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Article (Accepted Version)

Pienaar, Ilse S, Lee, Cecilia H, Elson, Joanna L, McGuinness, Louisa, Gentleman, Stephen M, Kalaria, Raj N and Dexter, David T (2015) Deep-brain stimulation associates with improved microvascular integrity in the subthalamic nucleus in Parkinson's disease. *Neurobiology of Disease*, 74. pp. 392-405. ISSN 0969-9961

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Deep-brain stimulation associates with improved microvascular integrity in the subthalamic nucleus in Parkinson's disease

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Abstract

Deep brain stimulation (DBS) of the subthalamic nucleus (STN) has become an accepted treatment for motor symptoms in a subset of Parkinson's disease (PD) patients. The mechanisms why DBS is effective are incompletely understood, but previous studies show that DBS targeted in brain structures other than the STN may modify the microvasculature. However, this has not been studied in PD subjects who have received STN-DBS. Here we investigated the extent and nature of microvascular changes in post-mortem STN samples from STN-DBS PD patients, compared to aged controls and PD patients who had not been treated with STN-DBS. We used immunohistochemical and immunofluorescent methods to assess serial STN-containing brain sections from PD and STN-DBS PD cases, compared to similar age controls using specific antibodies to detect capillaries, an adherens junction and tight junction-associated proteins as well as activated microglia. Cellular features in stained sections were quantified by confocal fluorescence microscopy and stereological methods in conjunction with in vitro imaging tools. We found significant upregulation of microvessel endothelial cell thickness, length and density but lowered activated microglia density and striking upregulation of all analysed adherens junction and tight junction-associated proteins in STN-DBS PD patients compared to non-DBS PD patients and controls. Moreover, in STN-DBS PD samples, expression of an angiogenic factor, vascular endothelial growth factor (VEGF), was significantly upregulated compared to the other groups. Our findings suggest that overexpressed VEGF and downregulation of inflammatory processes may be critical mechanisms underlying the DBS-induced microvascular changes.

Introduction

In the brains of Parkinson's disease (PD) patients, the degeneration of neurons in the substantia nigra pars compacta (SNpc) results in loss of dopamine (DA) content in the caudate nucleus and putamen, triggering downstream changes in the activity of the basal ganglia output pathways. These changes include increased activity of glutamatergic pathways originating from the subthalamic nucleus (STN) segment of the basal ganglia's indirect pathway that projects to the internal segment of the globus pallidus (GPi) and substantia nigra reticulata (SNr), resulting in PD motor symptoms (Bergman et al., 1990; Smith et al., 1998). Moreover, a hyperactive STN-SNpc pathway ensues, potentially reinforcing neurodegeneration of the SNpc dopaminergic neurons via glutamate-mediated excitotoxicity (Miller and DeLong, 1987; Bergman et al., 1994).

Such insights resulted in the development of deep brain stimulation (DBS) of the STN (STN-DBS), with studies showing a marked reduction in motor fluctuations and the disappearance of drug-induced dyskinesias in STN-DBS PD patients (Limousin et al., 1998). These patients also exhibited improved axial functions (Ngoga et al., 2014) and slower PD progression (Tagliati et al., 2010). This is in accordance with other studies (e.g. Temel et al., 2006), demonstrating that STN-DBS protected nigral neuronal loss in the 6-hydroxydopamine (6-OHDA) rat model of PD. Similarly, Wallace et al. (2007) observed that STN-DBS applied both before and after 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) administration to primates prevented further loss of SNpc dopaminergic neurons compared to sham-surgery controls.

Although STN-DBS as a therapeutic option has existed for 25 years, the mechanisms responsible for symptom improvement remain largely unknown. Recently, a role for the microvasculature in alleviating Parkinsonian syndrome following STN-DBS has been proposed (Nagai et al., 2012; Hill et al., 2013). This includes that DBS may enhance neurogenesis (Segi-Nishida et al., 2008; Warner-Schmidt et al., 2008). Related to this, Vedam-Mai et al. (2014) recently revealed that in PD patients, DBS stimulation induced neural stem cell proliferation, indicative of cellular plasticity, at both a local level and more distally, compared to normal and untreated PD brains. A further possibility is that DBS induces angiogenesis, possibly by upregulating the neurotrophic factors glial-derived neurotrophic factor (GDNF) and vascular endothelial growth factor (VEGF) (Glickstein et al., 2001; Hellsten et al., 2004; Wang et al., 2007; Lindvall and Wahlberg, 2008).

Blood vessel alterations have been implicated in the pathogenesis of several neurodegenerative disorders, including Alzheimer's disease (AD), PD, multiple sclerosis and amyotrophic lateral sclerosis (Lee and Pienaar, 2014). Here we used immunohistochemical and stereological methods to assess markers of the brain microvasculature including tight junction (TJ) complexes and adhesion molecules associated with the blood-brain barrier (BBB) (Ballabh et al., 2004; Abbott et al., 2010) within post-mortem STNs of STN-DBS PD patients, compared to non-stimulated PD and neurologically-intact controls. We provide the first evidence that STN-DBS result in significant microvascular changes, whilst identifying putative protein mechanisms to explain the observed angiogenic benefits.

Materials and methods

Subjects

Post-mortem human brain samples (non-stimulated PD, n = 7; PD STN-DBS, n = 5 and non-PD controls, n = 7) were provided by the Parkinson's UK Brain Bank at Imperial College London (http://www.parkinsons.org.uk/research/parkinsons_brain_bank.aspx) and the Banner Sun Health Research Institute Brain and Body Donation Program.

(BBDP, <https://www.brainandbodydonationprogram.org/>), Sun City, Arizona, USA. A summary of the demographic and clinical characteristics of the control, PD and PD STN-DBS subjects included in this study is presented in Table 1. Tissue was collected with informed consent by the donors via a prospective donor scheme according to the local Ethics Committee approval. Neuropathological examination was carried out on each case by an experienced neuropathologist.

