Supporting Information

Cicchetti et al. 10.1073/pnas.0904239106

SI Text

Supplemental Clinical Outcome. Significant cortical atrophy was noted on the preoperative MRI scan of patient 1 (B.L.). At the end of surgery, brain shrinkage because of cerebrospinal fluid loss was noted. The surface of the brain was ≈8 mm from the inner cortex of the skull. No subdural hematoma was noted on the immediate postoperative MRI scan. However, cortical shrinkage led to targeting error in the last needle tract (in the right caudate) (see Fig. S2). A 2-cm thick asymptomatic subacute subdural hematoma was noted on the localizing MRI scan done before the second operation and was drained uneventfully after completion of the contralateral transplant procedure. Follow-up MRI scans demonstrated complete resolution of the hematoma. The Unified Huntington's Disease Rating Scale (UHDRS) scores improved from 42 at baseline to 30 to 32 at 3 and 9 months postoperatively, but returned to baseline (1) by 18 months after surgery. Preoperative gait instability with falling improved for 2.5 years, whereupon a wheelchair was required and swallowing difficulties began to emerge. UHDRS scores stabilized until 3.5 years after surgery. Scores then progressively deteriorated to 52, 65, and 78 at 4, 6, and 7 years postoperatively. Cognitive function also progressively declined with Mini Mental State Evaluation (MMSE) scores worsening from 30 at both baseline and at 21 months postsurgery to 14 at 7 years postsurgery. She died at 67 years of age of an aspiration pneumonia complicated by a myocardial infarction 9 years postoperatively.

Patient 3 (M.C.) had an asymptomatic 1-cm cortical hemorrhage after her first operation, as well as a thin (3 mm) subdural hematoma without mass effect. She had postoperative confusion that resolved in 2 weeks. A 4-mm subdural hematoma was noted after the second operation, and the bilateral thin subdural hematomas both resolved on subsequent postoperative imaging without complications. Her preoperative UHDRS score of 33 remained unchanged until 10 to 12 months postsurgery, when scores improved to 28 and 21, respectively. Her preoperative balance difficulties and falling (once per month) improved postoperatively, and she did not fall for at least 2 years after surgery. Symptoms then worsened to a UHDRS score of 37 at 20 months after surgery and plateaued there until 3 years postoperatively. Cognitive function declined mildly with an MMSE score of 26 at baseline and 24 at 30 months postsurgery. Some depressive symptoms emerged at 2 years after surgery. The patient died at the age of 75 of a cardiorespiratory arrest as complication of end-stage HD, 10.5 years after transplantation.

Patient 5 (M.S.) was noted to have significant cortical and striatal atrophy on the preoperative MRI scan. She had 7-mm thick bilateral hygromas postoperatively, which increased to 1 cm after the second operation. She tripped at home, hitting her head 2 weeks after her second operation, with a 1- to 2-min loss of consciousness. A CT evaluation demonstrated conversion of her hygromas into subdural hematomas that required surgical drainage bilaterally. The hematomas were 2.5-cm thick on the left side and 1.0-cm thick on the right side at the time of surgery. The CT scan 1 month later demonstrated complete resolution of the subdural hematomas. She never improved back to baseline. Her UHDRS score was 27 before surgery and 31 after drainage of her subdural hematomas. She deteriorated to a score of 39 by 9 months postoperation that remained stable until 2.5 years after surgery, when she began a more rapid deterioration to a score of 53. By 5 years postoperatively, her UHDRS score was 64. MMSE scores declined from 27 at baseline to 16 at 5 years postsurgery. She died at the age of 68 of a cardiorespiratory arrest, secondary to end-stage HD, 9.5 years after surgery.

