α -Synuclein pre-formed fibrils impair tight junction protein expression without affecting cerebral endothelial cell function

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Abstract

Recently it has been shown that there is impaired cerebral endothelial function in many chronic neurodegenerative disorders including Alzheimer's and Huntington's disease. Such problems have also been reported in Parkinson's disease, in which α -synuclein aggregation is the pathological hallmark. However, little is known about the relationship between misfolded α -synuclein and endothelial function. In the present study, we therefore examined whether α -synuclein preformed fibrils affect endothelial function *in vitro*. Using a well-established endothelial cell model, we found that the expression of tight junction proteins, in particular zona occludens-1 and occludin, was significantly perturbed in the presence of fibril-seeded neurotoxicity. Disrupted expression of these proteins was also found in the postmortem brains of patients dying with Parkinson's disease. There was though little evidence *in vitro* of functional impairments in endothelial cell function in terms of transendothelial electrical resistance and permeability. This study therefore shows for the first time that misfolded α -synuclein can interact and affect the cerebral endothelial system, although its relevance to the pathogenesis of Parkinson's disease remains to be elucidated.

Keywords

α -Synuclein preformed fibrils; blood-brain barrier; transendothelial electrical resistance; permeability; zona occludens-1; occludin; Parkinson's disease

Figures 1-4 contain confocal microscopy pictures and will require color in print

Introduction

Neurodegenerative disorders such as Alzheimer's disease (AD), Parkinson's disease (PD), and Huntington's disease (HD), are all characterized by the presence of abnormal protein aggregates that are believed to contribute to the pathological process. Some of these misfolded proteins have been shown to interact with the cerebral microvasculature, leading to the hypothesis that the integrity and composition of the endothelial system may be involved in disease pathogenesis at some level. Indeed many studies have now demonstrated that the accumulation of these pathological proteins, including β -amyloid and Tau in AD patients, can damage cerebral vascular endothelial cells (Blanc et al., 1997, Eisenhauer et al., 2000, Kalaria and Hedera, 1995, Kovac et al., 2009 and Tamaoka et al., 1992) and by so doing disrupt the blood brain barrier (Hampel et al., 1995, Perlmutter, 1994, Zipser et al., 2007). Similarly in HD, mutant huntingtin pathology has recently been reported to involve all the major components of the cerebral vascular system in both patients and a transgenic animal model with associated endothelial disruption (Drouin-Ouellet et al., 2015).

Several reports have also suggested that there may also be problems within the endothelial system in PD, including one study that reported vascular degeneration in postmortem PD brain samples (Guan et al., 2013), and another that showed extravasation of proteins from the circulation into the PD striatum (Gray and Woulfe, 2015) with reduced endothelial P-glycoprotein efflux function (Bartels et al., 1995, Kortekaas et al., 2005). Pathologically, PD is defined by the presence of α -synuclein enriched Lewy bodies in many sites of the central nervous system including the nigrostriatal dopaminergic system (Spillantini et al., 1997), although exactly what

triggers the disease process remains to be elucidated. However it is known that α -synuclein is an intrinsically disordered protein which has innate neurotoxic and transmissible capabilities, especially when in the form of protofibrils. This ability of α -synuclein to act as a transmissible protein agent in PD has been the subject of a number of studies, given that it can easily be delivered experimentally as so called preformed fibrils (pff) (Luk et al., 2009, Luk et al., 2012, Peelaerts et al., 2015, Volpicelli-Daley et al., 2011).

However, it is unclear whether α -synuclein has any role either in maintaining endothelial function under normal conditions or in disrupting it under pathological conditions such as in PD. In the present study we therefore investigated what effect α -synuclein pff could have on the cerebral endothelial cell line (hCMEC/D3), which has been used previously to study the brain endothelial response to potential pathogens such as β -amyloid (Qosa et al., 2014, Weksler et al., 2005). In our new study these cells were co-cultured with astrocytes isolated from neonatal rodents or with primary human fetal cortical tissue, with or without α -synuclein pff, to determine what effects this had on the endothelial cells at a functional, cellular, as well as molecular level. Changes in endothelial protein expression seen in this *in vitro* system were then compared to those found in human postmortem material from PD patients.

