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LEVELS OF GLIAL CELL LINE-DERIVED NEUROTROPHIC FACTOR ARE DECREASED, BUT FIBROBLAST GROWTH FACTOR 2 AND CEREBRAL DOPAMINE NEUROTROPHIC FACTOR ARE INCREASED IN THE HIPPOCAMPUS IN PARKINSON'S DISEASE

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Abstract

Growth factors can facilitate hippocampus-based learning and memory and are potential targets for treatment of cognitive dysfunction via their neuroprotective and neurorestorative effects. Dementia is common in Parkinson's disease (PD), but treatment options are limited. We aimed to determine if levels of growth factors are altered in the hippocampus of patients with PD, and if such alterations are associated with PD pathology. Enzyme-linked immunoassays were used to quantify seven growth factors in fresh frozen hippocampus from ten PD and nine age-matched control brains. Western blotting and immunohistochemistry were used to explore cellular and inflammatory changes that may be associated with growth factor alterations. In the PD hippocampus, protein levels of the glial cell line-derived neurotrophic factor (GDNF) were significantly decreased, despite no evidence of neuronal loss. In contrast, protein levels of fibroblast growth factor 2 (FGF2) and cerebral dopamine neurotrophic factor (CDNF) were significantly increased in PD compared to controls. Levels of the growth factors epidermal growth factor (EGF), heparin binding epidermal growth factor (HB-EGF), brain-derived neurotrophic factor (BDNF) and mesencephalic astrocyte-derived neurotrophic factor (MANF) did not differ between groups. Our data demonstrate changes in specific growth factors in the hippocampus of the PD brain, which potentially represent targets for modification to help attenuate cognitive decline in PD. This data also suggests that multiple growth factors and direction of change needs to be considered when approaching growth factors as a potential treatment for cognitive decline.

Introduction

In addition to movement changes, mild cognitive impairment is present in up to 40% of early-stage PD patients (49, 61, 85), whilst frank dementia is present in 80% of patients 20 years post diagnosis (28). Treatment options for dementia in PD are limited (16, 23) but dementia is

associated with decreased quality of life (44, 45), increased incidence of nursing home admissions (67), increased carer burden (50) and increased mortality (46) in this disorder. The aetiology of cognitive decline in PD is incompletely understood but is reported to be associated with proteinopathology (42), dopaminergic dysfunction (30), cholinergic dysfunction (26, 64) and atrophy within the hippocampus (19, 60). This widely varying pathology suggests that endogenous factors which maintain hippocampal function may be altered in Parkinson's disease.

Growth factors represent potential therapeutic agents for neurodegenerative disorders including PD. Glial cell line-derived neurotropic factor (GDNF), cerebral dopamine neurotrophic factor (CDNF), mesencephalic astrocyte-derived neurotrophic factor (MANF) and fibroblast growth factor 2 (FGF2, also referred to as basic FGF) restore and protect dopaminergic neurons in the substantia nigra and striatum of rodent and non-human primate models of PD (17, 21, 81). Therapies based on increasing the bioavailability of these factors in the brain may therefore represent a novel approach to attenuate motor dysfunction in PD. Recent clinical trials of CDNF have been established to determine its safety and efficacy in attenuating nigrostriatal cell loss (11), although it is unknown if CDNF and other growth factors are also altered in other brain regions external to the basal ganglia in PD.

Additionally, GDNF therapy for movement dysfunction has progressed into clinical trials in PD (10, 12, 13), although no clinical benefit to GDNF therapy was reported in these studies. Notably, the subjects in these trials suffered from late-stage PD, while studies in rodents and primates suggest that earlier intervention and improved drug delivery results in better clinical outcomes (25, 63). Given that clinical signs of PD are suggested to occur only after significant nigral degeneration (5), the failure of these trials may therefore reflect a paucity of nigral cells on which GDNF can act. A more recent clinical trial has also found improved clinical PD symptoms after 80 weeks of GDNF therapy (91), compared to no significant improvement at 40 weeks (90). This also suggests that clinical trials of the efficacy of growth factors may need to consider a longer trial length in order to

produce clinically significant results. Ultimately, while GDNF therapy may not be suitable for treatment of PD motor symptoms, it may still represent a promising treatment for other brain regions with different patterns of neuronal loss in PD.

