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BASIC NEUROSCIENCES, GENETICS AND IMMUNOLOGY - REVIEW ARTICLE

Neuropathology of sporadic Parkinson disease before the appearance of parkinsonism: preclinical Parkinson disease

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Abstract Parkinson disease (PD) is no longer considered a complex motor disorder characterized by parkinsonism but rather a systemic disease with variegated non-motor deficits and neurological symptoms, including impaired olfaction, sleep disorders, gastrointestinal and urinary abnormalities and cardiovascular dysfunction, in addition to other symptoms and signs such as pain, depression and mood disorders. Many of these alterations appear before or in parallel with motor deficits and then worsen with disease progression. Although there is a close relation between motor symptoms and the presence of Lewy bodies (LBs) and neurites filled with abnormal α-synuclein, other neurological alterations are independent of LBs, thereby indicating that different mechanisms probably converge in the degenerative process. This review presents cardinal observations at very early stages of PD and provides personal experience based on the study of a consecutive series of brains with PD-related pathology and without parkinsonism, mainly cases categorized as stages 2–3 of Braak. Alterations in the substantia nigra, striatum and frontal cortex in pPD are here revised in detail. Early modifications in the substantia nigra at pre-motor stages of PD (preclinical PD: pPD) include abnormal small aggregates of α-synuclein which is phosphorylated, nitrated and oxidized, and which exhibits abnormal solubility and truncation. This occurs in association with a plethora of altered molecular events including increased oxidative stress, altered oxidative stress responses, altered balance of L-ferritin and H-ferritin, reduced expression of neuronal globin α and β chains in neurons with α-synuclein deposits, increased expression of endoplasmic reticulum stress markers, increased p62 and ubiquitin immunoreactivity in relation to α-synuclein deposits, and altered distribution of LC3 and other autophagosome/lysosome markers. In spite of the relatively small decrease in the number of dopaminergic neurons in the substantia nigra, which does not reach thresholds causative of parkinsonism, levels of tyrosine hydroxylase and cannabinoid 1 receptor are reduced, whereas levels of adenosine receptor 2A are increased in the caudate in pPD. Moreover, biochemical alterations are also present in the cerebral cortex (at least in the frontal cortex) in pPD including increased oxidative stress and oxidative damage to proteins α-synuclein, β-synuclein, superoxide dismutase 2, aldolase A, enolase 1, and glyceraldehyde dehydrogenase, among others, indicating post-translational modifications of PD-related proteins, and suggesting altered function of pathways involved in glycolysis and energy metabolism in the cerebral cortex in pPD. Current evidence suggests convergence of several altered metabolic pathways leading to chronic neuronal dysfunction, mainly manifested as sub-optimal energy metabolism, altered synaptic function, oxidative and endoplasmic reticulum stress damage and corresponding altered responses, among others. By understanding that these alterations occur at very early stages of PD and that neuronal fatigue and exhaustion may precede, for years, cell death and neuronal loss, we may direct therapeutic strategies towards the prevention and delay of disease progression starting at pre-parkinsonian stages of PD.

Keywords Preclinical Parkinson disease · Incidental Lewy body disease · Oxidative stress ·

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Endoplasmic reticulum stress · Haemoglobin · Mitochondria · Olfaction · Cerebral cortex · Striatum · Synuclein · Glycolysis

Introduction

Parkinson disease is clinically characterized by a complex motor disorder known as parkinsonism and is manifested principally by resting tremor, slowness of initial movement, rigidity and general postural instability. These symptoms are mainly due to the loss of dopaminergic neurons in the substantia nigra pars compacta, first the lateral tier followed by the medial region, leading to reduced dopaminergic input to the striatum, and accompanied by adaptive responses in the internal and external globus pallidus, subthalamus, thalamus and substantia nigra pars reticularis. Round, hyaline neuronal cytoplasmic inclusions called Lewy bodies (LBs), and enlarged aberrant neurites and threads are found in the parkinsonian substantia nigra (Forno 1996; Jellinger and Mizuno 2003). In addition to the substantia nigra, other nuclei are involved such as the locus coeruleus, reticular nuclei of the brain stem, and dorsal motor nucleus of the vagus, as well as the basal nucleus of Meynert, the amygdala and the CA2 area of the hippocampus. LBs and aberrant neurites as well are found in these locations (Forno 1996; Braak et al. 1999; Dickson 2001; Goedert 2001; Jellinger and Mizuno 2003; Braak and del Tredici 2008).

Cases with LB pathology in the brain stem without parkinsonism are considered incidental LB disease because they are unexpectedly discovered following appropriate post-mortem neuropathological study (Jellinger 2004, 2009; Saito et al. 2004). Whether these cases constitute pre-parkinsonian PD has been a matter of controversy for some years, as nobody can ensure that these cases would have progressed to parkinsonism if they had survived for longer times. However, the study of consecutive cases in large series and the recognition of several intermediate degrees of involvement of the brain stem, limbic structures and, eventually, the cerebral cortex make it clear that a prediction of preclinical PD (pPD) as an anterior stage of PD seems more than reasonable (Jellinger and Mizuno 2003; Braak and del Tredici 2008; Dickson et al. 2008).

LBs and neurites are composed of aggregates of normal, misfolded and truncated proteins, and ubiquitin, which are stored in the cytoplasm as non-degraded by-products of the degenerative process (Schults 2006; Wakabayashi et al. 2007; Leverenz et al. 2007; Xia et al. 2008). The main component of LBs and aberrant neurites is α-synuclein which is abnormally phosphorylated, nitrated and oxidized, has an abnormal crystallographic structure and abnormal solubility, and is prone to the formation of aggregates and insoluble fibrils (Spillantini et al. 1997; Wakabayashi et al. 1997; Baba et al. 1998; Hashimoto and Masliah 1999; Duda et al. 2000; Giasson et al. 2000; Fujiwara et al. 2002; Anderson et al. 2006).

Systematic study of cases with LB pathology has prompted a staging classification of PD based on the putative progression of LB pathology from the medulla oblongata (and olfactory bulb) to the midbrain, diencephalic nuclei, and neocortex (Braak et al. 2002, 2003, 2004). Stage 1 is characterized by LBs and neurites in the dorsal IX/X motor nuclei and/or intermediate reticular zone; there is also myenteric plexus involvement. Stage 2 affects the medulla oblongata and pontine tegmentum and covers pathology of stage 1 plus lesions in the caudal raphe nuclei, gigantocellular reticular nucleus, and coeruleus–subcoeruleus complex; the olfactory bulb is also involved. Stage 3 refers to pathology of stage 2 plus midbrain lesions, particularly in the pars compacta of the substantia nigra. Stage 4 includes basal prosencephalon and mesocortex (cortical involvement confined to the transentorhinal region and allocortex, and CA2 plexus) pathology in addition to lesions in the midbrain, pons and medulla oblongata. Stage 5 extends to sensory association areas of the neocortex and prefrontal neocortex. Stage 6 includes, in addition, lesions in first order sensory association areas of the neocortex and pre-motor areas; occasionally there are also mild changes in primary sensory areas and the primary motor field.

This classification shows an acceptable correlation between pathological findings and clinical data, mainly in a subgroup of cases with early onset and prolonged duration in which motor symptoms (parkinsonism) are clearly dependent on the damage of dopaminergic neurons in the substantia nigra pars compacta (Braak et al. 2002, 2005; Jellinger 2004; Wolters and Braak 2006). Thus, stages 1 and 2 are not accompanied by parkinsonism and they fulfil the criteria of pPD. Cases at stage 3 may present with parkinsonism depending on the amount of cell loss in the substantia nigra. More strictly, pPD occurs in cases with LB pathology limited to selected nuclei of the medulla oblongata and pons, and in cases with a few LBs in the substantia nigra but without substantial nigral neuron loss not surpassing a determined threshold, usually considered as 60% of the total population of dopaminergic neurons of the pars compacta (Jellinger and Mizuno 2003; DelleDonne et al. 2008).

Cumulative clinical evidence reveals that olfactory dysfunction, dysautonomia, sleep fragmentation, rapid eye movement behaviour disorder, mood and anxiety disorders, and depression may precede parkinsonian symptoms in a number of patients with PD clinically characterized by parkinsonism (Chaudhuri et al. 2005; Postuma et al. 2006; Iranzo et al. 2006; Siderowf and Stern 2008; Tolosa et al. 2007; Poewe 2007, 2008; Ziemssen and Reichmann 2007;

Herting et al. 2008; Ross et al. 2008; Natale et al. 2008). Whether these clinical symptoms are associated with LBs in selected regions of the central, autonomous and peripheral nervous systems is a matter of study.

α-Synuclein pathology affecting neurons and neurites occurs in the olfactory bulb and related olfactory nuclei at very early stages of cases with PD-related pathology (Daniel and Hawkes 1992; Pearce et al. 1995; Del Tredici et al. 2002; Braak et al. 2004; Bloch et al. 2006; Hubbard et al. 2007).

The cardiovascular autonomic system is also affected in pPD and PD, and alterations implicate both tyrosine hydroxylase-positive (extrinsic) and negative (intrinsic) nerves of the cardiac plexus (Iwanaga et al. 1999).