Material and methods

Ethics

Human fetal tissue was collected from routine termination of pregnancies at the Cambridge University Hospitals (Addenbrooke's) and experiments performed at the

John van Geest Centre for Brain Repair (University of Cambridge). All human tissue was collected under full ethical approval in accordance with the United Kingdom's Department of Health guidelines and local ethical approval (NRES Committee East of England - Cambridge Central, reference no. 96/085). Postmortem brain material was obtained from the Queen Square Brain Bank with full ethical committee approval and the tissue is stored under a licence from the Human Tissue Authority (reference no. 12198). Tissue was donated from registered prospective donors with informed consents. The postmortem human tissue analysis was conducted at the John van Geest Centre for Brain Repair, under full ethical approval in accordance with the United Kingdom's Department of Health guidelines and local ethical approval (Local Research Ethics Committee, reference no. 01/177). All animal embryos were harvested using Schedule 1 methods in accordance with the UK Animals (Scientific Procedures) Act 1986 and approved by the University of Cambridge Animal Welfare Policy.

Preparation of α -synuclein pff

Full length recombinant human α -synuclein was purchased from rPeptide (S-1001). Endotoxin levels were evaluated by the Limulus amebocyte lysate assay (Endotoxin Testing Services, Lonza Europe) and the amount expressed was negligible. The preparation of α -synuclein pff was carried out using the protocol published by Volpicelli-Daley et al., (2014). In short, monomers were resuspended in 10 mM Tris-HCl, 50 mM NaCl, pH 7.6 at a 5 mg/mL concentration. They were then placed on a 37 °C thermomixer, agitated for 7 days at 1,000 rpm, and then transferred to a -80 °C freezer. Immediately before use the pffs were diluted to 0.1 μ g/ μ L and sonicated with 60 pulses at 10% power for 60 sec (0.5 sec on, 0.5 sec off). For cell cultures, 20 μ L (139 pmol) of the sonicated pff, or equivalent amounts of α -synuclein monomers,

were used per well and added to the upper compartment, at the same time as the seeding of the endothelial cells. Half of the culture medium was replaced with fresh medium at the same time as the functional measurements were made.

Transmission electron microscopy

α -Synuclein pff samples, before and after sonication, were adsorbed onto glow discharged (GlowCube, Quorum Technologie Ltd, Laughton, UK.) 400 mesh, copper, carbon film grids (EM Resolutions, Saffron Walden, UK) for 30 sec, rinsed twice on DIW and negatively stained with 1.5% aqueous uranyl acetate for 30 sec. They were blotted on filter paper and viewed in a FEI Tecnai G2 (FEI Ltd, Oregon, USA operated at 200 kV). Images were recorded with a AMT XR60B camera running Deben software (AMT, Woburn, USA).

Cell cultures

hCMEC/D3 human brain endothelial cells (Cedarlane, CLU512) were cultured in T25 flasks coated with 150 μ g/mL rat Collagen I lower viscosity (R&D systems, 3443-100-01) for one hour. Endothelial cell media contained EBM-2 endothelial basal medium (Lonza 190860), 5% fetal bovine serum (FBS), 1% antibiotics (Life technologies 15240-0620), 1.4 μ M hydrocortisone (Sigma H0135), 5 μ g/mL ascorbic acid (Sigma A4544), 1% chemically defined lipid concentrate (Life Technologies 11905031), 10 mM HEPES (Life Technologies 15630106), and 1 ng/mL bFGF (Sigma F0291).

For primary astrocytic cultures, cortical tissue was dissected from postnatal day 3 C57/Ola mice and trypsinized in Hank's Balanced Salt solution without calcium and magnesium, 1% HEPES plus 0.25% Trypsin, at 37°C for 15 min in a shaking incubator at 100 rpm. To achieve a single cell suspension, trituration was performed by pipetting with a Pasteur pipette up and down 10 times. The resulting suspension was then centrifuged for 5 min at 1000 rpm and the pellet collected and plated into

poly-L-lysine-coated (PDL, 100 ug/mL, Sigma P5899) T75 flasks with 10 mL of astrocyte medium containing DMEM 1 g/L glucose (Life technologies 31885-023), 1 mM glutamine, 10% FBS, and 1% antibiotics. Shaking of astrocytes was performed to remove contaminating neurons and microglia and once a culture confluence of about 90% was reached, astrocytes were shaken at 80 rpm for 18 hours. Two days after shaking, the culture was treated with 20 μ M cytosine β -D-arabinofuranoside (Sigma C1768) for 4 days to remove proliferating cells such as microglia and fibroblasts. Astrocytes were used for experiments after about 6 weeks in culture.

For primary human neuronal cultures, human fetal cortical tissue from gestational age 61-70 day old aborted fetuses were dissected under sterile condition as published previously (Kuan et al., 2012). In short, the tissue was then mechanically triturated into a single cell suspension, before seeding onto PDL-coated glass coverslips treated with laminin (50 ug/mL, Sigma L2020) for 30 min, at a density of 100,000 viable cells per coverslip (12 mm diameter) in differentiation media composed of DMEM, 4.5 g/L glucose (Life Technologies 11965-084) supplemented with 10% FBS, 2% B-27 (Gibco 17504044), and 1% antibiotics. Cells were habituated in this endothelial cell medium for 24 hours before being co-cultured with the hCMEC/D3 cells. A schematic diagram to illustrate the different Transwell setups we adopted in the present study is shown in Fig. S1.