Emerging evidence suggests that cognitive dysfunction is associated with altered growth factors in the Alzheimer's disease (AD) brain (6, 15, 24, 68, 71, 76, 87). Studies of growth factors as candidate therapies in animal models of AD suggests these factors can reduce hippocampal pathology and restore memory function. For example, in rodent models hippocampal-targeted GDNF protects against AD-associated pathology (65), while CDNF improves long-term memory in a transgenic AD mouse model (40). FGF2 gene therapy reduces hippocampal plaque load, stimulates neurogenesis and restores spatial learning in a mutant mouse model of AD (39, 41). Together these data suggest that augmenting the growth factor microenvironment of the hippocampus can influence the development of abnormal pathology and associated hippocampus dysfunction in AD; this relationship may also be pertinent in PD, a disorder commonly associated with cognitive dysfunction. Here we quantified endogenous levels of growth factors and pathology in the hippocampus in PD compared with age-matched control cases to determine if growth factor support in this brain region is altered and associated with concomitant proteinopathology.

Materials and Methods

Growth factors GDNF, CDNF, MANF, FGF2, BDNF, epidermal growth factor (EGF) and heparin binding epidermal growth factor (HB-EGF) were chosen for inclusion in this study based on reports demonstrating these factors to be neuroprotective or neurorestorative for dopaminergic neurons (9, 17, 20, 21, 34, 36), or that these factors support learning and memory in animal models of AD (40, 41, 58, 65, 94). Hippocampal levels of these factors were quantified in ten cases of PD and nine age-matched normal controls. Hippocampal α-synuclein load and glial cell protein markers were also quantified to determine if altered growth factor levels were associated with disease pathology.

Tissue collection

This project was approved by the University of New South Wales Human Research Ethics Advisory Panel. Ten PD and nine age-matched control human brains were sourced by the Sydney Brain Bank and NSW Tissue Resource Centre. PD subjects were followed prospectively prior to death and clinical data regarding the severity of movement disorder was quantified annually. Severity of synucleinopathy was quantified according to Braak staging for α-synuclein pathology (5) and clinical symptoms of movement dysfunction using Hoehn and Yahr scaling (29). All PD cases had a clinical diagnosis of dementia and met pathological criteria for dementia according to Braak staging for neurofibrillary tangles (4) and CERAD plaque staging (Table 1) (52). All PD cases were treated with levodopa, while other anti-parkinsonian medications prescribed for the cohort included entacapone (four cases), selegiline (two cases), bromocriptine (two cases), cabergoline (two cases), pergolide (two cases), tolcapone (two cases) and amantadine (one case). Control cases revealed no clinical signs or symptoms of neurological or psychiatric disorders and neuropathological abnormalities were absent in all cases.

Brain tissues were prepared and sampled identically in all cases as previously described (88). For frozen tissues the head of the hippocampus, containing the cornu ammonis (CA) and dentate gyrus (DG), was dissected from a single coronal block located 0.5-1 cm anterior to the coronal block containing the lateral geniculate nucleus (Figure 1). Formalin-fixed, paraffin-embedded hippocampal tissue from the contralateral hemisphere to the frozen hippocampus was sampled from two cases for double immunofluorescence staining to characterise cell morphology.

Brain pH was determined by the NSW Tissue Resource Centre and Sydney Brain Bank upon collection of donated brains. Briefly, a 1-2 g segment of the lateral cerebellum was homogenized with a hand-held motorized homogenizer in 2.5 mL deionised water. A hand-held pH meter was then used to measure the pH of the homogenate and this reading recorded. Any pH readings below 5.0 or above 7.2 were re-measured to ensure accuracy.

ELISA

Frozen hippocampal tissue samples used to measure EGF, HB-EGF, FGF2 and BDNF were homogenized as previously described by Werry et al (88). BDNF levels were quantified in homogenates according to manufacturer protocol, while EGF, HB-EGF and FGF2 were quantified in supernatant following centrifugation at 14,000 *xg* for 30 min at 4°C.

Tissues for measurement of CDNF, MANF and GDNF were pestle-homogenised in 10x volume of homogenization buffer (137 mM NaCl, 20 mM Tris-HCl, 2.5 mM EDTA, 1% NP-40, 10% glycerol, 0.5mM sodium orthovanadate and cOmplete™ Mini Protease Inhibitor Cocktail (Roche); pH 8.0) on ice and quantified from supernatant following centrifugation at 12 000 *xg* for 20 min at 4°C.

