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Brain Insulin-Like Growth Factor and Neurotrophin Resistance in Parkinson's Disease and Dementia with Lewy Bodies: Potential Role of Manganese Neurotoxicity

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Abstract

Parkinson's disease (PD) and dementia with Lewy bodies (DLB) frequently overlap with Alzheimer's disease, which is linked to brain impairments in insulin, insulin-like growth factor (IGF), and neurotrophin signaling. We explored whether similar abnormalities occur in PD or DLB, and examined the role of manganese toxicity in PD/DLB pathogenesis. Quantitative RT-PCR demonstrated reduced expression of insulin, IGF-II, and insulin, IGF-I, and IGF-II receptors (R) in PD and/or DLB frontal white matter and amygdala, and reduced IGF-IR and IGF-IIR mRNA in DLB frontal cortex. IGF-I and IGF-II resistance was present in DLB but not PD frontal cortex, and associated with reduced expression of Hu, nerve growth factor, and Trk neurotrophin receptors, and increased levels of glial fibrillary acidic protein, α-synuclein, dopamine-β-hydroxylase, 4-hydroxy-2-nonenal (HNE), and ubiquitin immunoreactivity. $MnCl_2$ treatment reduced survival, ATP, and insulin, IGF-I and IGF-II receptor expression, and increased α-synuclein, HNE, and ubiquitin immunoreactivity in cultured neurons. The results suggest that: 1) IGF-I, IGF-II, and neurotrophin signaling are more impaired in DLB than PD, corresponding with DLB's more pronounced neurodegeneration, oxidative stress, and α-synuclein accumulation; 2) $MnCl_2$ exposure causes PD/DLB associated abnormalities in central nervous system neurons, and therefore may contribute to their molecular pathogenesis; and 3) molecular abnormalities in PD/DLB overlap with but are distinguishable from Alzheimer's disease.

Keywords

Central nervous system; dementia with Lewy bodies; human; insulin-like growth factor resistance; neurotrophin; Parkinson's disease; receptor-ligand binding

INTRODUCTION

Parkinson's disease (PD) is the second most common form of neurodegeneration in the Western hemisphere, exceeded in prevalence only by Alzheimer's disease (AD). PD is associated with a resting tremor, reduced voluntary movements, increased rigidity and stiffness, bradykinesia, and postural instability. Afflicted individuals exhibit a shuffling gait with “en bloc” turning, stooped posture, and dystonia. Motor impairments result in hypophonia, non-intelligible or monotonous speech, and dysphagia with eventual death from aspiration pneumonia. In addition, PD is frequently accompanied by neuropsychiatric and cognitive dysfunction

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including depression, abulia, hallucinations, delusions, paranoia, memory deficits, decline in executive function, and dementia. Finally, PD can be associated with sleep disorders such as insomnia, orthostatic hypotension, impaired proprioception, anosmia, increased musculoskeletal and neuropathic pain, and autonomic dysfunction resulting in urinary incontinence, nocturia, gastrointestinal dysmotility, altered sexual function, and weight loss [1,2]. Together, this constellation of central nervous system (CNS) abnormalities points toward a neurodegenerative process that targets many brain structures, beyond those integrally related to local nigrostriatal circuitry. When PD is associated with prominent and progressive neuropsychiatric symptoms and dementia, it is termed dementia with Lewy bodies (DLB). Alternatively, if the postmortem histopathological features show extensive overlap with AD, the entity may be termed, Lewy body variant of AD. Moreover, PD can be a component of other major neurodegenerative diseases including multiple systems atrophy, progressive supranuclear palsy, and corticobasal ganglionic degeneration.

Motor symptoms of PD are caused by degeneration of large pyramidal pigmented,dopaminergic neurons in the pars compacta zone of the substantia nigra, together with projection fibers to the striatum. Interruption of these pathways results in increased inhibition of neurons in the ventrolateral nucleus of the thalamus, and consequently, loss of excitatory input to the motor cortex, and attendant hypokinesia. In addition, PD/DLB exhibit degeneration of adrenergic neurons in the locus ceruleus with loss of projections to the thalamus. PD/DLB is characteristically associated with Lewy body formation in degenerating pigmented brainstem neurons. Lewy bodies are intraneuronal eosinophilic inclusions that contain aggregated and ubiquitinated α-synuclein protein [3-6]. Fundamental to the PD/DLB neurodegeneration cascade is impaired transport of α-synuclein-ubiquitin complexes from the endoplasmic reticulum (ER) to the Golgi [7], resulting in their accumulation in neurons, along with iron and copper, which enhance oxidative stress [8]. Iron accumulation and oxidative stress cause α-synuclein-ubiquitin complexes to aggregate and further accumulate, thereby exacerbating neuronal oxidative stress.

Like AD, the vast majority of PD represents sporadic occurrences of unknown etiology, whereas less than 10% of the cases are familial. Familial PD has been linked to mutations or polymorphisms in *PARK* genes that have been mapped to chromosomes 1, 2, 4, 6, 12, or X [9-14]. Of note is that *PARK1* encodes the α-synuclein, and PD arising from *PARK1* mutations has autosomal dominant inheritance [9,11,13]. The *PARK2* gene encodes Parkin protein, and mutations in *PARK2* account for a large percentage of familial PD [12,14,15]. *PARK5* encodes ubiquitin carboxyl-terminal hydroxylase L1 [16,17], which is of particular interest because of its role in ubiquitination and accumulation of α-synuclein-containing insoluble protein aggregates in PD/DLB CNS neurons. Despite these striking and somewhat informative familial linkages to PD, germline *PARK* gene mutations each account for relatively small percentages of the overall number of PD/DLB cases. Moreover, genetic factors alone are not likely to be sufficient to confer a PD/DLB phenotype since their effects are generally not manifested until individuals reach middle or old age. In essence, aside from aging, specific factors, i.e., “second hits” responsible for initiating critical events in the PD/DLB neurodegeneration cascade have not yet been determined [10,18,19].

Epidemiological and experimental data suggest that PD-type neurodegeneration may be mediated by environmental exposures to toxins, e.g., polychlorinated biphenyls (PCBs), paraquat, rotenone, and 1-methyl 4-phenyl 1,2,3,6-tetrahydropyridine (MPTP); pesticides, e.g., dieldrin and lindane; or transition metals, e.g., manganese and iron [20-22]. In addition, PD and α-synuclein pathology can be produced by head trauma [23,24] or chronic treatment with antipsychotic drugs that inhibit dopamine and promote dopamine receptor resistance [25,26]. More recently, interest in the role of exposures leading to altered metabolic states has grown due to emerging evidence that the risks for developing AD and PD have increased with

the epidemic rise in prevalence of obesity and type II diabetes [27]. In this regard, epidemiological studies demonstrated a significantly increased risk for developing PD in individuals with central obesity [28,29], whereas caloric restriction was found to ameliorate neurotrophin deficiency in an experimental model of PD [30]. Given the frequent clinical, pathological, and biochemical overlap between AD and PD/DLB, and evidence that AD is mediated by brain insulin and insulin-like growth factor (IGF) resistance and deficiency in humans and experimental animal models [31-34], it was of interest to determine if molecular abnormalities in brain insulin/IGF signaling mechanisms are also impaired in PD/DLB. Moreover, we extended our studies to examine expression of neurotrophin genes because of their prominent levels of expression in subcortical nuclei, which are commonly damaged by PD and/or DLB [35-38]. Finally, in light of the potential role of environmental factors, particularly manganese exposure, in the pathogenesis of PD/DLB [39-45], we investigated the extent to which molecular and biochemical abnormalities related to insulin/IGF signaling in PD/DLB could be reproduced experimentally in neurons exposed to $MnCl_2$.