Transendothelial electrical resistance (TEER) and permeability measurements

For both TEER and permeability measurements, the apical side of a 24-well plate containing Corning Transwell membranes with a pore size of 0.4 μ m (0.33 cm² culture area, Sigma CLS3470) was first coated with rat collagen I (R&D Systems 440-100-01) for 1 hour. For astrocytic co-cultures, the basal side of the membranes was coated with PDL, before 45,000 primary astrocytes were seeded onto the basal

side of the membrane for 24 hours in astrocyte medium. It was then replaced by endothelial cell medium for another 24 hours before hCMEC/D3 cells were trypsinized and seeded onto the apical side of the Transwell membranes at a density of 192,000 cells/cm², or 64,000 endothelial cells per Transwell. For neuronal co-cultures, primary cortical neurons seeded on coverslips were maintained in the differentiation medium for 3 days before switching to the endothelial cell medium for 24 hours. The hCMEC/D3 cells were then seeded as described above. Permeability and TEER measurements were taken at 4, 7, and 14 days after endothelial cell seeding.

For TEER measurements, the endothelial cell medium was replaced, with 900 μ L fresh medium in the lower compartment and 200 μ L in the upper compartment. A Millicell ERS-2 voltohmmeter (Millipore MERS00002) was used to measure TEER.

For permeability measurements, the endothelial cell medium was replaced with 400 μ L of fresh medium in the basal and 150 μ L in the upper compartment. Then 150 μ L of 5 mg/mL FITC-dextran (Sigma FD10S, average 10 kDa, and Sigma FD4, average 4 kDa) was added into the upper compartment, to give a final concentration of 2.5 mg/mL. After every 10 minutes, 50 μ L of medium was sampled from the lower compartment with fresh medium replacing it at the same time. The fluorescence of the lower compartment efflux samples was measured using the BMG FLUOstar Omega microplate reader. Permeability values were calculated as described previously (Cecchelli et al., 1999).

Immunofluorescence

For immunocytochemistry, cells were fixed either using 4% paraformaldehyde (-Tx100) or 4% paraformaldehyde, 4% sucrose, and 1% Triton X-100 (+Tx100) for 20 min. After blocking with 5% donkey serum for 1 hour, the coverslips were

immunostained for the endothelial marker zona occludens-1 (ZO-1, Life Technologies 33-9100, 1:500), astrocytic marker GFAP (Dako Z0334, 1:500), microglial marker Iba1 (Wako 019-19741, 1:1000), neuronal marker Tau (Thermo Scientific MN1000, 1:1000), α -synuclein Syn42 (BD Transduction 610787, 1:1000), or α -synuclein phosphor Ser129 (Wako 015-25191, 1:1000) at 4 °C overnight. Secondary antibodies (donkey anti-mouse Alexa Fluor® 488, Thermo Scientific A21202; donkey anti-mouse Alexa Fluor® 568, Thermo Scientific A10037; donkey anti-rabbit Alexa Fluor® 488, Thermo Scientific A21206; donkey anti-rabbit Alexa Fluor® 568, Thermo Scientific A10042) were incubated at room temperature for 3 hours, before coverslipping with vectashield antifade mounting media (Vector H-1500). Staining omitting primary antibodies was performed in all immunohistochemical experiments to serve as a negative control. Imaging was performed using a Leica TCS SP2 confocal microscope (Leica).

TUNEL staining

TUNEL staining, to detect apoptosis, was carried out using the 'DeadEnd™ Colorimetric Apoptosis Detection System' from Promega Corporation (G7360) exactly as described by the manufacturer.

Postmortem human brain tissue

Flash-frozen, postmortem human cerebellum from cases with pathologically confirmed PD and similarly aged control subjects was obtained from the Queen Square Brain Bank. The presence of Lewy body-like α -synuclein pathologies in the cerebellum has been described previously (Mori et al., 2003, Piao et al., 2003). Demographic details of the postmortem cases are provided in Table S1.

