Outcome of cell suspension allografts in a patient with Huntington’s disease

1Alexander Maxan*, 2Sarah Mason*, 1Martine Saint-Pierre, 2Emma Smith, 2Aileen Ho, 3Tim Harrower, 2Colin Watts, 2Yen Tai, 4Nicola Pavese, 1,5Julie C. Savage, 1,5Marie-Eve Tremblay, 6Peter Gould, 7Anne E. Rosser, 7Stephen B. Dunnett, 4Paola Piccini, 2Roger A. Barker*, 1,8Francesca Cicchetti*

*Equal first and last authors

1Centre de Recherche du CHU de Québec (CHUQ), Axe Neurosciences, Québec, QC, Canada, G1V 4G2; 2John van Geest Centre for Brain Repair and Department of Clinical Neuroscience, University of Cambridge, Cambridge, United Kingdom, CB2 0PY; 3Royal Devon and Exeter Hospital, Barrack Road, Exeter, Devon, EX2 5DW, 4Department of Medicine, Neurology Imaging Unit, Imperial College London, London, United Kingdom, W12 0HS; 5Département de médecine moléculaire, Université Laval, Québec, QC, Canada, G1K 0A6; 6Laboratoire de neuropathology, Hôpital de l’Enfant-Jésus-CHU de Québec, Québec, QC, G1J 1Z4; 7Brain Repair Group and BRAIN unit, Neuroscience and Mental Health Research Institute and School of Biosciences, Cardiff University, Cardiff, United Kingdom, CF10 3AX; 8Département de Psychiatrie & Neurosciences, Université Laval, Québec, QC, Canada, G1K 0A6

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Correspondence to either:
Francesca Cicchetti, Ph.D.
Centre de Recherche du CHU de Québec
Axe Neurosciences, T2-07
2705, Boulevard Laurier
Québec, QC, G1V 4G2, Canada
Tel #: (418) 656-4141 ext. 48853
E-mail: Francesca.Cicchetti@crchul.ulaval.ca

Roger A. Barker
Centre for Brain Repair
Forvie Site, Robinson Way
Cambridge, CB2 2P7, United Kingdom
Tel #: +44 (0)1223 337733
E-mail address: rab46@cam.ac.uk

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Abstract

For patients with incurable neurodegenerative disorders such as Huntington’s (HD) and Parkinson’s disease, cell transplantation has been explored as a potential treatment option. Here we present the first clinico-pathological study of a patient with HD in receipt of cell-suspension striatal allografts who took part in the NEST-UK multi-centre clinical transplantation trial. Using various immunohistochemical techniques, we found a discrepancy in the survival of grafted projection neurons with respect to grafted interneurons as well as a major ongoing inflammatory and immune responses to the grafted tissue with evidence of mutant huntingtin aggregates within the transplant area. Our results indicate that grafts can survive more than a decade post-transplantation but show compromised survival with inflammation and mutant protein being seen within the transplant site.
Introduction
Huntington’s Disease (HD) is an autosomal dominant neurodegenerative disorder that presents with a combination of motor, cognitive and psychiatric problems that progress over a 20-year period to death. It is currently incurable and while many therapies have been the subject of clinical trials, none have been shown to alter the natural history of this condition. More than twenty years ago, work commenced on a novel strategy that involved allografting foetal tissue into the striatum of patients with mid-stage disease to attempt to repair the damaged circuitry, as had been shown preclinically using excitotoxic lesions of the rat and monkey striatum. To date, seven small open-label studies of neural transplants have been conducted worldwide assessing the feasibility, safety, and tolerability of this procedure in patients with HD. This approach has yielded mixed results including post-mortem analysis of transplanted patients. Here we present long-term histological data on 1 of the 5 patients treated with a fetal striatal cell suspension allograft as part of the UK study.