Protein concentrations in supernatant were measured using a bicinchoninic acid assay (Thermo Scientific, Rockford, IL, USA) and levels of growth factor levels normalized to total amount of sample protein extracted. Supernatant protein levels were quantified using ELISA and assayed in triplicate (GDNF in duplicate) according to the manufacturer's protocol. For each factor, standard curves were run to identify a protein amount within the linear range of detection and ranged from 35-240 ug/sample. Acidification of tissue supernatant with 1 M HCl and re-neutralization with 1 M NaOH prior to analysis for GDNF (57) was performed to increase detectability (88).

Protein levels for CDNF and MANF were quantified using a custom designed double-antibody sandwich ELISA. Plates were coated (anti-CDNF monoclonal antibody clone 7D6 (Icosagen, Tartu, Estonia) in 1 µg/ml, 0.05 M carbonate coating buffer (pH 9.6) or goat anti-MANF polyclonal antibody (R&D Systems, Minneapolis, MN, USA), 1µg/ml in 0.05 M carbonate coating buffer (pH 9.6)) and incubated overnight at 4°C. Plates were blocked using 3% BSA in PBS for CDNF or 1% casein in PBS-T (PBS containing 0.05% Tween® 20) for MANF, for 2 h at room temperature then samples added and incubated with shaking at 4°C overnight. Detection antibody horseradish peroxidase (HRP)-linked mouse anti-CDNF clone 6G5 (Icosagen, Estonia; 1:1000) or HRP-linked mouse anti-MANF clone 4E12

(Icosagen, Estonia; 1:1000) was added and incubated with shaking for 5 h at room temperature. Substrate solution (Duoset ELISA Development system, R&D Systems, USA) was added and incubated for 20 min before 1 M sulfuric acid was added.

Immunohistochemistry

Immunohistochemistry was performed to investigate cellular morphology associated with altered neurotrophic factor protein levels in the hippocampus. A single 20 µm, formalin-fixed section was sourced from a subset of cases (five PD cases, five control cases indicated in Table 1). Sections were rehydrated, then cleared before antigen retrieval was carried out in citrate buffer (pH 6; Fronine, Sydney, Australia) with 0.05% Tween® 20 in a water bath at 95°C for 30 min. Sections were cooled before quenching endogenous peroxidases with 3% hydrogen peroxide in PBS and then blocked (CDNF: 10% normal horse serum in PBST; GDNF: 0.25% casein in PBST; FGF2: 10% normal goat serum) and incubated with primary antibody (Table S1) overnight. Sections were then incubated with biotinylated secondary antibody (Table S1) followed by Vector Elite Kit tertiary antibody complex (Vector Laboratories, Burlingame, CA, USA) before being visualised with 3,3'-diaminobenzidine (Sigma, USA) counterstained with cresyl violet, dehydrated and cover slipped with DPX mountant (VWR International Ltd, Radnor, PA, USA). Negative control sections were performed with primary antibodies omitted (data not shown).

Immunofluorescence

For further confirmation of cell types containing FGF, CDNF and GDNF, double immunofluorescence staining for astrocytic (glial fibrillary acidic protein; GFAP), microglial (ionized calcium-binding adapter molecule 1; Iba1) and neuronal (neuronal nuclei; NeuN) marker proteins using tyramide amplification was completed. Seven micron hippocampal sections from two representative cases (Table 1) were deparaffinated in xylene and rehydrated through an ethanol gradient before antigen retrieval in citrate buffer (pH 6.0) for 30 mins at 95°C. Sections were washed