SI Methods

Histochemistry. Sections were washed 3 times in PBS 0.1M before histochemical staining for NADPH-d (marker for nitric oxide containing striatal interneurons), AChE (enzyme catalyzing hydrolysis of the neurotransmitter acetyl choline in cholinergic neurons), and H&E (brain cytoarchitecture). For NADPH-d staining, sections were washed in PBS 0.1M pH 7.4, preincubated in 0.25% Triton X-100 in PBS for 10 min and transferred in a fresh solution of 0.25% Triton X-100, 0.05% of the reduced form of nicotinamide adenine dinucleotide phosphate (b-NADPH; Calbiochem), 0.02% nitro blue tetrazolium (Sigma) in PBS for 5 min at room temperature and then at 37 °C for 8 h. Sections were rinsed in PBS, mounted on gelatin-coated slides, and kept at 37 °C overnight. They were subsequently dehydrated in ascending grades of ethanol and coverslipped with DPX mounting media (Electron Microscopy Science).

For AChE staining, sections were washed 3 times in distilled water and incubated in 0.2% acetylthiocholine iodide (Sigma) in a stock solution (copper sulfate, glycine, magnesium chloride, maleic acid in 4% NaOH, 40% sodium sulfate). They were then rinsed 3 times in 40% sodium sulfate, and incubated in 10% ammonium sulfide for 2 min. After washes in distilled water, sections were counterstained with Kernechtrot red (J.T. Baker) for 1 min and rinsed in tap water. Slices were mounted on gelatin-coated slides, air-dried overnight, dehydrated in ascending grades of ethanol, and cover-slipped with DPX mounting media.

For H&E staining, sections were first mounted on gelatin-coated slides, air-dried overnight, and hydrated in 50% ethanol. They were then stained with hematoxylin (Fisher Scientific) for 40 sec, washed in running water for 5 min, and placed in differentiator solution (0.5% pure glacial acetic acid in 95% ethanol) for 1 min. Sections were washed using distilled water and counterstained with Eosin Y (Sigma) for 40 sec, dehydrated in ethanol and xylene baths (3 × 90% ethanol, 2 × 100% ethanol, 2× xylene) and cover-slipped with DPX mounting media.

Immunohistochemistry. Before immunostaining procedures, free-floating sections were washed 3 times in PBS 0.1M pH 7.4 and placed in 3% peroxide in 0.1M PB for 30 min at room temperature. For single immunostaining, sections were subsequently washed in PBS and then preincubated for 30 min at room temperature in a blocking solution containing, 0.1% Triton X-100 (Sigma) and 5% Normal Goat Serum (NGS, Wisent Inc.) diluted in PBS. Sections were incubated for 24 h at 4 °C in the same solution to which either anti-GFAP (Dako Canada,; 1:2,500) or anti-TH (Pel-Freez; 1:1,000), or for 48 h at 4 °C with anti-PV (Sigma; 1:1,000). Sections were then washed in PBS and incubated for 1 h at room temperature in the blocking solution to which biotinylated goat anti-rabbit (for GFAP and TH) or biotinylated goat anti-mouse (for PV) (Vector Laboratories; 1:1,500) was added. Following 3 washes in PBS 0.1M, sections were placed in a solution of avidin-biotin peroxidase complex (ABC Elite kit, Vector Laboratories) for 1 h at room temperature. Antibodies were revealed by placing the sections in Tris buffer solution containing 0.05% 3.3′-diaminobenzidine tetrahydrochloride (DAB, Sigma) and 0.1% of 30% hydrogen peroxidase at room temperature. Reaction was stopped by washing in 0.05M Tris buffer and subsequent PBS washes. Slices were

mounted on gelatin-coated slides, air-dried overnight, dehydrated in ascending grades of ethanol, xylene, and cover-slipped with DPX mounting media.

