \pm 2.7 and FVM in

Fig. 5 Foetal striatal primordia cells demonstrated immunoreactivity for GABA (A), GAD 65/67 (B) and MAP2 (C). Differentiated hNPCs demonstrated immunoreactivity predominantly for GABA (D), TUJ1 (E) and few cells were immunoreactive for GFAP (F) or nestin (G). Scale bar = 100 \mu m (A–G).
Although a one-way ANOVA of fibre density for groups of animals that received FVM grafts in the ST yielded a \( p = 0.0011 \) \( [F(9, 220) = 3.231] \), post hoc analyses demonstrated that the only groups in which there was a significant difference in fibre density was between those animals that received FVM in the ST and FSP in either the STN or SN and those that received FVM in the ST and differentiated hNPCs either in the SN or STN and SN. Importantly, there were no significant differences in TH-immunoreactive fibre density between animals that received FVM grafts in the ST alone compared to any of the other transplantation groups \( (P > 0.05) \).

Animals that received transplants of FSP cells demonstrated grafts that contained DARPP-32-immunoreactive cell bodies within the STN and/or SN (Fig. 7). Intrasubthalamic nucleus grafts were typically well-localized within the STN but in some cases extended into the cerebral peduncle. Intranigral grafts were mainly located within the SN pars reticulata (SNr) but in some cases extended into the cerebral peduncle or SN pars compacta (SNc). There was no statistically significant difference in the number of surviving DARPP-32-immunoreactive cells in the STN or SN of animals that received FSP grafts in the STN alone \( (3084 \pm 1361 \text{ cells in the STN}) \) or in combination with SN grafts \( (4738 \pm 1456 \text{ cells in the STN}) \), or in the SN alone \( (3720 \pm 585 \text{ cells in the SN}) \) or in combination with STN grafts \( (3524 \pm 920 \text{ cells in the SN}) \) \( [F(3, 20) = 0.3799, P = 0.7685] \). Similarly, there was no statistically significant difference in the DARPP-32-immunoreactive graft volumes in the STN or animals that received FSP grafts in the STN alone \( (0.376 \pm 0.24 \text{ mm}^3) \) or in combination with SN grafts \( (0.520 \pm 0.19 \text{ mm}^3) \), or in the SN alone \( (0.497 \pm 0.08 \text{ mm}^3) \) or in combination with STN grafts \( (0.430 \pm 0.12 \text{ mm}^3) \) \( [F(3, 20) = 0.1488, P = 0.9292] \).

Undifferentiated and differentiated hNPCs, regardless of the transplantation site, appeared as dense clusters of HuN-immunoreactive cells (Fig. 8A–D). In animals that received simultaneous hNPC grafts in the STN and SN, the grafts were discrete but some cells migrated to positions between the targets (Fig. 8A and B). Intrasubthalamic nucleus transplants of either undifferentiated or differentiated hNPCs were typically observed as clusters of cells, although cells also were observed in the zona incerta and cerebral peduncle (Fig. 8C). In the SN, the densest clusters of either undifferentiated and differentiated hNPCs were positioned in the SNr (Fig. 8D), but some cells migrated superiorly away from the graft nidus. No deformation of or mass effect in the STN or SN was observed in any of the transplanted animals. The host immune response to the hNPC grafts was assessed using immunohistochemistry for T lymphocytes and microglia. No lymphocytic infiltration of the grafts was observed (Fig. 9A–C) and microglia were observed throughout the brain sections and not in particular congregation within or around the grafts (Fig. 9D–F). This is consistent with post-mortem analyses of transplanted human FVM cell suspension grafts in which no host lymphocytic reaction and only a mild microglial reaction along the needle tracts was observed (Mendez et al., 2005, 2008).

