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Embryonic striatal grafts restore bi-directional synaptic plasticity in a rodent model of Huntington's disease

David M. Mazzocchi-Jones
University of Wollongong, davidmj@uow.edu.au

Mate Dobrossy
University Hospital Freiburg, Germany

Stephen Dunnett
Cardiff University, UK

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Embryonic striatal grafts integrate with the host striatal circuitry, forming anatomically appropriate connections capable of influencing host behaviour. In addition, striatal grafts can influence host behaviour via a variety of non-specific, trophic and pharmacological mechanisms; however, direct evidence that recovery is dependent on circuit reconstruction is lacking. Recent studies suggest that striatal grafts alleviate simple motor deficits, and also that learning of complex motor skills and habits can also be restored. However, although the data suggest that such 're-learning' requires integration of the graft into the host striatal circuitry, little evidence exists to demonstrate that such integration includes functional synaptic connections. Here we demonstrate that embryonic striatal grafts form functional connections with the host striatal circuitry, capable of restoring stable synaptic transmission, within an excitotoxic lesion model of Huntington's disease. Furthermore, such 'functional integration' of the striatal graft enables the expression of host-graft bi-directional synaptic plasticity, similar to the normal cortico-striatal circuit. These results indicate that striatal grafts express synaptic correlates of learning, and thereby provide direct evidence of functional neuronal circuit repair, an essential component of 'functional integration'.

Keywords

grafts, restore, bi, directional, synaptic, plasticity, rodent, model, embryonic, huntington, striatal, disease

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NEUROSYSTEMS

Embryonic striatal grafts restore bi-directional synaptic plasticity in a rodent model of Huntington's disease

David Mazzocchi-Jones,[1,2] Máté Döbrössy[3] and Stephen B. Dunnett[2]
[1]Graduate School of Medicine, University of Wollongong, NSW, 2522, Australia
[2]The Brain Repair Group, School of Biosciences, Cardiff University, Wales, UK
[3]Department of Stereotactic Neurosurgery, Laboratory of Molecular Neurosurgery, University Hospital Freiburg, Neurocentre, Freiburg, Germany

Keywords: circuit reconstruction, electrophysiology, functional connections, striatal grafts, synaptic plasticity

Abstract

Embryonic striatal grafts integrate with the host striatal circuitry, forming anatomically appropriate connections capable of influencing host behaviour. In addition, striatal grafts can influence host behaviour via a variety of non-specific, trophic and pharmacological mechanisms; however, direct evidence that recovery is dependent on circuit reconstruction is lacking. Recent studies suggest that striatal grafts alleviate simple motor deficits, and also that learning of complex motor skills and habits can also be restored. However, although the data suggest that such 're-learning' requires integration of the graft into the host striatal circuitry, little evidence exists to demonstrate that such integration includes functional synaptic connections. Here we demonstrate that embryonic striatal grafts form functional connections with the host striatal circuitry, capable of restoring stable synaptic transmission, within an excitotoxic lesion model of Huntington's disease. Furthermore, such 'functional integration' of the striatal graft enables the expression of host–graft bi-directional synaptic plasticity, similar to the normal cortico-striatal circuit. These results indicate that striatal grafts express synaptic correlates of learning, and thereby provide direct evidence of functional neuronal circuit repair, an essential component of 'functional integration'.

Introduction

Huntington's disease (HD) is a chronic neurodegenerative disorder primarily affecting the striatum, resulting in widespread loss of medium spiny projection neurons (MSNs), which constitute the majority population of striatal cells (Oorschot, 1996). The use of embryonic striatal grafts in the treatment of HD is currently at a trial stage within Europe and the USA, with preliminary results indicating an overall positive reduction of negative symptoms in some but not all patients (Bachoud-Levi *et al.*, 2000, 2002; Rosser *et al.*, 2002; Isacson, 2006; Reuter *et al.*, 2008). Experimental rodent models of HD, using excitotoxic lesions of the striatum, display anatomical (Schwarcz *et al.*, 1983) and behavioural (Dunnett & Iversen, 1982a,b) correlates, which share a symptomatic similarity with the human disorder (Beal *et al.*, 1986). Within such animal models it has been demonstrated that striatal grafts integrate with the host cortico-striatal circuitry, receiving afferent projections from the host prefrontal cortex, substantia nigra and raphé nucleus, and forming morphologically appropriate synapses (Clarke *et al.*, 1988; Wictorin *et al.*, 1989, 1990a; Clarke & Dunnett, 1993). Furthermore, it has been shown that striatal grafts project efferent fibres to the host globus pallidus and substantia nigra (Wictorin & Bjorklund, 1989; Wictorin *et al.*, 1990b, 1991, 1992), capable of influencing neurotransmission (Sirinathsinghji *et al.*, 1988).

Correspondence: Dr D. Mazzocchi-Jones, [1]Graduate School of Medicine, as above.
E-mail: davidmj@uow.edu.au

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Previous electrophysiological and neurochemical analyses of graft tissue have demonstrated that transplanted neurons display similar electrophysiological properties to the intrinsic cellular population, and that host neurons can signal patterned information to grafted cells (Rutherford *et al.*, 1987; Walsh *et al.*, 1988; Xu *et al.*, 1989, 1991b; Siviy *et al.*, 1993). Furthermore, it has been demonstrated both *in vitro* (Rutherford *et al.*, 1987; Walsh *et al.*, 1988; Siviy *et al.*, 1993) and *in vivo* (Xu *et al.*, 1989, 1991b) that transplanted striatal neurons (TSNs) respond to stimulation of the host cortex (Rutherford *et al.*, 1987; Walsh *et al.*, 1988; Xu *et al.*, 1989, 1991b; Siviy *et al.*, 1993) and thalamus (Xu *et al.*, 1991a). Although responses evoked from TSNs resemble those evoked from MSNs, some electrophysiological characteristics are unique to TSNs. Thus, TSNs have been shown to display smaller evoked amplitudes (Rutherford *et al.*, 1987; Walsh *et al.*, 1988; Xu *et al.*, 1991b), increased prevalence of inhibitory potentials (Rutherford *et al.*, 1987; Xu *et al.*, 1991b) and increased *N*-methyl-D-aspartate (NMDA) receptor efficacy (Walsh *et al.*, 1988; Siviy *et al.*, 1993). However, although it has been speculated that such characteristics will contribute to host–graft synaptic transmission, a detailed study of graft 'functional integration' has not been conducted.