3 times in 50% ethanol before endogenous peroxidases were quenched with 0.3% hydrogen peroxide in phosphate buffered saline (PBS). Sections were incubated in blocking solution (0.5% casein, 1% bovine serum albumin and 0.05% Tween® 20 in PBS) before overnight incubation at 4°C in the first primary antibody (Table S1) in blocking solution. Sections were then washed and incubated in appropriate HRP-conjugated secondary antibody (Table S1) in blocking solution before incubation in 1:50 Cyanine 5 (Cy5)- Tyramide amplification reagent according to the manufacturer's instructions (Perkin Elmer, Waltham , MA, USA). For fluorescent double staining, the protocol described above was repeated from peroxidase quenching in 0.3% hydrogen peroxide in PBS, using a second primary antibody and appropriate HRP-conjugated secondary antibody (Table S1). Due to low signal intensity sections stained for CDNF were further amplified via incubation in biotinylated anti-goat secondary antibody (1:1000, Vector Laboratories, USA) in blocking solution and Vector Laboratories ABC kit (1:500; both reagents) prior to tyramide amplification. Sections were then incubated in 1:50 Cyanine 3 (Cy 3)- Tyramide amplification reagents according to the manufacturer's instructions (Perkin Elmer, USA). Sections were washed, incubated in DAPI and coverslipped with 80% glycerol in PBS. Negative control sections were performed with either one or both primary antibodies or secondary antibodies omitted (data not shown). Acquisition of microscopy images was performed on a Zeiss LSM 710 confocal microscope (Zeiss, Oberkochen, Germany).

Semi-quantitative Neuronal Density Measurement

Single, slide-mounted sections (7 or 10 µm thick; sampled from the coronal level of the lateral geniculate nucleus [Figure 1]) stained with hematoxylin and eosin were obtained from the NSW Tissue Resource Centre for all cases and were scanned on an Olympus VS 120 slide scanner (Olympus Corporation, Tokyo, Japan) using extended focus imaging to produce a clear image of the entire section for neuronal quantification. Three sampling squares were placed randomly on the scanned image within the CA1 (300x300 µm), hilus (200x200 µm) and DG (100x100 µm) regions

using OlyVIA v.2.9.1 (Olympus Corporation, Tokyo, Japan). Within these defined regions of interest neurons were identified based on characteristic morphology; CA1 and hilus neurons exhibit pyramidal morphology with a large nucleus and a small, dense nucleolus while DG neurons are distinguishable by their ovoid, granular nucleus. Neurons were counted within each sampling square and neuronal density calculated using the sum of the neurons counted and the combined volume of each region of interest. This was standardized to a final measurement of neuronal number/mm^3 to normalize the differences in the area of the regions of interest and section thickness.

Western Blots

Glial cell protein markers (GFAP, IBA1 and human leukocyte antigen DR [HLA-DR]) and α-synuclein were quantified via western blotting to identify changes in cell populations and pathology respectively. Frozen hippocampal tissue were sonicated in 50 mM Tris-HCl (pH 7.5) buffer (10x volume) containing 125 mM NaCl, 5 mM EDTA disodium salt, 0.002% sodium azide, protease and phosphatase inhibitors (Roche, Germany) and supernatant collected following centrifugation at 120,000 *xg* for 2.5 h at 4°C. Pellets were resuspended in buffer containing 5% SDS, sonicated and centrifuged at 100,000 *xg* for 40 min at 25°C to collect a supernatant termed the SDS fraction.

Equal amount of protein from supernatant (30 µg) and SDS fractions (Iba1 and HLA-DR: 15 µg; GFAP: 30 µg) from each sample were separated on XT Bis-Tris precast polyacrylamide gels (Bio-Rad, Hercules, CA, USA) then transferred onto PVDF membrane (Millipore, USA). Membranes used for α-synuclein analysis were fixed with 0.4% PFA before immunoblotting. Membranes were blocked then incubated with specific primary antibodies (Table S2) overnight at 4°C. Membranes were incubated with appropriate secondary antibodies (Table S2) and then visualised using Clarity ECL detection (Bio-Rad, USA) according to the manufacturer's protocol using a ChemiDoc MP Imagining System (Bio-Rad, USA). Band intensity was analysed by densitometry using Image Lab™ Software 4.1 (Bio-Rad, USA) and normalized to β-actin (Millipore, USA).