Methods

Trial information
This patient was one of 5 who were part of the NEST-UK multicentre study which was initiated in 1998 to evaluate the safety and efficacy of bilateral fetal striatal transplantation in patients with mild HD (ISRCTN no 36485475). The operations were undertaken in Cambridge between 2000 and 2003 and this report deals with patient number 5, the only one to receive bilateral transplants of fetal striatal tissue in a single operation. The trial was approved by the Cambridge Regional Ethics Committee (REC ref: 95/086), as was the post-mortem analysis (REC ref:01/117).

Tissue preparation and neurosurgical procedures
Details of tissue procurement and preparation, immunosuppression, safety assessment and implantation have been fully reported elsewhere. The patient was followed up using the standard CAPIT-HD protocol.

Post-mortem histological evaluation
The brain was removed 37.3 hours after death and processed as described in. Sections were initially processed for histochemical analyses to assess graft location, survival and cyto-architecture. For this, 1 section of each graft was stained for acetylcholinesterase (AChE), as described.
Immunohistochemistry

For immunohistochemical stainings, the following primary antibodies were used: the neuronal marker MAP2 (rabbit anti-MAP2, Proteintech, 17490-1-AP, 1:500) or NeuN (mouse anti-NeuN, Millipore, MAB377, 1:2500) with the mutant huntingtin (mHtt) antibody (EM48, Millipore, MAB5374, 1:500). For striatal projection neurons, we used the calcium-binding protein calbindin (rabbit anti-CB, Abcam, Ab11426, 1:1000) and the Dopamine- and cAMP regulated neuronal phosphoprotein DARPP-32 (rabbit anti-DARPP-32, Cell Signaling, 2306, 1:1000). To identify grafted interneurons, we used calretinin (rabbit anti-CR, Swant 7699/4, 1:1000), parvalbumin (mouse anti-PV, Sigma, P3088, 1:1000) and choline acetyltransferase (Ms-anti-ChAT, Millipore MAB5270, 1:200), as previously described. We also performed an histochemical staining for nicotinamide adenine dinucleotide phosphate (NADPH-d) - a marker for nitric oxide containing striatal interneurons - following previously published protocols. The inflammatory/immune response was visualised using the microglial marker Iba1 (rabbit anti-Iba1, Wako, LAK4357, 1:500), the T helper cell marker CD4 (mouse anti-CD4, Leica, NCL-L-CD4-368, 1:500) and the natural killer and cytotoxic T cells CD8 (mouse anti-CD8, Leica, NCL-L-CD8-4B11, 1:500). Sections stained for immune cells (CD4 and CD8) were further pre-treated in a 10mM sodium citrate buffer solution at 80°C for 20 minutes. In some cases, immune cells were also counterstained with Nissl. Finally, a single immunohistochemistry staining for tyrosine hydroxylase (TH) (rabbit anti-TH, Pel Freeze Biologicals, P40101-150, 1:1000) was used to assess the dopaminergic innervation of the graft. In all cases, sections were incubated with appropriate secondary antibodies (Biotinylated Goat anti-mouse IgG, Vector, BA9200 1:500, Biotinylated Goat anti-rabbit IgG, Vector, BA1000 1:500) using the ABC Elite Vectastain® Kit (Vector Laboratories).

Image acquisition and quantification

Brightfield photomicrographs were taken using the Picture Frame software (Microbrightfield) attached to an E800 Nikon microscope (Nikon Instruments) and prepared for illustration using Adobe Photoshop CS5 and final figure panels assembled using Adobe Illustrator CS5.

All cell quantifications were performed on two sections of the cortex and striatum containing the graft. The perimeters of the grafted areas were delineated using the tracing contour option in Stereo Investigator (NeuroExplorer, version 10.0; Microbrightfield) as described in. To measure the cholinergic activity, images of the AChE stainings were processed with ImageJ software and P-zones, non
P-zones, and the host striatum were individually traced. These areas were measured for their mean gray values of color intensity.