Functionally, it has been clearly demonstrated that striatal grafts restore motor and cognitive performance within rodent models of HD (Dunnett & Iversen, 1982a,b; Isacson *et al.*, 1986; Dunnett *et al.*, 2000), and more recently in some but not all human patients (Bachoud-Levi *et al.*, 2000, 2002; Rosser *et al.*, 2002; Isacson, 2006; Reuter *et al.*, 2008). In particular, the cortico-striatal projection is

critically involved with the regulation of learning new motor skills and habits. Excitotoxic lesions of the rodent striatum, producing animal models of HD, result in the loss of previously trained motor skills and habits (Mayer *et al.*, 1992; Brasted *et al.*, 1999). Interestingly, striatal transplantation on its own is not sufficient to replace the lost skills and habits; rather, the animal must be retrained on the specific behavioural task, a phenomenon termed 'learning to use the graft' (Mayer *et al.*, 1992; Brasted *et al.*, 1999). Such relearning occurs over a time period similar to that seen in naïve animals when first presented with the task. Transfer of training experiments show that the relearning is specific to the grafted hemisphere (Brasted *et al.*, 1999), and includes recovery of complex functions, which delayed transplantation studies (Mayer *et al.*, 1992; Brasted *et al.*, 1999) show are dependant upon intact cortico-striato-pallidal systems (Dunnett, 1995). This suggests that grafts not only reintegrate into an intact graft–host system, but also that such functional connections provide the neural substrate for the observed relearning.

In the present study we demonstrate that embryonic striatal grafts restore baseline transmission and the expression of bi-directional synaptic plasticity at the host–graft cortico-striatal synapse, similar to that seen in the normal striatum. This study provides data demonstrating the 'functional integration' of striatal grafts, and provides a potential mechanism by which the grafted striatal cells can act as the substrate for the 'learning to use the graft' phenomena.

Materials and methods

Animals

A total of 175 male C57/BL6 mice (Harlan, UK) were used as control animals and hosts for transplantation (Table 1). All animals were housed six per cage in a temperature – (21°C) and humidity – (50%) controlled room, with a 12/12-h light–dark cycle (lights on during the day). All animals were given free access to food *ad libitum*. The experiments were undertaken in accordance with personal and project licences issued under the UK Animals (Scientific Procedures) Act 1986.

For graft experiments all donor tissue was collected from the PrP-L-EGFP-L transgenic mouse (Feil *et al.*, 1996). This transgenic mouse exhibits endogenous expression of green fluorescent protein (GFP) under control of the PrP promoter in all cells (Feil *et al.*, 1996).

Lesion surgery

Mice were anaesthetized in an induction chamber by inhalation anaesthetic (Isoflurane, Abbott, UK), using O_2 and NO as carrier gases, and placed in a stereotaxic frame (Kopf Instruments). Unilateral excitotoxic lesions of the dorsal striatum were made by injecting 2 × 0.24mL of 0.12M quinolinic acid (Cambridge Research Biochemicals), dissolved in 0.1M phosphate-buffered saline (pH = 7.4), at two depths within the same needle track in the left neostriatum. Each injection was administered over 1 min via a 30-gauge stainless steel cannula connected to a microdrive pump, with a further 2 min for diffusion. The injection coordinates were: A = +1.0, L = +1.8, V = 3.1 and 2.5 with measurements in millimetres anterior (A) to bregma, lateral (L) to the midline, vertical (V) below dura, and the nose bar set in the same horizontal plane as the interaural line. Following completion of the injections, the cannula was retracted, the wound cleaned and the skin sutured. Prior to regaining consciousness, animals received 5 mL glucose saline s.c. in the neck and 0.15 mL diazepam i.m. in the hind leg. For analgesia, 500 mg/L paracetemol was dissolved in the drinking water for 48 h following surgery.

Table 1. Total numbers of animals, slices prepared and recordings made

	Extracellular recordings			Intracellular recordings	
	No surgery	Lesion	Graft	No surgery	Graft
Animals	50	15	50	30	30
Slices	50	15	48	48	43
Evoked responses	30	0	40	28	30
LTP	24	0	22	18	18
LTD	15	0	14	18	18

LTP, long-term potentiation; LTD, long-term depression.

Harvesting of embryos and graft tissue

Time-mated pregnant female mice were killed at embryonic day (E) 13 by terminal anaesthesia with Sagatal (60 mg/kg, Rhone Merieux) and decapitation. The uterine horns were removed by sterile caesarian section and placed in 45 mL dissection medium containing 95.7% Dulbecco's modified Eagle medium (DMEM), 2.2% of 30% glucose, 1.6% of 7.5% $NaHCO_3$, and 0.5% 1M HEPES. Embryos were removed one at a time from the uterine horn for dissection in a sterile Petri dish. Crown–rump lengths were measured, and the embryos were then killed by decapitation. The brains were removed and the whole ganglionic eminence (WGE) was dissected bilaterally according to a standard protocol. The WGE tissue pieces were transferred to a sterile 1.5-mL Eppendorf tube containing dissection medium and kept on ice. Once the tissue pieces had settled they were washed 3–4 times with sterile dissection medium, then incubated at 37°C for 20 min in 1.5 mL trypsin/DNase solution containing 0.1% bovine trypsin (Worthington, UK) and 0.05% DNase (Sigma, UK) in dissection medium. Following incubation, the trypsin/DNase solution was removed and the tissue was washed four times with 200μL 0.05% DNase solution. The tissue was then mechanically dissociated using a Gilson pipette with yellow tip to obtain a cell suspension. Cell numbers and viability were calculated by the trypan blue dye exclusion method using a haemocytometer. Cell suspensions with viability of 95% or more were re-suspended in DNase solution and used for the transplant procedure.

Transplant surgery

Seven days following lesion the mice were brought back to surgery for transplantation of embryonic striatal cells. The cells were harvested and prepared on the day of transplantation. Host mice were anaesthetized with inhalation anaesthetic, and placed in a stereotaxic frame (as above). Each animal received 2 × 1-μL aliquots of graft suspension (approximately 200 000 cells/μL, 400 000 cells per graft), injected over 1 min via a 10-μL Hamilton syringe at each of the two lesion injection sites, A = +1.0, L = +1.8, V = 3.1 and 2.5. Following the injection, 2 min was allowed for diffusion before the syringe needle was withdrawn, the wound cleaned and the skin sutured. All animals received post-operative care, as above.