Confocal microscopy demonstrated that a proportion of transplanted differentiated hNPCs, both in the SN and STN, co-localized immunohistochemically with GABA (Fig. 8E), TUJ1 (Fig. 8E) and MAP2 (Fig. 8F) even up to 10 weeks post-transplantation. The overall proportion of transplanted cells that expressed GABA and TUJ1 was 44 ± 8.8 and 39 ± 9%, respectively, and was not significantly different (two-tailed \( t \)-test, \( t = 0.4071, \text{df} = 4, P = 0.7048 \)). Cells within the graft nidus were observed to be GABA and TUJ1 immunoreactive, whereas those cells

Fig. 6 Representative coronal sections of the striatum of rats that received intrastriatal FVM transplants demonstrated TH-immunoreactive grafts. Scale bar = 1000\( \mu \text{m} \) (A), 100\( \mu \text{m} \) (B).
that had migrated away from the deposit were not. No transplanted differentiated hNPCs demonstrated immunoreactivity for TH, serotonin or ChAT, and 18 ± 3.8% of the cells, typically those that had migrated away from the graft nidus, demonstrated immunoreactivity for GFAP. Although Ki67 immunoreactive cells were observed within the grafts (Fig. 8G), no tumour formation was observed in any animals. Transplanted undifferentiated hNPCs demonstrated immunoreactivity predominantly for nestin (Fig. 8H) and GFAP (66 ± 2.3%, Fig. 8I), but only 0.2 ± 0.2 and 1.7 ± 0.6% demonstrated immunoreactivity for GABA and TUJ1, respectively, and none were immunoreactive for TH, serotonin or ChAT, thus suggesting mainly astrocytic differentiation. Some cells demonstrated immunoreactivity for Ki67 (Fig. 8I), but no tumour formation was observed in any animals. A significantly greater proportion of transplanted differentiated hNPCs demonstrated immunoreactivity for GABA and TUJ1 (two-
A multitarget transplantation strategy for PD

Brain (2008), 131, 2106–2126

Downloaded from https://academic.oup.com/brain/article-abstract/131/8/2106/268174
by guest
on 30 April 2018
Electrophysiology studies

Whole-cell patch-clamp recordings of FSP cells in vitro were performed to investigate the degree to which they expressed the physiological properties of neurons (Fig. 10). The average passive membrane properties for the FSP cells were: resting membrane potential, $-57 \pm 2.2$ mV; whole cell capacitance, $25 \pm 3.4$ pF and input resistance, $290 \pm 44$ MΩ.

In voltage-clamp mode, steps (50 ms duration) from a holding potential of $-60$ mV to a range of test potentials between $-100$ and $+40$ mV demonstrated large amplitude, fast inactivating sodium currents (‘action currents’, Fig. 10C). Similar protocols demonstrated that cultured FSP cells also possessed non-inactivating outward (potassium) currents (Fig. 10D). In current–clamp mode, depolarizing current pulses (1 s duration) elicited trains of action potentials (Fig. 10E). As shown by frequency–current plots (Fig. 10F), the firing frequency increased linearly with increased current injection. In order to determine whether FSP cells could form GABAergic synaptic connections with each other, post-synaptic currents were also investigated in FSP cells (Fig. 10G, $n=16$). Spontaneous inward postsynaptic currents (PSCs) were observed at $-70$ mV, which reversed at $-40$ mV. To determine whether these PSCs were mediated by GABA$_A$ receptors, bicuculline (10 μM) was bath applied. This reversibly blocked the PSCs. These findings indicate that the FSP cells in culture receive GABAergic input. This input is depolarizing, as is often observed in neurons due to immature chloride transporters during development (Rivera et al., 1999; Payne et al., 2003).

Electrophysiological analyses of cultured differentiated hNPCs (Fig. 11) revealed quite different findings. These cells had similar resting potentials ($-61.8 \pm 0.97$ mV) and input resistances ($335 \pm 123$ MΩ) as the FSP cells, but significantly smaller whole cell capacitances ($4.7 \pm 0.8$ pF). Differentiated hNPCs demonstrated non-inactivating potassium
conductances (Fig. 11B) much smaller in amplitude than those measured in FSP cells (Fig. 10D). Further, the amplitude of the sodium conductances (Fig. 11C) were a small fraction of those recorded in FSPs, and in current-clamp the cells were never observed to fire action potentials (Fig. 11D, n = 12). Undifferentiated hNPCs demonstrated potassium conductances but sodium currents were never recorded. These cells also did not fire action potentials (data...
not shown, \( n = 7 \)). Spontaneous post-synaptic currents were never observed for either the differentiated or undifferentiated hNPCs.