Statistical Analysis

Demographic characteristics, levels of growth factors, glial and Lewy pathology markers in the diagnostic groups were examined using one-way analysis of variance and analysis of covariance (SPSS Statistics 20.0, SPSS Inc., Illinois, USA). Relationships between levels of each factor of interest and brain tissue pH, storage time and post-mortem interval (PMI) were investigated separately in each diagnostic group using linear regression. Differences in mean growth factor levels was analysed using an independent samples t-test. Number of cases analysed for each factor are presented in all figures and tables as numbers varied with tissue availability. Additionally, two data points greater than two standard deviations from the mean were removed prior to analysis of CDNF levels. Case numbers analysed for each growth factor as therefore as follows: EGF (Control *n*=9; PD *n*=10), HB-EGF (Control *n*=9; PD *n*=10), FGF2 (Control *n*=9; PD *n*=10), GDNF (Control *n*=6; PD *n*=7), CDNF (Control *n*=6; PD *n*=7), BDNF (Control *n*=7; PD *n*=10) and MANF (Control *n*=7; PD *n*=8). Relationships between growth factors, glial protein and pathology marker levels were investigated using linear regression. Semi-quantitative analysis of neuronal density was examined using an independent samples t-test, with one data point greater than two standard deviations from the mean removed prior to the analysis of the hilus (Control *n*=8; PD *n*=10). Significance level was set at $p \leq 0.05$ for all analyses.

Results

Age, PMI and brain pH did not differ significantly between control and PD cases (Table 1). Regression analysis demonstrated that protein levels of all growth factors investigated were not associated with brain pH, PMI, age or storage time in either control or PD cases. Similarly, protein levels of all growth factors were not associated in PD with either dementia-related functional and neuropathological scores (dementia duration, dementia rating, Braak tangle stage or CERAD plaque stage) nor with movement disorder-related functional and neuropathological scores (disease

duration, Hoehn and Yahr score or Braak α-synuclein stage) Freezer storage time was longer in the PD group (p=0.02; Table 1), however, levels of all proteins were independent of storage time in this group, suggesting no confounding effect. Levels of total α-synuclein quantified using immunoblotting were unchanged in the hippocampus in PD ($F_{(1,18)}$=1.05, p=0.32), however, levels of phosphorylated α-synuclein (phosphorylated at serine 129: pS129), specifically associated with PD pathology were significantly increased in the PD hippocampus ($F_{(1,17)}$=4.68, p=0.05; Figure 3E), confirming disease-associated pathology in the hippocampus of the PD cases.

Protein levels of neuronally-expressed neurotrophic factors are altered in the PD hippocampus in the absence of an alteration in neuronal density

Hippocampal levels of GDNF protein were significantly decreased (19%) in PD, compared with age-matched controls ($F_{(1,11)}$=5.55, p=0.04; Figure 2A). In contrast, CDNF protein levels ($F_{(1,11)}$=8.37, p=0.02; Figure 2D) were significantly increased (41%) in the PD hippocampus, compared with age-matched controls. GDNF-immunopositive cells expressed morphology consistent with that of neurons within the hippocampus of controls and PD cases (Figure 2B and 2C). CDNF-immunopositive cells similarly showed neuronal morphology in controls and PD (Figures 2E and 2F). Double immunofluorescence with the neuron marker protein NeuN confirmed the presence of GDNF (Figure 2G) and CDNF (Figure 2H) within cells of a neuronal phenotype. GDNF and CDNF staining was most marked in the neurons of the hilus and CA regions of the hippocampus. Less intense staining for CDNF was also observed in neurons in the granule cell layer of the DG, whereas no GDNF-positive cells were observed in DG granule neurons. Despite the significant alterations in hippocampal GDNF and CDNF protein levels observed here, overall density of neurons was unchanged in the hilus ($t_{(16)}$=1.806; p=0.090; Figure 2I), DG ($t_{(16)}$=-1.554; p=0.139; Figure 2J) and CA1 ($t_{(17)}$=1.319; p=0.205; Figure 2K). These data are consistent with previous quantitative measures of cell density in normal

aging (27, 73, 89), as well as with reports of hippocampal atrophy (7) without neuronal loss in PD (27, 37).

Hippocampal protein levels of FGF2 are increased in the PD hippocampus

Hippocampal levels of FGF2 were significantly increased (40%) in the PD hippocampus ($F_{(1,17)}$=7.47, p=0.01; Figure 3A) and FGF2-positive cells with glial morphology were found in both control and PD hippocampus (Figures 3B and 3C). Consistent with our previous finding that FGF2 is primarily present in glial cells, double immunofluorescence for FGF2 and cell type-specific protein markers demonstrated microglia ($Iba1^+$; Figure 3D) and astrocytes ($GFAP^+$; Figure 3E) strongly expressed FGF2, whereas only moderate staining was observed in neurons ($NeuN^+$; Figure 3F). Activated microglia, strongly expressing HLA-DR, were observed in the PD hippocampus however, very few of these cells were observed to be FGF2-positive.