Slice preparation

Mice were killed by cervical dislocation and decapitated. The brains were quickly removed and placed into ice-cold artificial cerebrospinal

fluid (aCSF; composition 126 mM NaCl, 2.5 mM KCl, 2.5 mM $CaCl_2$, 1.3 mM $MgSO_4$, 1mM $NaHPO_4$, 26 mM $NaHCO_3$ and 11 mM D-glucose) which was constantly bubbled with 95% O_2/5% CO_2 to maintain the pH at 7.4. During dissection and subsequent slicing this solution also contained 1mM kynurenic acid (Sigma Aldridge, UK), a broad-spectrum glutamate antagonist, to protect against excitotoxicity. Sagittal sections 300 μm thick were cut on a vibratome (Leica, GM). Slices were then transferred to a holding chamber containing aCSF at room temperature and incubated for at least 1 h prior to recording. To perform extracellular recording, slices were transferred to a submersion recording chamber. During the equilibration period and remainder of the experiment, slices were perfused with aCSF at 4mL/min at 31 ± 0.5°C. Slices were left for a further 5–10 min in the chamber to equilibrate before electrode placement.

Electrode placement

For all experiments, slices were viewed under ×50 magnification using an upright microscope (Olympus, UK) under bright-field phase illumination for positioning the electrodes and under incident ultraviolet illumination to visualize fluorescence emitted from GFP expression from grafted cells (Fig. 1).

For all experiments, stainless steel monopolar stimulating electrodes with a tip resistance of 6 MΩ (Intracel, UK) were placed in the host corpus callosum 1–2 mm from the graft site (Fig. 1). For all recordings, glass electrodes were produced from boroscilicate glass capillaries (Harvard Apparatus, UK), with an external diameter of 1.5 mm and an internal diameter of 0.86 mm, and pulled using a motorized micropipette puller (Sutter Instrument Co., USA), then filled with 1M KCl.

For extracellular recordings, the recording electrode was placed within the core of the graft region (region of dense GFP-positive cells) in a ventral location to the stimulating electrode. Control recordings were made from the intact striatum (in the absence of lesions and graft tissue was not present), with the electrode located in a similar location in mid-striatum, and at a similar distance ventral to the stimulating electrode. In pilot studies, we sought also to record from the lesioned striatum in the absence of grafts, and confirmed as expected that no cells exhibiting a positive response could be detected. Consequently, no data are presented from the lesioned striatum, and the responses of grafted cells are compared exclusively with responses from the intact striatal neurons located in similar positions on the contralateral side to lesions and grafts.

For intracellular recordings, sharp electrodes were used. GFP-positive TSNs were visualized under high-power magnification, impaled with the sharp electrode, then characterized for spike firing phenotype once impaled (Kita *et al.*, 1984), giving presumptive recordings of TSN activity. For control recordings, MSNs were identified visually via morphological characteristics, then characterized for spike firing phenotype once impaled (Kita *et al.*, 1984).

Stimuli and recording

In all cases, stimulus patterns were generated using a Master 8 pulse generator (Intracel) and a Neurolog stimulus isolator (Digitimer, UK). Responses were amplified using a differential amplifier (Warner Instrument Corp., USA), and Axoclamp (Axon Instruments, UK). Responses were monitored and stored for off-line analysis using custom software written in LabView (National Instruments, USA) running on a Macintosh G4 computer.

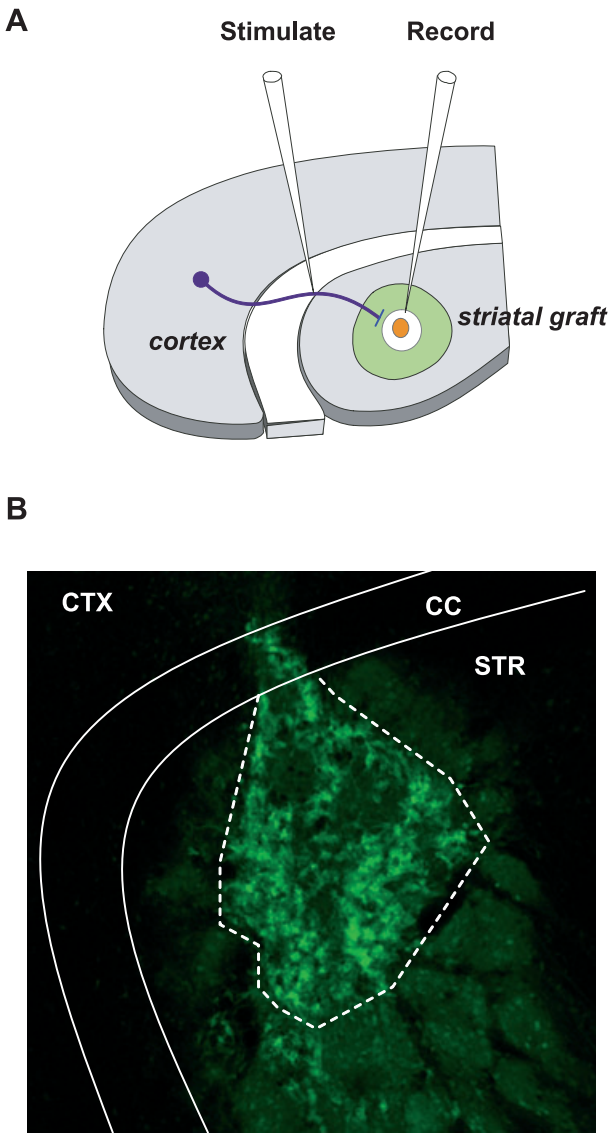

FIG. 1. The corticostriatal '*in vitro*' graft preparation. (A) Schematic representation of electrode positions within the striatal graft *in vitro* slice preparation. (B) Photomicrograph demonstrating the endogenous GFP fluorescence of the transplanted tissue within the striatum. CTX, cortex; CC, corpus callosum; STR, striatum.

In extracellular experiments, the initial slope of the field excitatory post-synaptic potential (fEPSP) was used as the measure of synaptic strength, although for intracellular experiments the amplitude of the EPSP was used (taken as the change in potential from baseline to the peak amplitude of the EPSP). Once a response had been gained, the slice was submitted to a 30-min rest period, where the slice was stimulated every 5–10 min, until the fEPSP slope or EPSP amplitude had stabilized.

Experimental protocol

Initially, an input/output (I/O) curve was computed. The fEPSP slope, or EPSP amplitude, was measured across a stimulus intensity of 1–10 mA, in steps of 1 mA. Four stimuli were given every 10 sec for each current intensity. Following I/O curve measurement, the slices were allowed a 5-min rest period. A stimulus intensity that evoked roughly 60% of the maximum response size was selected and stimuli

were delivered to the slice once every 20 s (square wave pulses, 0.2-ms duration) until a 20-min period of response stability was seen. Response stability was defined as a < 5% response size change over the 20-min period. This period is considered as 'baseline' as it reflects the fEPSP slope or EPSP amplitude prior to any changes brought about by tetanus or drug application.