Taken together, these data demonstrate that FSPs assumed many of the electrical properties of neurons. However, the inability of the hNPCs to fire action potentials precludes their classification as neurons, despite their immunocytochemical profile.

**Discussion**

Among the variables that likely influence the efficacy of clinical cell restoration strategies for Parkinson’s disease are the optimal sites and tissue types for transplantation (Olanow et al., 1996; Björklund et al., 2003). Placement of foetal dopaminergic cells in the ST can effectively ameliorate dopamine agonist-induced rotational asymmetry (Björklund and Stenevi, 1979), but complete restoration of complex sensorimotor behavioural deficits in animal models that are more reflective of clinical deficits exhibited by parkinsonian patients has not been achieved (Dunnett et al., 1987; Montoya et al., 1990; Abrous et al., 1993; Winkler et al., 1999; Baker et al., 2000; Freed et al., 2001; Mukhida et al., 2001). Increasing the number of dopaminergic cells transplanted into the ST (Mehta et al., 1998) or using a microtransplantation technique to implant cells over a larger number of sites in the ST so as to provide up to 80% of the normal dopaminergic reinnervation of the ST (Nikkhah et al., 1993; Olsson et al., 1995; Winkler et al., 1999) have not restored skilled forelimb use or forelimb motor function and akinesia in animals models of Parkinson’s disease. This may be related to the inability of foetal dopaminergic grafts in the ST alone to normalize activity in other basal ganglia structures affected in Parkinson’s disease. For instance, 6-OHDA lesion-induced down-regulation of substance P and dynorphin expression is only marginally affected in striatonigral projection neurons (Cenci et al., 1993; Winkler et al., 2003), and apomorphine-induced Fos-like expression and cytochrome oxidase activity persistently remain elevated in the STN (Nakao et al., 1998).

Despite its recognized central role in the pathophysiology of Parkinson’s disease (Henderson and Dunnett, 1998), to date only a few neural transplantation studies have targeted the STN. Grafting foetal dopaminergic cells into the STN, either alone or in conjunction with dopaminergic grafts in the ST and SN, was required to improve some measures of sensorimotor function but such recovery was incomplete (Mukhida et al., 2001). A similar multitarget dopaminergic transplantation strategy using murine-derived dopaminergic embryonic stem cells found that amphetamine-induced rotational asymmetry could be significantly reduced but dopaminergic STN grafts were insufficient to provide improvement in endurance in the
rotarod test, the only other behaviour examined (Inden et al., 2005). More recently, intrasubthalamic nucleus transplantation of hNPCs, which predominantly differentiated into nestin- and GFAP-immunoreactive cells, improved rotational asymmetry in parkinsonian rats, but its effects on more complex sensorimotor behaviours were not assessed (Anderson and Caldwell, 2007). The present study clearly demonstrates that forelimb akinesia and motor function, as determined by the stepping and cylinder tests, can be significantly improved in a rat model of Parkinson’s disease by intrastriatal grafting of dopaminergic cells with concurrent transplantation of GABAergic cells into the STN and/or SN. In some cases, this strategy facilitated normalization of scores in these tests. This is especially relevant for clinical transplantation strategies, since amelioration of these types of motor functions has been suggested to be a better predictor of the effects of transplantation in parkinsonian patients than assessment of rotational asymmetry (Metz and Whishaw, 2002; Georgievska et al., 2004; Casteñeda et al., 2005). Moreover, differentiated hNPCs could effectively be used as a standardized alternative to foetal GABAergic cells, which would be of practical benefit for clinical translation of this strategy.

