The amyloid precursor protein binds to β-catenin and modulates its cellular distribution

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GRAPHICAL ABSTRACT

ARTICLE INFO

Keywords:
Alzheimer’s disease
β-catenin
Amyloid precursor protein (APP)
GSK3
Wnt pathway

ABSTRACT

Accumulating evidence has shown that the processing of the amyloid precursor protein (APP) and the formation of amyloid-β are associated with the canonical Wnt/β-catenin signalling pathway. It was recently published that the drosophila homologue of APP is a conserved modulator of Wnt PCP signalling, suggesting a potential regulation of this pathway by APP. The aim of this study was to investigate the potential interaction of APP with the canonical Wnt pathway. APP overexpression in N2a cells led to alterations in the subcellular distribution of β-catenin by physically binding to it, preventing its translocation to the nucleus and precluding the transcription of Wnt target genes. In addition, studies in APP transgenic mice and human Alzheimer’s disease (AD) brain tissue showed the cellular co-localization of APP and β-catenin and binding of both proteins, suggesting the formation of physical complexes of APP and β-catenin, yet not present in healthy controls. Furthermore, a reduction in the levels of nuclear β-catenin was detected in AD brains compared to controls as well as a decrease in the expression of the inactive phosphorylated Glycogen Synthase Kinase 3 (GSK3) isoform. Therefore, these findings indicate a reciprocal regulation of Wnt/β-catenin signalling pathway and APP processing involving a physical interaction between APP and β-catenin.

Abbreviations: APP, amyloid precursor protein; BACE, beta-APP cleaving enzyme; GSK3, Glycogen Synthase Kinase 3; PCP, planar cell polarity; TCF/LEF, T-cell factor/lymphoid enhancer factor; TopFlash, TCF/LEF-Firefly luciferase

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https://doi.org/10.1016/j.neulet.2018.08.044
Received 10 July 2018; Received in revised form 20 August 2018; Accepted 29 August 2018
Available online 31 August 2018

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1. Introduction

According to the amyloid hypothesis, the main trigger of Alzheimer’s disease (AD) is the accumulation of amyloid-β (Aβ) peptides, which directly or indirectly initiates a series of pathological events, including glial activation, tau phosphorylation and neuronal loss. Aβ accumulation occurs due to increased generation and/or reduced clearance of Aβ. Understanding the cell pathways controlling these events is likely to be key to stopping the progression of the disease.

In the past decade, various reports have indicated that AD pathogenesis can lead to the dysfunction of the canonical Wnt/β-catenin signalling pathway [1,2]. For example, β-catenin levels were found diminished within the cytosol of cells treated with synthetic Aβ and thus the expression of the Wnt-associated genes was switched off [3]. Specifically, spatial memory impairments induced by hippocampal injection of Aβ fibrils in rats, as assessed in the Morris water maze, were prevented by treatments rescuing the Wnt signalling pathway [4]. These results suggest a close relationship between reduced levels of β-catenin in the cytosol and Aβ-induced synaptic toxicity (for a review, see [5]). More recently, it was shown that Wnt3a signalling is necessary for contextual fear memory acquisition and consolidation, in studies performed following the injection of antibodies against Wnt3a in adult mice [6].

The canonical Wnt pathway has been linked with the aetiology of both sporadic AD (SAD) and familial AD (FAD). Kawamura et al. [7] showed in 2001 that a mutation in a FAD-related gene, presenilin-1 (PS1) promoted an inhibitory effect on the Wnt / β-catenin pathway by enhancing the phosphorylation of β-catenin compared with the wild-type PS1 cells. This observation is consistent with previous findings [8,9] demonstrating that PS1 carrying an FAD mutation was able to form a physiological complex with β-catenin, and this interaction affected the levels, trafficking and stability of β-catenin in the cytosol.

Conversely, accumulating evidence has pointed towards a potential link between the Wnt/β-catenin pathway and AD pathogenesis via alterations in the activity of Glycogen Synthase kinase -3 (GSK3), which is an important regulator of Aβ generation and tau phosphorylation. GSK3 inhibition appears to be effective in reducing the production of Aβ by various mechanisms, including interfering with the γ-secretase cleavage of amyloid precursor protein (APP) [10] or, as we reported, affecting APP degradation by the autophagy/lysosomal pathway [11]. Additionally, we recently showed that the activation of the Wnt pathway by overexpression of the agonist Wnt3a or β-catenin results in a reduction in the transcription of BACE1 [12], by regulating the binding of the transcription factor TCF4 to the BACE1 gene promoter. These findings supported the suggestion that components of the Wnt signalling machinery are involved in AD.