METHODS

Human subjects

We obtained human postmortem brain tissue from eight controls, seven cases of PD, and eight cases of DLB from the Massachusetts General Hospital ADRC brain bank. Brains were harvested and stored as previously described [32]. All diagnoses were confirmed by standardized histopathological examination using guidelines established by the DLB Consortium [46]. The mean ages and sex ratios were similar for the three groups (Table 1). Fresh, snap-frozen samples of anterior frontal cortex and white matter (Brodmann Area 9-separately analyzed), amygdala, and lenticular nuclei (putamen + globus pallidus) were used to measure mRNA and protein expression. In addition, frontal cortex was used in competitive equilibrium receptor binding assays to detect insulin, IGF-I, or IGF-II resistance. Adjacent paraffin-embedded histological sections were used for immunohistochemical staining to detect α-synuclein, dopamine β-hydroxylase (DβH), 4-hydroxy-2-nonenol (HNE), or ubiquitin using previously described methods [32]. The brain regions selected for study represent targets of PD and/or DLB neurodegeneration [17,47].

Quantitative (q) RT-PCR assays

Total RNA was isolated with TRIzol (Invitrogen, Carlsbad, CA), reverse transcribed with random oligodeoxynucleotide primers, and the resulting cDNAs were used to measure mRNA expression by qRTPCR. Ribosomal 18S RNA was measured in parallel reactions to calculate relative abundance of each mRNA transcript [33,34]. PCR amplifications were performed in 20 μl reactions containing cDNA generated from 2.5 ng of original RNA template, 300 nM each of gene specific forward and reverse primer (Tables 2A and 2B), and 10 μl of 2× QuantiTect SYBR Green PCR Mix (Qiagen Inc, Valencia, CA). The amplified signals were detected continuously with the Mastercycler ep realplex instrument and software (Eppendorf AG, Hamburg, Germany) as previously described [48]. Annealing temperatures were optimized using the temperature gradient program provided with the Mastercycler ep realplex software. PCR primer pairs were designed using MacVector 9 (Cary, North Carolina), verified by BLAST Sequence analysis through the National Center for Biotechnology Information (NCBI) website and commercially synthesized (Sigma-Aldrich Co., St. Louis, MO).

Receptor binding assays

Competitive equilibrium binding assays were performed according to previously published methods [31,32]. Briefly, fresh frozen tissue was homogenized in NP-40 lysis buffer (50 mM Tris-HCl, pH 7.5, 1% NP-40, 150 mM NaCl, 1 mM EDTA, 2 mM EGTA) containing protease (1 mM PMSF, 0.1 mM TPCK, 1 μg/ml aprotinin, 1 μg/ml pepstatin A, 0.5 μg/ml leupeptin, 1

mM NaF, 1 mM $Na_4P_2O_7$) inhibitors. Protein concentration was determined using the bicinchoninic acid (BCA) assay (Pierce, Rockford, IL). Duplicate samples containing equivalent amounts of protein were incubated with 100 nCi/ml of [^{125}I] (2000 Ci/mmol; 50 pM) insulin, IGF-I, or IGF-II for 16 hours at 4°C. To measure non-specific binding, additional duplicate samples were identically prepared but with the addition of 0.1 μM unlabeled (cold) ligand. Bound radiolabeled tracer was precipitated with bovine gamma globulin and polyethylene glycol 8000. Radioactivity distributed in the supernatants (containing free ligand) and pellets (containing bound ligand) was measured in an LKB CompuGamma CS Gamma counter. Specific binding was calculated by subtracting fmol of nonspecific binding, i.e., amount bound in the presence of cold ligand, from the total fmol bound (absence of unlabeled competitive ligand). The results were analyzed and plotted using the GraphPad Prism 5 software (GraphPad Software, Inc., San Diego, CA).

Enzyme-Linked Immunosorbant Assays (ELISAs)

Immunoreactivity was detected in tissue or cellular homogenates using direct binding ELISAs as previously described [49]. Briefly, homogenates were prepared in radio-immunoprecipitation assay buffer (50 mM Tris-HCl, pH 7.5, 1% NP-40, 0.25% Na-deoxycholate, 150 mM NaCl, 1 mM EDTA, 2 mM EGTA) containing protease inhibitors as indicated earlier. Protein concentrations were determined using the BCA assay. Protein homogenates diluted to in Tris buffered saline (TBS) (40 ng in 100 μl) were adsorbed to the bottoms of the wells at 4°C. Non-specific binding was blocked with 3% bovine serum albumin (BSA) in TBS. Primary antibody (0.01–0.1 μg/ml) was applied for 1 hour at room temperature, after which immunoreactivity was detected with horseradish peroxidase (HRP)-conjugated secondary antibody (1:10000; Pierce) followed by Amplex Red (Molecular Probes, Eugene, OR). Fluorescence was measured (Ex 530/Em 590) in a SpectraMax M5 microplate reader (Molecular Devices Corp., Sunnyvale, CA). Negative controls included use of non-relevant monoclonal antibody (mAb) to Hepatitis B Surface antigen as the primary antibody, omission of the primary or secondary antibody, or coating of wells with 3% BSA instead of sample.

Primary rat cerebellar neuron cultures

Primary neuronal cultures were generated with postnatal day 8 rat pup cerebella [51] and maintained with Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal calf serum, 4 mM glutamine, 10 mM non-essential amino acid mixture (Gibco-BRL, Grand Island, NY), 25 mM KCl, and 9 g/L glucose. Five-day-old cultures were treated with 0, 10, 20 or 40 μM $MnCl_2$ for 48 hours, after which they were harvested for qRT-PCR, ELISA, or cellular ELISA studies. PCR primer pairs used in these experiments were published previously [51].

Cellular ELISA

A cellular ELISA was used to measure immunoreactivity directly in cultured cells (96-well plates) [50]. The only modification of the original protocol was that immunoreactivity was detected with the Amplex Red fluorophore (Ex 530/Em 590) (Pierce, Rockford, IL), and measured in a SpectraMax M5 microplate reader (Molecular Dynamics, Inc., Sunnyvale, CA). Cell density was assessed by measuring fluorescence after staining the cells with Hoechst H33342 (Ex360 nm/Em460 nm; Molecular Probes, Eugene, OR). The calculated ratios of fluorescence immunoreactivity to H33342 were used for inter-group comparisons. At least 8 replicate cultures were analyzed in each experiment.