Western blot

Cellular proteins from the endothelial cells or from postmortem brain tissues were extracted in a PBS buffer containing 1% Tx100 and Complete protease inhibitor (Roche 04693132001) using a dounce homogenizer. Total protein concentration was determined by the BCA assay (Life Technologies 23225). Fifteen micrograms of protein was loaded onto a 4-12% SDS-PAGE and electroblotted onto 20nm polyvinylidene difluoride membranes (GE 10600021). Membranes were then blocked using blocking buffer (1 M Tris at pH7.5, 5 M NaCl, 0.1% Tween 20, 5% skimmed dry milk) and incubated with primary antibodies in blocking buffer including ZO-1 (Life Technologies 33-9100, 1:1000), claudin-5 (Abcam 131259, 1:1000), occludin (Life Technologies 710192, 1:1000), tricellulin (Novus NB100-74633, 1:1000), or marveld3 (Aviva ARP44715_P050, 1:1000) at 4 °C overnight. The membranes were then rinsed in TBST (1 M Tris at pH7.5, 5 M NaCl, 0.1% Tween 20) and incubated with horseradish-peroxidase conjugated secondary antibodies for 1hr at room temperature. The membranes were developed with the SuperSignal™ West Pico chemiluminescence method (Life Technologies 34080). Chemiluminescence was detected by exposing the membrane in the Bio-Rad UV-imaging suite from 5 to 30 min. Optical densities of the bands were normalized to β -actin and quantitated using Image J 1.47v.

Measurement of inflammation-related cytokines

Culture media from EC-neuronal co-culture experiments was collected at 14 days after endothelial cell seeding, freeze dried immediately and stored in -20 °C before use. A panel of key inflammation-related cytokines was measured using Meso Scale Discovery (Rockville) electrochemiluminescent immunoassays, specifically V-PLEX human proinflammatory panel 1 (IFN γ , IL-1 β , IL-2, IL-4, IL-6, IL-8, IL-10, IL-12p70,

IL-13, and TNF α). Assays were run according to the manufacturer's instructions. Samples were typed in duplicate.

Statistical analysis

Normality of data was assessed using Shapiro-Wilk test before statistical analysis. Datasets that were not normally distributed (e.g. gender ratio) were analysed using the Mann Whitney test, while all other quantitative data was analysed using univariate ANOVA, with the significance level set at a conventional threshold of $p < 0.05$ for two-tailed tests. A *post-hoc* Bonferroni test was used as appropriate. Partial correlational analysis was also employed to study the relationship between two continuous variables. All the analysis was performed using SPSS Release 16.0.0.

Results

The barrier function of the endothelial cell line is not affected by incubation with α -synuclein pff

In order to assess the barrier function of the hCMEC/D3 cells and its possible modulation by the presence of α -synuclein pff in the culture medium, we seeded hCMEC/D3 cells on the upper compartment and measured both the TEER and permeability to FITC-dextran. A schematic diagram to illustrate the different Transwell setups we adopted in the present study is shown in Fig. S1. As the number of days *in vitro* (DIV) increased, there was, as expected, a progressive increase in TEER (Fig. 1A) and a corresponding reduction in permeability (Fig. 1B), indicating an improved barrier function of the hCMEC/D3 cells over time in line with previously published studies (Qosa et al., 2014, Weksler et al., 2005).

The presence of fibrillar assemblies of the α -synuclein pff was confirmed using transmission electron microscopy (TEM) both before and after sonication (Fig. 1C). hCMEC/D3 cells were cultured for 14 days with the pffs as their toxic effects have been reported after this duration of exposure (Volpicelli-Daley et al., 2014, Volpicelli-Daley et al., 2011). We also found, in line with previous reports using human umbilical vein endothelial cells (HUVECs) and umbilical artery smooth muscle cells (HASMCs) (Tamo et al., 2000), that α -synuclein (syn42) was found to be constitutively expressed at a low level in the hCMEC/D3 cells (Fig. 1D, top right). After Tx100 fixation, insoluble inclusion-like α -synuclein stainings could though be distinctively observed in the incubated cells (Fig. 1D, bottom left), while endogenous α -synuclein in the hCMEC/D3 cells was solubilized and could not be detected (Fig. 1D, bottom right). In addition, we found no apparent difference in the level of expression of the tight junction protein ZO-1 with pff treatment (Fig. 1D, right).

The barrier function of the endothelial-astrocytic co-culture is not affected by incubation with α -synuclein pff

The blood brain barrier is formed by endothelial cells that receive direct support from astrocytes, and it has previously been demonstrated that co-cultivation of the hCMEC/D3 cells with primary rodent astrocytes improves their barrier function in the Transwell system (Hsuchou et al., 2010). We therefore seeded primary rodent astrocytes onto the basal side, and hCMEC/D3 cells onto the apical side of the Transwell membrane. Similar to the endothelial mono-cultures, the TEER significantly improved over time in the astrocytic co-culture, but the presence of pff again did not lead to any detectable changes in barrier function (Fig. 2A and 2B). Using confocal microscopy we were unable to detect the presence of α -synuclein in

glial fibrillary acidic protein (GFAP)-positive astrocytes (Fig. 2C), in line with what others have previously reported (Lee et al., 2010).