Together, these observations can be summarized as follows: (a) variegated non-motor deficits and neurological symptoms, covering impaired olfaction, sleep disorders, gastrointestinal and urinary abnormalities and cardiovascular dysfunction, in addition to other symptoms and signs as pain, depression and mood disorders, may occur at early stages of the disease. (b) A relationship between neurological symptoms and Lewy pathology exists, but it is not clear whether the severity of morphological lesions correlates with clinical symptoms. (c) Olfactory tests, polysomnographic studies and MIBG myocardial scintigraphy in combination may be used to discover early signatures of the disease (Stiasny-Kolster et al. 2005).

Lack of correlation between α-synuclein aggregates and impaired function is dramatically represented in relation with cognitive and mental functions. Retrospective clinical and pathologic studies have shown that there is no relationship between LB stage and severity of cognitive impairment in advanced stages of PD (Parkkinen et al. 2005; Weisman et al. 2007; Parkkinen et al. 2008; Jellinger 2008, 2009).

These and other studies have led to the visualization of PD as a systemic disease in which functional deficits are not exclusively related with LB pathology in neurons and neurites. This is dramatically manifested in relation with cerebral cortex involvement in PD in which variegated pathological molecular events converge to alter neuronal function (Ferrer 2009a).

Neuropathological examination of pPD in a cohort of consecutive cases in a general population

Over the last 10 years (2001–2010), 115 cases of LB-related pathology were recovered in a consecutive series of cases subjected to post-mortem study in a general hospital (Institut de Neuropatologia, Hospital Universitari de Bellvitge). These were classified as stages 1–2: 24%, stages 3–4: 42%, stages 5–6: 26%, and atypical: 8%. Interestingly, mixed pathology was very common. AD-related pathology was found in 81% of cases (40% stages I–II of Braak, 46% corresponding to stages III–IV, and 14% to stages V and VI), argyrophilic grain disease and other tauopathies in 24%, vascular pathology in 28%, and other pathological conditions in 12%. Some cases had multiple pathologies (mainly LB disease, AD and vascular pathology including lacunae, multi-infarct encephalopathy, haemorrhages and demyelination of the centrum semi-ovale reminiscent of Binswanger encephalopathy). These figures are similar to those already reported in other series (Jellinger 2004, 2008, 2009, 2010; Braak et al. 2006; Markesbery et al. 2009; Beach et al. 2009; Dickson et al. 2010).

The distribution of PD-related pathology in our series has a gradient with age; cases with stages 5 and 6 are older as a group than cases at stage 4. However, a number of cases at stages 1 and 2 were remarkably aged. More than half were older than 75, indicating that, within the same age interval, some patients had suffered from fully developed PD whereas others had remained at pre-motor stages. These findings are inline with the observation that, at least some cases of incidental LB disease could represent preclinical PD, arrested PD, or a partial syndrome due to a lesser burden of causative factors (Frigerio et al. 2009).

For the present purposes, parkinsonism (including slight motor symptoms, revealed by relatives following an unbiased post-mortem interview in cases that had not been diagnosed during life) occurred in 68% of the cases. Excluding atypical cases, pPD was seen in 28 cases of stages 1–2, 10 cases of stage 3, and 3 cases of stage 4. Morphological studies of the substantia nigra were carried out in this group. In none of these cases did nerve cell loss in the substantia nigra exceed 50% of the total number of dopaminergic neurons as revealed by quantitative studies using tyrosine hydroxylase immunohistochemistry. A summary of antibodies used for immunohistochemistry, and single- and double-labelling immunofluorescence and confocal microscopy, is given in Table 1.

Molecular studies in the frontal cortex were restricted to 20 pPD cases with no associated pathology or with accompanying AD-related changes stages I–II, to exclude cases in which cortical pathology could interfere with changes interpreted to be exclusive of pPD. Control samples were from cases with no neuropathological changes or with AD-related pathology stages I–II. Methods employed were mono- and bi-dimensional gel electrophoresis and western blotting, immunoprecipitation and functional assays (Dalfó et al. 2004, 2005; Dalfó and Ferrer 2008; Muntané et al. 2008; Martínez et al. 2010).

The present observations are mainly focused on the substantia nigra pars compacta, caudate and cerebral cortex (frontal cortex area 8) in pPD. The olfactory bulb and tract also deserve a brief comment.

Table 1 Antibodies, species, source and dilution used for immunohistochemistry and immunofluorescence in the present study

Antibody	Species	Manufacturer	Dilution
ATP-synthase α	Mouse	BD Biosciences (Madrid, Spain)	1:1,000
Casein kinase II	Rabbit	Stressgen (bioNova, Madrid, Spain)	1:100
COX7A2	Mouse	Abnova (Taipei, Taiwan)	1:50
eif2α	Rabbit	Abcam (Cambridge, UK)	1:100
eif2α-P	Rabbit	Cell signalling (Izasa, Barcelona, Spain)	1:100
Ferritin	Goat	Affinity Bioreagents (bioNova, Madrid, Spain)	1:500
Haemoglobin (β chain)	Mouse	Abcam (Cambridge, UK)	1:200
8-Hydroxy-2 deoxyguanosine	Mouse	Abcam (Cambridge, UK)	1:200
LAMP-1	Rabbit	Sta. Cruz (Quimigen, Barcelona)	1:100
LAMP-2	Rabbit	Sta. Cruz (Quimigen, Barcelona)	1:500
LC-3	Rabbit	Cell signalling (Izasa, Barcelona, Spain)	1:100
Neuroketals	Goat	Chemicon (Millipore, Madrid, Spain)	1:200
Oxphos	Mouse	Mitosciences (Eugene, Oregon, USA)	1:1,000
Phospho-p38	Rabbit	Cell signalling (Izasa, Barcelona, Spain)	1:100
p62	Rabbit	BIOMOL (Quimigen, Madrid, Spain)	1:500
Peroxiredoxin-2	Rabbit	Abcam (Cambridge, UK)	1:1,000
VDAC, porin	Mouse	Calbiochem (bioNova, Madrid, Spain)	1:300
SOD-1 (Cu/Zn)	Mouse	Novocastra (Newcastle, UK)	1:50
SOD-2 (Mn)	Rabbit	Stressgen (bioNova, Madrid, Spain)	1:100
α-Synuclein	Mouse	Novocastra (Newcastle, UK)	1:100
α-Synuclein	Rabbit	Chemicon (Millipore, Madrid, Spain), epitope 111–131	1:500
α-Synuclein	Rabbit	Abcam (Cambridge, UK), epitope 11–26	1:100
α-Synuclein	Guinea pig	Calbiochem (bioNova, Madrid, Spain), epitope 123–140	1:50
α-Synuclein	Mouse	ATGen (Montevideo, Uruguay), epitope 61–90	1:100
Nitrated α-synuclein	Mouse	Zymed (San Francisco, CA, USA)	1:300
Phospho-α-synuclein	Mouse	WAKO (Rafer, Madrid, Spain)	1:500
Transferrin	Rabbit	Affinity Bioreagents (bioNova, Madrid, Spain)	1:300
Ubiquitin	Rabbit	DAKO (Barcelona, Spain)	1:500
8-dOHG	Mouse	Abcam	1:50
Tyrosine hydroxylase	Mouse	Chemicon	1:1,000
CB-1 receptor	Rabbit	Frontier Science	1:200

Tissue sections were pre-treated with formic acid for 3 min and then with 10 mM sodium citrate buffer pH 6 for 20 min at 95°C

The substantia nigra in pre-motor stages of PD

It is classically considered that the substantia nigra in pre-motor stages of PD is not morphologically affected or damaged to an extent not surpassing a threshold currently accepted as involving less than 60% of the total of dopaminergic neurons of the substantia nigra pars compacta. Using the staging nomenclature of PD-related pathology in our series, pre-motor or incidental PD may correspond to stages 1–3. Surprisingly, parkinsonian symptoms have been reported in stage 2 of Braak (Jellinger 2009). This is not our experience: parkinsonian symptoms and signs appear at stage 3 in our series and stage 3 may be also encountered in pPD cases in which decline of substantia nigra dopaminergic neurons does not exceed 50% of the total. Since vascular pathology is not rare in ours and in other series, we cannot rule out the possibility of primary vascular lesions in the striatum may account for motor deficits in cases with PD-related pathology stage 2 in other series.

α-Synuclein in the substantia nigra

An important issue is the relative amount of α-synuclein in the substantia nigra pars compacta when compared with other brain regions. Studies in normal human brain are puzzling since it is not clear whether the substantia nigra and striatum had the lowest (Rockenstein et al. 2001) or the highest levels (Solano et al. 2000) of α-synuclein. Regarding PD, comparative studies of control and diseased

brains are not illuminating. α-Synuclein mRNA levels in the PD substantia nigra have been reported to be unmodified, increased, or decreased when compared with age-matched controls (Rockenstein et al. 2001; Wirdefeldt et al. 2001; Kingsbury et al. 2004; Chiba-Falek et al. 2006). Cleaved (truncated) fragments of α-synuclein are prone to aggregate in vitro and have been considered prime components in LBs (Murray et al. 2003; Li et al. 2005).