Levels of hippocampal EGF ($F_{(1,17)}$=4.16, p=0.06), HB-EGF ($F_{(1,17)}$=0.12, p=0.73), BDNF ($F_{(1,15)}$=0.46, p=0.51) and MANF ($F_{(1,13)}$=0.28, p=0.61) did not vary between PD and control cases.

Association between growth factors, glial and pathology markers.

Levels of FGF2 and CDNF co-varied for the cohort as a whole (r^2=0.559, p=0.003), however this relationship reflected an association in the control (r^2=0.938, p=0.001), but not PD, cases (r^2=0.118, p=0.451). Levels of FGF2 and GDNF (r^2=0.011, p=0.74), and levels of GDNF and CDNF (r^2=0.109, p=0.749) were not associated. There were no further associations between CDNF and GDNF (Control r^2=0.622, p=0.113; PDD r^2=0.024, p=0.771) or FGF2 and GDNF (Control r^2=0.486 p=0.124; PDD r^2=0.004, p=0.894) when the cohorts were analysed separately.

As FGF2 is strongly expressed by glial cells and levels of this protein are increased in the PD hippocampus, levels of glial cell markers were investigated via immunoblotting (Figure 3G). Total

hippocampal levels of the astrocytic marker GFAP ($F_{(1,15)}$=2.26, p=0.15), microglial marker Iba1 ($F_{(1,12)}$=0.36, p=0.56) and activated microglial marker HLA-DR ($F_{(1,17)}$=0.48, p=0.49) were unchanged in PD compared with age-matched controls. FGF2 levels were, however, significantly associated with GFAP levels in the cohort as a whole (r^2=0.34, p=0.02) and this association was preserved when the PD cases were analysed separately (r^2=0.44, p=0.05; Figure 3H). Total α-synuclein levels were not altered in PD, and no association was found between any of the growth factors investigated and either total or pS129 α-synuclein protein levels.

Discussion

GDNF-based treatment strategies for attenuating movement dysfunction have been the subject of clinical trials (22, 43, 74), although the failure of these studies to produce clinical improvement has hampered the study of growth factors as potential treatments for PD. However, this does not preclude the use of growth factor-based therapies in other brain regions such as the hippocampus, which we report does not experience neuronal loss in PD and may therefore prove more responsive to growth factor therapies than the degenerating substantia nigra.

While the neuroprotective and neurorestorative effects on dopaminergic cells has been well-reported, GDNF in other brain functions, such as cognition and memory, has been little explored. GDNF contributes to normal hippocampal development (32) and hippocampal GDNF levels are maintained throughout the healthy human lifespan (88), suggesting this protein may have an ongoing effect on the regulation of hippocampal function. In contrast to the healthy aged hippocampus, we found a significant decrease in GDNF in PD.

GDNF co-localised exclusively to neuronal markers in immunofluorescence staining however we found no apparent neuronal loss in the hippocampal CA1, hilus and dentate gyrus, suggesting that a reduction in hippocampal GDNF in PD does not simply reflect the death of neurons containing

this protein, but rather a potential reduction of GDNF per neuron and thus the involvement of other disease-related processes other than apoptotic or necrotic activation. Reduced synaptogenesis may underlie hippocampal atrophy in PD, which demonstrates an overall loss of volume (7) without neuronal loss (27, 37), as described in previous studies. Previous rodent studies demonstrate a role of GDNF in driving axonal and dendritic sprouting in the hippocampus (32) and basal ganglia (66), suggesting that it may also contribute to structural reorganisation and plasticity of hippocampal cells in addition to, or as a component of, neuroprotection or neurorestoration. Alterations in synaptogenesis and plasticity are strongly associated with cognitive decline in rodents and humans (35, 70). The observed reduction in GDNF in the hippocampus in PD is therefore consistent with reduced neuroplasticity, whereby reduced GDNF contributes to cognitive decline by attenuating synaptic function.