Interestingly, it was recently demonstrated that the drosophila homologue of APP is a conserved modulator of the Wnt planar cell polarity (PCP) signalling pathway, suggesting that APPs are part of the membrane protein complex upstream of this particular Wnt signalling [13]. Given the ability of APP to form complexes with components of the Wnt calcium pathway and form part of the membrane protein complex upstream of Wnt signalling, it was thereby of great interest to explore whether APP can bind to other substrates and affect the canonical Wnt signalling. One potential binding candidate is β-catenin, because changes in its subcellular distribution have great impact on target gene activation. β-catenin has been reported to localise in the cell membrane, the cytoplasm, and the cell nucleus [14,15]. Therefore, it is important to understand the physiological role of APP and to determine whether it might contribute to AD pathogenesis possibly by altering β-catenin distribution and affecting the activity of the Wnt signalling pathway.

This study aimed to understand the functional interaction and physiological connection between the canonical β-catenin signalling pathway and APP by investigating if and how APP overexpression affects the expression and cellular distribution of β-catenin. We hypothesize that overexpression of APP might contribute to AD pathogenesis through alterations in the subcellular distribution of β-catenin, resulting in abnormal activation of the Wnt/β-catenin pathway. Here we observed that APP binds and co-localizes with β-catenin and this leads to alterations in the subcellular distribution and nuclear function of β-catenin.

2. Materials and methods

2.1. Materials and antibodies

Antibodies used were polyclonal anti-β-catenin and monoclonal anti-phosphorylated β-catenin from Sigma, 6E10 (against Aβ1-16) and 4G8 (against Aβ17-24) from Covance, 5313 against APP N-terminus (generously provided by Prof. Christian Haass, Munich, Germany), rabbit R1(57) against anti-APP COOH-terminal (a gift from Dr Mehta, Institute of Basic Research in Developmental Diseases, New York), and rabbit c-Myc from Santa Cruz. Monoclonal GSK3β was from Biosource and polyclonal P-GSK3β(S9) was from Cell Signalling. Secondary fluorescent antibodies were Alexa Fluor 488 and 594 goat anti-rabbit/mouse. The rest of chemical reagents were obtained from Sigma and Invitrogen (Thermofisher).

2.2. Mouse brains

APP23 transgenic mice that overexpressed human APP with the Swedish double mutation (from Novartis) were used for this study. This animal model shows a robust formation of amyloid plaques in brain pathology, initially in the frontal cortex and rapidly extending to the hippocampus from six months of age [16]. Brain tissue homogenates and brain sections were obtained from two 9 months old female transgenic and 2 wild-types of the same age and sex. All the animal procedures were approved by the U.K. Home Office and were in accordance with the Animals (Scientific Procedures) Act of 1986.

2.3. Human brains

Human brain samples consisted of frontal cortex tissue obtained from autopsies at London Neurodegenerative Diseases Brain Bank and the Huddinge Brain Bank, in accordance with the laws and the permission of the ethical committee. The control group included 16 subjects who died either of non-neurological diseases or traffic accidents and had no history of long-term illness or dementia (mean age 83 ± 2 years, 9 M and 7 F). The sporadic AD group included the frontal-cortex samples from 19 patients with clinically and pathologically confirmed AD (mean age 83 ± 2 years; 8 F and 11 M). Brains were kept at -80 °C until used.

2.4. Cell culture and transfection

A mouse neuroblastoma cell line, N2a cells was used in this study. N2a cells were cultured in normal Dulbecco’s modified Eagle medium (DMEM) supplemented with 10% foetal bovine serum (FBS) and penicillin / streptomycin mix. All cells were grown in a 5% CO₂ incubator at 37 °C. Cells were transfected using the transfection reagent Turbofect (Thermo scientific). Plasmids constructs used as follows: wild-type β-catenin cDNA (from Hans Clevers), Wnt3a cDNA (Addgene, US), APPwt and APPsw cDNA (from Efraïm Levy), and superTOP at and superFOP-FLASH at reporter constructs (obtained from Richard Killick).