The enhancement of behavioural improvements observed here is likely related to the combined effects of the striatal dopaminergic and subthalamic and nigral GABAergic grafts. Amelioration of rotational behaviour, observed in the present study in all animals that received FVM striatal transplants regardless of the type of tissue transplanted in other basal ganglia structures, has been shown to be related to the ability of intrastratial foetal dopaminergic transplants, which by themselves are unable to reinervate the STN and SN (Baker et al., 2000; Mukhida et al., 2001), to increase dopaminergic output in the lesioned ST (Dunnett et al., 1988). Restoration of complex sensorimotor behaviour was observed only in animals that received both intrastratal transplants of FVM cells as well as intrasubthalamic nucleus and/or intranigral transplants of FSP cells or differentiated hNPCs, which maintained a GABAergic phenotype despite transplantation into these non-neurogenic regions of the CNS, and not with transplantation of undifferentiated hNPCs, which differentiated into astrocytes. Moreover, despite the differences in their electrophysiological properties, both GABAergic cell types were efficacious in promoting behavioural recovery when transplanted in conjunction with striatal dopaminergic grafts. Transplanted foetal-derived neurons have exhibited electrophysiological properties typical of mature cells in situ in other studies (Sorensen et al., 2005) to an extent which has not been observed in analyses of transplanted neural precursor cells (Kim et al., 2002; Schulz et al., 2004; Wernig et al., 2004; Brederlau et al., 2006). In congruence with the results of the present study in which FSP cells demonstrated electrophysiological characteristics of neurons whereas differentiated hNPCs exhibited only much smaller sodium conductances that precluded their ability to fire action potentials, this suggests that the ability of transplanted neural precursor cells to impart beneficial behavioural effects may be related more to their ability to secrete neurochemicals that affect the function of neighbouring cells rather than their mature synaptic integration into host neural circuitries.

The greater functional improvement observed in hemiparkinsonian animals with dopaminergic and GABAergic grafts in the present study therefore may have been related to restoration of dopaminergic function to the ST and, in part, to inhibition of the STN and/or SN by GABAergic grafts. Inhibitory effects of GABAergic grafts have been previously demonstrated. First, FSP cells, over 98% of which express GAD (Campbell et al., 1995), transplanted into the adult brain differentiate into GABAergic neurons (Wichterle et al., 1999) and intracerebral microdialysis of these grafts has demonstrated that they release GABA into surrounding tissue and restore GABA overflow in an impulse-dependent manner to levels that approximate that of the normal ST (Campbell et al., 1993). Second, neural precursor cell-derived GABAergic cells can express GABA and influence the level of local GABA-mediated synaptic inhibition after intracerebral transplantation (Álvarez-Dorado, 2006). Third, intranigral transplantation of either FSP cells or cells engineered to secrete GABA improved skilled forelimb function, paw-reaching and forelimb akinesia in hemiparkinsonian rats (Winkler et al., 1999) and tremulous jaw movements in a rat model of Parkinson’s disease tremor (Carlson et al., 2003), respectively, by increasing tonic release of GABA in the SNr and thus downregulating the overactivity of SNr neurons. Attenuation of neuropathic pain associated with spinal nerve root ligation-induced loss of dorsal horn inhibitory interneurons was similarly ascribed to GABA production by intraspinally transplanted GABAergic hNPCs (Mukhida et al., 2007). Therefore, in the present study, GABA released in the STN and/or SN by foetal- or differentiated hNPC-derived grafts may have hyperpolarized neurons in these structures and decreased their firing, consistent with the suggested mechanism of action of GAD gene therapy in parkinsonian patients and animals (Kaplitt et al., 2007). Winkler and colleagues (1999) found that intranigral FSP grafts did not induce any changes in striatal neuropeptide expression, and thus postulated that the behavioural effects of the grafts were due to direct GABAergic input to the SNr that modulated striatal outflow involving the SNr without altering neuronal function in the ST. Together with the results of the present study, this suggests that intrasubthalamic nucleus and/or intranigral foetal- or hNPC-derived GABAergic grafts can influence the net functional output of the basal ganglia by modifying striatal output in combination with intrastratal FVM grafts in the hemiparkinsonian rat. Theoretically, based on the mechanism of action of 6-OHDA in the animal model, the effects due to the loss of dopamine should be improved by restoration of dopamine function alone. Indeed, this has been demonstrated at least in part (Abrous et al., 1993; Baker et al., 2000; Dunnett et al., 2005).
et al., 1987; Freed et al., 2001; Montoya et al., 1990; Mukhida et al., 2001; Winkler et al., 1999), however, the addition of GABAergic grafts to basal ganglia targets can provide additional benefit in improving sensorimotor function.