It is also important to note that our finding of reduced GDNF in the hippocampus is not necessarily reflective of a pathological change affecting the hippocampus itself. GDNF is reported to be delivered to neuronal soma via retrograde transport (81), therefore cells containing GDNF may be representative of either GDNF-receiving, or GDNF-producing cells. Given that *GDNF* mRNA is present in the human hippocampus (31, 80) and specifically in hippocampal neurons in both rodents and humans (62, 72), it is likely that the neurons found to be immunopositive for GDNF in this study may be producing rather than receiving GDNF. If so, reduced levels of GDNF may not only affect the hippocampus itself, but also other brain regions which innervate it and which may be dependent on receiving GDNF from hippocampal neurons. For example, the cholinergic basal forebrain and noradrenergic locus coeruleus directly innervate the hippocampus (51, 69), contribute to cognitive function (8, 55) and degenerate in PD (26, 93). GDNF is also suggested to be neuroprotective of these cells (59, 92). Ultimately, these combined findings may indicate that alterations in GDNF affect hippocampal function via a reduction in endogenous neuroprotection as well as failing to adequately maintain connectivity between the hippocampus and other brain regions. Future studies into the

role of GDNF on hippocampal function are needed to further elucidate alternative pathways via which GDNF may affect hippocampal function and therefore contribute to cognitive change in PD.

Together with the observed decrease in GDNF expression, we found significant increases in the expression of both the neuron-associated CDNF and glia-associated FGF2, demonstrating for the first time that CDNF is present in hippocampal neurons. Both CDNF and FGF2 exhibit neuroprotective and neurorestorative properties for dopaminergic neurons (1, 2, 48, 78, 97); the reported increase in both factors in this study may therefore reflect a similar function towards glutamate neurons in the hippocampus, evidenced by consistent neuronal density in PD cases compared with controls. CDNF is thought to modulate protein folding in the endoplasmic reticulum, preventing the accumulation of misfolded proteins which may then induce endoplasmic reticulum stress and stress-induced apoptosis (84, 96). Phosphorylated α-synuclein (77) and α-synuclein oligomers (14) are known inducers of endoplasmic reticulum stress, which has been well-characterised in the PD brain (83, 86). Here we found significantly increased levels of phosphorylated α-synuclein in the PD hippocampus, thus we speculate that increased levels of CDNF in this brain region reflects a protective response by these stressed hippocampal neurons. Interestingly, we found no significant difference in MANF levels between PD and control cases. Given that the structure and function of CDNF and MANF are closely related (47), this may indicate an alternative role of CDNF in the progression of PD pathology, warranting further investigation in future studies.

We also found a significant increase in FGF2, in contrast to the PD midbrain (54, 82) or healthy aged hippocampus (88). FGF2 is involved in the regulation of astrocytic and microglial reactivity and neuroinflammation (3, 79); here we observed a positive association between FGF2 and GFAP expression in the PD hippocampus which may be reflective of these mechanisms. However, we also observed consistent levels of microglial and cell reactivity protein markers in PD and control cases, which does not support a marked inflammatory response in this brain region in our cases of advanced PD. The lack of gliosis in the hippocampus of PD patients would be consistent with previous reports of an attenuated inflammatory response in late stage PD (53, 75). Alternatively,

increased levels of FGF2 are found in AD hippocampus and co-localise to senile plaques (18, 38), however we found no association between FGF2 levels and severity of either AD or PD proteinopathology in the PD hippocampus. Given that FGF2 is not strongly associated with glial reactivity markers in this study, we suggest that an alternate mechanism underlies the increase in FGF2 expression seen in this study. FGF2 is a modulator of long-term potentiation and synaptic plasticity in hippocampal neurons (33, 95), both of which are important for learning and memory (56, 95). This is similar to the previously described role of GDNF, indicating that FGF2 may be upregulated as a compensatory response to buttress synaptic function in the absence of GDNF. This is supported by work demonstrating the importance of growth factors in combination in order to exert a neuroprotective effect (36).

Despite initially disappointing clinical outcomes, GDNF has demonstrated potential as a treatment option to attenuate dopaminergic cell death. In this study we aimed to determine if this factor or others may similarly represent a tractable and effective treatment option for cognitive decline, which is a common and debilitating consequence of PD. We found significant reductions in levels of GDNF but increased levels of the CDNF and FGF2 in the PD hippocampus, in the absence of apparent neuronal loss. These data suggest that alterations in GDNF protein may contribute to cognitive decline in PD, although the lack of apparent neuronal loss suggests that hippocampal GDNF exerts its neuroprotective function differently to GDNF within the dopaminergic midbrain. Given that GDNF-based treatments are already in Phase 2 clinical trials, we suggest that these treatment options may also be suitable for further studies of GDNF supplementation for the management of cognitive decline in PD.