Attempts to produce synaptic plasticity within both grafted and control tissue were made via high-frequency stimulation (HFS). The HFS comprised theta-burst stimulation in six trains, each of ten bursts at 5 Hz and each burst consisting of four pulses at 100 Hz, with a pulse width of 0.4 ms and an inter-train interval of 20 s. Following tetanus, slices were stimulated at baseline levels (0.05 Hz), and recording continued for a further 65 min. Successful expression of synaptic plasticity was defined as a greater than 5% change in response size, lasting at least 60 min following HFS (Table 1).

For pharmacological characterization, either 30μM of the NMDA receptor antagonist APV (Sigma Aldrich), or 3μM of the D2 receptor antagonist sulpiride (Sigma Aldrich) were added to the aCSF perfusate solution.

Data analysis and statistics

Data were analysed off-line. All data were normalized to a baseline level, which was defined as the average response size over the 20 min of baseline stimulation. These normalized values were expressed as a percentage of the baseline response (baseline = 100%) and averaged into 2-min bins. Results are presented as the mean ± SEM.

All data, irrespective of successful expression of synaptic plasticity, were included for analysis of the mean level of expression of long-term potentiation (LTP) and long-term depression (LTD). Within the two treatment groups (graft and control) the proportion of recordings successfully expressing either LTP or LTD is summarized in Table 1.

Multi-factorial analyses of variance were conducted on the data using the Genstat statistical package (v.10.1; VSN International, Hemel Hempstead, UK), with *post-hoc* tests correcting for multiple comparisons as appropriate.

Results

Intracellular and extracellular *in vitro* slice recordings were obtained from a total of 175 animals across three surgical groups: control, lesion and graft (Table 1).

All control recordings were obtained from animals that had no lesion or graft surgery performed on them. Responses evoked from the control striatum appeared consistent with those previously reported (Calabresi *et al.*, 2000).

Slices prepared from animals that had received unilateral excitotoxic lesions displayed a consistent lesion of the dorsal-lateral striatum, from which we were unable to elicit any form of synaptic response from the lesion loci in any of the cases examined (n = 15).

Eighty mice were transplanted with embryonic striatal tissue. Following preparation for *in vitro* recording, 72 displayed clearly identifiable graft regions similar to those seen in Fig. 1B.

Extracellular recording of host–graft basal transmission and synaptic plasticity

Beginning at 4 weeks post-transplantation it was possible to record fEPSPs from the graft region, following basal stimulation of the host corticostriatal fibres (Fig. 2A). Responses evoked from the graft region appeared visually similar to those recorded from the normal

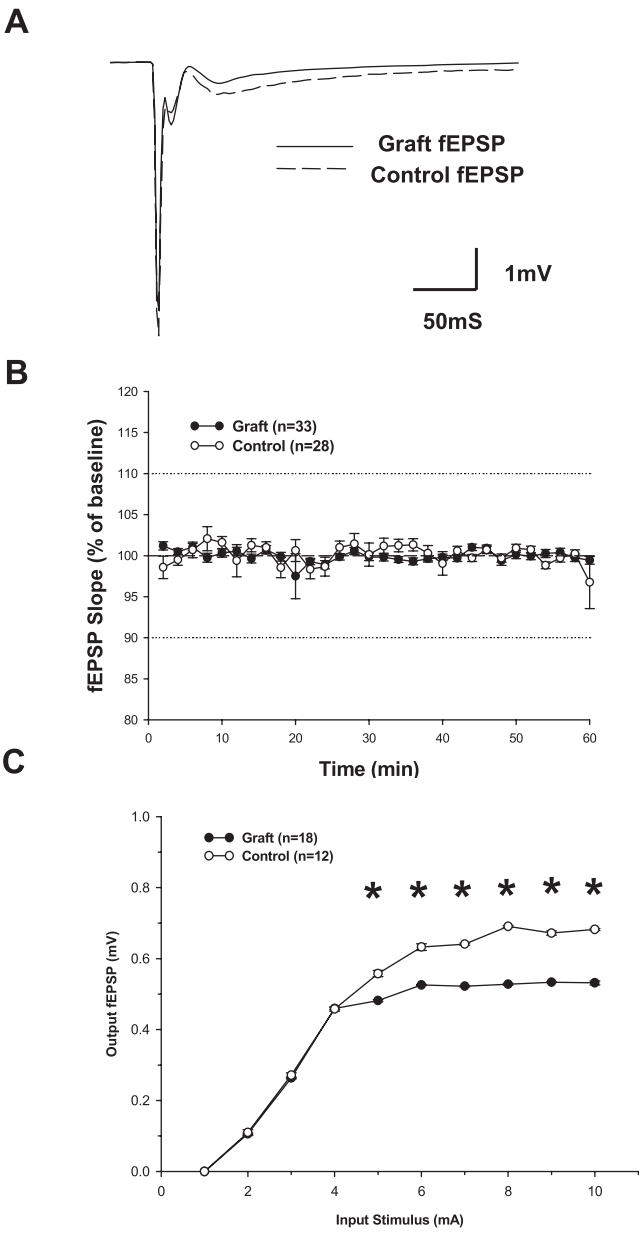

FIG. 2. Extracellularly recorded basal synaptic characteristics. (A) Example traces of control and graft fEPSPs. (B) Normalized data demonstrating graft (n = 33) and control (n = 28) recordings following basal stimulation. (C) I/O curves; stepped increases in input current (Input) are plotted against the size of the fEPSP (Output) in graft (n = 18) and control (n = 12) recordings. fEPSP, field excitatory post-synaptic potential. *P < 0.001.

striatum, albeit consistently smaller than those seen in control recordings. However, both control and graft basal recordings demonstrate that following consistent basal stimulation there is no significant difference in size of the normalized fEPSP between graft and control recordings over time (Groups × Time, $F_{29,1711}$ = 1.18, P = 0.233), indicating that the host–graft cortico-striatal synapse is able to sustain long-term synaptic transmission. In order to assess the efficacy of host–graft baseline transmission, I/O curves were generated from both control and graft recordings, and these demonstrate that under normal conditions, and below 5-mA stimulation, control and graft recordings showed no significant difference in the size of the fEPSP (Fig. 2C). However, at stimulation intensities of 5 mA and above, there was a highly significant difference between the size of the fEPSP evoked