Sources of reagents

Human recombinant [^{125}I] insulin, IGF-I, and IGFII were purchased from Amersham Biosciences (Piscataway, NJ). Unlabeled recombinant human insulin, IGF-I and IGF-II were purchased from Bachem (King of Prussia, PA). Antibodies to Hu, myelin-associated

glycoprotein-1 (MAG-1), glial fibrillary acidic protein (GFAP), growth associated protein-43 (GAP-43), α-synuclein, DβH, 8-hydroxy-2′-deoxyguanine (8-OHdG), ubiquitin, HNE, and β-actin were purchased from AbCam (Cambridge, MA). ATPLite assay kit was obtained from Perkin-Elmer (Waltham, MA). Amplex Red and Hoechst H33342 dye were purchased from Molecular Probes (Eugene, OR). All other fine chemicals and reagents were purchased from CalBiochem (Carlsbad, CA) or Sigma-Aldrich (St. Louis, MO).

Statistical analysis

Data are depicted as means ± S.E.M. in the graphs. Inter-group comparisons were made using one-way analysis of variance (ANOVA) with the post-hoc Dunn's multiple comparison test of statistical significance. In addition, trend lines were analyzed using Deming linear regression. Statistical analyses were performed using GraphPad Prism 5 software (GraphPad Software, Inc., San Diego, CA). Significant P-values and trends are indicated over the graphs.

RESULTS

Pathologic shifts in cell populations in PD and DLB (Table 3)

Previous studies demonstrated significant pathological shifts in brain cell populations that correlated with neuronal and oligodendroglial cell loss, gliosis, and neuroinflammation in AD [31]. To determine if PD and DLB were associated with similar abnormalities, we measured relative mRNA levels of Hu neuronal ribosomal RNA binding protein [52,53]; myelin-associated glycoprotein-1 (MAG-1), a marker of oligodendrocytes; GFAP, which is expressed in astrocytes; endothelin-1 (ET-1), a marker of endothelial cells; and ionized calcium binding adaptor molecule-1 (IBA-1), a gene expressed in activated microglia [54,55]. The ng quantities of mRNA were normalized to 18S rRNA in corresponding samples. mRNAs isolated from different brain regions were analyzed simultaneously. The studies demonstrated that Hu expression was significantly reduced relative to control in PD and DLB frontal cortex and amygdala, and in DLB but not PD frontal white matter and basal ganglia. MAG-1 expression was significantly reduced in PD and DLB fontal white matter and basal ganglia, and in DLB frontal cortex. GFAP expression was significantly increased in all brain regions of DLB cases, but only in subcortical nuclei, i.e., amygdala and basal ganglia in PD. ET-1 expression was significantly reduced in DLB cortex, white matter, and basal ganglia, and in PD basal ganglia. Finally, evidence of neuroinflammation was detected in PD frontal cortex, white matter, and basal ganglia, and DLB basal ganglia. Within each brain region, the mean levels of 18S rRNA were similar among the groups.

PD and DLB associated abnormalities in expression of insulin, IGF-I, and IGF-II polypeptide and receptor genes

qRT-PCR studies detected mRNA transcripts corresponding to insulin, IGF-I, and IGF-II polypeptides and receptors in control, PD, and DLB samples (Table 4). Insulin gene was most abundantly expressed in the amygdala followed by frontal cortex and white matter. IGF-I was most abundantly expressed in white matter followed by amygdala, and similarly lower levels were observed in frontal cortex and basal ganglia. IGF-II was the least abundantly expressed of the three growth factors, and the patterns of expression mirrored those of insulin, except that IGF-II transcripts were not detected in basal ganglia. Insulin gene expression was significantly reduced in PD and DLB white matter and amygdala. IGF-I expression was not reduced in PD/DLB, and instead was significantly elevated in PD frontal cortex. IGF-II expression, like insulin, was significantly reduced in PD and DLB frontal white matter. With regard to the receptors (R), IGF-IR was most abundant, followed by IGF-IIR, and then insulin-R. Insulin-R expression was significantly reduced in PD and DLB white matter, and in DLB amygdala. IGF-IR mRNA was significantly reduced in DLB frontal cortex and frontal white matter, and in PD frontal white matter. IGF-IR expression was significantly increased in PD and DLB

amygdala. IGF-IIR expression was significantly reduced in DLB frontal cortex, but increased in the amygdala and basal ganglia relative to control.

DLB is associated with impaired IGF-IR and IGF-IIR binding

Effective ligand binding is critical to insulin and IGF signaling cascades, such that many downstream effects of impaired insulin and IGF signaling have been reported in relation to human CNS diseases [31,32], and experimental *in vivo* and *in vitro* models [34,51,56]. Importantly, impairments in insulin/IGF receptor binding have been linked to reduced neuronal survival, energy production, glucose utilization, and acetylcholine homeostasis [56]. We performed equilibrium binding assays to determine if the alterations in IGF-I and IGFII receptor expression in the frontal cortex were associated with reduced ligand binding to those receptors. Competitive equilibrium binding studies demonstrated similar levels of specific insulin-R binding in control, PD, and DLB, but significantly reduced levels of IGF-IR and IGF-IIR binding in DLB relative to control frontal cortex (Fig. 1). The modest reductions in IGFIR, and IGF-IIR binding in PD did not reach statistical significance.

Altered neurotrophin and neurotrophin receptor gene expression in PD and DLB (Table 5)

Altered expression of neurotrophins, including brain derived neurotrophic factor (BDNF) and nerve growth factor (NGF), and their receptors, including TrkA and p75, has been reported in PD and DLB [57-59]. However, the potential relationship between altered neurotrophin or neurotrophin receptor expression and impairments in insulin/IGF signaling in neurodegenerative diseases has not yet been explored. To focus our investigations, we measured mRNA levels of the TrkA, TrkB, and p75 neurotrophin receptors, and NGF and BDNF neurotrophins in frontal cortex by qRT-PCR. Other neurotrophins and receptors, including TrkC, NT3, and NT4-5, were excluded because exploratory studies revealed minimal levels of these genes in control brains (data not shown). We detected reduced expression of TrkA (NGF receptor), TrkB (BDNF receptor), and NGF, but not p75 (low affinity NGF receptor) in DLB, significantly reduced expression of NGF and BDNF in PD.

Protein studies of PD and DLB neurodegeneration

Levels of immunoreactivity corresponding to Hu, MAG-1, GAP-43, β-actin, HNE, ubiquitin, α-synuclein, and DβH were measured in frontal cortex by ELISA with Amplex Red fluorescence (Table 6). Corresponding with the qRT-PCR results, Hu and MAG-1 expression were significantly reduced in DLB relative to control. HNE, a marker of lipid peroxidation and oxidative stress was significantly increased in DLB. In addition, the mean levels of α-synuclein and DβH were increased in both PD and DLB relative to control. No significant alterations in GAP-43, a marker of neuritic growth, ubiquitin, or β-actin, were observed in PD or DLB. Immunohistochemical staining studies confirmed the increased levels of DβH (Fig. 2A–2C), α-synuclein (Fig. 2D–2F), and HNE (Fig. 2G–2I) in DLB and PD relative to control frontal cortex. In addition, α-synuclein- and ubiquitin-positive Lewy body inclusions (Fig. 2F, 2L) were detected in DLB cortical neurons. Increased ubiquitin immunoreactivity was not detected in DLB samples by ELISA because the extraction methods employed would not have solubilized proteins integral to Lewy bodies.