Our recent studies have shown consistently lower levels of α-synuclein protein expression in the human substantia nigra and nucleus basalis of Meynert when compared with other brain regions independent of age and pathology. Phosphorylated α-synuclein at Ser129 is increased in the same regions, thus correlating with the total amount of α-synuclein. Additionally, truncated α-synuclein is naturally observed in control and diseased brains, correlating with the total amount of α-synuclein (Muntané et al., in preparation).

α-Synuclein pathology and Lewy inclusions in the substantia nigra in pPD

Phosphorylated α-synuclein at Ser129 is considered a key step in the development of α-synuclein aggregates (Anderson et al. 2006). Phosphorylated α-synuclein at Ser129 co-localizes with α-synuclein in punctuate Lewy-type aggregates in the substantia nigra at stage 3 of Braak (Fig. 1a–c). It is difficult to ascertain the amount of phosphorylated α-synuclein of the total α-synuclein on the basis of immunohistochemistry using antibodies that may have different binding capacities to their corresponding substrates. However, double-labelling studies suggest that phosphorylated α-synuclein at Ser129 predominates in LB lesions in pPD. Similar pattern was seen in affected nuclei of the medulla oblongata and affected neurons in the substantia nigra in pPD. Casein kinase II, one of the enzymes that phosphorylates α-synuclein at Ser129 (Ishii et al. 2007; Waxman and Giasson 2008), is currently found in LB in the substantia nigra in advanced stages of PD (Ryu et al. 2008). Yet casein kinase II is also observed in neurons with early α-synuclein inclusions not sequestered by LBs in pPD (Fig. 1d–f), indicating that increased casein kinase II expression is associated with increased α-synuclein phosphorylation at early stages of PD-related pathology. Other activated kinases, the functions of which have not been clearly identified in PD, have also been reported in association with LBs. One of them, active p38 (p38-P), is accumulated in cytoplasmic granules in the vicinity of LBs or in association with irregular-shaped or diffuse α-synuclein deposits in a percentage of substantia nigra pars compacta neurons in PD (Ferrer et al. 2001) and in a few neurons in pPD at stage 3 (Fig. 1g–i). In addition, nitrated α-synuclein is also found in small granules in dopaminergic neurons before the appearance of LBs in pPD. Finally, oxidative damage of α-synuclein has also been found using biochemical methods in the substantia nigra in pPD (Dalfó and Ferrer 2008). These observations indicate that modifications of α-synuclein, including oxidation, nitration and phosphorylation at Ser129, are early events in the substantia nigra at stage 3 of PD and occur in neurons with punctuate α-synuclein aggregates.

Truncated α-synuclein variants, detected with antibodies raised against different C- and N-terminal epitopes of α-synuclein, are components of punctate α-synuclein aggregates and LBs (Fig. 2).

These observations indicate that altered expression of proteins is not restricted to LBs, at least at early stages of LB formation. Rather, altered expression of proteins is seen in the vicinity but not within LBs in pPD. On the other hand, the structure of LBs is not homogeneous but different forms of α-synuclein are differentially incorporated into LBs.

Mitochondria dysfunction

Deficiencies in complex I subunits of the respiratory chain and impaired activity of the electron transport chain are well-known abnormalities in the substantia nigra in sporadic PD with parkinsonism (Mizuno et al. 1989; Parker et al. 1989; Schapira 2008; Onyango 2008).

Early studies showed no modifications in the activity of mitochondrial complexes II, III and IV in pPD (Jenner et al. 1992; Dexter et al. 1994). More especially, complex I activity in pPD was normal or reduced to intermediate levels among controls and cases with PD (Jenner et al. 1992; Dexter et al. 1994). However, studies in total homogenates do not permit the detection of modifications restricted to individual neurons. The use of immunofluorescence with appropriate markers offers limited information, as oxphos, porin, cytochrome *C* oxidase subunits and ATP synthase immunoreactivities are preserved in neurons of the substantia nigra pars compacta in stage 3 of Braak, independent of the presence or absence of α-synuclein inclusions (data not shown). Whether subunits of mitochondrial complex I are oxidatively damaged, functionally impaired and misassembled in pPD substantia nigra as reported in PD (Keeney et al. 2006) is not known.

Oxidative stress

Reduced glutathione levels are decreased in the substantia nigra in pPD and they are considered one of the first alterations in the substantia nigra in PD (Jenner et al. 1992; Jenner 1993, 1998; Dexter et al. 1994; Zeevalk et al. 2008).

Oxidative damage in the substantia nigra is already present in pPD at stages 2 and 3 of Braak (Dalfó et al.

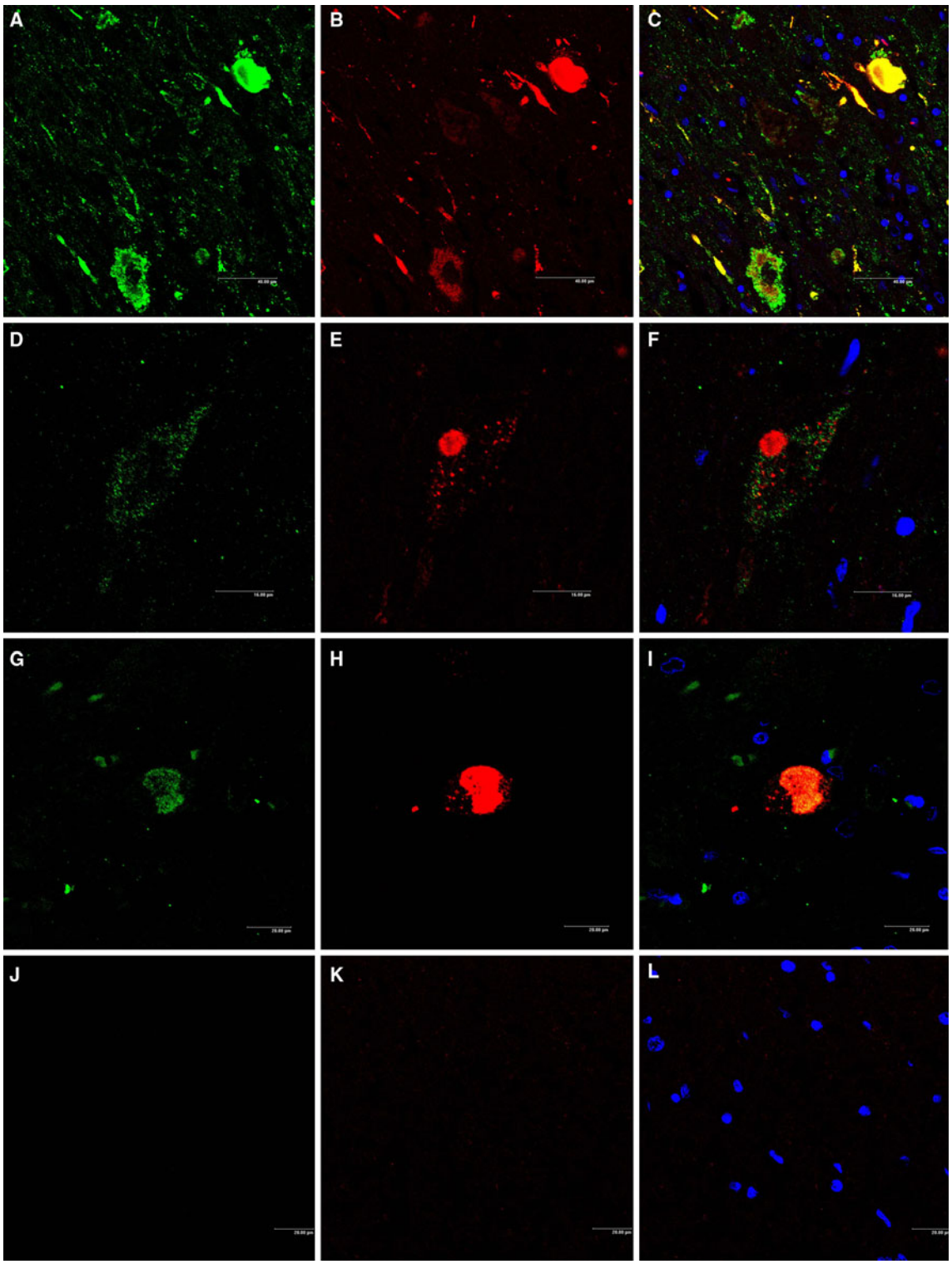

Fig. 1 Double-labelling immunofluorescence and confocal microscopy of α-synuclein (*green*) and α-synuclein PSer129 (*red*) (**a–c**), casein kinase II (*green*) and α-synuclein (*red*) (**d–f**), and p38 kinase-P (*green*) and α-synuclein (*red*) (**g–i**) in the substantia nigra pars compacta in pPD stage 3. α-Synuclein co-localizes with α-synuclein PSer129 in several but not all punctiform, neuritic and cytoplasmic aggregates. Casein kinase II and p38 kinase-P are expressed in the cytoplasm of neurons with α-synuclein aggregates. p38-P also co-localizes with synuclein in LBs. **j–l** Negative controls without primary antibodies