2.5. Immunocytochemical staining

N2a cells were seeded on glass coverslips in 12- well plates, permeabilised and fixed in methanol at -20 °C for 10 min. Afterwards, they were washed in phosphate buffered saline (PBS) pH 7.3 and
blocked with 1% bovine serum albumin (BSA) in PBS. To detect the co-localisation of APP and β-catenin, double immuno-staining protocol was performed. The antibodies 4G8 at 1/500 dilution and anti-β-catenin at 1/500 were incubated in blocking buffer for 1 h. Following this, coverslips were incubated with the secondary antibodies, immunofluorescent goat anti-rabbit 488 IgG and goat anti-mouse 594 IgG. The coverslips were mounted with Vectashield containing DAPI from Vector Labs.

2.6. Immunohistochemical staining

Double immuno-labeling of brain tissue is a powerful tool to co-localise different proteins in the brain and to document the pathogenesis of AD. Brain sections from APP23 mice and wild-type mice and post-mortem human brain sections were processed for immunofluorescence double-label staining and confocal laser scanning-microscopy analysis.

The protocol for staining mouse and human brain sections was similar. The brain samples were permeabilised in 0.25% Tris-buffered saline (TBS) /Triton X-100, blocked in 10% fetal bovine serum (FBS) /1% BSA in 0.1% TBS-Triton X-100, and incubated in 1% FBS/0.1% BSA in 0.01% TBS-Triton X-100 with primary antibodies overnight at 4 °C for 48 h. After several washes with TBS, incubation with the fluorescent secondary antibodies for 1 h, the samples were mounted with Vectashield containing DAPI from Vector Labs.

2.7. Protein extraction

N2a cells and brain samples were homogenised using radio-immunoprecipitation assay (RIPA) buffer (1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 50 mM Tris–HCl, and 150 mM NaCl, pH 7.2) plus a cocktail of protease inhibitors (Compete, Roche). Cell lysates were centrifuged at 13,000 rpm for 10 min at 4 °C. Protein levels were determined in the supernatants using the Bradford method.

According to the detected concentration, 100 μg of protein samples were used in immuno-precipitation analysis.

2.8. Subcellular fractionation

Brains were homogenized and left on ice for 10 min. in hypotonic buffer (10 mM Tris pH 7.4, 1 mM EDTA, 1 mM EGTA) containing Roche Complete* protease inhibitor cocktail and sodium fluoride and sodium orthovanadate, two established phosphatase inhibitors. Then, samples were centrifuged at 5,000RPM for 5 min. to pellet the nuclear fraction. The supernatant containing the membrane and cytosol was transferred to a fresh tube and subsequently centrifuged at 13,000RPM for 45 min. The pellet containing the membrane fraction was kept for further analysis. The cytosolic fraction was then precipitated by adding 20% trichloroacetic acid (TCA) in a 1:1 ratio to give a final concentration of 10%. After incubating for 45 min. on ice, this fraction was spun at 13,000RPM for 30 min. The pellet was washed firstly in ice cold 90% acetone and then 100% acetone, spinning at 13,000RPM for 10 min. between the washes. The final pellet was resuspended in 2X loading buffer 1, adding tris-HCl pH8 to neutralise the acid. All steps of this procedure were carried out on ice, to avoid protein degradation.

2.9. Immunoprecipitation analysis

Co-immunoprecipitation (coIP) was performed to analyse the binding between APP and β-catenin. Extracted proteins (i.e., samples of N2a cells, mouse brain and human brain) were immunoprecipitated with antibodies against β-catenin or p-β-catenin and Protein A Sepharose beads (Invitrogen) overnight for 4 °C. The protein complexes were boiled in sample buffer (See Blue® plus prestained standard by Novex) and separated by 10% SDS-polyacrylamide gel electrophoresis (PAGE) to polyvinylidene fluoride membranes that were subjected to immunoblot analysis. Primary antibody against APP (6E10) was used at 1/1000 in 5%BSA/0.5%NaNO3 in TBST and detected with anti-mouse HRP secondary antibodies in 5% fat-free milk in TBST. To develop the film, ECLTM reagents and autoradiography film (GE Amersham, UK) were used in an automated developer.