PD cases (either those that had received STN-DBS or non-stimulated PD cases) had a clinical history of PD and were also selected based on the absence of dementia and the presence of at least 2 of the cardinal clinical signs of PD, as well as histological evidence of Lewy bodies/ alpha-synuclein deposition, pigmented neuronal loss in the SNpc and being Levodopa responsive. In all STN-DBS PD cases the DBS electrode was chronically implanted and it was more than a year between placement and death.

Normal control subjects were chosen based on the pathology reports that classified the cases as being within the normal range for their age and not presenting with any signs of neuropathology or histological abnormalities. This included the absence of atrophic changes in the mesencephalic regions, no evidence of plaques or vascular amyloid and the lack of detection of neurofibrillary tangles or excessive glial staining following an immunoperoxidase tau stain. Moreover, in the normal controls, there was no indications of cortical or nigral Lewy body formation nor of infarcts, with the small arteries that appeared void of sclerotic changes. Unless specified, the cause of death was bronchopneumonia.

Moreover, in none of the three case groupings were cases included for any of the three study groups if the neuropathological report mentioned that evidence was found of ischemia, indicative of a stroke-like episode. In addition, cases were excluded where mention was found in the clinical notes that a patient had received chronic treatment with drugs known to potentially influence the circulation, including anti-hypertensives, anti-inflammatories or steroids.

Histopathological staining and STN delineation

STN-containing formalin-fixed, paraffin-embedded blocks from PD, control and STN-DBS PD cases were serially cut at 6 μ m using a microtome (Microm International, Waldorf, Germany) and mounted onto SuperFrost™ slides (Thermo Fisher Scientific, Runcorn, UK). Standard haematoxylin and eosin (H&E) and luxol fast blue (LFB) staining, the latter for detecting myelin sheaths, were used for general morphological analysis, to accurately identify the STN and for revealing the electrode tract in the STN-DBS PD patient cohort.

The STN boundary was delineated on H&E and LFB stained sections as a discrete, compact structure located just medial to the peduncular portion of the internal capsule and superiolateral to the SN. The extent of the nucleus was marked on the slides with a permanent marker pen, used for overlaying to define the nucleus on the serial immunostained sections (Fig. 1A).

For single-antigen immunohistochemistry, tissue sections were dewaxed with xylene, rehydrated with a series of graded ethanols (EtOH), before washing well in distilled water. Sections were then incubated in 3% (w/w) hydrogen peroxide (H₂O₂, Sigma-Aldrich, Poole, UK) in PBS for 20 min at room temperature (RT) to block endogenous peroxidases. Antigenic epitopes were 'unmasked' to reduce nonspecific background staining and increase antibody labelling. For antigen retrieval, the sections were immersed in 10 mM sodium citrate buffer (pH 6.0) and heated in a steam cooker for 20 min. After heating, the sections were washed and cooled to RT under running tap water and rinsed in Tris-buffered saline (TBS). For blocking non-specific binding sites, the sections were incubated for 1 h at RT in 10% normal horse serum (NHS, Vector Laboratories, Peterborough, UK), diluted in PBS.

Each STN set of serial sections (n = 32) was immunostained for vascular endothelial marker, glucose transporter isoform 1 (GLUT-1, 1:150, Millipore, Watford, UK, #400060) (Lax et al., 2012) and β subunit of the major histocompatibility complex (MHC)-II receptor (HLA-DP/ DQ/DR), serving as a microglia marker (1:200, DAKO Cytomation, Glostrup, Denmark, #CR3/43). The primary antibody was applied to the sections overnight at 4 °C. The following day, after washing in PBS, sections were incubated for 2 h at RT in horse anti-mouse secondary antibody (1:200, Vector Laboratories) for visualising the microglia and horse anti-rabbit (1:200, Vector Laboratories) for identifying microvessels. This was followed by incubation in avidin-biotin complex (ABC) elite complex (Vector Laboratories) for 30 min at RT. Immunoreactivity was visualised with the chromogen 3,3'-diaminobenzidine (DAB, Vector Laboratories), applied for 10 min at RT. Sections were then dehydrated through graded EtOH, cleared in 2 changes of xylene, before being mounted in DPX (Sigma-Aldrich) and applying glass coverslips. For each staining protocol, a negative control was included, by omitting the antibody (images not shown).

Capillary endothelial cell layer thickness and inner diameter

Micrograph images of capillaries (n = 386, neurological controls; n = 410, PD and n = 396, STN-DBS PD) were taken with a 60 $\times$ /1.40 N.A. oil-immersion objective with a Nikon Eclipse 50i light microscope (Nikon Inc., Surrey, UK), fitted with a 24-bit digital camera (Qimaging Corporation, Vancouver, Canada), utilising ImageJ software (Rasband, U.S. National Institutes of Health, version 1.4). From cross-sectional images, the average endothelium thickness and inner diameter (in μ m) was estimated as previously described (Lax et al., 2012). Only microvessels $\leq 10 \mu$ m in diameter (considered as capillaries) were included

in the analyses (Burke et al., 2014), whilst those of diameter $> 10 \mu$ m were considered to be venules and were therefore excluded from analyses (Tata and Anderson, 2002). The thicknesses of the endothelial cell layer were only measured where the limits of the

endothelial laminae were clearly defined and if the outer lamina did not show splitting. Measurement of each vessel was repeated 3× per microvessel.