**Statistical analysis**

For all cell quantifications, an unpaired Student’s t-test was performed using Prism (6.0; Graph-Pad Software Inc., La Jolla, CA).

**Results**

**Clinical course**

The patient first noticed problems in 1995, at the age of 37, with a slight change in his mood and his family became aware of his problems in 1997 when he developed nightmares and depression. He had a family history for HD and went on to have a positive genetic test with an expansion of 47 CAG repeats in exon 1 of the huntingtin gene. In 2003, he was selected for neural grafting and underwent a bilateral transplant procedure without complications and was followed up according to the CAPIT-HD protocol until his death in 2015. His clinical history showed no obvious change in his disease course after grafting, either clinically or on PET imaging, as detailed in 4.

**Post-mortem graft evaluation**

*Graft location and cytoarchitecture*

Macroscopically grafts were easily identified. In total, six grafts were located in the left hemisphere with 2 in the caudate, and 4 in the putamen (Figure 1A-B’) while on the right only 1 and 2 grafts in these structures were found respectively (Figure 1C-E).

*General graft health*

All the typical phenotypes of striatal interneurons were observed within the grafted tissue including cells positive for CR, PV, NADPH and ChAT (Figure 1F-I). They showed healthy morphologies and extensive dendritic arborizations. Stereological quantifications for each of these cell types revealed similar counts within the transplanted area in comparison to the host striatum for CR, PV, and NADPH interneurons (Figure 1F’, G’, H’ and I’). Despite rare examples of healthy MAP2 staining within the graft (Figure 1J), the presence of DARPP-32-positive cells was almost exclusively seen within the host striatum (Figure 1K). Grafted projections neurons, as also identified with CB, were largely necrotic (Figure 1L,L’) and there were fewer cells within the transplant area. Due to the tested antibody being discontinued, we
used the AChE staining to measure the cholinergic enzyme levels within the P-zones and compared them to the host striatum, and they looked similar (Figure 1I').

The inflammatory-immune response to cell suspension grafts

A ring of densely packed, maximally hyperactive microglia surrounded each of the transplanted sites (Figure 2A, B), and was also present within the graft itself. Throughout this ring, the spectrum of microglial activation was represented from dystrophic (Figure 2C, D), clustered (Figure 2E) and rod (Figure 2H) cells. Grafted regions lacking striatal markers (non P-zones), had qualitatively much lower microglial density, though the microglial morphology and Iba1 intensity remained consistent with a pro-inflammatory phenotype (Figure 2F, G). As senescence continues, these processes become fragmented and severe dystrophy was confirmed by spherical microglial cell bodies with no processes, surrounded by small dots of cytoplasm apparently disconnected from the soma (Figure 2I). The outer borders of the graft contained more dystrophic microglia, with gnarled, beaded processes (Figure 2J, K). The host tissue, on the other hand, was almost entirely populated by dystrophic and non-functional microglia. The p-zones contained the highest ratio of ramified microglia and the fewest dystrophic cells (Figure 2L), although there were still many more hyperactive cells than normally present in healthy tissue.

Graft innervation and presence of HD-related pathology

The grafts received modest innervation, as evidenced by the sparse dopaminergic fibres seen near the transplants (Figure 3A, B). Several mHtt+ aggregates were seen within the graft site and at the graft/host interface (Figure 3C, D). The mHtt+ aggregates were not only seen within the extracellular matrix of the grafted area, as previously reported in solid tissue transplants 16, but they were also found within cell bodies in the grafts (Figure 3F, G, H, L). A particularly interesting observation was that some mHtt+ aggregates were found along, or even within, the dendritic tree of cells within the transplant (Figure 3J, K). Notably, striatal host cells expressing mHtt+ extended their dendritic trees into the grafts (Figure 3I, I').