Other sections were immunohistochemically processed with nickel-intensification of DAB to enhance the chromogen signal. These sections were treated in a similar manner as described above except that the main buffer solution was composed of PBS 0.2M pH 7.4 and 1% BSA (Sigma) was added as a blocking agent in the primary and secondary antibodies, as well as in the ABC solutions. Sections were incubated 48 h at 4 °C with either anti-CB (Sigma; 1:2,500), CD4 (Serotec; 1:250), CD8 (Serotec; 1:200), HLA-DR (Serotec; 1:200), ubiquitin (Calbiochem; 1:250), synaptophysin (Calbiochem; 1:500), EM48 (provided by X.J. Li, Emory University; 1:2,000) or anti-CR (Swant; 1:2,500) and 1 h with biotinylated goat anti-mouse (for CB) or goat anti-rabbit (for CR) (Vector Laboratories; 1:1,500). After incubation with ABC, sections were washed twice in acetate imidazole 0.2M pH 7.2 followed by Ni-DAB solution (dH_2O, sodium acetate 1M pH 7.2, imidazole 0.2M pH 9.2, nickel-sulfate 6.H_2O, DAB, H_2O_2 30%). Immunohistochemical reaction was terminated by washes in acetate imidazole 0.2M (pH 7.2) followed by 0.2M PBS rinses. Slices were mounted on gelatin-coated slides, air-dried overnight, dehydrated in ascending grades of ethanol, and cover-slipped with DPX mounting media. In these experiments, immunohistochemical controls included omission of the primary or the secondary antibody, which completely abolished the immunostaining.

Other sections were processed for double immunohistochemistry to visualize neuronal nuclei (NeuN) and microglia (Iba-1) or calcium binding protein (CB) and vGlut1. After overnight incubation at 4 °C with an antibody against Iba-1 (Wako Chemicals; 1:1000) or 48 h at 4 °C with an antibody against vGlut1 (Sigma; 1:500), the sections were extensively washed in PBS and incubated for 1 h at room temperature in a PBS solution containing biotinylated goat anti-rabbit IgG (Vector Labs; dilution 1:1,500), Triton X-100 (0.1%), BSA (1%), and NGS (5%). After further washing in PBS, the sections were placed in a solution containing ABC (Elite kit; Vector Labs) for 1 h at room temperature. The bound peroxidase was revealed with nickel-intensified DAB as the chromogen. After immunostaining for Iba-1 or vGlut1, the sections were reincubated overnight at 4 °C with a NeuN antibody (Chemicon; 1:1,000) or 48h at 4 °C with an antibody against CB (Sigma; 1:2,500). The incubation procedures were the same as above, except that the incubation time in secondary antibody, goat anti-mouse (Vector Labs; 1:1,500), was 2 h and sections were revealed using DAB (Sigma) and 0.01% hydrogen peroxide in 0.05 M Tris-imidazole (pH 7.2) at room temperature. The reaction was stopped after 10 to 15 min by extensive washing in PBS. Controls included omission of either one of the primary antibodies to exclude cross-reactivity of the secondary antibodies.

Sections intended for electron microscopy were prepared as above for vGlut1 immunohistochemistry, but for single immunostaining only, without Triton X-100 in all solutions and using DAB as the chromogen. After revelation, these sections were osmicated, dehydrated in ethanol and propylene oxide, and flat-embedded in Durcapan (Fluka). Rectangular pieces within the grafted P-zone were removed from the flat-embedded vGlut1-immunostained sections, glued to the tip of resin blocks, and sectioned ultrathin (80 nm) with a Reichert Jung ultramicrotome. These sections were collected on bare 150 mesh copper grids, stained with lead citrate, and examined with a Phillips CM100 electron microscope (60 kV, Philips Electronique).

Assessment of Graft Volume and Location. Nissl staining, as described previously (2), was used to perform the 3-dimensional reconstruction of transplantation sites using Neurolucida modeling software (Microbrightfield) attached to a E800 Nikon microscope (Nikon Instruments) (see below). Two distinct sets of calculations were performed in relation to graft volume. The first calculations, which are found in Table 1 of Fig. 1, depict the volumes of the entire corpus striatum (putamen, caudate and the globus pallidus). Representative serial sections (1 in 10) were compared to equivalent sections from the corpus striatum of a control brain. The second set of volumetric measurements was used to evaluate the degree of brain shrinkage of patients 1 and 5. For this measurement, serial sections of the putamen of the transplant recipient were compared to equivalent serial sections of the putamen in an age-matched control brain.