Immunofluorescence staining

To examine for abnormal leakage of blood vessels, a dual immunofluorescence protocol was followed for co-localising GLUT-1 with endogenous IgG, a plasma-derived protein. Heat-mediated antigen retrieval was performed on the sections by microwaving sections in sodium citrate buffer (10 mmol/l sodium citrate (Sigma-Aldrich), 0.05% Tween 20 (Sigma-Aldrich), pH 6). The primary antibody, purified mouse anti-human IgG (γ chain specific, 1:300, Southern Biotech, Birmingham, Ala, USA, clone: JDC-10) was co-applied with rabbit anti-GLUT-1 primary antibody (dilution 1:200, Dako Cytomation), left overnight at 4 °C. The IgG antibody was visualised by labelling with green-fluorescent Alexa Fluor 488 dye (1:200, Life Technologies, Paisley, UK), whilst GLUT-1 was tagged with orange-fluorescent Alexa Fluor 546 dye (Life Technologies). The sections were rinsed in 0.5% Sudan Black B (Sigma-Aldrich) in 70% EtOH, before being given a final rinse under running tap water. Finally, the tissue sections were mounted and glass coverslipped, using fluorescent mounting media (Vector laboratories).

A dual immunofluorescence protocol was performed to detect the vascular endothelial cell layer, comprising the structural component of the microvessels, by using the endothelial cell marker, GLUT-1, together with a range of surrounding TJ and adhesion proteins. In addition, dual staining for GLUT-1 alongside a primary antibody for detecting vascular endothelial growth factor (VEGF) was performed on a different set of serial STN-containing sections. To enable random sampling, three 6 μm formalin-fixed paraffin-embedded sections per case, which had been cut at equally spaced intervals of a minimum of 36 μm, was stained for each respective antigen, but always in combination with GLUT-1, which served for identifying the microvascular structure.

Briefly, sections were dewaxed and rehydrated as described above. A similar pretreatment procedure was followed as used for immunofluorescent detection of IgG-GLUT-1. The sections were then washed well in distilled water before incubation with 10% normal donkey serum (Sigma-Aldrich) for 60 min at RT. The serum was tipped off before application of the primary antibody for either zonula occludens-1 (ZO-1, 1:150; monoclonal mouse, Life Technologies, CA, USA, #339100), occludin (1:150; monoclonal mouse, Life Technologies, #331500), claudin-5 (1:150, monoclonal mouse, Life Technologies, #352500) and VE-cadherin (1:200, monoclonal mouse, Millipore, MABT129). In a separate series of sections, monoclonal mouse anti-human VEGF (1:150, Invitrogen, Karlsruhe, Germany, #AHG0114) was applied. In each case, the antibody was applied simultaneously with an antibody for detecting GLUT-1 (1:150, polyclonal rabbit; Millipore, #400060-50UG), diluted in TBS containing 0.1% Triton-X and left to incubate overnight at 4 °C.

The sections were then washed 3 × 5 min with TBS prior to incubation with appropriate secondary antibodies for 1 h at 37 °C. These were green donkey anti-mouse IgG Alexafluor 488 dye (Invitrogen, #A-11008) for visualising all TJ proteins and VEGF and orange-fluorescent donkey anti-rabbit IgG Alexafluor 546 (Invitrogen, #A10040) for visualising GLUT-1.

To minimize autofluorescence, sections were then incubated in 0.5% Sudan Black B (Sigma-Aldrich) in 70% EtOH for 5 min in a dark room, for binding lipofuscin present in neurons (Romijn et al., 1999). The solution was washed off the sections by rinsing them under running tap water for 5 min. Finally, the sections were mounted with Vectashield mounting medium (Vector laboratories), coverslipped with glass slides and then allowed to dry overnight at 4 °C. The sections were stored at 4 °C until confocal image collection. Controls were included where sections were treated in exactly the same way, but the primary antibody was omitted (images not shown).

Capillary length

For each experimental group, at least 8 high magnification (a 60 ×/1.4 NA oil-based objective lens) images were taken from each serial section fluorescently-stained for GLUT-1 (see the section below) per case. Digital images were taken with a TCS SP5 II confocal laser scanning microscope (Leica Microsystems, Wetzlar, Germany), fitted with a Leica DFC 320 digital camera. The images were digitally viewed and collected with LAS EZ image analysis software. Longitudinally-viewed capillaries were collected from the sections that had been fluorescently co-stained for GLUT-1 along with the various TJ markers. Using ImageJ software, individual capillaries were then traced along the length using the same confocal z-stacked images compiled for TJ protein signal intensity analysis. The length of each capillary was measured by tracing lines alongside both outermost layers of each capillary, starting from clearly visible capillary tips (Fig. 2D, arrowheads). The lengths of the two boundary lines of each vessel were then averaged to yield a single value, given as μm.

Stereological assessment of microglia and capillaries

An estimate of activated microglia and capillaries in the STN of all groups was obtained by unbiased design-based stereological cell-counting techniques using Image-Pro® Plus stereology software v. 6.2 (Media Cybernetics). Sections were viewed using a Nikon Eclipse E800 microscope (Nikon Inc., Surrey, UK) fitted with a computer-controlled motorized stage and a 3-CCD digital camera (JVC, Tokyo, Japan). From section montages, tiled at ×10 magnification, the STN was delineated, using the H&E stained sections (the first of the series) as guide. From these images, the counting area (A) was calculated using Cavalieri's principle and a 350 × 350 μm volume grid. The DAB-stained MHCII⁺ cells were recognised by the more compact phagocytic morphology of this cell-type, representative of stages C & D, in accordance with a scale established by Kanaan et al. (2008). In contrast, chromogen-based visualisation of GLUT-1 on the endothelial luminal membrane, the abluminal membrane and the cytoplasmic compartment, allowed for reliable identification of microvessels (Lax et al., 2012).