Manganese exposure produces molecular indices of PD/DLB neurodegeneration in vitro

Primary cerebellar neuron cultures generated from rat pups were treated with 0–40 μM $MnCl_2$ for 48 hours, and then analyzed for cell density, energy metabolism (ATP production), immunoreactivity, and insulin/IGF polypeptide and receptor expression. $MnCl_2$ exposure caused dose-dependent reductions in cell density and ATP production such that at the highest dose used, both indices were approximately 50% lower than control (Fig. 3). Direct binding and cellular ELISAs were used respectively to measure immunore-activity in protein

homogenates and directly in cultured neurons. $MnCl_2$ treatment significantly reduced Hu neuronal expression, and increased α-synuclein, DβH, and ubiquitin immunoreactivity (Fig. 4). MnCl2 treatment also caused dose-dependent increases in 8-OHdG, an index of DNA damage that is increased in PD [60] (Fig. 4). β-actin was not significantly modulated by $MnCl_2$ exposure. Finally, qRT-PCR studies demonstrated that increasing doses of $MnCl_2$ treatment resulted in increased levels of insulin gene expression, but decreased levels of insulin receptor, IGF-I receptor, and IGF-II receptor expression (Fig. 5), reflecting insulin and IGF resistance.

DISCUSSION

Pathological shifts in gene expression corresponding to different brain cell populations in PD and DLB

The frontal lobe, amygdala, and basal ganglia are targets of neurodegeneration in PD and/or DLB. The studies herein provide evidence that PD and DLB are associated with significant neuronal loss in all three structures, based upon decreased levels of Hu mRNA, a neuronal-specific gene. The lower levels of Hu in DLB compared with PD correspond with the greater severity of neurodegeneration in DLB. Reduced levels of MAG-1 mRNA in DLB and PD frontal white matter reflect oligodendroglial loss, corresponding with an earlier finding of white matter atrophy in both diseases [61]. Loss of frontal white matter projecting fibers would interrupt circuitry between subcortical or brainstem nuclei and the frontal cortex. In addition to oligodendroglia degeneration, another potential cause of frontal white matter fiber degeneration in PD/DLB is neuronal loss in the amygdala, since fibers emanating from the basolateral nucleus project to the frontal cortex [62,63].

As expected, degeneration in cortex and white matter were accompanied by increased GFAP expression, i.e., gliosis. In DLB, the reduced levels of the vascular endothelial gene, ET-1, suggest that loss of vascular elements may contribute to neurodegeneration and dementia by compromising perfusion, nutrient supply, and energy metabolism. Finally, increased IBA-1 expression in PD and DLB is consistent with the concept that neuroinflammation mediates the PD/DLB neurode-generation cascade, similar to the findings in AD [31]. Altogether, these studies demonstrate that neurodegeneration in both PD and DLB results in loss of multiple cell types, including neurons, oligodendroglia, and endothelial cells, similar to the findings in AD [31]. Therefore, additional research is needed to understand the increased vulnerability of non-neuronal cell types in major neurodegenerative diseases. The relevance of this point is reinforced by the previous finding of white matter atrophy in the early stages of AD and DLB [64,65].

Altered expression of genes mediating insulin and IGF signaling in brain

The abnormalities gene expression that are directly pertinent to the integrity of insulin and IGF signaling in brain, overlapped with those observed in AD, but most importantly, were distinct. In contrast to AD [31,32], impairments in insulin and insulin receptor expression were confined to frontal white matter and amygdala and did not involve the frontal cortex in DLB or PD. On the other hand, DLB overlapped with AD with respect to the significant reductions in IGF-I and IGF-II receptor expression and receptor binding in frontal cortex. The absence of such abnormalities in PD is consistent with the minimal or absent cortical neurodegeneration, and further suggests that dementia in AD and DLB are mediated in part by impairments in cortical IGF signaling mechanisms. In PD and DLB white matter, both insulin and IGF-II mRNAs were reduced, while the IGFI and IGF-II receptor mRNAs were increased. Since insulin, IGF-1, and IGF-II signaling mechanisms overlap and cross-talk, the relative preservation of IGF-I mRNA vis-à-vis increased IGF-I and IGF-II receptor expression may have sufficed to sustain some aspects of neuro-cognitive function in DLB.

Further evidence of brain IGF resistance in DLB

The selective impairment of IGF-I and IGF-II receptor binding with preservation of insulin receptor binding distinguishes DLB from AD in which binding to all three receptors is reduced, but the abnormalities in insulin receptor binding dominate [31,32]. In PD, IGF-I and IGF-II receptor binding were intermediate between control and DLB, corresponding with the extent of neurodegeneration. IGF-I has a key role in neuronal survival, while IGF-II sustains mesenchymal elements, including astrocytes and vascular cells [66]. Therefore, in DLB, impaired IGF-I signaling caused by reduced ligand binding, could account for the significant losses in neurons and oligodendroglia, whereas impaired IGF-II signaling may reflect degeneration of vascular elements. Although IGF-II can stimulate neuronal survival and function by activating insulin or IGF-I signaling pathways [66], the finding of IGF-II resistance indicates that this potential secondary support measure deteriorates in DLB.

Role of neurotrophin resistance and deficiency

Neurotrophin and neurotrophin receptor abnormalities have been implicated in PD, DLB, and other neurodegenerative diseases [67,68]. For example, neurotrophin withdrawal may contribute to olfactory and neuropsychiatric dysfunction in PD and DLB [62]. The present study demonstrated significant reductions in TrkA and TrkB in DLB frontal cortex, and significant reductions in NGF in both DLB and PD. Since TrkA is the receptor for NGF and TrkB is the receptor for BDNF, the results suggest that DLB is associated with BDNF and NGF resistance and NGF deficiency in the frontal lobe. The absence of cortical neurotrophin receptor resistance in PD helps distinguish PD from DLB using molecular methods. On the other hand, the reduced NGF expression in both PD and DLB suggest that NGF withdrawal may be pivotal in the pathogenesis of both diseases. These results also suggest that from a molecular diagnostics approach, DLB and AD overlap with respect to neurotrophin receptor loss and resistance [67-69], but differ in that AD is not associated with cortical neurotrophin deficiency [70,71].

Correlates of neurodegeneration

We used ELISAs to measure HNE, ubiquitin, α-synuclein, and DβH immunoreactivity, with the expectation that this approach will aid in the eventual establishment of quantitative biochemical assays for distinguishing DLB from PD, and possibly AD. HNE immunoreactivity reflects lipid peroxidation with formation of cytotoxic aldehydes, and its levels are increased in various neurodegenerative diseases [72-74]. HNE covalently modifies and cross-links neuronal cytoskeletal proteins, forms pyrrole adducts with proteins, disrupts of neuronal calcium homeostasis, perturbs mitochondrial function, and promotes caspase-activated cell death [75]. The finding of increased HNE immunore-activity in the frontal cortex distinguishes DLB from PD, and links DLB-associated impairments in cognition and memory to chronic CNS oxidative stress. The finding of increased α-synuclein immunoreactivity in both PD and DLB frontal cortex, despite the absence of cortical Lewy bodies in PD, was unexpected. Since the protein extracts would not have dissolved Lewy bodies, which are composed of aggregated and ubiquitinated α-synuclein complexes, the increased levels of cortical α-synuclein immunoreactivity measured in PD and DLB most likely reflect increased levels of the soluble protein, consistent with the findings by immunohistochemical staining. Although DβH activity was previously shown to be reduced in PD cerebrospinal fluid [76], our finding of significantly increased cortical levels of DβH in both PD and DLB, as shown by ELISA and immunohistochemical staining, suggests a possible compensatory response to declining ability to convert dopamine to noradrenaline [77].