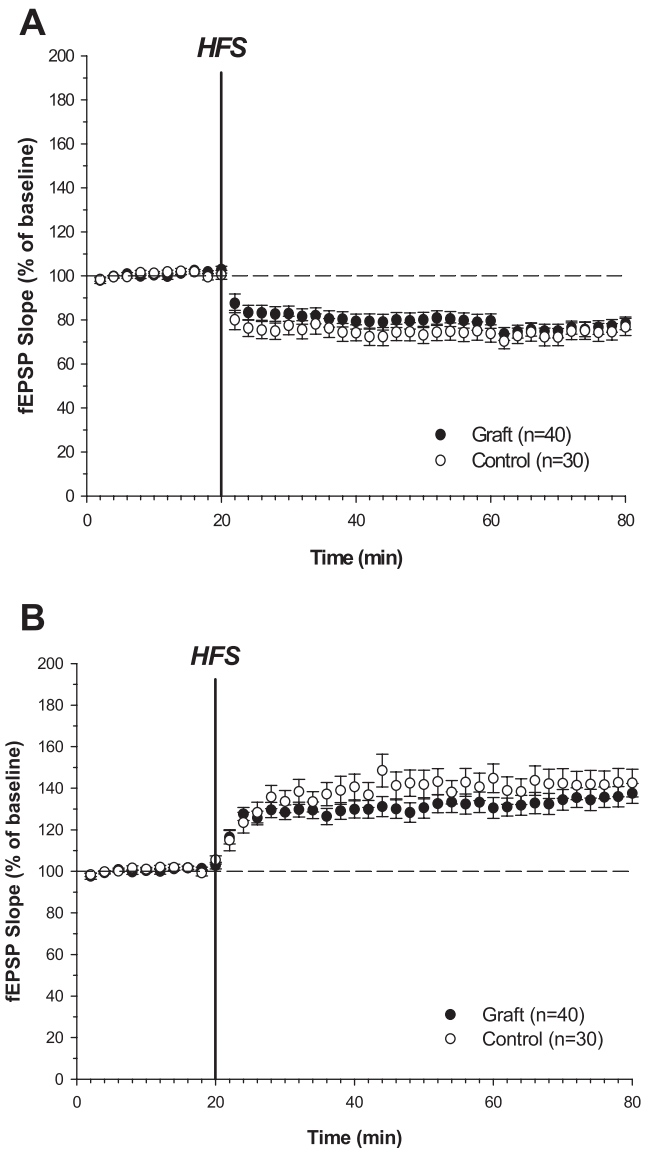

FIG. 3. Extracellularly recorded synaptic plasticity. (A) Expression of LTD in graft (n = 40) and control (n = 30) recordings. (B) Expression of LTP in graft (n = 40) and control (n = 30) recordings. HFS, high-frequency stimulation.

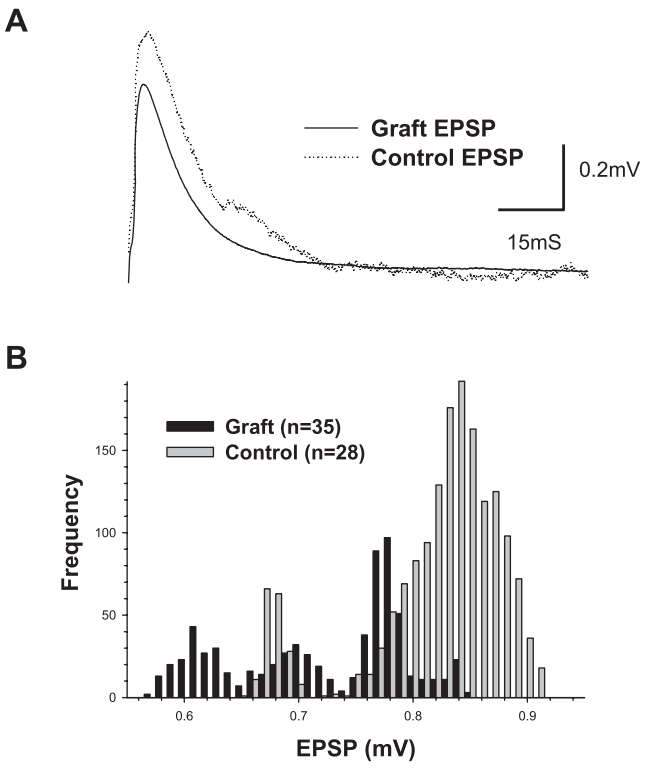

FIG. 4. Intracellularly recorded basal synaptic characteristics. (A) Example traces of MSN and TSN EPSPs. (B) Histogram representing the frequency of the absolute size of EPSPs recorded from graft (n = 35) and control (n = 28) neurons. EPSP, excitatory post-synaptic potential.

from control and graft recordings (Groups, $F_{1,28} = 1742.79$, $P < 0.001$), with control fEPSPs being of a greater size than those evoked from the grafts.

Extracellular recording of bi-directional synaptic plasticity

Under physiologically normal conditions, HFS of the cortico-striatal fibres resulted in the expression of LTD in 15 of 30 control recordings and 14 of 40 graft recordings, rates which did not differ between the two groups [$\chi^2_1 = 1.03$ (n = 70), $P = 0.31$].

LTD evoked from both control and graft recordings display a significant reduction in the size of the fEPSP when compared with their respective baselines (Fig. 3A; Groups × Time, $F_{39,2652} = 120.96$, $P < 0.001$), with no significant difference in the level of depression seen between graft and control recordings (Groups, $F_{1,68} = 0.87$, $P = 0.35$).

As previously demonstrated by Calabresi *et al.* (2000), in the normal striatum, removal of magnesium from the aCSF perfusate solution resulted in the preferential expression of LTP following HFS in 24 of 30 control recordings, and 22 of 40 graft recordings (Fig. 3B). Both control and graft recordings display a significant level of potentiation when compared with their baseline levels (Time, $F_{39,2652} = 56.50$, $P < 0.001$), with no significant difference in the level of potentiation between control and graft recordings (Groups, $F_{1,68} = 1.33$, $P = 0.253$).

Intracellular recording of host–graft basal synaptic characteristics and plasticity

As with extracellular recordings, it was possible to record EPSPs from TSNs beginning at 4 weeks after transplantation. TSNs were identified via their endogenous fluorescence, and intracellular recordings were made using sharp electrodes. Responses evoked from TSNs appeared visually similar to those recorded from MSNs (Fig. 4A). The frequency distribution of plots demonstrate that TSNs and MSNs display multi-modal expression of EPSPs (Fig. 4B), with TSNs displaying a significantly higher distribution of EPSP size than MSNs ($\chi^2_1 = 1297.81$, $P < 0.01$).