2.10. Luciferase dual reporter assays

Changes in TCF (T-cell factor) transcription factors binding sites (TOPFlash) or mutant TCF-binding site (FOPFlash) activities were determined by using Firefly luciferase reporter genes and co-transfected with cytomegalovirus–Renilla luciferase normalizing construct in a 1:800 ratio. TOP and FOP-FLASH reporter assays were carried out on a luminescence reader (Berthold-Wallac) following the protocol for the Promega dual reporter assay *. The data were normalised to the Renilla luciferase expression to allow for varying transfection efficiencies.

2.11. Statistics

An unpaired student’s t-test or a one-way ANOVA were used to show any significant statistical difference between the means of two or more groups respectively. A Dunnett’s post-hoc test was used to assess the significance in reference to the control group.

3. Results

3.1. APP affects β-catenin subcellular distribution in N2a cells

To evaluate whether APP expression affected the distribution of β-catenin in the cells, we used N2a cells transfected with β-catenin only and N2a cells transiently transfected with both APPsw and β-catenin. Confocal microscopy analysis revealed that N2a cells overexpressing β-catenin only (Fig. 1B) showed high levels of β-catenin in the nucleus, as indicated by co-localisation with DAPI staining. In contrast, the distribution of β-catenin changed to a more cytoplasmic pattern in cells overexpressing APP (Fig. 1F). In addition, double staining with a polyclonal anti-β-catenin antibody and a monoclonal anti-mouse 4G8 antibody for APP revealed a co-localisation of APP with cytoplasmic β-catenin, particularly in the Golgi apparatus (Fig. 1H), suggesting that they may bind to each other.

Following this, we performed experiments of co-immunoprecipitation to determine the binding between both proteins. We observed a robust co-precipitation of endogenous β-catenin and APP by performing immunoprecipitation with antibody against β-catenin and western blot with antibody 6E10 against APP (Fig. 2A). A very weak band was detected in cells that did not overexpress APP, which could be related to endogenous APP.

To ascertain whether the change in intracellular localisation of β-catenin accounted for any functional variation, we transfected N2a cells with reporter genes harbouring TCF binding sites (TOPFlash) or a mutant TCF binding site (FOPFlash). This reporter gene detects changes in the transcriptional activity of the canonical Wnt/β-catenin pathway. N2a cells were transiently transfected with Wnt3a and reporter genes TOP flash or FOP flash with and without overexpressing APPsw or APPwt, to confirm that this effect was independent of the APP mutation. Interestingly and in support with our previous results showing reduced β-catenin in the nucleus in cells overexpressing APP, there was a major decrease in the TOP flash signal in cells transfected with APPsw or APPwt and Wnt3a compared to that of cells transfected with only Wnt3a cDNAs (Fig. 2B). In contrast, the statistical analysis of FOP signal showed no significance differences between cells overexpressing APPsw and cells overexpressing Wnt3a only.

3.2. APP binds to β-catenin in vivo

We next investigated whether the binding of β-catenin and APP
occurs in vivo, first in brain homogenates from APP23 mice compared with wild-type controls. The immunofluorescence double staining performed using the same antibodies as above was carried out on brain sections from APP23 Tg mouse model harbouring the APP Swedish mutation (n = 2) and wild-type mice (n = 2). We used this mouse model because it does not overexpress PS1, which has been previously shown to bind β-catenin. Confocal microscopy analysis showed co-localization of cytoplasmic β-catenin and APP in the cell body of neurons within the cortex, similar to the results obtained in N2a cells transfected with APP and β-catenin. Fig. 3A showed specific co-localisation of β-catenin and APP in the cytosol and at the cell membrane, but not in the nucleus of the neurons in the cortex.

Interestingly, and in agreement with the studies of co-localisation by immunofluorescence analysis, we observed a marked binding of β-catenin to APP by performing immunoprecipitation with an antibody against phosphorylated β-catenin and western blot with antibody 6E10 against APP in homogenised brain sample from APP23 mice model. No binding was detected in wild-type mice as control counterparts, as illustrated in Fig. 3B.