Finally, groups of T helper cells (stained for cluster of differentiation 4, CD4) (Figure 3M-N) and cytotoxic T cells (stained for cluster of differentiation 8, CD8) (Figure 3O-R) were found forming clusters within the grafts, indicating an ongoing immune and inflammatory response at the time of death. Of significance, the genetic product of HD, mHtt, was also identified within infiltrating T helper cells (Figure 3R).
Discussion

This is the first histological report on striatal cell suspension allografts originating from the UK NEST striatal transplantation HD project (NEST-HD) 5,17. Post-mortem evaluation revealed that (i) it was easy to find striatal transplants within which grafted interneurons were largely spared in contrast to the grafted projection neurons that were rarely seen; (ii) there was a striking microglial response around the graft; iii) there were mHtt aggregates within the grafted tissue as well as within cells of the transplanted area and finally, (iv) there was infiltration of immune cells within the grafted tissue that also contained mHtt, both of which may have further contributed to poor graft survival. These observations are similar to those that we have reported previously in HD patients who receiving solid tissue pieces but importantly there were notable differences in that we saw a much more marked infiltration of immune cells in the cell suspension grafts along with more florid mHtt cellular pathology within the transplant sites.

This latter observation relating to mHtt expression within the grafts requires further comment. One could argue that the cells expressing mHtt within the boundaries of the grafted areas are host cells that have migrated into the transplant. Alternatively, one could speculate that innervation of the grafts by host striatal neurons containing mHtt+ results in the transport of the pathological protein from the host to the graft, given that mature striatal cells have little if any migratory capacity 18. Unfortunately, the underlying explanation for this finding cannot be explored in this context, as the technical limitations inherent to human post-mortem analysis does not allow one to make mechanistic conclusions. Additionally, other routes of transport should be considered in light of the fact that mHtt+ was seen, although rarely, within CD8+ cells. The significant infiltration of CD4+ and CD8+ cells, notable within the grafted areas, may have indeed been facilitated by the more complex and efficient vascularization that we saw within the cell suspension grafts (data not shown).

In conclusion, this study again highlights that fetal striatal allografts can survive long-term in the human HD brain. However, while interneurons within them survive, projection neurons degenerate, and this is all associated with inflammation around and in the transplant as well as the expression of mHtt pathology at the graft site. The relevance and mechanistic consequences of these observations awaits clarification but raises questions as to whether cell-based approaches for repairing the HD brain can ultimately repair the dysfunctional networks seen in this condition 16,19-26.
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Figure 1. Graft location, cytoarchitecture and grafted cell survival. Macroscopic identification of the transplants based on AChE staining revealed a total of three grafts in the left hemisphere (delineated by dotted lines): one in the upper caudate nucleus (A, A') and two in the putamen (A, A'', B, B') that occupied between 7.2 and 9.9% of the total striatal area. Cell suspension grafts were observable as clusters closely resembling P-zones and NP-zones (A-B'). An additional three grafts were located in the right hemisphere (delineated by dotted lines): one in the caudate nucleus (C), and two in the putamen (D-E) that were much smaller and occupied less than 1.2% of the total striatal area. Immunohistochemical staining for striatal interneurons included CR, PV, NADPH and ChAT (F-I). High power photomicrographs highlighting the CR (F) and PV (G) staining of cells within a p-zone of the graft as well as examples of grafted neurons expressing NADPH-d (H), or ChAT (I). All grafted interneurons showed a rather healthy morphology with extensive dendritic arborizations. Stereological cell counts revealed a similar number of CR- (F'), PV- (G'), NADPH- (H') and ChAT- (I') immunolabeled cells in the grafted area vs. the host striatum. Similar immunohistochemical staining approaches were used to identify grafted projection neurons and included MAP2, DARPP-32 and CB (K-L'). While certain p-zones displayed a restricted number of healthy MAP2 staining cells (J), DARPP-32+ projection neurons were typically absent in the grafted tissue but frequently found within the host striatum (K). In contrast to interneurons, detectable CB-immunoreactive projection neurons were largely necrotic (L, L'). Stereological cell counts revealed a striking difference between the number of MAP2- (J'), DARPP-32- (K'), and CB- (L') immunolabeled elements in the grafted area vs. the host striatum. Scale bars: A, B, C, D, E = 1.25 mm; A', B' = 250 µm; A'' = 20 µm. F = 100µm; G = 250µm; H, I = 25µm; J = 25µm; K = 50 µm; L = 250µm; L' = 50 µm. Abbreviations: Acetylcholinesterase, AChE; CB; Calbindin; CD, caudate nucleus; ChAT, choline acetyltransferase; CR; calretinin; dopamine- and cAMP-regulated neuronal phosphoprotein, DARPP-32; globus pallidus internal segment, GPi; globus pallidus external segment, GPe; microtubule associated protein 2, MAP2; nicotinamide adenine dinucleotide phosphate, NADPH; PV; parvalbumin putamen, PUT.