Estimation of the Striatal Zones (P-Zones) Within the Grafts. Volumetric evaluation of graft size (Cavalieri method) as well as P-zone and non-P-zone areas for patients 1 and 5 were explicitly performed using the right hemisphere of both patients 1 and 5. Both grafts and P-zones were delineated using Nissl staining and the Tracing Contours option in the Stereo Investigator software, version 5.0 (Microbrightfield). Areas for either P-zones or non-P-zones were calculated using Contour Measurements option.

Three-dimensional reconstruction. Three-dimensional reconstruction was performed using the Serial Section Reconstruction method provided by the Neurolucida software, version 6.0 (Microbrightfield). Briefly, the caudate, putamen, and transplant sites were traced using the Tracing Contours function for each section. Subsequently, each tracing was imported into the NeuroExplorer software, where the drawings were aligned to complete the 3-dimensional reconstruction. This procedure allowed calculation of structure and graft volumes, which took into account section thickness (40 μm).

1. Vonsattel JP, et al. (1985) Neuropathological classification of Huntington's disease. *J Neuropathol Exp Neurol* 44:559–577.

2. Freeman TB, et al. (2000) Transplanted fetal striatum in Huntington's disease: phenotypic development and lack of pathology. *Proc Natl Acad Sci USA* 97:13877–13882.

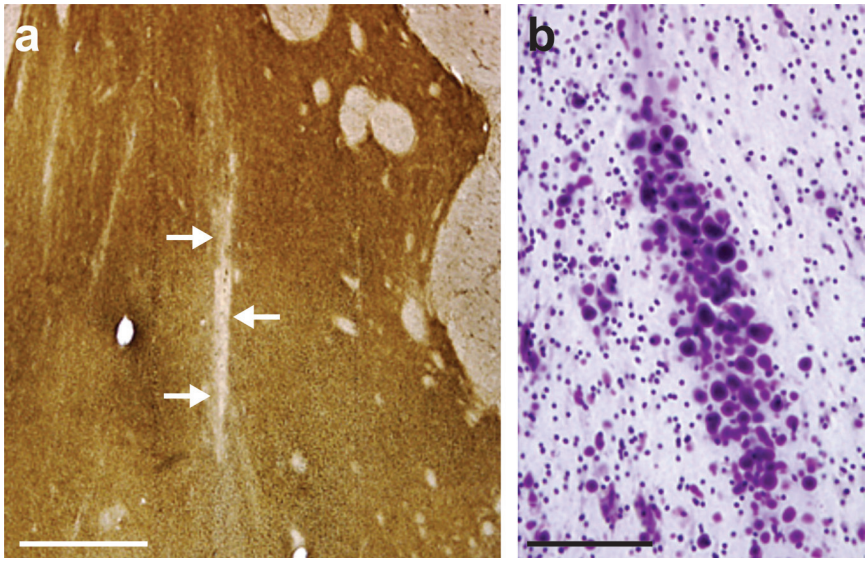
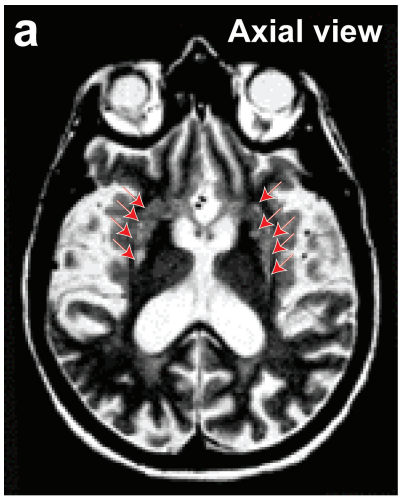
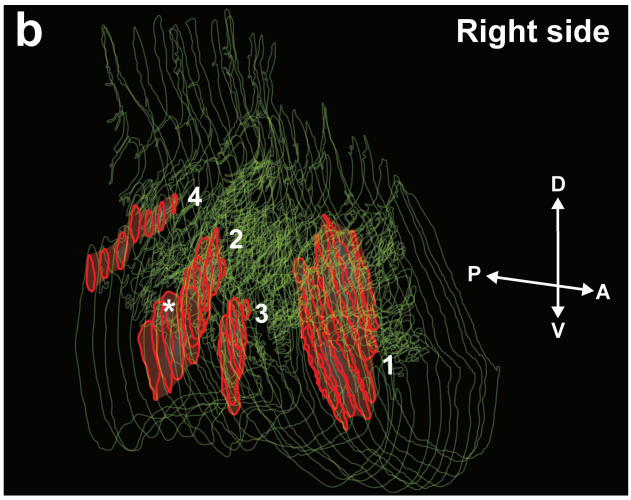
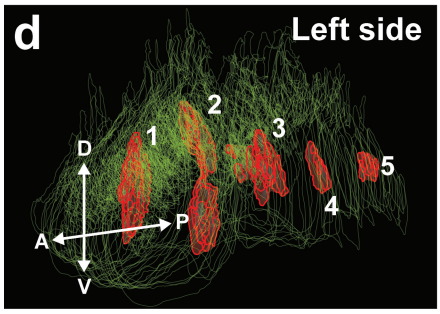