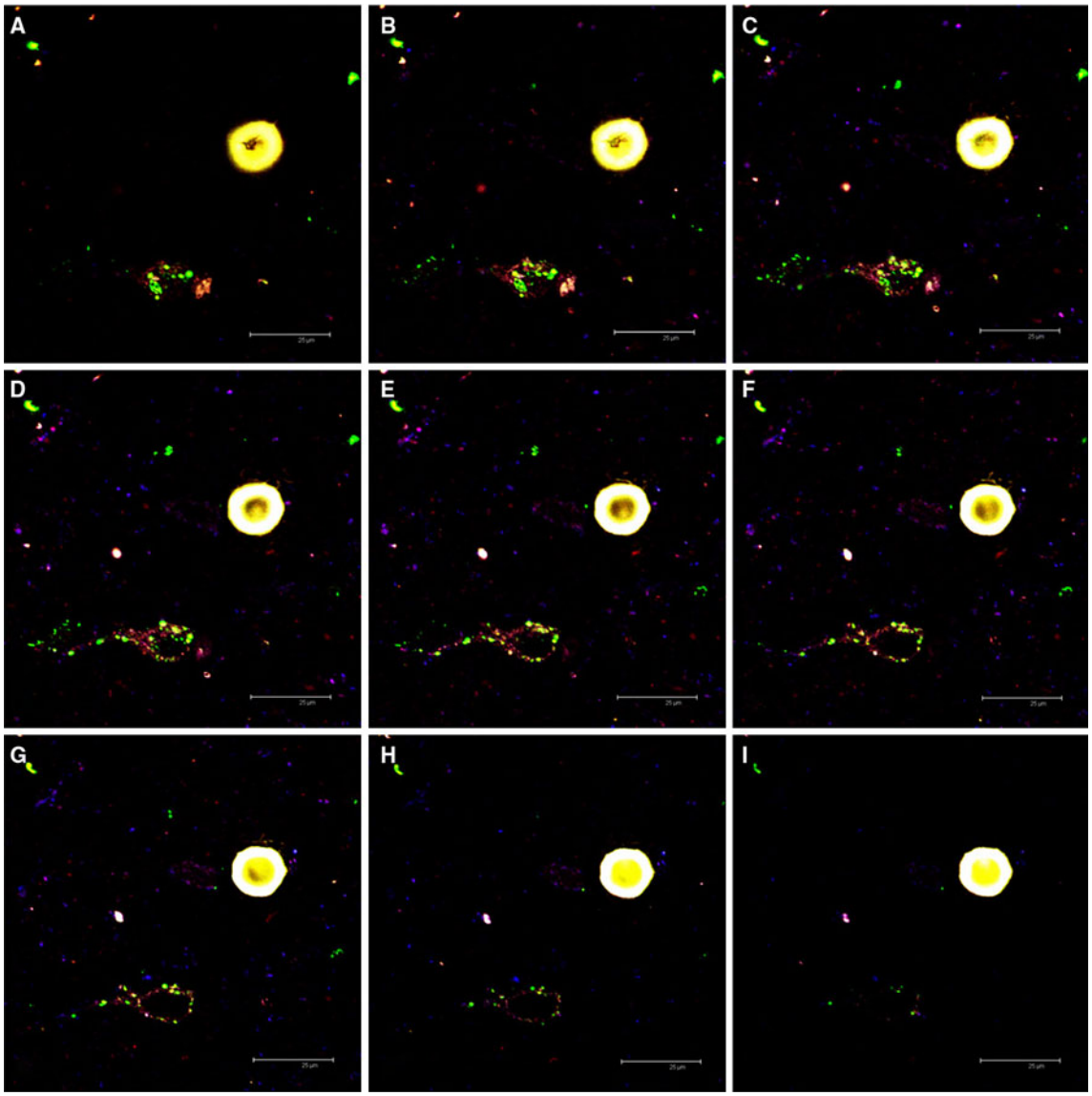

Fig. 2 Triple-labelling immunofluorescence and confocal microscopy of α-synuclein inclusions in the substantia nigra in pPD. Triple labelling with antibodies directed to the NAC region (AB9, *green*), N-terminal (AB2, *red*) and C-terminal (AB8, *blue*) of α-synuclein show slight differences in the staining of α-synuclein inclusions and LBs in the substantia in pPD. Several punctiform inclusions are differentially stained with the different anti-α-synuclein antibodies whereas co-localization of all three antibodies is found in LB (*white* in the merge construction)

2005). This is further documented by increased neuroketal (NKT) immunofluorescence in neurons with α-synuclein deposits and by the presence of NKTs in LBs (Fig. 3a–c). Neuroketals are a class of compounds that result from the oxidation of docosahexaenoic acid (DHA), a membrane polyunsaturated fatty acid especially vulnerable to free radical attack, which is present in large amounts in the brain. Therefore, the presence of neuroketals indicates oxidation of DHA in substantia nigra dopaminergic neurons at early stages of PD.

Oxidative stress responses are also augmented in neurons with α-synuclein pathology. Expression of peroxiredoxin 2, superoxide dismutase 1 (SOD1) and SOD2 is increased in neurons with α-synuclein inclusions (Fig. 3d–l). In addition to cytoplasmic increase in SOD1 and SOD2, these proteins can also be sequestered in LBs (Fig. 3j–l). It is interesting to have in mind that SOD2 has been reported oxidatively damaged in PD (Choi et al. 2005; Dalfó et al. 2005). Thus, proteins produced to counteract oxidative stress are, in turn, oxidatively damaged and sequestered into LBs.

In the line evidencing early oxidative damage in the substantia nigra in pPD is the observation of lipoxidative damage of α-synuclein in pPD (Dalfó and Ferrer 2008). Therefore, available observations show that oxidative

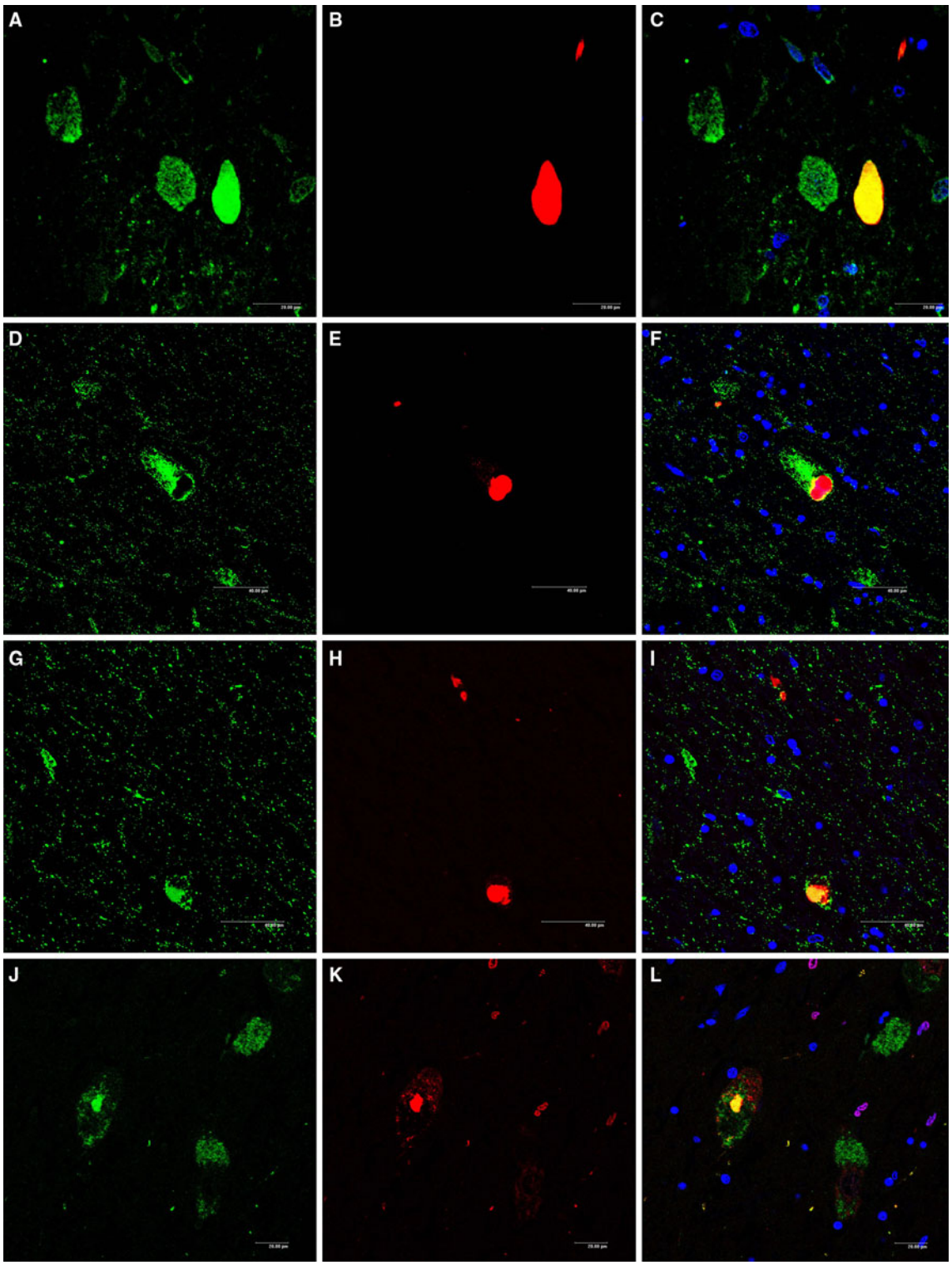

Fig. 3 Double-labelling immunofluorescence and confocal microscopy of neuroketal (*green*) and α-synuclein (*red*) (**a**–**c**), peroxiredoxin (*green*) and α-synuclein (*red*) (**d**–**f**), superoxide dismutase 2 (*green*) and α-synuclein (*red*) (**g**–**i**), and superoxide dismutase 1 (*green*) and α-synuclein (*red*) (**j**–**l**) in the substantia nigra pars compacta in pPD stage 3. Markers of oxidative damage and markers of oxidative stress responses are expressed in neurons of the substantia nigra. In addition, neuroketal, SOD1 and SOD2, but rarely peroxiredoxin accumulate in LBs

damage is an early event in PD and that oxidative damage in the substantia nigra in stage 2 precedes the formation of LBs in pPD substantia nigra.