Potential role of neurotoxicants in the pathogenesis of PD/DLB

In vitro experiments showed that $MnCl_2$ exposure impairs survival, energy metabolism, insulin, IGF-I and IGF-II receptor expression, reflecting insulin/IGF resistance, and it also increases α-synuclein, HNE, and DβH immunoreactivity in CNS neurons. These results overlap extensively with our findings with respect to PD/DLB, and strongly support the hypothesis that environmental neurotoxicants contribute to the pathogenesis of these diseases. Another point of novelty in this study was the direct comparison between the molecular and biochemical abnormalities associated with trophic factor deficiency, trophic factor receptor resistance, neurotransmitter dysfunction, and oxidative stress in PD and DLB, as well as in relation to an agent that is thought to contribute to the pathogenesis of sporadic PD/DLB. Further studies are needed to extend our understanding of the mechanisms by which transitional metals such as manganese and iron promote or exacerbate PD/DLB neurodegeneration.

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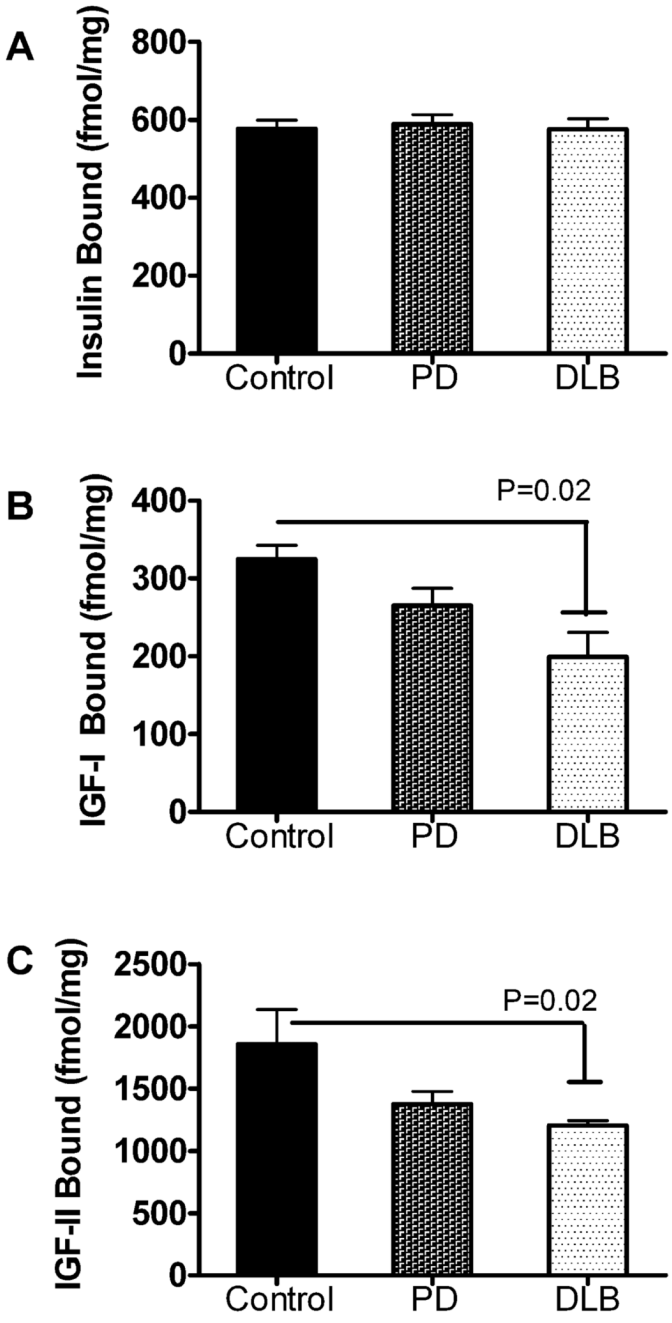

Fig. 1.

Impaired brain IGF receptor binding in DLB. Competitive equilibrium binding to insulin, IGF-I, and IGF-II receptors was measured in anterior frontal cortex of brains with normal aging (control), PD, or DLB. Membrane protein extracts were incubated with 50 pM [^{125}I]-labeled insulin, IGF-I, or IGF-II as tracer, in the presence or absence of 100 nM cold ligand. Membrane bound tracer was precipitated and radioactivity associated with the pellets (bound ligand) or remaining in the supernatants (free ligand) was measured in a gamma counter. Specific binding (fmol/mg) was calculated using the GraphPad Prism 5 software. Graphs depict the mean ± S.E.M. of results obtained for (A) insulin, (B) IGF-I, and (C) IGF-II specific binding. Data

were analyzed statistically using ANOVA with post hoc significance tests. Significant P-values are indicated over the bar graphs.

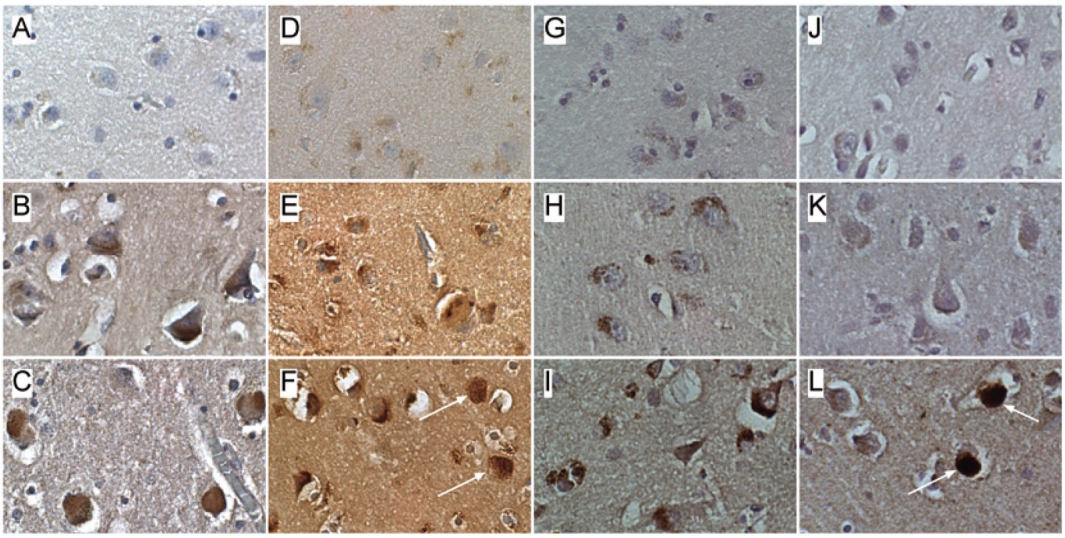

Fig. 2.