Fig. S1. Necrotic grafts. Examples of necrotic grafts found in the putamen of patient 3 stained with (*a*) AChE (*arrows*) and (*b*) Nissl staining. (Scale bars: *a*, 250 μm; *b*, 50 μm.)

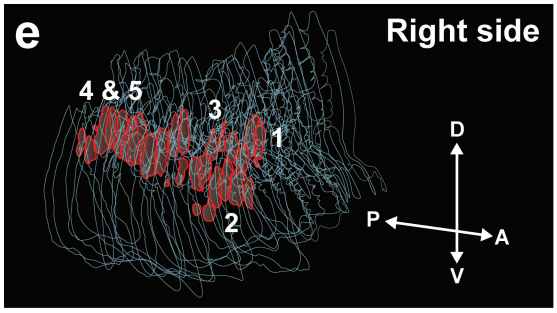


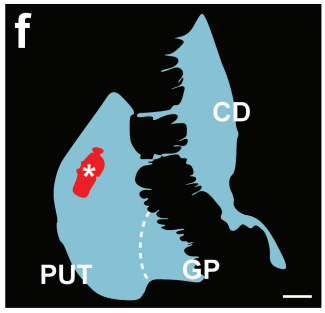




Graft sites Right side	Total volume (mm^3)	% of total volume
1	24.2	1.1
2	21.8	1.3
3	9.1	0.4
4	2.8	0.1
Total % of graft volume within corpus striatum		2.9

Graft sites Left side	Total volume (mm^3)	% of total volume
1	33.4	0.9
2	28.8	0.7
3	37.7	1.0
4	39.7	1.0
5	9.3	0.2
Total % of graft volume within corpus striatum		3.8





Graft sites Right side	Total volume (mm^3)	% of total volume
1	4.24	0.2
2	11.7	0.7
3	18.0	1.1
4 & 5	36.5	2.3
Total % of graft volume within corpus striatum		4.3

Fig. S2. Graft locations. (*a*) Axial view of an MRI scan performed 24 h after the first surgery of patient 1 showing localization of graft sites (*red arrows*) in both hemispheres. (*b–f*) Computer-generated 3-dimensional reconstruction of transplantation sites, based on a virtual representation of the Nissl staining of a series of 1 in 10 coronal sections of 2 of the 3 postmortem hemispheres analyzed in the present study (patients 1 and 5). Data derived from patient 1 is illustrated in (*b*) through (*d*) and has been colored in green. Patient 5 is illustrated in (*e*) and (*f*) and is colored in blue. Transplant sites in both cases are depicted in red. All putamenal grafts but not all caudate grafts were located. Total graft volume in patients 1 and 5 was 3 to 4% and 4.3% of the total host striatal volumes, respectively. (Scale bars in *c* and *f*, 1 mm.) A, anterior; CD, caudate; D, dorsal; GP, globus pallidus; P, posterior; PUT, putamen; STR, striatum; V, ventral.