The barrier function of the endothelial cells is transiently affected by incubation with α -synuclein pff in a neuronal co-culture

We then co-cultured the hCMEC/D3 cells with primary human fetal cortical cells, which predominantly, but not exclusively, consisted of neurons. As neurons do not form direct contact with the endothelial cells, primary cortical cells were seeded on coverslips in the bottom of the lower compartment. In contrast to what was observed for endothelial mono-cultures or the endothelial-astrocytic co-cultures, there was no significant effect of time on either TEER or permeability (Fig. 3A-C). To our surprise the barrier function of the hCMEC/D3 cells was in fact compromised in the primary cortical co-culture paradigm, as indicated by a reduced TEER and altered permeability. Incubation with pff further impaired the TEER ($p=0.012$ on DIV4, $p=0.001$ on DIV7), although only transiently ($p>0.05$ on DIV14) (Fig. 3A).

α -Synuclein pff treatment is neurotoxic to human fetal cortical neurons

As both monomeric α -synuclein and Tau expression was solubilized after Tx100 fixation, the presence of insoluble α -synuclein inclusions could only be observed in cells incubated with pff (Fig. 3D, bottom). To validate whether the pff were able to induce apoptotic cell death (Mahul-Mellier et al., 2015), we quantified the degree of DNA fragmentation in primary cortical cells and observed a 4-fold elevation in the number of TUNEL-positive cells (Fig. 4A-B). We also demonstrated that prolonged incubation with pff induced α -synuclein phosphorylation at serine 129 (Fig. 4D),

which is associated with the formation of the pathological species (Volpicelli-Daley et al., 2011), and thus presumably explains its toxicity in this culture system.

Finally we attempted a tri-culture system by seeding the hCMEC/D3 cells to the apical side, primary rodent astrocytes onto the basal side of the Transwell membrane, and primary fetal human cortical cells below the Transwell membrane. However neither the TEER nor permeability was affected by pff incubation for 14 days *in vitro* in this co-culture set up (Fig. S2).

α -Synuclein pff treatment disrupts the expression of tight junction proteins in the endothelial-neuronal co-culture

Although there was no evidence to suggest that pff induced consistent impairments of endothelial barrier function using this Transwell-based model, we examined whether prolonged pff incubation affected the expression of tight junction proteins on the hCMEC/D3 cells. Compared to the endothelial mono-culture condition, the expression levels of ZO-1, claudin-5 and tricellulin, but not occludin, were significantly elevated in primary cortical co-cultures (Fig. 5), while the expression of marveld3 was below the detection threshold in the endothelial mono-culture. In the primary cortical co-culture paradigm, incubation with α -synuclein monomers did not significantly alter the expression of all tight junction proteins as opposed to controls. In contrast, pff incubation induced a significant reduction in both occludin ($p=0.001$) and ZO-1 ($p=0.001$) expression in primary cortical co-cultures after 14 days *in vitro* (Fig. 5). Occludin is an integral membrane protein that directly regulates the tight junction paracellular permeability, while ZO-1 resides on the cytoplasmic membrane surface of intercellular tight junctions and is involved in tight junction assembly

(Abbott et al., 2006). However, expression of claudin-5, which is another integral membrane protein modulating paracellular permeability, was not significantly affected by pff incubation (Fig. 5). Similarly, expression of tricellulin and marveld3, which both contain a conserved four transmembrane MARVEL domain as is seen with occludin (Raleigh et al., 2010), were not noticeably perturbed by chronic pff treatment (Fig. 5).

α -Synuclein pff-mediated down-regulation of tight junction protein expression was not associated with any changes in inflammation-related cytokines

As α -synuclein is a potent activator of microglia leading to the production of proinflammatory cytokines (Kim et al., 2013), it would be critical to know whether this regulates the expression of tight junction proteins (Capaldo and Nusrat, 2009). We therefore investigated whether prolonged pff incubation altered the level of proinflammatory cytokines in culture, which could have been released by the endothelial cells (Verma et al., 2006), or the residual microglial cells in the primary cerebral culture (Fig. 4C). Using the multiplex electrochemiluminescent assays, we found no significant treatment effects on the level of ten inflammation-related cytokines in the culture medium of the EC-neuronal co-cultures (Table 1). This argues against the possibility that pff incubation reduces endothelial ZO-1 and occludin expression by upregulating proinflammatory cytokines.