Patterns of DβH, α-synuclein, HNE, and ubiquitin immunoreactivity in control, PD, and DLB frontal cortex. Paraffin-embedded histological sections were immunostained to detect (A-C) DβH, (D-F) α-synuclein, (G-I) HNE, or (J-L) ubiquitin in (A,D,G,J) control, (B,E,H,K) PD, or (C,F,I,L) DLB frontal cortex. Immunoreactivity was detected with biotinylated secondary antibody, avidin-biotin horseradish peroxidase complex reagents, and diaminobenzidine as the chromagen (brown precipitate). Sections were lightly counter-stained with hematoxylin and preserved under coverglass. Globular (F) α-synuclein or (L) ubiquitin positive neuronal cytoplasmic inclusions (arrows) distinguish DLB from PD and control. Increased DβH, α-synuclein, and HNE immunoreactivity distinguish DLB and PD from control.

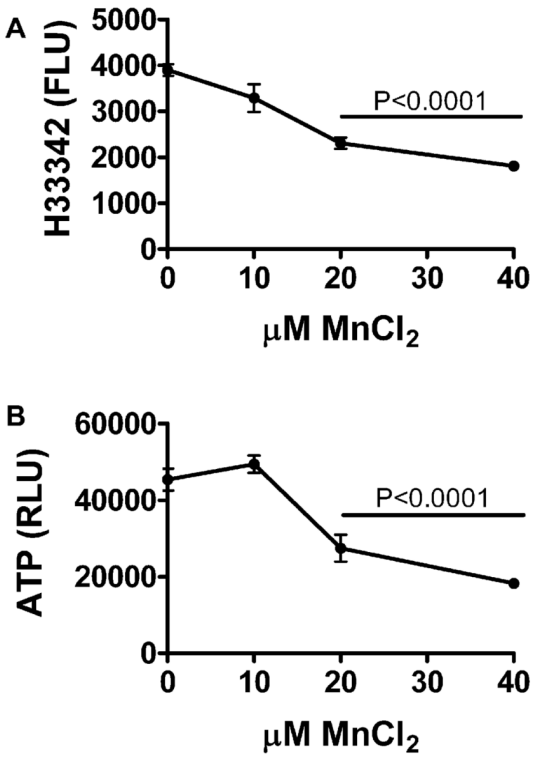

Fig. 3.

Manganese exposure impairs neuronal viability, energy metabolism, and Hu neuronal gene expression in CNS neurons. Rat primary cerebellar neuron 96-well cultures were treated with 0, 10, 20, or 40 μM $MnCl_2$ for 48 hours. (A) To measure cell density, cells were fixed, stained with Hoechst H33342, and fluorescence intensity (Ex340 nm/Em360 nm) was measured in a SpectraMax-5 microplate reader. (B) To measure energy metabolism, live cultures were analyzed using the ATPLite assay, and luminescence was quantified in a TopCount machine. (C) Hu immunoreactivity was measured by cellular ELISA using the Amplex Red soluble fluorophore. Graphs depict the mean ± S.E.M. fluorescent light units (FLU) or relative light units (RLU) measured in 12 replicate assays per $MnCl_2$ dose. Results were analyzed using the Deming linear regression analysis. Significant trends are indicated within the panels.

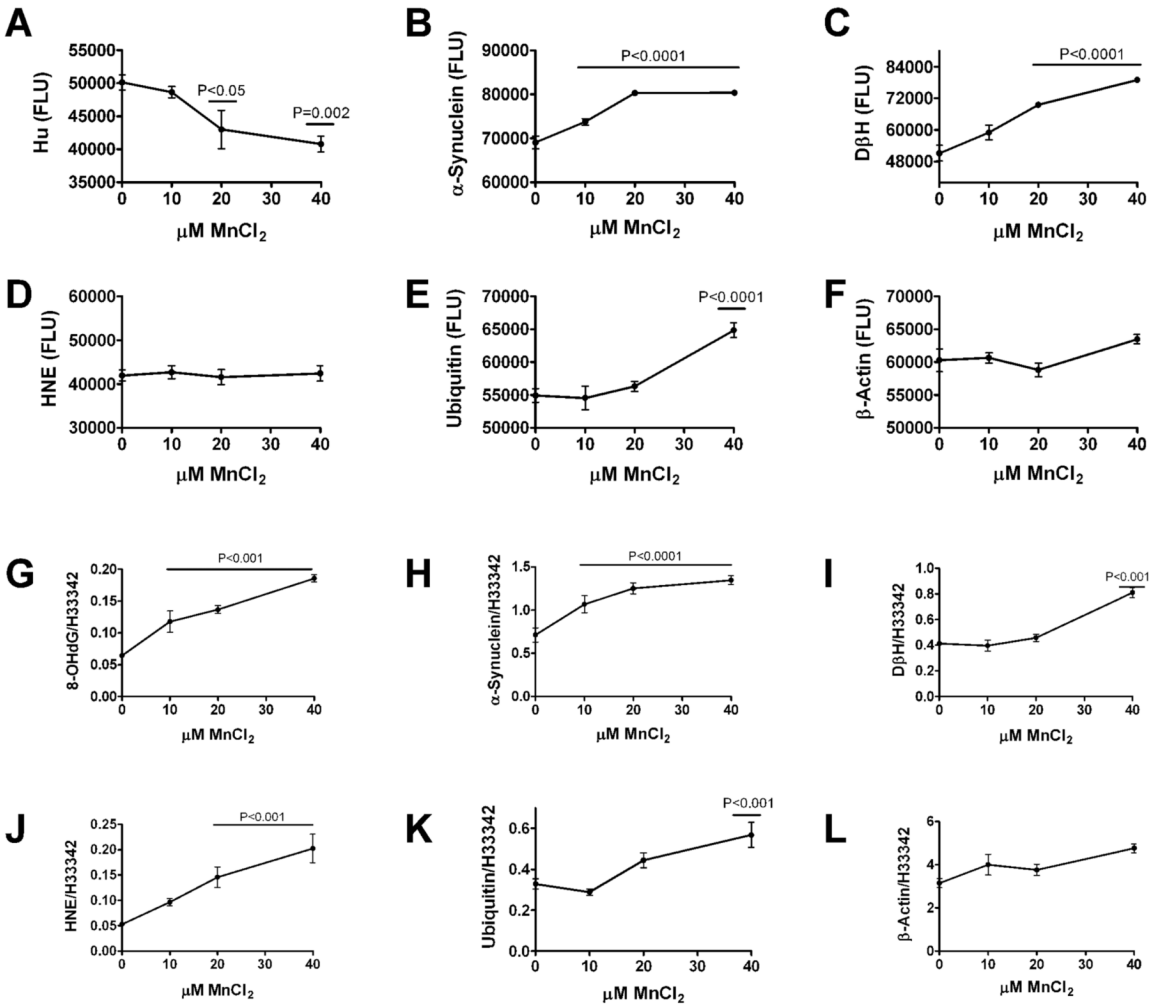

Fig. 4.