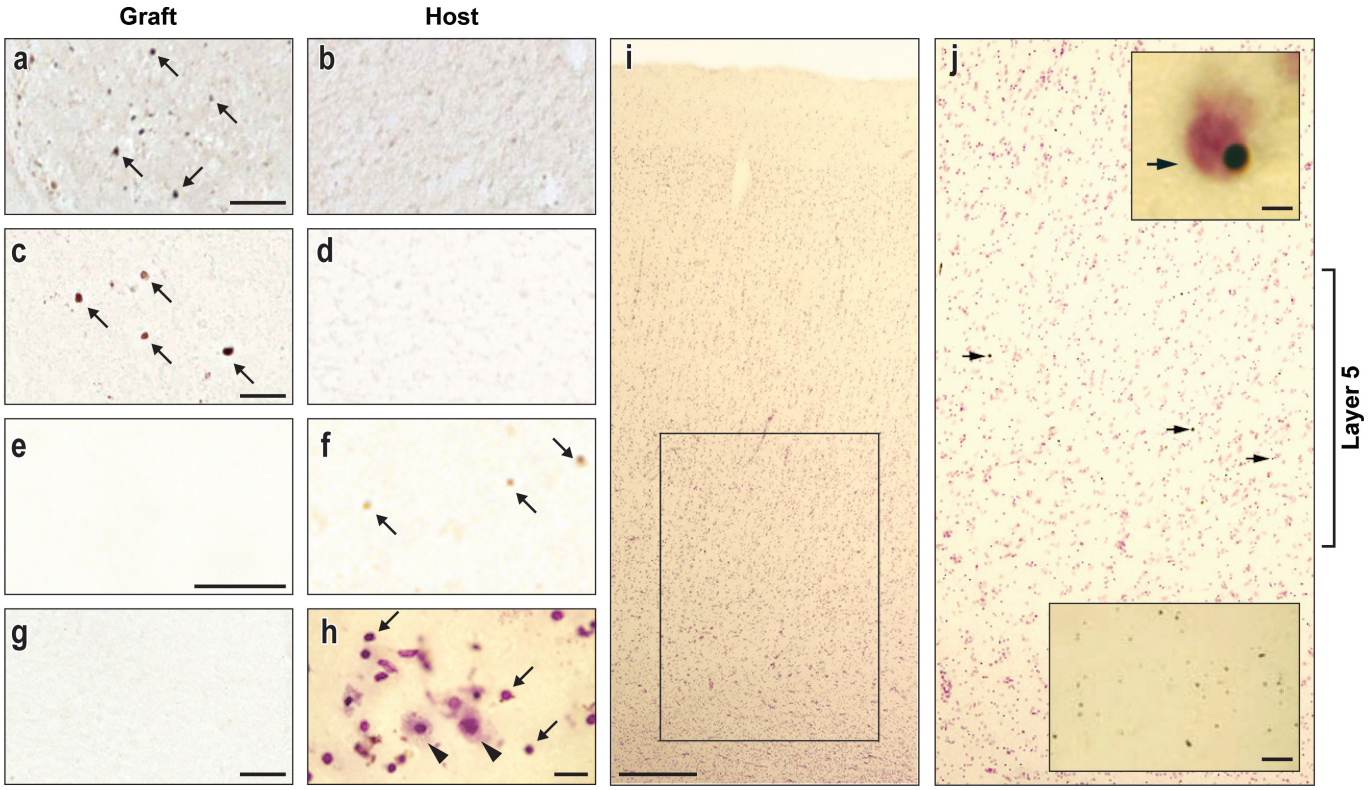


Fig. S3. Immune responses to grafts. (*a*) Photomicrographs of CD8 immunostaining (cytotoxic suppressor T-cell subset) demonstrated a greater CD8 immune response within the transplant (*arrows*) compared to the host (*b*). A similar difference was found for CD4 (subpopulation of T lymphocytes) immunostaining, where CD4-positive staining was seen within the graft (*c*, *arrows*) but not within the host (*d*). Neither ubiquitin staining, showing protein aggregates (*e* and *f*, *arrows*), nor EM48 (*g* and *h*), identifying the abnormal huntingtin mutant protein (*arrows*) within Nissl-stained cells (*arrowheads*), showed expression in the graft (*e* and *g*) as compared to the host putamen (*f* and *h*), where the abnormal *huntingtin* gene is expressed. These photomicrographs were taken from patient 1 but similar observations were made for patient 5. (*i* and *j*) EM48 expression was also pronounced in layer 5 of the cortex (*arrows* in *j*) (pictures taken from patient 3). Photomicrograph in (*j*) represents a higher magnification of (*i*) (see *Inset*). Depicted in (*j*) is also a higher magnification of the layer 5 distribution of nuclear inclusions (*Bottom Inset*) and higher magnification of a doubly stained neuronal nuclear inclusion (Nissl/EM48) (*Top Inset*). (Scale bars: *a* and *b*, 100 μm; *c* and *d*, 50 μm; *e* and *f*, 50 μm; *g*, 25 μm; *h*, 14 μm; *i*, 300 μm; *Insets j*: *Top*, 6.25 μm; *Bottom*, 50 μm.)

Table S1. Patient characteristics

	Patient 1 (B.L.)	Patient 3 (M.C.)	Patient 5 (M.S.)
Gender	Female	Female	Female
CAG repeats	42	42	42
Grades	3	3	2
Time from diagnosis (years)	5	12	5
Symptom duration (years)	8	17	9
Age at transplantation (years)	58	64	59