Three serial STN sections, equidistally spaced 42 μm apart/ immunomarker was available per subject. The number of microglia/ microvessels showing immunopositivity for the respective markers was counted at $\times 20$ magnification, using a $200 \times 200 \mu\text{m}$ systematic uni- form random points experimental grid containing a $125 \times 125 \mu\text{m}$ counting frame. The number of microglia/ microvessels in the STN was estimated using the formula: $N = (1/\text{area sampling fraction}) \times \text{total cell count}$. The area sampling fraction equaled the area of the number of frames counted/A. The density (capillaries or microglia/ mm^2) in the STN was calculated by dividing estimated cell number (N) by the area of the outlined STN for each case, with the mean across cases used as the final value representing the patient group (Schmitz and Hof, 2005). The density of activated microglia/capillaries is expressed as the estimated number of cells/ mm^2 .

Quantification of immunofluorescence

The fluorescence intensity (FI) values of ZO-1, occludin, claudin-5, VE-cadherin and VEGF were calculated as previously described (Lax et al., 2012). Images taken of ZO-1, occludin, claudin-5, VE-cadherin and VEGF, in each instance co-stained with GLUT-1, were acquired using a TCS SP5 II confocal laser scanning microscope (Leica Microsystems, Wetzlar, Germany), equipped with LAS EZ (Leica Microsystems, v.3.0) im- aging software and fitted with a Leica DFC 320 digital camera. The digital images were captured using a $\times 63$ magnification objective lens of numerical aperture /1.30. Three sections per case (48 μm equidistance apart) was analysed per protein (ZO-1, occludin, claudin-5 and VE- cadherin).

The STN was manually outline by copying the STN boundary line (delineated from H&E and LFB stained sections taken from each case) onto the glass coverslip of the fluorescently-stained slides. The slides were in serial arrangement, with the H&E stained section placed first, followed by an LFB-stained section, and then the various fluorescently- stained sections.

Image capture was restricted to within the boundaries of the outlined STN and was collected by proceeding clockwise with 6–10 non-overlapping images per section and choosing a random starting position. Z-stacked imaging (three different levels, every 1.8 μm) was performed across the length, breadth and depth of each specimen. Automated maximum intensity projection 16-bit (508×508 pixels) file images were generated for each channel, the exposure times (400 ms), gain and offsets for each image acquisition being identical. Furthermore, identical conditions of laser excitation (488 for FITC Alexa 488 green and 546 for FITC Alexa 546) and an emission peak of 518 nm and 568 nm, respectively were maintained for all fluorescent images collected. Prior to capturing, the background fluorescence (nonspecific fluorescence of the tissue) of each image was set to a barely detectable level by adjusting the gain of the charge-coupled de- vice camera. Digital images were imported into ImageJ software and the outline of vessels were traced manually on the image by an investi- gator blinded to treatment groups. The integrated optical density (IOD) relevant to the area occupied by cells stained positively by a particular antibody (ROI) was calculated for each individual vessel. This unit of measure is representative of the intensity of fluorescence (Lax et al., 2012).

IgG extravascular deposits as an index of enhanced BBB permeability (Chen et al., 2009; Cristante et al., 2013) were detected using the same fluorescence microscopic hardware and software as for quantifying the TJ-, adherens proteins and VEGF as well as a similar objective lens for capturing the projection z-stack images, which was reconstructed by

using the confocal microscope software. From these images, the IgG immunopositive signal outside the GLUT-1-stained blood vessels was subjected to threshold processing by using ImageJ software's Integrated Density measurement tool, before analysing the IgG-positive fluorescent signal. Analysis to determine the level of extravascular deposits of IgG, synonymous with capillary leakage, was performed on 10–12 randomly selected fields of view of the STN, in 3 non-adjacent, serially-cut sections (spaced 42 μm apart).

Statistical analyses

Values are expressed as the mean $\pm$ standard error of the mean (S.E.M.). The distribution of datasets was tested using the Shapiro-Wilk normality test. All statistical analyses were performed using Statistical Package for Social Sciences (SPSS) software (IBM SPSS, version 19). All data comparisons were made using a 2-way-ANOVA procedure, with the three study groups (control, PD and STN-DBS) and individual serving as factors. Significance levels were defined using the following commonly implemented guidelines throughout the analyses: $P < 0.001^{***}$, extremely significant, $P = 0.001\text{--}0.01^{**}$ highly significant and $P = 0.01\text{--}0.05^{*}$ significant.

Results

Capillary density

The mean capillary density in control subjects was 168 ± 4.2 (Figs. 1B, D). However, this was reduced (by 26%) to a significant extent in non-STN-DBS PD subjects (125 ± 2.3 , Figs. 1B, D). In STN-DBS in PD subjects, capillary density was increased by 20% compared to control values (202 ± 6.7 , Figs. 1B, D).

Microglial density

Different from the ramified status of quiescent microglia, activated cells displayed the distinctive morphology, being round and having a typical morphology of amoeboid cells (Fig. 1E). The mean number of activated microglia in the STN was 157 ± 2.8 in controls, whilst this was significantly upregulated ($\sim 98\%$, Fig. 1C) in PD cases. Microglial den- sity was lower (by 67%) in STN-DBS PD cases compared to PD without DBS (Fig. 1C). However, in STN-DBS PD, microglial density was still ele- vated (by 26%) compared to the level measured in controls ($P < 0.001$, Fig. 1C).

Capillary endothelial cell layer thickness

The average thickness of capillary endothelial cells of controls was $0.95 \mu\text{m} \pm 0.04$ (Figs. 2A, C), similar to previously reported values measured in the cerebellum of normal control subjects (Lax et al., 2012). However, significant thinning (by 19%, $P < 0.001$, Figs. 2A, C) of the endothelial layer was noted in PD cases. In contrast, there was a striking thickening, exceeding the thicknesses in controls (by 35%, $P < 0.001$, Figs. 2A, C) was observed in STN-DBS PD patients.