Down-regulation of tight junction protein expression in postmortem PD brains

To validate whether the changes we found in endothelial tight junction proteins mediated by pff is clinically relevant, we examined the expression of these proteins in the postmortem brain of PD subjects (n=12), compared to controls (n=6). There

were no significant differences in age, gender ratio, and postmortem delay between groups (Table S2). Compared with the control brains, there was a significant reduction in the levels of occludin (Fig. 6B) and ZO-1 (Fig. 6C) proteins in the PD tissue, consistent with that observed in the endothelial-neuronal co-cultures. Multiple bands were observed with anti-ZO-1 antibody that ranged from 250 kDa to 150 kDa, in line with that observed in the intestinal biopsy materials previously by Clairembault et al., (2015), and so the total integrated intensity was normalized to β -actin to allow for us to undertake a differential expression analysis. Similar to our *in vitro* findings, the level of claudin-5 expression was not affected by PD pathology (Fig. 6D). Although there was in general no significant difference in tricellulin expression between patients and controls (Fig. 6E), two bands were seen with the tricellulin antibody which spanned around 60kDa in control subjects, while in some patients only the heavier band was detectable (Fig. 6A). Similarly, although there was no significant difference in marveld3 expression as a group between patients and controls (Fig. 6F), there was a pronounced elevation in marveld3 expression in several PD patients (Fig. 6A). This however did not appear to correlate with any clinical measures in PD patients in the partial correlation analysis (Fig. S3). Similarly we were unable to identify any associations between the levels of other tight junction protein and clinical measures in these PD cases (Fig. S3).

Discussion

Our study has demonstrated that α -synuclein pff treatment *in vitro* can downregulate the expression of tight junction proteins, including ZO-1 and occludin, in a human endothelial cell line. Similar down-regulation of tight junction protein expression was also observed in postmortem brain tissue from PD patients. Such changes however

did not lead to significant impairments in endothelial barrier function, as measured used the TEER or permeability.

Our observation that the expression of occludin and ZO-1 is significantly reduced in PD patient brains, as well as in brain endothelial cells after prolonged α -synuclein pff incubation *in vitro*, is in line with a recent report showing that in PD patients, the expression of occludin in the intestinal epithelial barrier is also disrupted in the presence of α -synuclein pathology (Clairembault et al., 2015). The lack of a functional alteration in the endothelial cell line in our study, in spite of a profound down-regulation of tight junction proteins in particular occludin, may be explained by the different physiological functions of these proteins. For instance, claudin-5 and occludin are both involved in the regulation of paracellular permeability in the endothelial tight junctions, as evidenced by the fact that there is severe impairment in endothelial barrier function in claudin-5 knockout mice embryos (Nitta et al., 2003). In contrast, results from similar knockout studies with occludin indicate that it is not essential for barrier function during development (Saitou et al., 2000, Saitou et al., 1998). This indicates that although occludin plays a central role in regulating the function of tight junctions that are already established, it has a dispensable role during tight junction formation. As such our culture system involves the formation and maturation of tight junctions between hCMEC/D3 cells as the cells reach confluence (Fig. 1 and 2), and thus pff mediated impairments of occludin and ZO-1 expression could have been compensated for by the relatively sparing of claudin-5, tricellulin, and marveld3 expression. This possibility is supported by a previous study, demonstrating that marveld3 can to some extent compensate for the loss of occludin in maintaining barrier function in a Transwell epithelial model (Raleigh et al., 2010).

Alternatively it is possible that the sensitivity of the cellular system we employed may be insufficient to address subtle changes in endothelial function induced by pff toxicity. Although the hCMEC/D3 cell line has been repeatedly demonstrated to be a simple and robust system to model the blood brain barrier *in vitro* (Hatherell et al., 2011, Hsuchou et al., 2010, Qosa et al., 2014, Weksler et al., 2005), recently better “tighter” models have been used which have greater sensitivity to drug or pathogen screening. For instance, primary cultures of rat brain capillary endothelial cells have a reported TEER of around $300 \Omega \times \text{cm}^2$ (Kovac et al., 2009), while a human pluripotent stem cell derived brain microvascular endothelial cells has been developed with a TEER of up to $3600 \Omega \times \text{cm}^2$ (Lippmann et al., 2014). In addition, the Transwell system we adopted is a static system while endothelial cells *in vivo* are constantly subject to blood flow (Naik and Cucullo 2012) and physiological sheer stress, which have both been shown to affect endothelial barrier functions (Santaguida et al., 2006). Furthermore the Transwell system is essentially a two dimensional assay without capillary-like features such as luminal compartments, and the effects of the different cell types may have affected the overall functional efficacy of our Transwell system. For example, the transient decrease in TEER observed in the endothelial-neuronal co-culture modality may be due to the induced pff toxicity on fetal cortical cells after 7 days *in vitro* (Volpicelli-Daley et al., 2014). Such a change may however have been masked in the tri-culture model, since the presence of astrocytes increased resistance and decreased permeability of endothelial cell monolayers by nearly 2-fold, compared to endothelial cells alone.