Manganese exposure causes neurodegeneration and oxidative stress. Rat primary cerebellar neuron cultures were treated with 0, 10, 20, or 40 μM $MnCl_2$ for 48 hours. Protein homogenates were used to measure (A) α-synuclein, (B) DβH, (C) HNE, (D) ubiquitin, or (E) β-actin by ELISA (see Methods). Immunoreactivity was also measured by cellular ELISA in 96-well cultures. Cells were fixed *in situ*, permeabilized, treated to prevent non-specific binding, and incubated with primary antibodies to detect (F) 8-OHdG, (G) α-synuclein, (H) DβH, (I) HNE, (J) ubiquitin, or (K) β-actin. Immunoreactivity was detected with horseradish peroxidase conjugated secondary antibody and Amplex Red fluorophore. Fluorescence light units (FLU) were measured in a SpectraMax-5 microplate reader (Ex 561 nm/EM 584 nm). For cellular ELISAs, immunoreactivity was normalized to Hoechst H33342 fluorescence. Graphs depict the mean ± S.E.M. levels of immunoreactivity as a function of $MnCl_2$ dose. Trend lines were analyzed using the Deming linear regression model. P-values corresponding to trends significantly different from zero are shown in each panel.

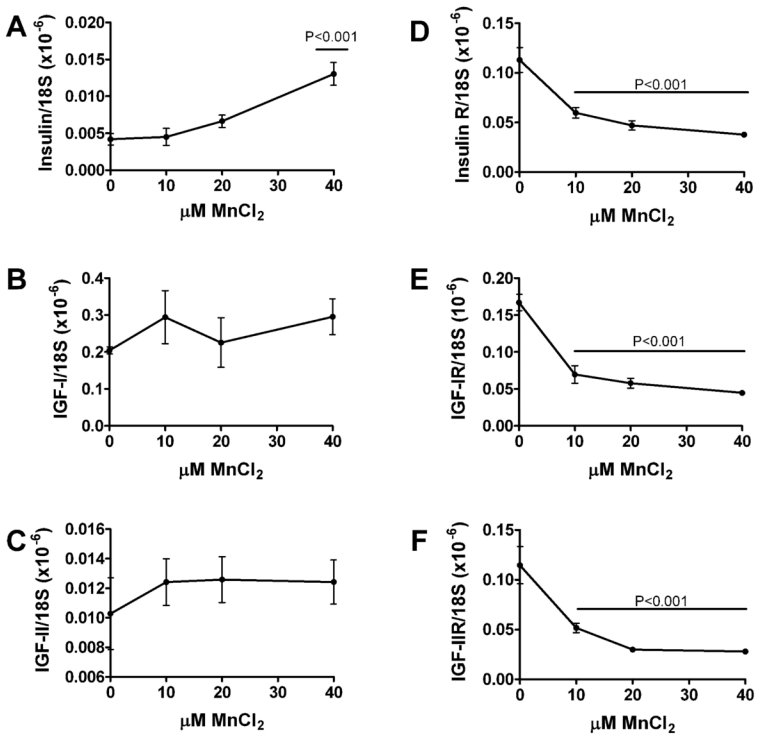

Fig. 5.

Manganese exposure inhibits IGF-I and IGF-II receptor expression in primary CNS neuron cultures. Rat primary cerebellar neuron cultures were treated with 0, 10, 20, or 40 μM $MnCl_2$ for 48 hours. RNA extracted from the cultured cells was reverse transcribed and used to measure (A) insulin, (C) IGF-II, (B) insulin receptor (R), (C) IGF-I, (D) IGF-IR, and (F) IGF-IIR by qRT-PCR analysis. The mRNA levels were normalized to 18S rRNA measured in the same samples. Graphs depict the mean ± S.E.M. relative levels of gene expression as a function of $MnCl_2$ dose. Trend lines were analyzed using Deming linear regression. Significant P values are indicated in the panels.

Table 1

Patient population profile

	Control	PD	DLB	P-Values
Number	8	7	8	
Age (yrs)*	79.9 ± 5.2	83.4 ± 2.1	81.7 ± 1.7	NS
Male/Female	5/3	6/1	5/3	NS
Brain Wt (gm)*	1293 ± 51	1311 ± 94	1001 ± 383	NS
PMI (hrs)*	9.5 ± 2.0	8.6 ± 1.4	8.4 ± 1.8	NS

PD = Parkinson's disease; DLB = dementia with Lewy Bodies; PMI = postmortem interval; NS = not significant.

* ± S.D.

Table 2

Human primer pairs for quantitative RT-PCR

mRNA	Sequence (5′→3′)	Position	Size (bp)
Insulin	TTC TAC ACA CCC AAG TCC CGT C	189	134
	ATC CAC AAT GCC ACG CTT CTG C	322	
Insulin R	GGT AGA AAC CAT TAC TGG CTT CCT C	1037	125
	CGT AGA GAG TGT AGT TCC CAT CCA C	1161	
IGF-I	CAC TTC TTT CTA CAC AAC TCG GGC	1032	147
	CGA CTT GCT GCT GCT TTT GAG	1178	
IGF-I R	TAC TTG CTG CTG TTC CGA GTG G	295	101
	AGG GCG TAG TTG TAG AAG AGT TTC C	395	
IGF-II	CTG ATT GCT CTA CCC ACC CAA G	996	76
	TTG CTC ACT TCC GAT TGC TGG C	1071	
IGF-II R	CAC GAC TTG AAG ACA CGC ACT TAT C	403	132
	GCT GCT CTG GAC TCT GTG ATT TG	534	
IBA-1	GCT GAG CTA TGA GCC AAA CC	2	199
	TCG CCA TTT CCA TTA AGG TC	200	
ET-1	AGA GTG TGT CTA CTT CTG CCA CCT G	517	144
	CAT CTA TTC TCA CGG TCT GTT GCC	660	
MAG-1	TGG AAG CCA ACA GTG AAC GG	1128	104
	TTG AAG ATG GTG AGA ATA GGG TCC	1231	
Hu	ATG GAG CCT CAG GTG TCA AAT G	246	113
	TTG CTG TCA TCT GTG GTT GCC	358	
GFAP	TAT GAG GCA ATG GCG TCC AG	738	130
	AGT CGT TGG CTT CGT GCT TG	867	
Trk1	TCA TCT TCA CTG AGT TCC TGG AGC	980	103
	AGC GTG TAG TTG CCG TTG TTG	1082	
Trk2	CGG GAA CAT CTC TCG GTC TAT G	1282	185
	TTG CTG ATA ACG GAG GCT GG	1466	
P75	ACC TCA TCC CTG TCT ATT GCT CC	862	78
	TCC ACC TCT TGA AGG CTA TGT AGG	939	
NGF	ATA CAG GCG GAA CCA CAC TCA G	46	174