Post-operative latency (years)	9	10.5	9.5
Causes of death	Aspiration pneumonia complicated by a myocardial infarction	Cardiorespiratory arrest as a complication of end-stage HD	
Immunosuppression	CsA 6 mg/kg/day 7 days before first operation continuing for 14 days after second operation, then 2 mg/kg/day for 6 months		
Number and location of transplants	1 L caudate	2 L caudate	1 L caudate
	4 L putamen	6 L putamen	4 L putamen
	1 R caudate	2 R caudate	1 R caudate
	4 R putamen	6 R putamen	5 R putamen
Surgical complications	Asymptomatic subacute subdural hematoma after first operation	Asymptomatic cortical hemorrhage and thin subdural hematoma after first and second operations	Bilateral hygromas post-operatively. Conversion of her hygromas into subdural hematomas requiring surgical drainage

Data from ref. 1 and subsequent clinical evaluation.

1. Hauser RA, Sandberg PR, Freeman TB, Stoessl AJ (2002) Bilateral human fetal striatal transplantation in Huntington's disease. *Neurology* 58:1704, author reply 1704.

Table S2. Clinical outcomes

	Patient 1 (B.L.)	Patient 3 (M.C.)	Patient 5 (M.S.)
UHDRS score pre-operation	42	33	27 (31 after subdural hematoma drainage)
UHDRS score post-operation	30 (3 mo)	28 (10 mo)	39 (9 mo)
	32 (9 mo)	21 (12 mo)	53 (2.5 yrs)
	40 (18 mo)	37 (20 mo)	64 (5 yrs)
	52 (4 yrs)		
	65 (6 yrs)		
	78 (7 yrs)		
MMSE score pre-operation	30	26	27
MMSE score post-operation	30 (21 mo)	24 (30 mo)	16 (5 yrs)
	14 (7 yrs)		

MMSE, Mini Mental State Evaluation; UHDRS, Unified Huntington's Disease Rating Scale.
Data from ref. 1 and subsequent clinical evaluation.

1. Hauser RA, Sandberg PR, Freeman TB, Stoessl AJ (2002) Bilateral human fetal striatal transplantation in Huntington's disease. *Neurology* 58:1704, author reply 1704.