Capillary length

Differences observed between the patient groups for STN capillary length was less striking than the thickness increase seen in STN-DBS PD samples compared to other groups, with values for PD patients ($91 \mu\text{m} \pm 2.86$) decreasing by only 3% (Figs. 2B, D). However, a small but significant increase was evident in the STN-DBS group ($109 \mu\text{m} \pm 4.6$; STN-DBS PD vs. control: 14%, STN-DBS PD vs. PD: 16%, Figs. 2B, D).

Serum IgG expression

To distinguish between IgGs within the lumen of the microvessels and those which may have leaked from the vessels, we co-stained with a GLUT-1 antibody. In controls, serum IgGs were principally restricted in the lumen of vessels (Figs. 3A, B). However, in PD patients we observed widespread extravascular IgG staining (32% increase), indicating BBB impairment, compared to controls (Fig. 3A). In particular, the IgG depositions appeared as halos around the vessels, forming a clear concentration gradient, with darker staining seen in the centre of the halo. Interestingly, the level of extravascular IgG decreased in STN-DBS PD cases (by 32%, Fig. 3A), compared to PD and similar to that observed in controls (Figs. 3A, B).

TJ (ZO-1, occludin and claudin-5) and adhesion protein IOD levels

Immunofluorescence for the TJ proteins ZO-1 (Fig. 4B) and occludin (Fig. 5B), claudin-5 (Fig. 6B) and the adhesion protein VE-cadherin (Fig. 7B) revealed that in the STN of all PD cases there was a redistribution of these proteins, with extrusion of the proteins from the microvessel walls into the parenchyma. In addition, obvious thinning and fragmentation of the TJ proteins were seen in PD cases, which was absent in either the control or STN-DBS PD cases. Statistical analyses performed on FI values for the various proteins, revealed the following results: ZO-1 expression in control cases revealed filamentous immunoreactivity along

the vessel endothelium (Fig. 4B). However, in PD cases disruption of ZO-1 localisation was seen, with more punctate pattern of ZO-1 distribution, indicative of discontinuation TJs along the blood vessels (Fig. 4B). This disrupted pattern was no longer evident in STN-DBS cases, being instead comparable to the pattern observed in control cases. STN ZO-1 fluorescence levels were significantly reduced in the PD cases compared to controls, but were markedly elevated above control levels in the STN-DBS PD cases (Fig. 4A).

In control specimens, occluding was continuously expressed, aligning with vascular endothelial cells (Fig. 5B). Similar to ZO-1, occludin immunoreactivity in the STN was markedly downregulated in PD subjects (Figs. 5A, B), yet upregulated in STN-DBS PD subjects ($P < 0.001$, Figs. 5A, B), compared to controls. Occludin expression in PD cases appeared non-continuous and punctate, indicating the absence of a function BBB.

Immunofluorescent distribution of claudin-5 was continuous within the STN microvessels of controls, but appeared more fragmented in the microvessels of PD cases (Fig. 6B). In fact, in PD cases many capillaries lacked detectable claudin-5 immunoreactivity. However, in the STN of DBS PD cases, merged claudin-5/GLUT-1 images noted strong localisation and alignment of claudin-5 (green) with the endothelial cells (red). The pattern of results obtained for claudin-5 IOD values was similar to IOD analyses of ZO-1 and occludin with marked reduction in claudin-5 immunofluorescence in the PD cases compared to controls, whilst in the STN-DBS PD cases there was small but significant increase in claudin-5 immunofluorescence above control levels (Fig. 6A).

In control STN, expression and localisation of VE-cadherin, a protein of vital importance for maintaining and controlling endothelial cells, appeared continuous and intact along vessel walls (Fig. 7B). This was more pronounced in the STN-DBS PD cases; however, PD microvessels showed scant labelling of VE-cadherin, appearing discontinuous and scattered along the vascular wall (Fig. 7B). VE-cadherin expression showed a similar pattern to that of TJ proteins, with PD cases demonstrating a marked reduction in immunofluorescence compared to controls. In the other hand, in STN-DBS cases, VE-cadherin immunofluorescence levels were elevated above that seen in control cases (Fig. 7A).

GLUT-1 IOD levels

As seen in the representative confocal images taken of isolated microvessels in control cases (Fig. 8C, top panel), GLUT-1 is expressed by microvessel endothelial cells was similar to what was previously described (Lax et al., 2012), whilst in the STN-DBS PD cases there was a marked elevation of GLUT-1 expression compared to controls (Fig. 8A). Overall, the GLUT-1 immunosignal was weak in PD specimens, compared to controls and significantly downregulated compared to the STN-DBS PD group (Fig. 8A).

VEGF IOD levels

Expression of the growth factor VEGF largely overlapped with GLUT-1 immunoreactivity (Fig. 8C, merged images) in both control and STN-DBS PD cases. However, VEGF co-localisation with GLUT-1 was decreased in the PD cases (Fig. 8C), with a more diffuse

pattern of localisation seen throughout the vessel. This transpired into a significant reduction in VEGF immunofluorescence in PD cases, but still a marked increase in the STN-DBS cases compared to controls (Fig. 8B).

Discussion

The BBB comprises of specialised brain capillary endothelial cells, connected by intercellular tight junction (TJ) complexes, perivascular cells, astrocytic end-feet and a thick extracellular matrix (Ballabh et al., 2004). The belt of endothelial TJs in cerebral capillaries is the primary regulators of the cross-talk between the brain and the periphery (Abbott et al., 2010). The TJ-associated protein occludin was the first integral membrane protein to be identified within the TJs of endothelial cells (Furuse et al., 1993). Although its structural and functional role in the TJ barrier remains to be elucidated, its relevance for barrier formation against macromolecule passage has been highlighted in several studies. This includes work by Wittchen et al. (1999), who revealed that occludin influences paracellular permeability of ions and proteins in epithelial and endothelial cells by interacting directly with the actin cytoskeleton via the ZO TJ-associated proteins. The ZO proteins provide the structural basis for assembling multiprotein complexes at the cytoplasmic surface of intercellular junctions.