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The data that support the findings of this study are available from the corresponding author upon reasonable request.

Conflict of Interest Statement

MS holds stock in Herantis Pharma Ltd and Mobidaig Ltd. GMH holds stock in Cochlear Ltd and NIB Holdings. MS and PL are inventors of a patent owned by Herantis Pharma Ltd. MS is a consultant for Genecode Ltd and Amarantus Ltd. MS is on the advisory boards for Heidelberg University Neuroscience Center, Aarhus University Dandrite Center, Helsinki Institute of Information Technology and Göttingen University Neuroscience Center. GMH is on the advisory board for the Danish Research Institute of Translational Neuroscience, Aarhus University. CSW is on the advisory board for Lundbeck Australia Pty Ltd and in collaboration with Astellas Pharma Inc., Japan.

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Age (y)	Sex	PMD (h)	Tissue pH	Storage time (wk)	Disease duration (y)	Hoehn and Yahr Scale	Braak α-synuclein Staging	Dementia Duration (y)	Dementia Severity[&]	Braak Tangle Staging	CERAD Plaque Staging	Cause of Death
Control												
67[#]	M	29	7.06	61	-	-	0	-	-	0	0	Coronary artery atherosclerosis
73[#]	M	9	6.52	122	-	-	0	-	-	0	0	Metastatic chondrosarcoma
73	F	45	6.86	176	-	-	0	-	-	0	0	Atherosclerotic cardiovascular disease
80	M	12	6.5	82	-	-	0	-	-	0	0	Emphysema
81	M	29	6.57	156	-	-	0	-	-	0	0	Cardiac failure
85[#]	F	10	6.63	181	-	-	0	-	-	0	0	Respiratory failure, pneumonia
86[#]	F	14.5	6.36	230	-	-	0	-	-	0	0	Septicaemia, peripheral vascular disease
87	F	24	6.37	9	-	-	0	-	-	0	0	Acute peritonitis
88[#]	M	9	6.36	183	-	-	0	-	-	0	0	Pneumonia, chronic obstructive airways disease
Mean												
80 ± 2.5	-	20.2 ± 4.1	6.6 ± 0.1	136.3 ± 21.7	-	-	-	-	-	-	-	
PDD												
69[#]	M	5	5.99	160	17	5	V	4	1	2	0	Bronchopneumonia
72	M	29	6.75	296	7	2	IV	4	3	0	0	Cardiorespiratory failure
75[#]	M	9	6.77	160	14	5	V	5	3	4	1	Cardiorespiratory failure
78	M	6	6.25	260	24	4	V	1	1	0	0	Metastatic carcinoma
80	M	17	6.52	288	11	5	VI	3	1	0	2	Cerebrovascular accident^
83	F	32	6.23	208	14	5	V	2	1	3	1	Ischaemic heart disease
83[#$]	F	7	6.69	168	14	5	V	2	3	1	3	Pneumonia
84	M	7	6.74	252	17	4	IV	2	1	3	0	Infected pelvic ulcer
85[#$]	F	26	6.58	124	17	4	V	4	1	1	2	Cerebrovascular accident^
90[#]	M	5	6.42	196	15	5	V	1	2	0	0	Cardiac failure
Mean												
79.9 ± 2.0	10	14.3 ± 3.4	6.5 ± 0.1	211.2 ±	15 ± 1.4	-	-	2.8 ± 0.4	1.7 ± 0.9	-	-	

									18.9*

Values shown are mean ± SEM. PMD- Post Mortem Delay; F- female; M- male; PDD- Parkinson's disease dementia

* $p \leq 0.05$ compared with control group.

[#] Cases used for immunohistochemistry.

[$] Cases used for immunofluorescence.

^ Cerebrovascular accident was noted as cause of death, however pathologically there were no signs of infarction or significant vascular disease in brain tissues.

[&] For dementia severity 1=mild, 2=moderate and 3=severe.