Since its identification as the main component in Lewy bodies (Spillantini et al., 1997), the role of α -synuclein in the pathogenesis of PD has been the subject of extensive investigation. Although it is still not clear what the exact physiological functions of this protein are in the normal brain, it is clear that α -synuclein has a very high propensity to form oligomers and pff (Uversky 2007). Several studies have demonstrated that these pff can directly induce neurodegeneration both *in vitro* and *in vivo*, while exogenously prepared pff can recruit endogenous α -synuclein and transform them into pathological species (Luk et al., 2009, Luk et al., 2012, Volpicelli-Daley et al., 2011). Exactly how such pff-mediated propagation and neurodegeneration relates to the onset and pathogenesis of PD remains to be elucidated, but there is increasing evidence to suggest that misfolded α -synuclein can gain access to the central nervous system through peripheral routes. This has been shown when pff is administered intramuscularly (Sacino et al., 2014), intragastrically (Holmqvist et al., 2014), or following a direct injection of virally-mediated α -synuclein to the vagus nerve (Ulusoy et al., 2013). This has led to the hypothesis that PD may begin in the periphery, which is supported by the fact that disrupting the vagal connection through vagotomy is associated with a decreased risk for subsequent PD (Svensson et al., 2015). Furthermore it has recently been shown that systemically administered pff can directly cross the blood brain barrier and mediate pathological changes including microglial activation in the brain (Peelaerts et al., 2015). Although hydrophilic macromolecules are normally prevented from crossing the blood brain barrier, results from our study show that α -synuclein pff can downregulate tight junction proteins, which can help explaining such entry and may have implications for the pathogenic spread and evolution of this

disorder. This in turn may have therapeutic implications for agents given peripherally to treat PD.

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Contributions

W.L.K. conceived the study. W.L.K., N.B., P.V.M., C.H.W. and R.A.B. designed the study. W.L.K., N.B., X.H., N.M., R.W and J.N.S. conducted the experiments, W.L.K., R.W., and C.H.W. analyzed the results. W.L.K., N.B., C.H.W., and R.A.B. wrote the manuscript, all authors approved the manuscript, P.V.M. and R.A.B. supported the study.

Competing interests

The authors declare no competing financial interests.

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Figure captions

Figure 1: Effects of 14 day α -synuclein pff incubation *in vitro* on hCMEC/D3 cells. As expected, both the transendothelial electrical resistance (A), and permeability (B), improved over time in culture ($p=0.001$), but neither measures were significantly affected by the presence of pff in the culture medium. Values represent the mean $\pm$ SEM of three independent experiments performed in hexaplicates. ***

$p \leq 0.001$. (C) Representative TEM images showing the fibrillar structures of pff before and after sonication. (D) Representative pictures on the expression of tight junction protein ZO-1 (green) and intracellular α -synuclein (red). Immunostaining with ZO-1 revealed both cytoplasmic expression as well as tight junction formation at cellular borders with staining along cell-cell contacts. Nuclei were counterstained with Hoechst (blue). Bar represents 100 nm in (C) and 30 μ m in (D).

Figure 1 can be a 1.5-, or 2-column fitting image.

Figure 2: Effects of α -synuclein pff incubation for 14 days *in vitro* on hCMEC/D3-astrocyte co-cultures. As expected, the transendothelial electrical resistance (A), but not permeability (B), significantly improved over time ($p=0.001$), but neither measure was significantly affected by the presence of pff in the culture medium. Values represent the mean $\pm$ SEM of two independent experiments performed in hexaplicates. *** $p \leq 0.001$. (C) Representative pictures on the expression of intermediate filament GFAP (green) in astrocytes and the lack of α -synuclein stainings (red) within such cells. Nuclei were counterstained with Hoechst (blue). Bar represents 15 μ m.

Figure 2 can be a single, or 1.5-column fitting image.

Figure 3: Effects of α -synuclein pff incubation for 14 days *in vitro* on hCMEC/D3-neuronal co-cultures. In the endothelial-neuronal co-cultures there were no significant changes in either TEER or permeability over time. However there was a transient reduction of transendothelial electrical resistance in the presence of pff (A), although this was not reflected in the transendothelial permeability using either 10 kDa (B), or 4 kDa FITC-dextran (C). Treatment with monomeric α -synuclein (+mono)

was always used as an additional control. Values represent the mean $\pm$ SEM of three independent experiments performed in hexaplicates. * $p\leq 0.05$, *** $p\leq 0.001$. (D) Representative pictures on the expression of total Tau-positive neurons (green) and α -synuclein (red). Nuclei were counterstained with Hoechst (blue). Bar represents 30 μ m.