Claudin-5 forms and regulates endothelial cell junctions (Nitta et al., 2003) to control vascular permeability and leukocyte recruitment into tissue. In addition, the TJ-associated adhesion protein, vascular endothelial cadherin (VE-cadherin) regulates cell survival, migration and polarity by maintaining interactions between vascular endothelial cells (Deiana and Vestweber, 2013).

Unlike AD, where vascular degeneration comprises a well-described pathological feature, potentially contributing to disease pathogenesis (Marchesi, 2011; Zlokovic, 2011), information on the nature and extent of vascular pathology in PD remains limited. All cases used in the current study were idiopathic PD and not vascular PD (diffuse white matter lesions and/or subcortical infarcts); accounting for 2.5–5% of PD cases (Gupta and Kurivilla, 2011).

Previous work had shown that capillary changes could occur up- stream of microvascular remodelling. This includes work conducted in mice to show that capillary remodelling occurs through the formation of 'new loops' (Heinzer et al., 2008; Harb et al., 2013). In the case of Harb et al. (2013), individual microvessels were studied in the intact brains of mice, using high-resolution confocal imaging and long-term time-lapse two-photon microscopy across the lifetime of the animal. Pertinent patterns of change were discerned, with vessel formation and elimination that continued throughout life. The study described new vessel formation as a long-term adaptive response to metabolic challenges, particularly in young adult mice compared to older animals, where an absence of such plasticity-related responses was seen. Worth noting also are the topographic differences seen in vascular supply, distribution and density of brain capillaries in cortical and hippocampal structures, with distinct pathophysiological responses, as described by Cavaglia et al. (2001). Using a combined immunocytochemical–confocal microscopy–microangiographic method for investigating regional interactions between cerebrovasculature and parenchymal cells, the group validated this method for morphological and functional evaluation of vascular patterns and BBB intactness in the mouse CNS. Application of the technique revealed divergent sensitivity to ischemia/reperfusion, in terms of vascular density between two hippocampal subfields (Cavaglia et al., 2001).

In other work, Burke et al. (2014) examined post-mortem tissue taken from hippocampi to assess whether microvascular morphology, specifically length density and diameter, was affected in different dementias, placing particular emphasis on post-stroke dementia. The study reported an increase in microvessel length density (cumulative vessel length per unit tissue volume) in AD and post-stroke dementia cases, suggesting that either an increase in angiogenesis or else the formation of new microvessel loops form in response to cerebral hypoperfusion. In addition, in non-demented post-stroke post-mortem brain tissue, vessel diameter was significantly increased compared to demented post-stroke cases. In light of these findings, the authors suggested that an increased perfusion surface between blood and brain, associated with increased microvessel length density and increased vessel diameter, may induce remodelling of microvessels during hypoxia (Burke et al., 2014). Taken together, this result suggests that the sum total of hippocampal microvascular changes may be an important determining factor for whether post-stroke survivors develop dementia or not.

Previous studies on brain microvessel pathology in PD cases re- vealed comorbid atherosclerosis and subclinical impairment of brain vessels may contribute to PD mortality (Rektor et al., 2012), whilst others reported degenerative vascular morphology in multiple brain regions in PD patients (Guan et al., 2012). The current study offers a comprehensive description of degenerative vascular-related changes seen in the STN of PD patients compared to controls. Our results agree with others reporting decreased expression of the TJ proteins occludin and ZO-1 in the striatum of PD post-mortem brains (Chen et al., 2008). In addition, we found evidence of claudin-5 and VE-cadherin protein downregulated expression in PD-affected STN compared to control cases. However, rescue of depleted ZO-1, occludin, claudin-5 and VE-cadherin levels were seen in the STN of STN-DBS PD samples compared to PD ones. Moreover, confocal microscopy in the PD STN-DBS cases revealed that the belt of fluorescently-stained TJ- and adhesion protein was intact and continuous, similar to those seen in control cases.

The endothelial linings of capillaries are only one cell layer thick, but fulfil a vital role in maintaining integrity of the BBB, whilst preventing harmful substances from entering neuronal cells (Bradbury, 1993). In the current study, several morphological changes affected the micro- vasculature in PD STNs compared to controls. In addition to a general loss of vessels, as noted from microvessel stereology, thinning of the microvessel endothelial cell layer and shortening of microvessels were observed in PD STNs. Since GLUT-1 was used as a protein marker for identifying the microvascular endothelial cells, it is reasonable to assume that the loss of the GLUT-1 protein, responsible for a constant supply of glucose to both the vascular cells and the neurons may have contributed to vascular degeneration.