Having obtained convincing co-staining results in cells and mouse brain overexpressing APP, the distribution of β-catenin and its potential interaction with APP was also examined in brain tissue from sporadic AD patients. Likewise, we observed limited staining of β-catenin in the nucleus of cells within the frontal cortical region, and robust staining of specific co-localised cytoplasmic β-catenin and APP in the frontal cortex of AD brains (Fig. 3C).

We then sought to determine whether APP was binding to β-catenin in human brains and whether this interaction was altered in AD patients. The same protocols and antibodies were followed using homogenised brain sample from two sporadic AD patients and two normal healthy controls. Immunoblot analysis revealed that APP immunoprecipitated with anti-phospho-β-catenin antibody only in AD patients and not in the normal ageing brain (Fig. 3D).

3.3. APP binding to β-catenin affects its subcellular localization in AD brains

To confirm the reduced nuclear staining of β-catenin observed in sporadic AD brains, we performed subcellular fractionation of frontal-cortex tissue from 6 non-demented (ND) control patients (3 M, 3 F, 85 ± 8 year old) and 7 SAD patients (3 M, 4 F, 81 ± 5 years). The expression of β-catenin was analysed by Western blotting and it was normalised to the loading control of each fraction. There was no significant change in total β-catenin levels within the nuclear fraction in AD cases compared to healthy controls (Fig. 4A). However, no significant changes on total β-catenin forms were detected in the membrane and cytoplasmic fractions (Fig. 4A).

To determine whether there were additional alterations in the function of the Wnt/β-catenin canonical pathway, we examined the expression of GSK3 isoforms (α and β) and Ser-9 phosphorylated-GSK3β (P-GSK3(S9)) in human postmortem brain lysates (frontal cortex) from a group of 12 sporadic AD patients and 10 age-matched and healthy controls. The expression of GSK3α and GSK3β was assessed by immunoblotting with anti-GSK3α and anti-GSK3β antibodies. The expression of these isoforms was normalised to loading control with β-actin. A significant decrease in the expression of β-catenin was observed in AD brains, suggesting a reduction in the canonical Wnt/β-catenin pathway in AD brains.

Fig. 1. APP affects β-catenin subcellular distribution. A–D. Immunofluorescence staining of N2a cells transiently transfected with β-catenin cDNA, showing nuclear localization of β-catenin (green) and DAPI (blue). E–H. Immunofluorescence staining of N2a cells transiently transfected with APPsw and β-catenin cDNA, showing anti-β-catenin staining (green) in the cytoplasm and co-localization with APP antibody (red) (Bars represent 10 μm). Confocal images were captured on a Leica TCS-SP MP microscope and processed using Image J. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Fig. 2. APP binds β-catenin and affects the transcriptional activity of the canonical Wnt pathway. A. Co-immunoprecipitation analysis of cells transfected with β-catenin and APP, showing IP with β-catenin and blot with 6E10 against APP B. TOP/FOP-FLASH luciferase assay showing a significant decrease in the transcriptional activity of β-catenin in N2a cells overexpressing APP with wild-type or with the Swedish mutation (APPsw) (n = 4–12). All cells were transfected with Wnt3a cDNA. Columns represent mean ± SEM. Asterisk represents significant differences between TOP signal and control (determined by one-way ANOVA with Dunnet’s posthoc test; ***p < 0.001).
non-demented controls. Our results are in agreement with previous publications, showing a decrease in the levels of inactive p-GSK3β isoform in AD brains compared to healthy controls (Fig. 4F) and overall no changes in the expression of unphosphorylated GSK3 isotypes (Fig. 4D, E). These results indicate that the AD brains present less β-catenin in the nucleus and more active GSK3β compared to healthy individuals of the same age, suggesting a dysfunction of the canonical Wnt pathway in AD.

4. Discussion

Over the last decade, there has been increasing evidence suggesting that the Wnt/β-catenin pathway is dysfunctional in AD brains, reporting reductions in some of the components of the pathway [2,17]. It was reported that APP appears to down-regulate β-catenin expression by increasing its degradation [1], suggesting a role for APP in sustaining neuronal function by preventing cell cycle reactivation and...
maintaining synaptic integrity. Given the proposed functional connection between APP and β-catenin, the main goal of the present study was to determine whether overexpressed APP affects the subcellular distribution of β-catenin in vitro and in vivo, and if so, whether APP physically binds to β-catenin and affects its transcriptional function in the Wnt signalling pathway.