As with extracellular recordings, under normal physiological conditions HFS resulted in the expression of LTD in 18 of 28 control recordings, and 18 of 30 graft recordings, which did not differ in frequency ($\chi^2_1 = 0.59$, $P = 0.44$). LTD evoked from both control and graft recordings displayed a significant reduction in the size of the EPSP (Fig. 5A; Time, $F_{39,2184} = 143.18$, $P < 0.001$) compared with baseline recordings, with no significant difference in the level of depression between control and graft recordings (Groups, $F_{1,56} = 0.10$, $P = 0.751$).

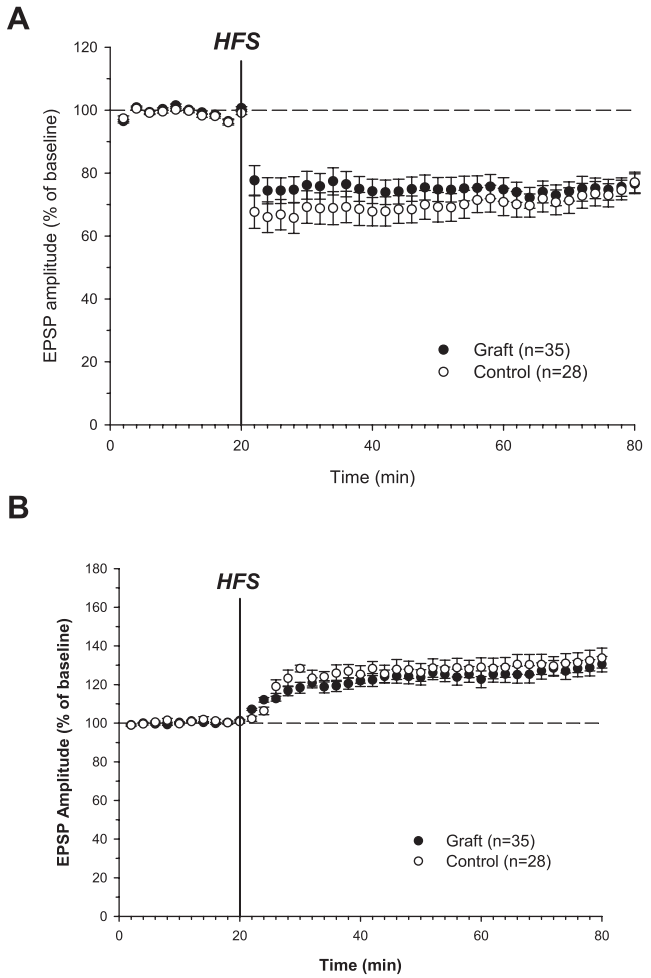

FIG. 5. Intracellularly recorded synaptic plasticity. (A) Expression of LTD in graft (n = 35) and control (n = 28) recordings. (B) Expression of LTP in graft (n = 35) and control (n = 28) recordings. HFS, high-frequency stimulation.

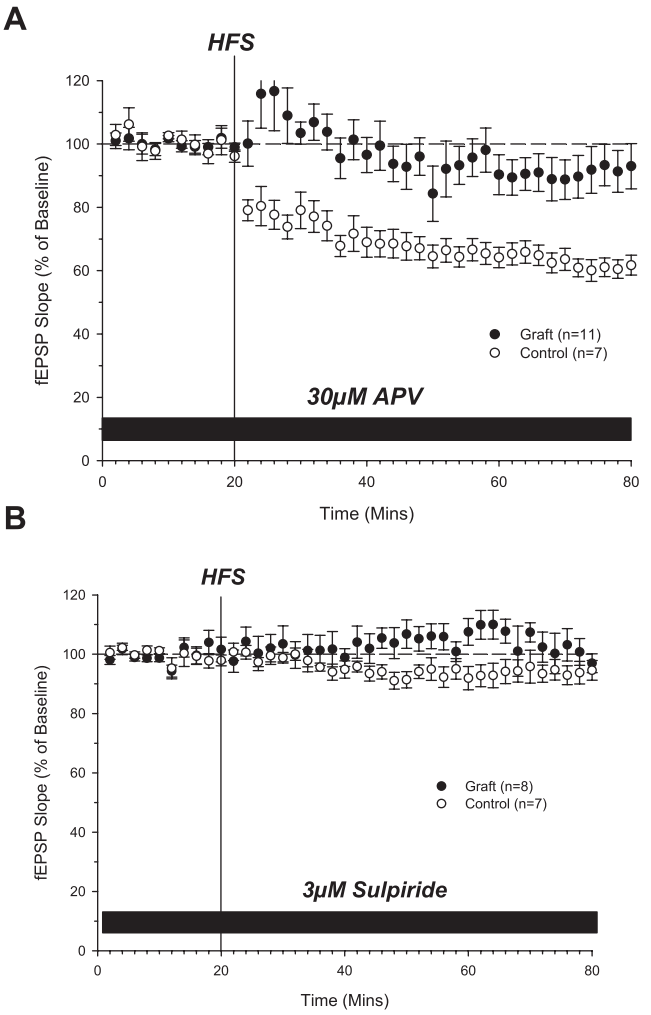

FIG. 6. Monoaminergic and glutamatergic characterization of graft synaptic plasticity. (A) Failure to express LTP in graft (n = 11) and control (n = 7) recordings, following application of 30 μM APV. (B) Failure to express LTD in graft (n = 8) and control (n = 7) recordings, following application of 3 μM sulpiride. HFS, high-frequency stimulation.

Similarly, following removal of magnesium from the aCSF perfusate, HFS resulted in the expression of LTP from 18 of 28 control recordings, and 18 of 30 graft recordings ($\chi^2_1 = 0.59$, $P = 0.44$). LTP evoked from both control and graft recordings displayed a significant increase in the size of the EPSP (Time, $F_{39,2184} = 135.68$, $P < 0.001$), with no significant difference in the level of potentiation between control and graft recordings (Groups, $F_{1,56} = 0.14$, $P = 0.823$).

Glutametergic and monoamergic characterization of graft synaptic plasticity

Pharmacological characterization of graft synaptic plasticity was carried out to ascertain that graft synaptic plasticity replicated the glutamatergic and monoaminergic characteristics previously reported in the normal striatum (Calabresi *et al.*, 2000).

Removal of magnesium from the aCSF perfusate and application of 30μM APV abolished the expression of LTP within both control (n = 7) and graft (n = 11) recordings (Fig. 6A), similar to the effects of this drug previously reported in the normal striatum (Calabresi *et al.*, 2000).