Further biochemical studies have shown increased levels of neuroketals in the substantia nigra in cases with PD-related pathology stages 1 and 2 (Fig. 4), thus further supporting that oxidative damage to specific lipids in the substantia nigra occurs at very early stages of PD before the appearance of regional α-synuclein aggregates. These observations indicate early oxidative damage to lipids and certain proteins. In addition, evidence of DNA damage in the substantia nigra in pPD is shown in Fig. 4.

Iron

Iron plays an important role in the regulation of several enzymatic activities and in redox modulation. Iron dyshomeostasis has been observed in PD (Andersen 2004; Salazar et al. 2006; Hirsch 2006; Barnham and Bush 2008; Youdim 2008). Neuromelanin has a protective function under physiological conditions contributing to iron homeostasis (Double 2006). However, neuromelanin lipoxidation, altered α-synuclein binding to neuromelanin and increased levels of L-ferritin in neuromelanin have harmful effects on dopaminergic cell fate (Halliday et al. 2005; Double and Halliday 2006; Zecca et al. 2006, 2008; Tribl et al. 2009). Iron is increased in the substantia nigra in PD and it is stored in glial cells and macrophages at the time that neuromelanin decreases in vulnerable neurons (Gerlach et al. 2006; Fasano et al. 2006). In addition, increased iron levels have been found in individual neurons in PD (Oakley et al. 2007). As a result, increased iron levels, and particularly increased levels of labile iron, may increase iron-mediated oxidative damage and contribute to redox imbalance in PD (Wang et al. 2008; Chinta and Andersen 2008; Wypijewska et al. 2010).

No evidence of alteration in iron, copper, manganese or zinc in the pPD substantia nigra was noted in early studies (Jenner et al. 1992). However, recent observations have shown that L-ferritin concentration in the substantia nigra is lower in pPD (and PD) when compared with controls, whereas H-ferritin in PD is higher than in pPD and controls, thus indicating fine abnormalities in iron metabolism in the substantia nigra at early stages of PD (Koziorowski et al. 2007).

Neuronal globin α and β chain in the substantia nigra in pPD

Recent studies have demonstrated the presence of globin α and β in neurons in the mouse, rat and human brains, using robust combined methods including transcriptome analysis (cDNA microarrays and nanoCAGE) of laser-captured micro-dissected neurons, quantitative transcriptase-polymerase chain reaction (RT-PCR), in situ hybridization and immunohistochemistry with a panel of different specific antibodies (Biagioli et al. 2009; Richter et al. 2009). Nerve cells also express erythropoietin receptor, erythropoietin and hypoxia-inducible factor Hif1α (Matsuda et al. 1994; Digicaylioglu et al. 1995).

Globin α and β is present in dopaminergic neurons of the substantia nigra, and its expression is reduced in vulnerable neurons in parallel with abnormal α-synuclein deposition (Fig. 5). In contrast, the expression of neuroglobin and erythropoietin receptor is preserved in the same cells. These preliminary data point to the possibility that decreased globin α and β may play a role in the pathogenesis of PD.

Endoplasmic reticulum stress

Accumulation of misfolded proteins can trigger a reticulum stress response which is manifested by the activation of certain markers such as pancreatic endoplasmic reticulum kinase (PERK) and eukaryotic initiation factor 2 α (eIF2α). Phosphorylated PERK and phosphorylated eIF2α expression is increased in dopaminergic neurons of the substantia nigra in advanced stages of PD (Hoozemans et al. 2007). Increased eIF2α and phosphorylated eIF2α are also found in neurons of the substantia nigra pars compacta, they partly co-localize with α-synuclein inclusions in pPD (Fig. 6). Together, these observations support the idea that endoplasmic reticulum stress plays a role in the pathogenesis of early stages of PD (Wang and Takahashi 2007).

Protein degradation: the ubiquitin–proteasome system and autophagy in iPD

LBs and neurites are manifestations of impaired protein degradation in vulnerable cells. Since protein degradation putatively occurs via cytosolic proteases, chaperone-mediated autophagy (CMA), ubiquitin–proteasome system (UPS) and micro-, macroautophagy, all these mechanisms have been analysed in experimental models of PD. However, much less is known about what happens in PD.

Several in vitro studies have shown impaired UPS function in PD (Olanow and McNaught 2006). In favour of impaired UPS function is the cardinal early observation that ubiquitin occurs in Lewy inclusions and that p62 immunoreactivity appears in association with abnormal α-synuclein inclusions at early stages of LB (Kuusisto et al. 2003). However, the sequence of events is obscure. Morphological evidence in PD neurons shows fine α-synuclein deposition in the cytoplasm of selected neurons. Ubiquitin and p62 decorates some of but not all these inclusions

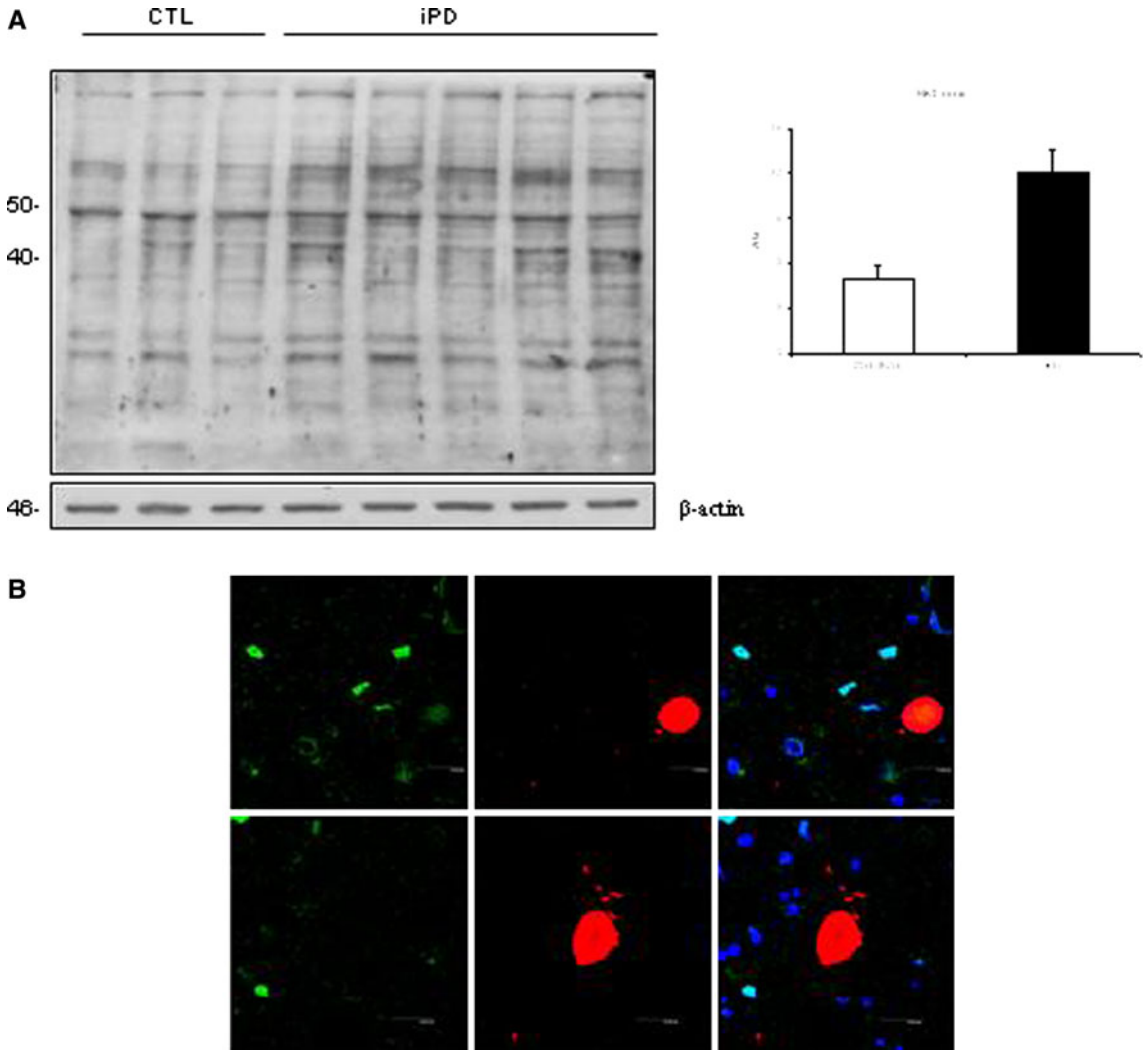

Fig. 4 **a** Increased neuroketal expression in total homogenates of the substantia nigra in pPD (*iPD*) compared with controls (*CTL*). Accompanying diagram illustrtes significant differences $P < 0.001$ (Student's *t* test). **b** Increased oxidative damage to nucleic acids of glial cells in the substantia nigra of pPD at stage 3. Double-labelling immunofluorescence and confocal microscopy to 8-hydroxyguanine (8-dOHG) (*green*) and α-synuclein (*red*) shows green staining in the nuclei of several glial cels but not in neurons with α-synuclein inclusions. Nuclei (*blue*) are stained with TO-PRO