Our results show that in a normal situation, β-catenin is located in the nucleus, regulating the transcription of target genes in the canonical Wnt pathway. However, in cells overexpressing APP we found that β-catenin distribution changes to a more cytoplasmic location and co-localizes with overexpressed APP. This effect was observed not only in N2a cells transiently transfected with APPsw, but also in brain samples of APP23 transgenic mice and human sporadic AD patients, demonstrating a robust physical interaction of APP with β-catenin. In addition, physiological complexes of proteins were detected in co-immunoprecipitation analyses, by precipitation with antibodies against β-catenin and β-APP and blotting with an anti-APP antibody, showing that this interaction occurs also in vivo in APP23 Tg mice brain and human AD post-mortem brains. These findings support our hypothesis that APP affects the subcellular distribution of β-catenin through physical binding. As β-catenin plays a major role in cell-cell adhesion along with the caderhins [17] one could argue that this difference may be due to differences in the confluence of the cells. However, the confluence between APP-transfected and un-transfected cells was the same.

Our results contrast with the observations of Chen and Bodles [1], who reported that expression of wild type APP or of familial AD APP mutants in primary neurons down-regulated β-catenin in membrane and cytosolic fractions, and did not appear to affect nuclear β-catenin or β-catenin-dependent transcription.

Moreover, in our study, we show that overexpressed APP was indeed able to regulate β-catenin nuclear function, leading to substantial changes in the transcriptional activity of β-catenin. Differential TOPflash signals were found in cells expressing APPsw by Luciferase reporter analysis, indicating that overexpression of APP significantly reduced the activation of the Wnt/β-catenin pathway. No differences in FOPflash signal confirms the specificity of this effect. This is in line with the observations of Chen and Bodles [1] showing that in APP knockout CA1 pyramidal cells, accumulation of β-catenin was associated with the up-regulation of cyclin D1, a downstream target of β-catenin signalling. Therefore, our results support our hypothesis that APP overexpression not only alters the subcellular distribution of β-catenin but also modulates the Wnt/β-catenin signalling pathway.

Various studies have provided examples of regulated protein nuclear localisation by cytoplasmic anchoring, suggesting an association between protein-protein physical interactions with nuclear function of transcription factors. First, apart from β-catenin, APP was shown to bind and affect the localisation of other transcription factors. The binding of β-catenin to APP that we have observed is very similar to findings of Fe65, an adaptor protein interacting with β-APP located in the nucleus previously reported in COS7 cells. Interestingly, APP has been reported to affect the intracellular localisation of Fe65 by anchoring to the plasma membrane, preventing its nuclear translocation, which contributes to AD pathogenesis [18]. Similarly, another example of such a switch of the dual roles of β-catenin in gene transactivation and in cell-cell adhesion is Wnt 4, a member of the Wnt protein family involved in GSK3β-independent (i.e., non-canonical) Wnt pathway. Bernard et al. [19] reported a role of Wnt 4 in redirecting β-catenin to the cell membranes in vitro and in vivo, leading to prohibition of β-catenin binding to TCF and reduced expression of Wnt genes in the nucleus.

Taking these findings together, we can infer longer acting consequences on AD pathogenesis resulting from the APP-β-catenin interaction. Specifically, we propose that down-regulation of nuclear β-catenin by binding to APP results in a suppression of the transcription of Wnt target genes, increasing BACE1 transcription and thereby enhancing Aβ generation from APP β-site cleavage. Elevated Aβ in turn inhibits Wnt signalling pathway as suggested above. Indeed, we demonstrated in the present study that GSK3β inactive form is reduced in AD brain, therefore indicating the inhibition of the pathway.

In conclusion, our results show that APP regulates the canonical Wnt pathway and that the interaction between APP and β-catenin is increased in AD patients and transgenic APP models, with potential functional implications on gene transcription. Future research should focus on how pharmacological and molecular methods can be used to modulate the binding between APP and β-catenin.

Acknowledgements

We thank Prof. Steve Gentleman (Imperial College London) for his helpful editing of our manuscript. MHG fellowship was funded by the Imperial College Medical Research Council Doctoral Training Centre. We also thank the London Neurodegenerative Diseases Brain Bank and the Huddinge Brain Bank for the donation of the human brains used in the present study.

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