The downregulated expression of GLUT-1 measured here in the non- surgical PD STN specimens implies decreased glucose availability in surrounding neurons. Related to this, others have shown that GLUT-1 protein expression in brain capillaries is

reduced in the brains of AD patients, although this didn't associate with changes in the GLUT-1 mRNA structure (Mooradian et al., 1997) or the levels of GLUT-1 mRNA transcripts (Wu et al., 2005). Together with studies showing that the BBB surface area available for glucose transport is substantially reduced in AD (Bailey et al., 2004; Wu et al., 2005), these findings suggest that AD-affected brains are subject to a chronic shortage in energy metabolites due to GLUT-1 deficiency at the BBB. Related to this, in the current

For the current study, it was significant to measure the changes in the STN, as the nucleus is considered to play a key role in the pathophysiological origin of the Parkinsonism i.e. STN hyperactivity seen in MPTP-lesioned parkinsonian monkeys (Miller and DeLong, 1987). Subsequently, STN-DBS was introduced for improving akinesia in advanced PD (Pollak et al., 1993). However, despite encouraging clinical trial results and the now routine use of STN-DBS, its mechanisms of action remains unclear. The finding that STN-DBS induces bursting activity in SNpc dopaminergic neurons implies that disinhibition of STN neurons, resulting from a DA lesion, could induce excitotoxic damage to the STN's output nuclei, including the SNpc (Wichmann and DeLong, 2003). However, many factors can contribute to the downstream mechanisms of this excitotoxic neurodegeneration, including BBB dysfunction (Ting et al., 1986). Our results indicate that STN-DBS reverses the extent of vascular pathology in PD case.

Our observations contribute towards better understanding that the microvasculature of the STN undergoes a degree of degeneration in PD and that levels of proteins that modulate this process are modified through DBS stimulation. Potential therapeutic leads stand to be gained from studying BBB alterations, for developing putative therapeutic intervention strategies. In particular, targeting of the vasculature may provide a possible strategy for stimulating a system, for endogenously repairing the damaged brain. It is our hope that the current findings will instigate further research into the mechanisms underlying vascular changes following DBS, to enhance mobilisation of the vasculature for treating neurodegenerative diseases, including but not limited to PD.

Abbreviations

Alzheimer's disease, AD; Blood-brain barrier, BBB; Deep brain stimulation, DBS; 3,3'-diaminobenzidine, DAB; Dopamine, DA; Ethanol, EtOH; Ethylenediaminetetraacetic acid, EDTA; Fluorodeoxyglucose, FDG; Fluorescence intensity, FI; Glial cell line-derived neurotrophic factor, GDNF; Globus pallidus, GPi; Glucose transporter isoform 1, GLUT-1; Haematoxylin and eosin, H&E; 6-hydroxydopamine, 6-OHDA; Immuno-globulin G, IgG; Integrated optical density, IOD; Interleukin, IL; Junction associated proteins, JAMs; L amino acid transporters, LAT; Levodopa, L-DOPA; Luxol Fast Blue, LFB; Major histocompatibility complex, MHC; 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine, MPTP; Non-pathologic control, npc; Not significant, n/s; Normal horse serum, NHS; Parkinson's disease, PD; Phosphate-buffered saline, PBS; PBS containing 0.05% Triton X-100, PBSX; Positron emission tomography, PET; Reactive oxygen species, ROS; Standard error of the mean, S.E.M.; Substantia nigra pars compacta, SNpc; Substantia nigra reticulata, SNr; Subthalamic nucleus, STN; Tight junction, TJ; Tris-buffered saline, TBS; Vascular endothelial cadherin, VE-cadherin; Vascular endothelial cells, VECs; Vascular endothelial growth factor, VEGF; versus, vs.; Zonula occludens, ZO.

Funding

ISP holds an Imperial College London Jnr. Research Fellowship. This study received grant support from the Rosetrees Trust.

Acknowledgments

Post-mortem human brain tissue samples were kindly provided by the Banner Sun Health Research Institute, Sun City, Arizona, USA and the Parkinson's UK Tissue Bank, Imperial College London, London, UK, funded by the UK Parkinson's Disease Society.

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Table 1
Clinical and demographic characteristics of all cases included. Results are shown as the mean $\pm$ S.E.M.

	Control (n = 7)	PD non-stimulated (n = 7)	PD STN-DBS (n = 5)
Sex ratio (M/F)	4:3	4:3	2:3
Age of PD symptom onset (years)	N/A	62 $\pm$ 4.32	74 $\pm$ 5.15
Age at death (years)	77 $\pm$ 12.13	76 $\pm$ 6.84	80 $\pm$ 1.17
Disease duration	N/A	15 $\pm$ 6.28	24 $\pm$ 4.37
PMI (h)	10 $\pm$ 1.13	10 $\pm$ 1.5	6 $\pm$ 1.5
Braak staging	N/A	II; III; IV	IV; III; IV

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Fig. 1. (A) A representative photograph of the stimulating electrode tract placed within the STN of a PD patient. Outlines indicate the anatomical location of the STN in relation to major surrounding structures, including the SN. (B) Stereological quantification of the number of capillaries (per mm² $\pm$ S.E.M.) in the STN of all subject groups. All comparisons proved extremely significant ($P < 0.001$). (C) Stereological quantification of activated microglia (per mm² $\pm$ S.E.M.) in the STN of all subject groups. Again, all comparisons proved extremely significant ($P < 0.001$). (D) Representative light microscopic images of GLUT-1 immunopositive capillaries of each subject group illustrate the loss of vessels seen within the STN of PD cases compared to controls, whilst an increased number of vessels, exceeding those of control case samples, were seen within the STN of STN-DBS cases. 20 $\times$ magnification, scale bar: 100 μ m. (E) Representative light microscopic images of fully activated microglia, which mediate an inflammatory response, shown in the STN of the three subject groups. Images illustrate the increased cell counts made in the STN of PD samples, with significantly lower counts made in the STN of STN-DBS PD and control cases. 20 $\times$ objective, scale bar: 50 μ m; 60 $\times$ objective, scale bar: 10 μ m, for inserts.