Table 3

Pathological shifts in gene expression corresponding to distinct cell populations in PD and DLB brains

mRNA	CONTROL		PD		DLB	
FRONTAL CORTEX						
Hu	0.310	±0.018	0.162	±0.088*	0.087	±0.022*
MAG	0.673	±0.124	1.083	±0.282	0.179	±0.041**†
GFAP	1.265	±0.556	26.61	±21.34	62.85	±25.88***
IBA	0.012	±0.009	0.117	±0.089	0.010	±0.003*
ET1	0.375	±0.063	0.367	±0.137	0.091	±0.036
FRONTAL WHITE MATTER						
Hu	0.214	±0.034	0.223	±0.052	0.035	±0.006*
MAG	4.66	±0.912	0.731	±0.148*	0.538	±0.155*
GFAP	1.114	±0.210	29.95	±7.689*	25.18	±8.708*
IBA	0.008	±0.003	0.120	±0.038*	0.094	±0.050
ET1	0.452	±0.017	0.312	±0.051	0.217	±0.079*
AMYGDALA						
Hu	0.437	±0.038	0.108	±0.055***	0.047	±0.012***
MAG	1.999	±0.134	2.200	±0.515	1.639	±0.399
GFAP	0.792	±0.156	49.97	±5.327***	29.34	±3.654***
IBA	0.052	±0.016	0.084	±0.014	0.087	±0.013
ET1	0.317	±0.039	0.337	±0.055	0.373	±0.071
BASAL GANGLIA						
Hu	0.035	±0.002	0.030	±0.007	0.009	±0.002**
MAG-1	1.350	±0.061	0.413	±0.081	0.373	±0.092
GFAP	5.973	±0.520	10.54	±1.773*	17.79	±5.922*
IBA	0.0003	±2E-06	0.063	±0.016***	0.033	±0.006***
ET1	0.434	±0.083	0.085	±0.018*	0.149	±0.012*

MAG-myelin associated glycoprotein; GFAP-glial fibrillary acidic protein; IBA-allograft inhibitory factor; ET-endothelin. Gene expression was measured by qRT-PCR analysis with values ($\times 10^{-6}$) normalized to 18S rRNA measured in parallel reactions. Inter-group comparisons were made using repeated measures ANOVA with the post hoc Dunn's multiple comparison test.

* P<0.05,

** P<0.01,

*** P<0.005 relative to control;

† P<0.05 or better relative to PD.

Table 4

Altered expression of insulin and IGF polypeptide and receptor genes in PD and DLB

mRNA	CONTROL		PD		DLB	
FRONTAL CORTEX						
INR	0.006	±0.0003	0.003	±0.001	0.004	±0.001
IGF1R	28.97	±2.44	12.94	±4.925	3.31	±1.277**†
IGF2R	0.677	±0.048	0.553	±0.162	0.168	±0.059**†
Insulin	0.001	±0.0002	0.001	±0.0003	0.0004	±0.0002
IGF1	0.0002	±1E-05	0.001	±0.0001	0.0001	±3E-05
IGF2	3.3E-5	±2.1E-5	5.0E-5	±2.2E-5	0.00	±0.00
FRONTAL WHITE MATTER						
INR	0.006	±0.0004	0.001	±0.0003***	0.001	±0.0004***
IGF1R	43.5	±8.315	2.648	±0.291***	2.72	±0.842***
IGF2R	0.395	±0.009	0.324	±0.129	0.401	±0.127
Insulin	0.001	±7E-05	3E-05	±1E-05***	2E-05	±9E-06***
IGF1	0.001	±0.0002	0.001	±0.0004	0.001	±0.0002
IGF2	0.0002	±0.00001	3.7E-6	±9.6E-7***	7.2E-6	±5.2E-6***
AMYGDALA						
INR	0.0134	±0.002	0.005	±0.001**	0.004	±0.002**
IGF1R	2.437	±0.477	30.43	±11.87**	25.94	±7.56**
IGF2R	0.527	±0.105	0.979	±0.241***	1.839	±0.622***
Insulin	0.017	±0.002	0.005	±0.003**	0.003	±0.001**
IGF1	0.002	±0.0002	0.002	±0.001	0.002	±0.001
IGF2	0.0002	±0.0001	0.0002	±0.0001	0.0004	±0.0002
BASAL GANGLIA						
INR	0.0007	±0.0001	0.0004	±0.0002	0.001	±0.0002
IGF1R	0.7054	±0.0668	0.6963	±0.095	0.959	±0.210
IGF2R	0.0301	±0.0035	0.0867	±0.0301	0.229	±0.102*
Insulin	8E-06	±3E-06	3E-06	±3E-06	4E-06	±2E-06
IGF1	0.0004	±3E-05	0.0003	±8E-05	0.0005	±0.0001

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mRNA	CONTROL		PD		DLB	
IGF2	0.000	±0.000	0.000	±0.000	0.000	±0.000

IN-insulin; IGF-insulin-like growth factor; R-receptor. Gene expression was measured by qRT-PCR analysis with values ($\times 10^{-6}$) normalized to 18S rRNA measured in parallel reactions. Inter-group comparisons were made using repeated measures ANOVA with the post hoc Dunn's multiple comparison test.

*P<0.05,

**P<0.01,

***P<0.005 relative to control;

†P<0.05 or better relative to PD.

Table 5

Altered neurotrophin and neurotrophin receptor expression in PD and DLB frontal cortex

mRNA	CONTROL		PD		DLB	
TrkA	0.372	±0.041	0.185	±0.055	0.058	±0.017***
TrkB	97.05	±15.36	108.3	±87.55	15.88	±5.794**
$p75^{NGF}$	3.749	±0.945	3.056	±0.785	3.15	±0.979
BDNF	32.28	±3.203	10.48	±3.094*	22.79	±4.254
NGF	25.3	±5.394	2.47	±0.436***	6.63	±2.761**

Trk-tyrosine kinase receptor; $p75^{NGF}$-low affinity NGF receptor; BDNF-brain derived neurotrophic factor; NGF-nerve growth factor. Gene expression was measured by qRT-PCR analysis with values ($\times 10^{-9}$) normalized to 18S rRNA measured in parallel reactions. Inter-group comparisons were made using repeated measures ANOVA with the post hoc Dunn's multiple comparison test.

*P<0.05,

**P<0.01,

***P<0.005 relative to control;

†P<0.05 or better relative to PD.

Table 6

Increased indices of neurodegeneration and oxidative stress in PD and DLB frontal cortex

PROTEIN	CONTROL		PD		DLB	
Hu	77164	±1974	76146	±3065	60308	±4672***
MAG	60823	±4366	46577	±4959	28859	±3793***
β–actin	75449	±1188	79048	±1282	76205	±1677
GAP-43	43049	±3186	33536	±1795	35941	±3238
α–synuclein	11508	±1646	29642	±3501***	22384	±1657***
D*β*H	23254	±1122	32649	±2583*	31563	±2031*
UBQ	64742	±1871	65516	±2169	68132	±1229
HNE	46470	±3519	57480	±4809	60555	±1480*

MAG-myelin-associated glycoprotein; GAP-43-growth associated protein-43; D*β*H-dopamine beta hydroxylase; UBQ-ubiquitin; HNE-4-hydroxy-2-nonenal. Immunoreactivity was measured in direct binding ELISAs using HRP-conjugated secondary antibody and Amplex Red soluble fluorophore. Values reflect arbitrary fluorescence light units (Ex 530 nm/Em 590 nm). Statistical comparisons were made using repeated measures ANOVA with the post hoc Dunn's multiple comparison test.

* P<0.05,

** P<0.01,

*** P<0.005 relative to control.