The local mechanism of action of the STN and SN grafts in the present study is unlikely due to non-GABAergic-mediated factors. For example, although inhibition of the STN concurrently with neurotrophin nigrostriatal dopaminergic lesions prevents parkinsonian symptomatology by either increasing the expression of TH in remaining SNc dopaminergic neurons (Paul et al., 2004) or protecting against their loss (Piallat et al., 1996; Luo et al., 2002; Wallace et al., 2007), this is unlikely to be the case here since rats were transplanted only after complete unilateral 6-OHDA lesions. Although lesions of the STN can improve motor function in Parkinson’s disease, STN and SN grafts in the present study did not disrupt the morphology of these structures, which also did not have the appearance associated with lesions (Paul et al., 2004). This is consistent with other studies employing STN or SNr grafts (Anderson and Caldwell, 2007; Mukhida et al., 2001; Winkler et al., 1999). Moreover, behavioural recovery occurred gradually over 9 weeks, which is consistent with graft-derived effects (Anderson and Caldwell, 2007). Lastly, it is unlikely that the effects of the FSP grafts were mediated by non-GABAergic cells present within the tissue (Winkler et al., 1999), since these grafts were similar in efficacy to those of differentiated hNPC grafts, which contained cells immunoreactive for GABA and not for any of the other neurochemical phenotypes analysed.

The attenuation of parkinsonian symptomatology observed in this study using both dopaminergic and GABAergic grafts agrees with current understanding of basal ganglia circuitry. Although the etiology of STN overactivity is controversial (Levy et al., 1997; Parent and Cicchetti, 1998), decreased levels of GABA and increased levels of glutamate in the STN associated with dopaminergic degeneration are thought to be due to decreased activity of the inhibitory pallidostubalamic pathway (Albin et al., 1989; Parent and Hazrati, 1995b) and increased glutamate release from the corticosubthalamic pathway (Magill et al., 2001), respectively. Other anatomical studies also reveal a direct role for GABA in the STN. The STN receives GABAergic input from the globus pallidus pars internus, STN neurons are enriched in messenger RNA encoding the high-affinity GABA transporter (Augood et al., 1999), and GABA is known to have a role in modulating the activity of STN neurons by influencing their firing rates, patterns of discharge and bursting activity (Hamani et al., 2004). GABAergic grafts in the present study therefore may have improved sensorimotor behaviours by restoring GABAergic inhibitory input to the STN and SNr and, thus, tonic inhibition on thalamic and brain stem premotor nuclei (Faull and Mehler, 1978; Kha et al., 2001).

In this regard, the results of the present study are consistent with Parkinson’s disease clinical and animal studies in which GABAergic inhibition of the STN or SN also had therapeutic effects. Overexpression of GAD in the STN led to the synthesis and activity-dependent release of GABA from subthalamic nerve terminals that changed STN output from excitatory to inhibitory (Lee et al., 2005), suppressed the firing of SNr neurons (Lu et al., 2002) and ameliorated Parkinson’s disease behaviours (Lu et al., 2002; Lee et al., 2005; Kaplitt et al., 2007). Among the mechanisms suggested for the decreased firing of STN neurons after DBS (Beurrer et al., 2001; Benazzouz et al., 2004) is their functional inactivation due to the release of GABA in the STN (Boraud et al., 1996; Dostrovsky et al., 2000; Maurice et al., 2003) and SNr (Windels et al., 2000; Maurice et al., 2003) and activation of presynaptic GABAergic axon terminals (Meissner et al., 2005). In pharmacological studies, inhibition of STN activity by microinjection of the GABA A agonist muscimol (Levy et al., 2001; Mehta and Chesselet, 2005) or the glutamate receptor antagonist 2-methyl-6-(phenylethynyl)-pyridine (Phillips et al., 2006) improved parkinsonian motor symptoms. Inhibition of the SNr with either muscimol (Finn et al., 1997; Wichmann et al., 2001) or the GABA uptake antagonist β-alanine (Ishiari et al., 2004) have improved tremor and akinesia, whereas decreasing inhibition to the SNr using bicuculline worsened motor functions in rats with dopamine depletion in the basal ganglia (Correa et al., 2003).