Fig. 2. (A) Mean endothelial cell thickness (μm) of capillaries ($\pm$ S.E.M.) in the STN of the three subject groups. The comparisons control vs DBS and PD vs DBS proved extremely significant ($P < 0.001$), whilst control vs PD was highly significant ($P = 0.004$), indicating that DBS induced a pronounced thickening of this cell layer in the STN capillaries of STN-DBS PD cases. (B) STN capillary length (μm) in all three subject groups ($\pm$ S.E.M.). On average, capillaries were longer in STN-DBS PD subjects, compared to control ($P = 0.004$) and PD subjects ($P = 0.001$). (C) Representative light microscopic images clearly illustrate endothelial cell thickening in STN-DBS PD cases, compared to controls, and was particularly prominent when compared to PD cases that had not received STN-DBS. $100\times$ magnification, scale bar: $5\mu\text{m}$. (D) Representative confocal images of longitudinally-viewed capillaries stained with the GLUT-1 antibody for viewing the single layer of endothelial cells lining the vasculature, illustrates the increased lengthening of microvessels in the STN of STN-DBS compared to control and PD cases. $20\times$ oil-based objective, scale bar: $25\mu\text{m}$.

Fig. 3. (A) The mean FI values of GLUT-1 and serum IgG fluorophore-labelled antibodies, with values that were normalised to that of the control group ($\% \pm$ S.E.M.). Comparisons of control vs. PD and PD vs. STN-DBS PD proved extremely significant ($P < 0.001$), whilst control vs. STN-DBS PD was not ($P > 0.05$). Such lowered serum IgG extravascular expression seen in STN-DBS PD samples, indicates that DBS prevented the excessive plasma protein leakage from STN capillaries into the brain parenchyma, as seen in PD samples, suggesting that DBS intervention improves BBB integrity. (B) Confocal microscopic images reveal marked amelioration of IgG (green) leakage surrounding the GLUT-1 immunopositive capillaries (red) in STN-DBS PD cases, compared to controls and PD cases. Merged images are shown in the right panels, with yellow indicating co-localisation. 60 $\times$ oil-based objective, scale bar: 25 μ m.

Fig. 4. (A) Mean FI values calculated for the ZO-1 fluorophore-labelled antibody ($\pm$ S.E.M., normalised to control values) was significantly elevated in STN-DBS PD compared to control cases ($P < 0.001$) and was even more pronounced when compared to PD cases ($P < 0.001$). (B) Confocal microscopic images reveal double-immunofluorescence staining of STN microvessels with an antibody specific to the vascular endothelium cells, GLUT-1 (red) and ZO-1 protein (green), surrounding the endothelium cell layer. Merged images are shown in the panels farthest to the right, with yellow indicating co-localisation. In both the controls and STN-DBS PD sections, the microvessels show a continuous and linear labelling of ZO-1. However, in PD cases, the TJ proteins are discontinuous along the inner endothelial cells of the microvessels. 40 $\times$ oil-based objective, scale bar: 50 μ m.

Fig. 5. (A) Mean FI values calculated for the occludin fluorophore-labelled antibody ($\% \pm$ S.E.M., normalised to control values) was significantly elevated in STN-DBS PD cases, compared to control cases ($P < 0.001$), this difference being even more pronounced when comparing STN-DBS PD to PD samples ($P < 0.001$). (B) Representative confocal microscopic images of STN sections, stained for GLUT-1 (red), reveal the capillary endothelial cell layer alongside occludin (green). Merged images are shown in the panels farthest to the right, with yellow indicating co-localisation. Occludin expression, as a possible determinant of TJ permeability is found to be abundant in STN-DBS PD and control cases, with the immunosignal forming a continuous linear expression pattern. In PD sections, the linear strands of the occludin immunosignalling seen in the other groups were severely disrupted. 40 $\times$ oil-based objective, scale bar: 50 μ m.

Fig. 6. (A) Immunofluorescence analysis of Claudin-5 ($\% \pm$ S.E.M., normalised to control values) was extremely significantly upregulated in STN-DBS PD serial sections, compared to PD cases ($P < 0.001$), whilst also being significantly elevated compared to controls ($P = 0.035$). (B) Representative confocal microscopic images reveal GLUT-1-stained endothelial cells (red), alongside claudin-5 immunoreactivity (green), showing visibly enhanced claudin-5 expression in the STN of STN-DBS PD and control cases compared to PD ones that have not received STN-DBS intervention. The merged images, shown in the panels farthest to the right, indicate co-localisation (yellow). 40 $\times$ oil-based objective, scale bar: 50 μ m.

Fig. 7. (A) The mean FI values of the fluorophore-labelled VE-cadherin antibody ($\% \pm$ S.E.M., normalised to control values) was increased in the STN of STN-DBS PD cases, with differences that were particularly pronounced compared to PD cases. All possible comparisons made between the three groups showed extremely significant differences ($P < 0.001$). (B) Confocal microscopic images GLUT-1-stained endothelial cells of the capillaries (red), alongside VE-cadherin (green). The merged images are given in the panels farthest to the right, to indicate co-localisation (yellow). The images clearly illustrate the increased FI signalling of VE-cadherin seen in STN-DBS PD cases, compared to PD and controls, appearing particularly prominent in the representative STN-DBS PD stained sections. 40 $\times$ oil-based objective, scale bar: 50 μ m.

Fig. 8. (A) The mean FI values of the fluorophore-labelled GLUT-1 antibody ($\% \pm$ S.E.M., normalised to control values) were upregulated extremely significantly in STN-DBS PD cases compared to PD cases ($P < 0.001$) and significant compared to controls ($P = 0.022$). (B) For VEGF FI measurements made in the capillaries of the STN ($\% \pm$ S.E.M., normalised to control values), extremely significant differences were seen for all possible group comparisons ($P < 0.001$). (C) The confocal images give representative examples to illustrate the results obtained from GLUT-1 (red) and VEGF (green) FI quantification. Merged images are shown in the panels farthest to the right, with yellow indicating co-localisation of the two proteins. 40 $\times$ oil-based objective, scale bar: 50 μ m.