suggesting that abnormal synuclein deposition is a first step followed by ubiquitination of α-synuclein and association of p62 to polyubiquitinated proteins (Kuusisto et al. 2003; Nakaso et al. 2004). More recent observations have provided evidence of altered expression of components of the UPS in PD (Chu et al. 2009). Moreover, some of them appear to be modified by oxidation thus resulting in lost of function (Choi et al. 2004; Chung et al. 2004; see later). Therefore, altered degradation by the UPS of abnormal α-synuclein and several hundred proteins associated with LBs does probably depend on multiple factors (Shamoto-Nagai et al. 2007; Chu et al. 2009).

The role of autophagy, including CMA and macroautophagy in PD, is postulated mainly on the basis of observations in cell models (Stefanis et al. 2001; Cuervo et al. 2004; Engelender 2008; Martínez-Vicente et al. 2008; Xilouri et al. 2008, Vogiatzi et al. 2008; Mak et al. 2010). Little is known, however, about the role of autophagy in human PD. Several morphological studies have shown that neuromelanin in the substantia nigra is associated with the autophagosome/lysosome system (Anglade et al. 1997; Tribl et al. 2006) thus providing a possible subtle link between α-synuclein and lysosomes in the substantia nigra under normal conditions and in PD. Certain autophagy markers are altered in the substantia nigra in PD (Pan et al. 2008). More straightforward in the present context is the observation that the expression and localization of proteins linked with autophagy and

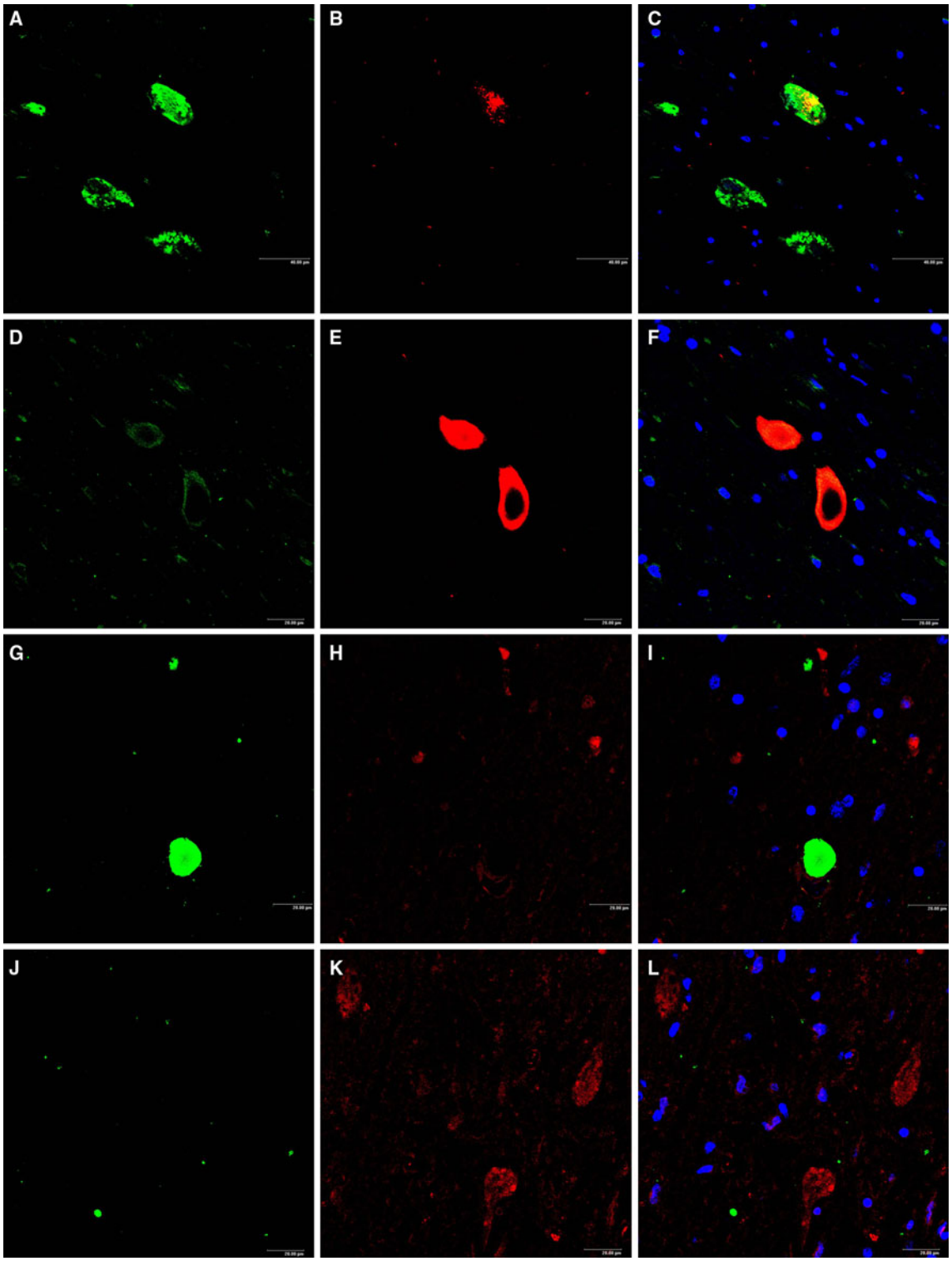

Fig. 5 Double-labelling immunofluorescence and confocal microscopy of ferritin (*green*) and α-synuclein (*red*) (**a–f**), α-synuclein (*green*) and globin α-chain (*red*) (G-L) in the substantia nigra pars compacta in pPD stage 3. Ferritin is decreased in neurons with α-synuclein inclusions (compare **a–c** with **d–f**). Similarly, globin α-chain expression is reduced in neurons with α-synuclein inclusions (compare **g–i** with **j–l**)