Clinical implications
Targeting the ST with dopaminergic grafts and the STN and SNr with GABAergic grafts in Parkinson’s disease represents a novel and rational manner of addressing the dopaminergic deficiency in the ST and the pathological overactivity of the STN and SN and, thus, improving the efficacy of standard intrastral dopaminergic transplantation strategies. Additionally, graft-derived inhibition of the STN and SNr may avoid some of the disadvantages associated with other methods to modulate STN and SNr function, such as the temporally limited effects (Levy et al., 2001; Mehta et al., 2005) and exacerbation of motor deficits described with direct administration of GABA agonists into the basal ganglia (Mehta et al., 2005), and the costs (Bin-Mahfoudh et al., 2003) and technical, cognitive and psychiatric complications of DBS (Oh et al., 2002; Constantoyannis et al., 2005; Hamani et al., 2005; Hamani and Lozano, 2006; Parsons et al., 2006; Temel et al., 2006). Although FSP cells can be used as a source of GABAergic cells for transplantation, hNPCs expanded under controlled conditions in bioreactors and differentiated into a GABAergic phenotype could be used, since the beneficial effects of the grafts may be related to their ability to tonically secrete GABA rather than their functional integration into host circuitries. The present study supports...
A multitarget transplantation strategy for PD

the use of GABAergic hNPCs as an adjunct to foetal striatal dopaminergic transplantation strategies for Parkinson’s disease in the clinical setting, however, further surveillance will be required to ensure that hNPC transplantation is without adverse effects over the long-term. Modulation of basal ganglia structures other than the ST, STN and SN also may be necessary to more completely restore motor function in Parkinson’s disease. For example, the classical model of basal ganglia physiology predicts that the globus pallidus pars internus (GPI) becomes hyperactive in Parkinson’s disease (DeLong, 1990), and this has been confirmed in both experimental models (Filion and Tremblay, 1991) and in Parkinson’s disease patients (Hutchison et al., 1994; Merello et al., 1999). Consistent with this, pallidotomy (Lang et al., 1997; Lozano et al., 1995), GPI DBS (Dostrovsky et al., 2000; Kumar et al., 2000) and injection of a GABA agonist into the GPI have been shown to ameliorate parkinsonian symptomatology (Penn et al., 1998), and recently this structure has become a target of interest for neural transplantation (Bartlett and Mendez, 2005). Targeting appropriate basal ganglia structures for transplantation in Parkinson’s disease with appropriate tissue types may be of critical importance in cell restoration strategies to optimize clinical outcomes.

Acknowledgements

This project was supported by funding from the Capital Health Research Foundation (IM), Stem Cell Network (IM, LAB) and the Natural Sciences and Engineering Research Council (AS, LAB). KM was supported by fellowships from the Congress of Neurological Surgeons (Margot Anderson Foundation Fellowship in Brain Restoration Research), Dalhousie Medical Research Foundation (Clinical Research Fellowship), Parkinson Society Canada (Basic Research Fellowship) and Nova Scotia Health Research Foundation (Student Research Award) and a Killam Memorial Scholarship. The authors gratefully acknowledge the technical assistance of Alia Mukhida, Damaso Sadi, Ruperto Ullalia, Craig Moore and Dr. Amanda Wintink.

References

Dunnett SB, Whishaw IQ, Rogers DC, Jones GH. Dopamine-rich grafts ameliorate whole-body asymmetry and sensory neglect but not independent limb use in rats with 6-hydroxydopamine lesions. Brain Res 1987; 415: 63–78.
A multitarget transplantation strategy for PD