Figure 2. Inflammatory response to cell suspension grafts. Series of double and triple immunostainings to visualize the inflammatory response to the grafted cells. A-B. Double immunohistochemical staining for Iba1 using nickel-intensified DAB (microglial cells in black) and NeuN as revealed using the chromogen DAB (neuronal cell in brown) in selected grafts. B-B'. Representative images of the clear gradient in microglial response intensity (C) as well as various microglial morphologies within and surrounding the grafts. C-K. Higher power magnification of grafted areas, showing microglial activation at the graft-host border (C, E), within P-zones (D), NP-zones (F), clusters of activated cells (G), and host cells (H), illustrating the intensity of the microglial response as well as the various phenotypes of the activated cells. Pro-inflammatory microglia are identifiable by the increased Iba1 reactivity and shortened process length (E). Highly dystrophic microglia in the host were often missing processes entirely (H), or otherwise displaying beaded, broken processes (I). Slightly activated microglia (J) and rod cells (K) were present within non P-zones. L. Pie charts detailing the percentages of microglial cell populations found at different structures or graft interfaces. Scale bars: A, B = 1mm; B' = 50µm; B'' = 35µm; C, D, E, I = 30µm; F = 80µm; G = 20µm; H, J, K = 15µm.

Figure 3. Graft innervation, presence of HD-related pathology and T-cell response to cell suspension grafts. A-B. Tyrosine hydroxylase immunostaining demonstrated modest innervation of the grafts by the dopaminergic system of the host. In some grafts (C), as well as at the border of the grafts (D), the presence of mHtt was abundant. Within the grafted area, mHtt was observable both within the extracellular matrix (E, F) and also within the nucleus of a number of cells within the graft site (F-H, chevrons). In addition to being localized within cell bodies of the host (I, I'), mHtt was observable within
(J, K) and along dendritic paths of cells within the graft or within cells that had a complex arborisation (L). Both CD4 (M, N) and CD8 staining (O-R) revealed clusters of infiltrating cells within the grafts and around blood vessels found within the transplants. On rare occasions, mHtt was seen within CD8+ cells (R). Scale bars: A = 175µm; B-C = 100µm; D, I, J, K = 50µm; E, F, I’, L = 25µm; G = 35µm; H = 20µm. M, N, O = 60µm; P = 100µm; Q = 30µm; R = 8µm. Abbreviations: CD4, cluster of differentiation 4; cluster of differentiation 8, CD8; mutant huntingtin protein, mHtt; TH, tyrosine hydroxylase.
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Author Contributions
SLM and RAB were involved in the study concept and design. AM, MS-P, ES, AH, TH, CW, YT, NP, PP, JS, M-ET and PG were involved in data acquisition and analysis. AM, SLM, RAB and FC were involved in drafting of the manuscript and figures.

Potential Conflicts of Interest
The authors declare no conflicts of interest.
References


Figure 2

Microglial response

Figure 3

TH innervation | mHtt expression within the graft

Immune response

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