Figure 3 can be a 1.5-, or 2-column fitting image.

Figure 4: α -Synuclein pff incubation for 14 days *in vitro* induced apoptosis and α -synuclein phosphorylation in primary fetal cortical cells. Representative pictures of the number of TUNEL-positive cells in the hCMEC/D3-neuronal co-cultures, incubated with monomeric α -synuclein or with pff (A). There was an approximately 4-fold increase in the number of apoptotic cells ($p=0.001$) (B). The primary cortical culture, while consisting mainly of neurons, also contained some GFAP- (green) and Iba1- (red) positive astrocytes and microglia cells, respectively (C). Prolonged incubation with pff increased the expression of phosphor Ser129 (red) in Tau-positive neurons (green), which is considered pathological (D). Values represent the mean $\pm$ SEM of two independent experiments performed in hexaplicates*** $p\leq 0.001$. Nuclei were counterstained with Hoechst (blue). Bar represents 30 μ m.

Figure 4 can be a 1.5-, or 2-column fitting image.

Figure 5: Effects of α -synuclein pff incubation for 14 days *in vitro* on the expression of endothelial tight-junction proteins. Expression levels of ZO-1 ($p=0.001$), claudin-5 ($p=0.001$), and tricellulin ($p=0.001$), but not occludin, were significantly upregulated when co-cultured with primary fetal neurons. No marveld3 expression could be detected in the hCMEC/D3 mono-culture. Representative blot images are shown in

(A). The level of ZO-1 ($p=0.001$) and occludin ($p=0.001$), but not the other tight junction proteins, was significantly reduced by pff treatment in the co-culture paradigm (B). In contrast, monomeric α -synuclein treatment did not significantly affect the expression of all tight junction proteins examined. Values represent the mean $\pm$ SEM of two independent experiments performed in hexaplicates *** $p\leq 0.001$ vs. EC only group, +++ $p\leq 0.001$ vs. EC+N -pff and EC+N +mono groups.

Figure 5 can be a single, or 1.5-column fitting image.

Figure 6: Altered tight junction protein expression in postmortem PD brains which was not associated with disease stages. The level of occludin ($p=0.03$) (A, B) and ZO-1 ($p=0.001$) (A, C), but not claudin-5, tricellulin, or marveld3 (A, D-F), was significantly reduced in the PD postmortem brain tissue, as oppose to healthy control subjects. Representative blot images are shown in (A). Values represent the mean $\pm$ SEM, * $p\leq 0.05$, *** $p\leq 0.001$, the level of tight junction protein expression was normalized to β -actin expression and adjusted for age, gender, and postmortem delay (B-F).

Figure 6 can be a 1.5-, or 2-column fitting image.

Cytokines	EC+N -pff mean $\pm$ SEM (pg/mL)	EC+N +mono mean $\pm$ SEM (pg/mL)	EC+N +pff mean $\pm$ SEM (pg/mL)	p-value
IFN γ	6.72 $\pm$ 1.40	5.31 $\pm$ 0.36	4.82 $\pm$ 1.25	0.171
IL10	0.67 $\pm$ 0.31	0.40 $\pm$ 0.26	0.49 $\pm$ 0.24	0.481
IL12p70	0.86 $\pm$ 0.19	0.88 $\pm$ 0.42	0.96 $\pm$ 0.37	0.892
IL13	2.62 $\pm$ 0.62	4.41 $\pm$ 1.42	2.50 $\pm$ 1.03	0.470
IL1 β	1.73 $\pm$ 0.11	2.41 $\pm$ 0.55	1.36 $\pm$ 0.49	0.584
IL2	4.19 $\pm$ 1.88	3.27 $\pm$ 1.36	4.83 $\pm$ 2.12	0.502
IL4	0.63 $\pm$ 0.44	0.40 $\pm$ 0.31	0.43 $\pm$ 0.39	0.677
IL6	213.75 $\pm$ 71.87	192.09 $\pm$ 64.35	152.47 $\pm$ 75.00	0.587
IL8	752.94 $\pm$ 199.01	1211.38 $\pm$ 468.26	859.24 $\pm$ 405.58	0.248
TNF α	1.37 $\pm$ 0.45	2.02 $\pm$ 1.06	1.22 $\pm$ 0.54	0.353

Table 1: Expression of inflammation-related cytokines in the culture medium of EC-neuronal co-cultures after 14 days *in vitro*. Results were derived from three independent experiments performed in hexaplicates.

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6