Under physiologically normal conditions, application of 3μM sulpiride abolished the expression of LTD, within both control (n = 7) and graft (n = 8) recordings (Fig. 6B), similar to the effects of this drug previously reported in the normal striatum (Calabresi *et al.*, 2000).

Discussion

The term 'functional integration' has been used to refer to both whole-animal behavioural changes associated with grafts and to anatomical and physiological aspects of the grafts within the host environments. In the current study the term is used in the anatomical and physiological sense, considering: (i) whether the grafted embryonic striatal neurons reconnect with the host; (ii) whether they demonstrate physiological properties akin to endogenous striatal neurons to establish synaptic contacts with the host striatal neurons capable of facilitating neuronal transmission; and (iii) whether they provide a substrate for restoring behavioural responses utilizing similar neural processes that are employed within the neuronal circuitry of the intact brain. In the present study we demonstrate that embryonic striatal grafts not only restore tonic baseline transmission within the lesioned striatum, but also permit the expression of bi-directional synaptic

plasticity. We suggest that the graft-mediated expression of synaptic plasticity offers a neuronal substrate for the behavioural plasticity observed in the phenomenon of 'learning to use the graft' (Mayer *et al.*, 1992; Brasted *et al.*, 1999). Therefore, whilst the observations presented in this study are of importance in demonstrating that striatal grafts are anatomically connected and physiologically active, we consider that these results provide, for the first time, direct evidence of a neural substrate at the level of cortico-striatal synaptic plasticity to underlie the previously observed functional observations of graft effects on motor learning and memory.

'Functional integration' of embryonic striatal grafts

It is well established that embryonic striatal grafts can integrate anatomically within the host striatal circuitry (Wictorin *et al.*, 1992; Clarke & Dunnett, 1993; Fricker-Gates *et al.*, 2001), and that this integration correlates with the observed restoration of behavioural performance across an array of tests which impinge on cortico-striatal function (Fricker *et al.*, 1997; Brasted *et al.*, 1999; Dobrossy & Dunnett, 2005). Yet, direct evidence that this integration involves the restoration of physiological correlates of neuronal function has hitherto been lacking.

'Functional integration' of embryonic striatal grafts requires the restoration of a number of correlates of synaptic function. In line with previous findings (Rutherford *et al.*, 1987; Walsh *et al.*, 1988; Xu *et al.*, 1989, 1991a,b; Siviy *et al.*, 1993), we observed that it is possible to detect the continuous expression of both EPSPs from TSNs and fEPSPs from the graft region following continuous basal stimulation of the host cortico-striatal fibres (Fig. 2). Previous analyses of host–graft cortico-striatal transmission have shown that the graft is responsive to host cortical stimulation, but questions have been raised regarding the stability of these connections (Rutherford *et al.*, 1987; Walsh *et al.*, 1988; Xu *et al.*, 1989, 1991a; b; Siviy *et al.*, 1993). We demonstrate that host–graft cortico-striatal recordings display stable amplitudes during constant low-frequency stimulation over periods of up to 60 min (Fig. 2B), demonstrating functional stability of the excitatory responses evoked from the graft, similar to that seen in normal striatal circuitry. However, although host–graft basal cortico-striatal transmission appears similar to that observed in normal striatal circuitry, we note an interesting difference in the ability of the two systems to respond to higher stimulus intensities (Fig. 2C). I/O curves were generated to ascertain the efficacy of baseline transmission (Fig. 2C). The responses elicited from the graft reach saturation point at a lower input current than control recordings (Fig. 2C). The mechanisms underlying this difference in transmission still remain unclear. However, it is important to note that as with previous findings (Rutherford *et al.*, 1987; Walsh *et al.*, 1988; Xu *et al.*, 1989, 1991a,b; Siviy *et al.*, 1993), we also show that EPSPs recorded from graft neurons are characteristically smaller than those observed from MSNs (Fig. 4B). It is therefore possible that the lower saturation of the fEPSP response to high stimulation intensities may be reflective of the smaller response size. Our findings pose an interesting question as to how a lower input saturation point may affect the pattern of signalling through the entire host–graft cortico-striatal circuit. Unfortunately, the answer to this question falls outside of the scope of the present study. It is important to note that our present findings both confirm and enhance previous studies of host–graft synaptic connectivity, thus providing further evidence to support the 'functional integration' of the graft cortico-striatal synapse within the host striatal circuitry.

Embryonic striatal grafts display bi-directional synaptic plasticity

Since its first description almost a decade ago, the phenomenon of 'learning to use the graft' has remained one of the strongest sources of evidence for 'functional integration' of striatal grafts into the host-striatal circuitry (Mayer *et al.*, 1992; Brasted *et al.*, 1999). However, direct evidence that such an effect is governed by the re-formation of functional cortico-striatal synapses is lacking. Furthermore, the expression of re-learning within striatal grafts has been theorized to involve the expression of synaptic plasticity, which is widely accepted as the most appropriate cellular correlate and putative substrate for 'Hebbian' learning (Bliss & Lomo, 1973). Additionally, recent evidence suggests that impaired striatal synaptic plasticity may provide a cellular mechanism for the observed cognitive symptoms in HD (Di Filippo *et al.*, 2007). Therefore, although restoration of synaptic plasticity to lesioned striatum is important, true repair of the HD-like degeneration will require expression of similar levels and mechanisms of plasticity, as seen in the normal striatum (Calabresi *et al.*, 2000; Di Filippo *et al.*, 2007).

We demonstrate here, for the first time, that embryonic striatal grafts are capable of expressing bi-directional synaptic plasticity following HFS of the host cortical fibres. Beginning at 4 weeks post-transplantation it is possible to induce both LTD and LTP within embryonic striatal grafts, depending on the tissue environment. Within the normal striatum, HFS results in a periodic expression of LTD. We found a 50% (15 of 30 recordings) probability of inducing LTD from control recordings following HFS. Failure to induce LTD was not confined to either particular slices or particular animals. In many cases, there were recordings from one site within the striatum that failed to display LTD, whereas other sites within the same slice did yield LTD following HFS. Therefore, it would appear that induction of LTD within the normal striatum is critically dependent on the location of recording, which presumably relates at least in part to the topography of cortico-striatal connections. Within embryonic striatal grafts the probability of expressing LTD following HFS was 35% (14 of 40 recordings), somewhat lower than that seen in control recordings from the normal striatum, but not significantly so. Unlike control recordings, moving the location of the recording and stimulating electrode often failed to yield LTD. Therefore, although it is clear that obtaining LTD from striatal grafts is much more difficult than within the normal striatum, evidence for the mechanisms underlying this is limited to electrophysiological observations. We hypothesize that the differences may relate not only to the pattern and extent of corticostriatal connections reformed in the grafts, but also to the fact that most 'striatal' grafts derived from the ganglionic eminence contain patches of striatal-like neuronal populations, interspersed with areas of non-striatal cell types originating from the same area of the embryonic forebrain (Graybiel *et al.*, 1989). Following transplantation, functional innervation of the graft is time-dependent and varied across the numerous striatal afferents (Wictorin *et al.*, 1988, 1989). Furthermore, innervation of the graft does not occur homogenously, with various regions of the graft receiving different levels of afferent innervation across the post-transplantation period. Anatomical studies have clearly shown that innervation of the outer regions of the graft is established around 4 weeks after transplantation. Following this period, innervation of the graft begins to reach the innermost regions over 3–6 months (Wictorin *et al.*, 1988, 1989). During the present study, LTD is first sought from the innermost regions of the graft, before moving to the other regions should LTD fail to be expressed. Although success is not guaranteed from the outer regions of the graft, the probability of gaining LTD is increased. Additionally, it has been shown that within striatal grafts only 50–80% of the corticostriatal afferents form anatomically correct