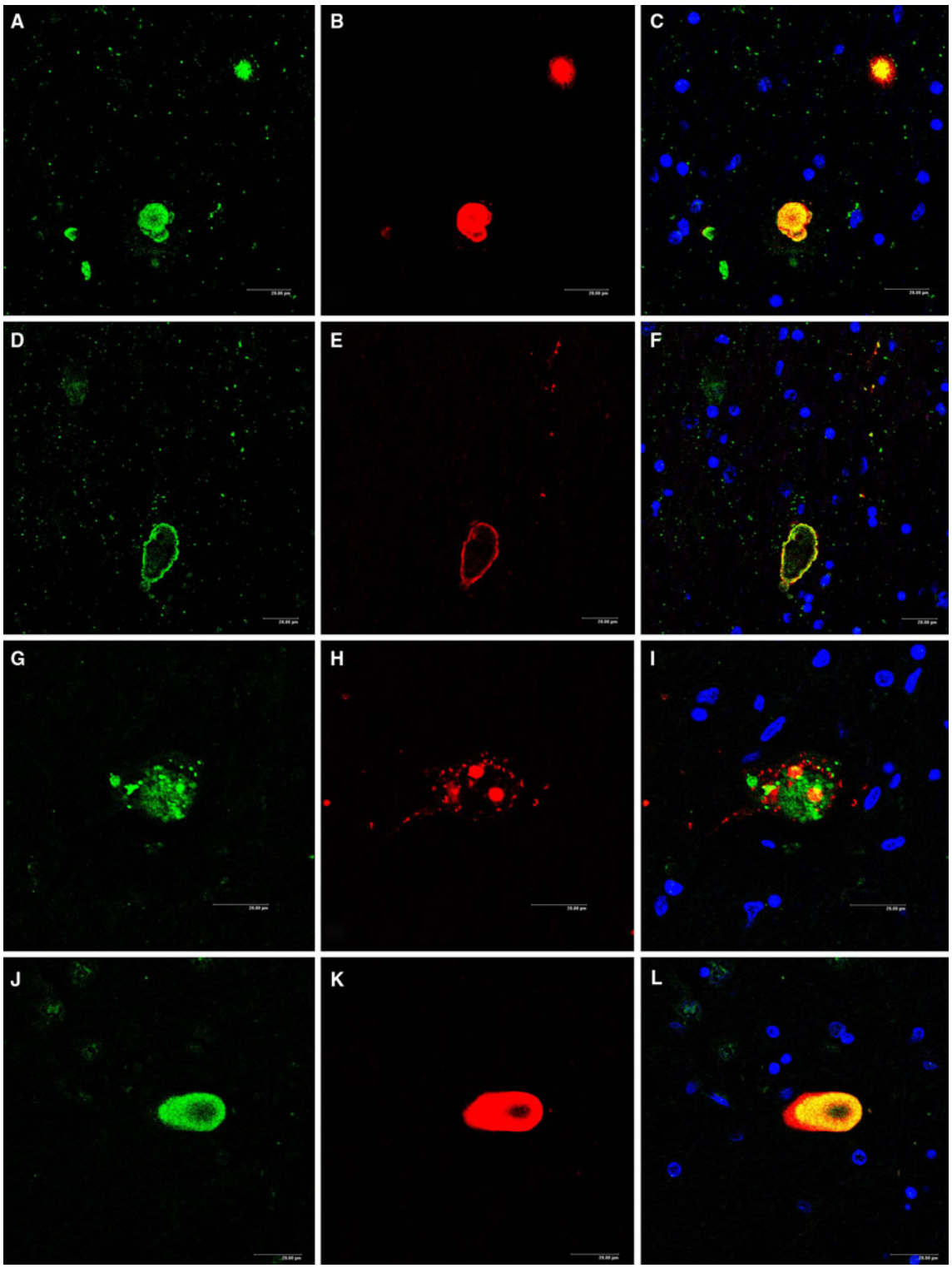

Fig. 6 Double-labelling immunofluorescence and confocal microscopy of eif2α (*green*) and α-synuclein (*red*) (**a–f**), and eif2α (*green*) and eif2α-P (*red*) (**g–l**) in the substantia nigra pars compacta in pPD stage 3. eif2α co-localizes with α-synuclein inclusions (**a–f**). The majority of eif2α is phosphorylated (**g–l**) thus suggesting activation of the reticulum stress responses

lysosomes as microtubule-associated protein 1 light chain 3 alpha (LC3), lysosomal-associated protein 1 (LAMP1) and LAMP2 (Klionsky et al. 2008) are altered in pPD (Fig. 7). In the same line, recent findings suggest that CMA activity is reduced in PD brain (Alvarez-Erviti et al. 2010).

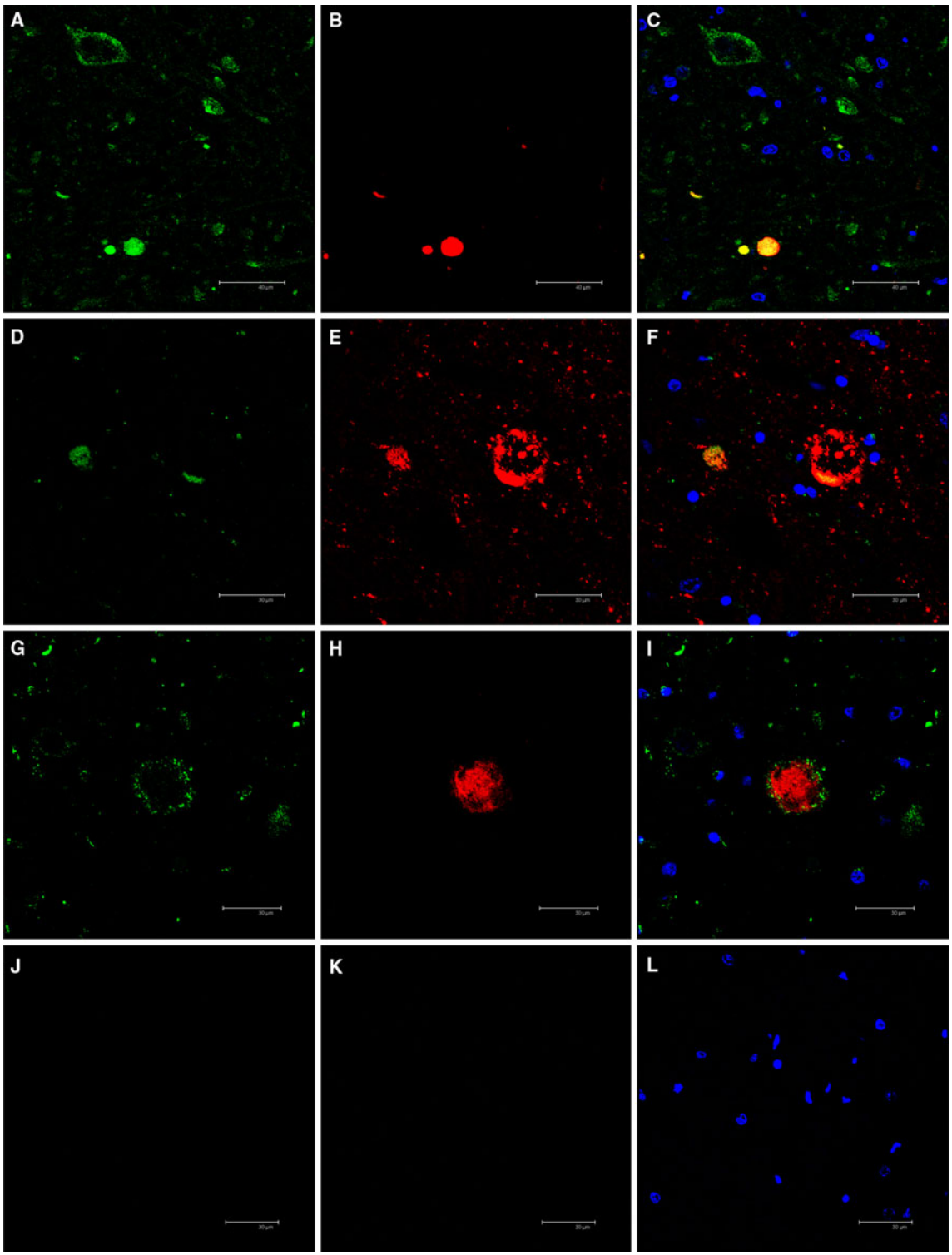

Fig. 7 Double-labelling immunofluorescence and confocal microscopy of microtubule-associated protein 1 light chain 3 alpha (LC3) (*green*) and α-synuclein (*red*) (**a–c**), lysosomal associated protein 2 (LAMP2) (*green*) and α-synuclein (*red*) (**d–f**), LAMP1 (*green*) and α-synuclein (*red*) (**g–h**) in substantia nigra pars compacta in pPD stage 3. Markers of autophagy are expressed in association with α-synuclein aggregates. **j–l** Negative controls processed without primary antibodies

In summary, experiments in vitro strongly support the idea of altered UPS and autophagosome/lysosome function in models of altered α-synuclein turnover and clearance, but precise information about the amount of α-synuclein cleared by the UPS and the CMA systems is not known in human disease.

The basal ganglia in pPD

Even in the absence of motor symptoms, loss of neurons in the substantia nigra translates into loss of dopaminergic innervation and consequent remodelling of the putamen. This is clearly illustrated by the loss of tyrosine hydroxylase (TH) immunoreactivity and loss of vesicular monoamine transporter 2 (VMAT2) in the putamen in pPD, with values that are intermediate between those in normal individuals and in parkinsonian stages of PD (DelleDonne et al. 2008; Dickson et al. 2008) (Fig. 8). These morphological aspects are also sustained by observations using serial metabolic imaging with [(18)F]-fluorodeoxyglucose positron emission tomography (PET) in sporadic cases (Tang et al. 2010). Multitracer PET scans are, therefore, useful tools to unveil dopaminergic dysfunction in patients with suspected pPD.

The balance of other molecules is also modified in the striatum in pPD. Leu-encephalin levels are reduced in the putamen and undetectable in the substantia nigra in PD, but Leu-encephalin levels are only barely reduced in the putamen in pPD (Fernandez et al. 1996).

Adenosine receptors 2A (A_{2A}Rs) in the striatum are practically restricted to GABAergic neurons projecting from the caudate and putamen to the external globus pallidus, which also expresses dopamine D_2 receptors (D_2Rs) (Fuxe et al. 2007). A_{2A}Rs expression levels in the striatum are increased in PD and correlate with motor symptoms (Varani et al. 2010). Recently, we have seen increased A_{2A}R expression levels in the caudate in cases with pPD stage 3 of Braak, thus indicating that this is an early abnormality of the denervated striatum (Buira et al. 2010).

Finally, early and pre-symptomatic stages of PD are associated with desensitization/downregulation of type 1 cannabinoid (CB1) receptors, whereas advanced stages are characterized by up-regulatory responses of CB1 receptors (García-Arencibia et al. 2009). Whether down-regulation of CB1 receptors at early stages is pathogenic remains to be elucidated; this may constitute an important putative target for therapeutic intervention. Decreased CB1 receptor expression is also observed in the caudate in pPD, showing that this is a very early alteration in the course of PD (Fig. 8).