synapses with transplanted striatal neurons (Wictorin *et al.*, 1989; Xu *et al.*, 1989). This raises important questions about whether graft maturity plays a role in the ability to express LTD, and whether these anatomically incorrect synapses hinder the expression of LTD. These issues lie outside of the scope of the present study, but warrant further investigation by, for example, examining slices with grafts prepared at various post-transplantation periods.

Although recordings from striatal grafts display a lower probability of expressing LTD than those seen in control recordings, there is no significant difference in the level of depression observed in recordings from striatal grafts when compared with those from the normal striatum. It is important to note, however, that the size of the fEPSP recorded from striatal grafts is lower, on average, than that recorded from the control striatum. Thus, although the fEPSPs recorded from striatal grafts are smaller than those recorded post-tetanus in controls, both responses decrease in size by the same proportion following HFS. Successful expression of LTD occurs to similar levels to that seen within control recordings. Therefore, it would appear that within striatal grafts the host–graft corticostriatal synapse is not only functional to host stimulation but is also plastic to changes to host stimulation. Furthermore, we demonstrate that application of the D2 receptor antagonist sulpiride blocks the expression of LTD within grafts. This observation in the grafted slices replicates observations in the normal striatum, suggesting LTD in both striatal grafts and the normal striatum is dependent upon a similar D2-mediated mechanism.

As with LTD, it is possible to express LTP from striatal grafts from 4 weeks following transplantation. One interesting observation is that following removal of magnesium the probability of obtaining synaptic plasticity, in this case LTP, is somewhat higher (55%, 22 of 40 recordings) than the probability of obtaining LTD under normal physiological conditions, although again the difference was not significant. This is interesting as it might have been expected that the factors responsible for the lower probability of expressing synaptic plasticity from striatal grafts under normal physiological conditions would also affect the probability of expressing synaptic plasticity under conditions where magnesium is removed. If such factors do contribute to the ability to express LTP in the absence of magnesium, there are a number of possible reasons for the higher probability of obtaining synaptic plasticity.

Under magnesium-free conditions the NMDA receptor is chronically active, and therefore freely able to contribute to baseline transmission and LTP induction. The induction of LTP is critically dependent on the internal calcium concentration, which in turn is critically dependent on calcium flow into the neuron. Chronic activation of NMDA receptors results in a large increase in calcium current into the neuron, and therefore increases the cell's ability to potentiate following HFS. Furthermore, increased calcium entry into the post-synaptic neuron would make the neuron more responsive to weaker inputs. Therefore, weak stimulation intensity may not be sufficient to induce LTD, yet still be sufficient to induce LTP, due to the higher potential of the post-synaptic neurons to be potentiated following removal of magnesium. This increased potentiation effect can be seen as a positive drift in the baseline values during low-frequency baseline recordings, prior to LTP induction. Additionally, it is important to note that a number of studies have shown that TSNs display a higher degree of excitability than the MSN of the normal striatum (Siviy *et al.*, 1993). Therefore, it is possible that an increased NMDA excitability may be contributing to the increased probability of expressing synaptic plasticity in magnesium-free conditions. Furthermore, we demonstrate that application of the NMDA receptor antagonist APV blocks the expression of LTP within grafts. However, it is interesting to note that whilst the normal striatum shows a clear expression of LTD (under such conditions), application of APV does not cause a similar expression of LTD within striatal grafts.

The demonstration of host–graft bi-directional synaptic plasticity is a significant discovery. The crux of 'learning to use the graft' is that the graft provides the cellular substrate for re-learning previously learnt motor skills and habits lost post-lesion, and restored following transplantation (Mayer *et al.*, 1992; Brasted *et al.*, 1999). Critical to this concept is the recognition that an animal trained to perform a motor skill or habit, which is disrupted by a lesion of the striatum, does not show recovery after transplantation until it has undergone further training to relearn the lost skill or habit. As the normal striatum is known to be involved in motor learning and habit formation, this observation suggests that reformation of corticostriatal connections is not sufficient to enable the graft to perform the lost motor responses, but rather that the new neurons replaced by transplantation provide a new circuitry for motor learning and are the substrate within which the habit or skill is represented. The transplanted neurons not only restore lost connections but are integrated as a functional component within a plastic neuronal system necessary to learn and perform skilled actions. Thus, these data indicate restoration of a complex level of neuronal processing and circuit repair that exceeds simple expectations for neuronal replacement at the level of tonic pharmacological signal or information relay. Although transfer of training has been used behaviourally to suggest that the relearning required the presence of the graft circuitry, and the known organization of basal ganglia circuits suggests that the grafted striatal neurons could provide a necessary replacement for lost striatal projection neurons as a substrate for relearning, the present physiological observations provide the first direct evidence that they do exhibit bi-directional plasticity at the physiological level of a type considered necessary to represent new motor learning and the performance of skilled motor habits. Furthermore, these findings suggest that neurological rehabilitation following clinical transplantation in patients with neurodegenerative disease could improve the functional outcome and optimize the benefits of such intervention.

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Abbreviations

aCSF, artificial cerebrospinal fluid; DMEM, Dulbecco's modified Eagle medium; EPSP, excitatory post-synaptic potential; fEPSP, field excitatory post-synaptic potential; GFP, green fluorescent protein; HD, Huntington's disease; HFS, high-frequency stimulation; I/O, input/output; LTD, long-term depression; LTP, long-term potentiation; MSN, medium spiny projection neuron; TSN, transplanted striatal neuron; WGE, whole ganglionic eminence.

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