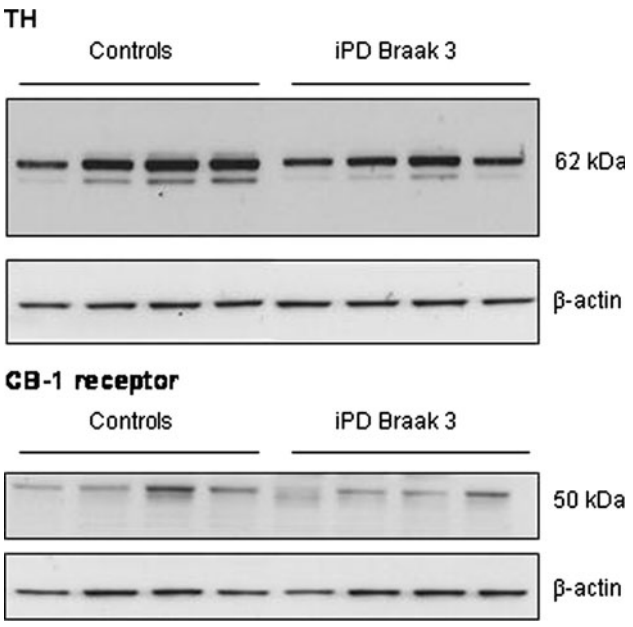

Fig. 8 Expression levels of tyrosine hydroxylase (*TH*) and cannabinoid 1 receptor (*CB-1 receptor*) in the caudate nucleus in controls and in pPD (*iPD Braak 3*). Reduced expression of TH (62 kDa) and CB-1 receptor (52 kDa) is observed in diseased cases when compared with controls ($P < 0.01$, Student's t test)

The olfactory bulb and tract in pPD

In the present series, the morphological study of the olfactory bulb and tract revealed the presence of small numbers of neurons and neurites bearing α-synuclein aggregates which are also recognized with antibodies against nitrated α-synuclein and against phospho-specific α-synuclein Ser129 antibodies (Fig. 9). However, it is hard to assume that such a small number of structural anomalies may account for the sophisticated olfactory deficits that include not only loss of olfaction but also altered discrimination of odours with increased perception in some circumstances. It may be hypothesized that, as in other regions, olfactory alterations in pPD and PD are the result of more complicated settings resulting from several molecular deficits.

The cerebral cortex in pPD

Altered behaviour, apathy and depression may occur in patients with pPD (Poewe 2008; McKinlay et al. 2009; Pedersen et al. 2009). The molecular substrates of such alterations are scarcely known but pieces of knowledge are rapidly growing (Ferrer 2009a).

Brain cortex and mitochondrial O_2 uptake and complex I activity are significantly lower in PD, whereas mtNOS activity, cytochrome content, expression of SOD2, mitochondrial mass, and oxidative damage are significantly higher in the frontal cortex in PD. The decreases in tissue and mitochondrial O_2 uptake and in complex I activity are considered the consequences of mitochondrial oxidative damage in the cerebral cortex in PD (Navarro et al. 2009). Unfortunately, no similar data are available in pPD.

Recent observations have shown abnormal lipid composition in the frontal cortex in pPD which is manifested

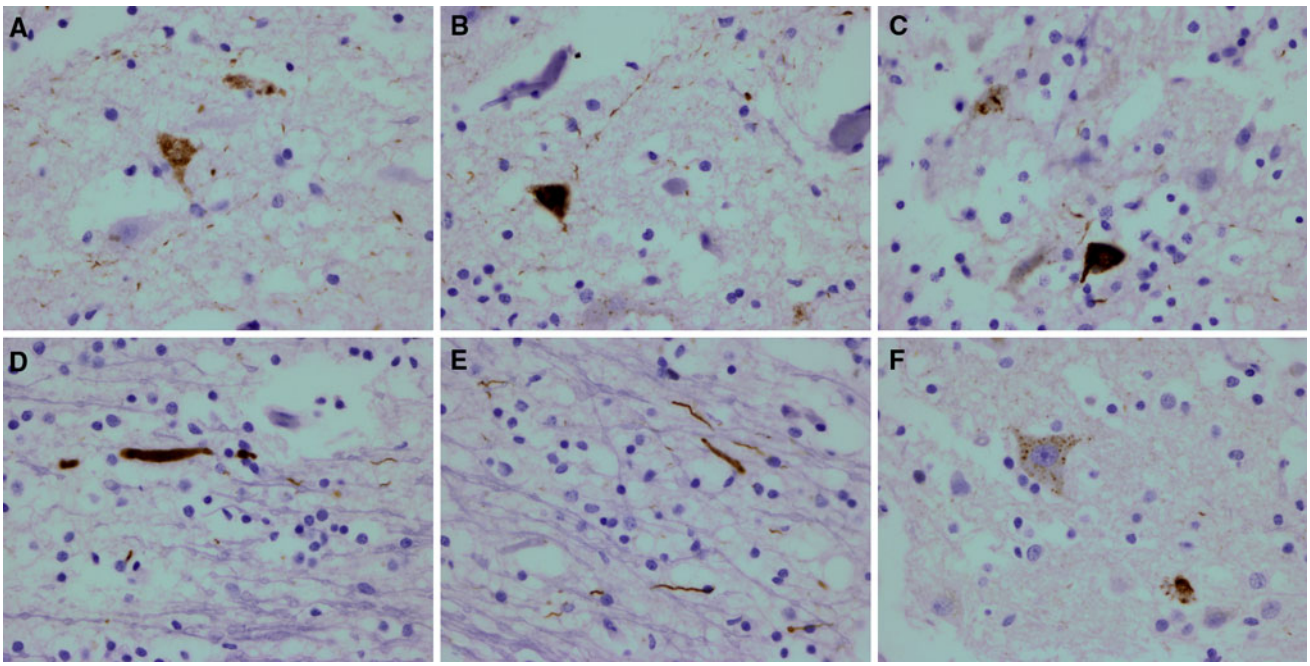

Fig. 9 Images of the olfactory bulb at stages 2 and 3 of Braak. Scattered neurites and cell bodies are immunoreactive with anti-α-synuclein antibodies. Paraffin sections slightly counterstained with haematoxylin

with significantly increased expression levels of the highly peroxidizable DHA and increased peroxidability index (Dalfó et al. 2005). More dramatically, several key proteins are targets of oxidative damage in the frontal cortex, as revealed by bi-dimensional gel electrophoresis, redox proteomics and mass spectrometry, in pPD including α-synuclein, β-synuclein and SOD2 (Dalfó et al. 2005; Dalfó and Ferrer 2008). In addition, increased oxidative damage of aldolase A, enolase 1 and glyceraldehyde dehydrogenase (GAPDH), all of them involved in glycolysis and energy metabolism, is found in the frontal cortex in pPD (and PD as well) (Gómez and Ferrer 2009). Other oxidatively damaged proteins are phosphoprotein enriched in astrocytes 15, SH3 domain binding glutamic acid-rich protein like, ubiquitin-conjugating enzyme E2N-like, prohibitin, proteasome subunit Y and thioredoxin. Finally, cortical synapses are abnormal in pPD (and PD), as tau phosphorylation and α-synuclein phosphorylation are increased in synaptic-enriched fractions of frontal cortex homogenates (Muntané et al. 2008).

Concluding comments

Until recently, PD was considered to be a movement disease mainly characterized by the loss of dopaminergic neurons and occurrence of LBs in the remaining cells in the substantia nigra. This point of view has served to drive major advances in the treatment of patients with PD, including pharmacological and neurosurgical therapeutic interventions geared to balancing dopamine deficits and related neurotransmitters and modulators in the nigrostriatal system. However, PD is a systemic disease affecting several distinct neuronal populations of the central and peripheral nervous system, with the appearance of motor symptoms in a substantial number of patients as the top of a plethora of non-motor signs and symptoms affecting the heart, gastrointestinal tract, olfaction, sleep and mental functions. Moreover, neuropathological and clinical studies have shown only a partial correlation between regional α-synuclein inclusions and corresponding neurological deficits at early stages of the disease, and no clear relation between LBs, and motor and cognitive impairment. Cumulative evidence in recent years indicates that mitochondrial dysfunction, oxidative stress and oxidative damage to crucial proteins, post-translational modifications of proteins, and endoplasmic reticulum stress converge in the pathogenesis of PD. This is further supported by proteomic studies showing dysregulation of proteins involved in energy metabolism, oxidative stress, signal transduction, electron transport and detoxification pathways in PD (Srivastava et al. 2010). Furthermore, the use of redox proteomics has revealed that protein altered by oxidative damage may have an impact on protein function in spite of apparently normal total levels of the protein (Martínez et al. 2010). Importantly, some of these changes occur at very early stages of PD-related pathology in areas with no accompanying Lewy-like inclusions, such as the substantia

nigra in stage 2, and the striatum and cerebral frontal cortex in stages 2 and 3 of Braak. Furthermore, although still patchy, several pieces of information suggest that failure in energy metabolism may have an additive deleterious effect on neuronal function. The term 'exhausted neuron' was applied in the context of Alzheimer disease to describe the particular situation of neurons working under suboptimal conditions of reduced energy production, increased energy demands and oxidative damage (Ferrer 2009b). This term can also be applied to pPD and PD in which neurons suffer from a convergence of intrinsic and external deficiencies that may impair neuronal function and eventually result in cell death. Recognition of this scenario helps us understand day-to-day variations in neurological deficits, fluctuations, apparent transient recoveries and subtle mining of neuronal integrity in neurodegenerative disease. More importantly, many of these alterations may be targets of therapeutic intervention geared to delaying disease progression.

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