Madhavi Thomas
Editor

Inflammation in Parkinson's Disease

Scientific and Clinical Aspects

Foreword by
Stanley H. Appel

Springer

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ISBN 978-3-319-08045-1 ISBN 978-3-319-08046-8 (eBook)
DOI 10.1007/978-3-319-08046-8
Springer Cham Heidelberg New York Dordrecht London

Library of Congress Control Number: 2014945339

Printed on acid-free paper

Springer is part of Springer Science+Business Media (www.springer.com)

I would like to dedicate this volume to my mentors, Stanley H. Appel, Joseph Jankovic, and Thomas N. Chase.

Foreword

Neuroinflammation is a prominent pathological hallmark of Parkinson's disease (PD), but, more often than not, neuroinflammation has been considered the consequence rather than the cause of PD. The many timely chapters in this book expand our understanding of the role of neuroinflammation in the pathogenesis of neuronal injury in PD, and strengthen the evidence that neuroinflammation is not merely the consequence but can actively mediate neuronal injury. In PD as in other neurodegenerative diseases, including Alzheimer disease and amyotrophic lateral sclerosis, neuroinflammation fans the flames of neurodegeneration. These neurodegenerative diseases present predominantly as sporadic and, to a lesser extent, inheritable disorders. The etiology of the sporadic forms of disease is largely unknown, but discovery of specific mutations responsible for inheritable forms of each of these disorders has revealed the central role of misfolded proteins in sporadic as well as familial disease. Dysfunction in multiple molecular pathways has been implicated in neurodegeneration, including autophagy and ubiquitin-proteasomal pathways, mitochondrial function, production of reactive oxygen species (ROS), alterations in axonal transport, and impaired RNA metabolism. All are key events that can initiate and promote neurodegeneration. The heterogeneity of the clinical phenotypes might be attributable at least in part to the multiplicity of these compromised pathways. The key question is whether compromise of any or all of these perturbations within neurons is sufficient to cause neuronal death. Data from ALS transgenic mouse models suggest that expression of a genetic defect in superoxide dismutase (mSOD1) solely in motor neurons does not lead to clinical disease. Furthermore, expression solely in microglia also does not cause motor neuron injury or give rise to disease, nor does expression solely in astrocytes. Expression of the mutant gene and misfolded proteins must be present in motor neurons and be communicated to glia to cause motor neuron injury, progressive disease, and shortened survival. Thus, in the animal model of ALS, dysfunction of the multiple compromised pathways within neurons is not sufficient to cause neuronal cell death; motor neuron injury is non-cell autonomous and disease progression depends on a well-orchestrated involvement of motor neurons and glia, and subsequently T lymphocytes.

A similar scenario appears relevant to PD. Ever since the report that a mutation in α-synuclein gave rise to familial PD, there has been an explosion of evidence documenting the central role of misfolded α-synuclein in the pathophysiology of both motor and non-motor signs as well as dopaminergic and non-dopaminergic systems in PD, prompting the delineation of PD as an α-synuclein proteinopathy. The pathological hallmark of PD is the Lewy body, an α-synuclein-containing intraneuronal cytoplasmic inclusion; a central role for misfolded α-synuclein has also been reinforced by reports that increased copy number of the α-synuclein gene can cause familial parkinsonism. In PD as in ALS, non-cell autonomous pathways can be implicated, which means that signaling between neurons and glia may be required to promote progressive neuronal injury and accelerated death. Regardless whether intraneuronal accumulation of misfolded and aggregated α-synuclein proteins leading to mitochondrial dysfunction, increased ROS, and altered RNA processing are early events in neuronal injury, cell death depends on the well-orchestrated participation of non-neuronal cells including microglia and astrocytes. Neuroinflammation is the hallmark of this non-cell autonomous process and depends on neuron-glial-T lymphocyte signaling, fostered and initiated by the compromised intra-neuronal pathways. In experimental models signals from injured neurons are initially communicated to microglia, members of the innate immune system, and subsequently to astrocytes, thereby coordinating a communal response to neuronal injury. In response to alterations in the activation states of the innate immune microglia and astrocytes, T lymphocytes, as members of the adaptive immune system, join the process by infiltrating the CNS at sites of neuronal injury.

The potential contribution of neuroinflammation to the pathogenesis of PD is supported by a number of reports. A genome-wide association study (GWAS) associated PD with a highly polymorphic region of HLA-DR, previously reported with numerous inflammatory disorders. HLA-DR immunoreactive microglia are prominent in the SN of PD patients, and are known to interact with T lymphocytes. Degeneration of the ventral midbrain DA neurons in PD is accompanied by the presence of activated microglia with increased inducible nitric oxide synthase (iNOS), TNF-α, IL-1β, IFN-γ, and nuclear translocation of NF-κB. In PD, activated microglia exhibit a pro-inflammatory M1 phenotype. Increased lipid peroxidation, as well as carbonyl- and nitrotyrosine-modified proteins, is also observed in CNS nigral tissue. Activated microglia that surround DA neurons are noted in postmortem tissues of human subjects that have previously developed symptoms and signs of parkinsonism following exposure to the neurotoxin MPTP.

In vitro and experimental in vivo studies provide evidence that misfolded α-synuclein itself may contribute to the neuronal-glial signaling that promotes neurodegeneration. Normal monomeric and aggregated forms of α-synuclein secreted from DA neurons can convert M2 anti-inflammatory microglia to M1 pro-inflammatory microglia, enhancing secretion of ROS and cytokines and leading to neuronal cell death, while inhibitors of these ROS attenuate in vitro DA neurotoxicity. Following expression of the human α-synuclein protein in transgenic mice, insoluble α-synuclein aggregates accumulate in nigral neurons, and render DA neurons more vulnerable to LPS-induced microglia-mediated neurotoxicity.

DA neurons exposed to MPP+ have increased accumulation and secretion of misfolded and aggregated α-synuclein, but the MPP+-mediated DA neuronal injury was not sufficient to cause neuronal death; microglia are necessary to deliver the coup de grace by releasing free radicals and pro-inflammatory cytokines. The increased release of misfolded and aggregated α-synuclein from injured DA neurons has a self-propagating effect causing further oxidation and further release of α-synuclein from DA neurons, and further amplifying DA neurodegeneration. In addition, intracerebral inoculation of preformed α-synuclein fibrils in non-transgenic mice spreads in a prion-like fashion, spreading severe dopaminergic cell injury.

T lymphocytes also participate in the ongoing inflammatory process. Both CD4+ and CD8+ T lymphocytes are present in the SN of PD patients. Although end-stage autopsy tissue cannot define the functional role of T lymphocytes, experimental animal models have provided meaningful insights. In MPTP-treated mice, T lymphocytes enhance cytotoxicity. SCID, Rag1−/−, or TCR−/− mice lacking T lymphocytes are relatively resistant to MPTP-induced SN dopaminergic cell degeneration; in mice lacking CD4+ T lymphocytes, MPTP caused significantly less DA cell death. Thus, MPTP-mediated DA cell killing requires CD4+ T lymphocytes as well as microglia.

T lymphocytes can be neuroprotective as well; transplantation of T lymphocytes from mice immunized with the immunomodulatory drug glatiramer acetate (GA) attenuated the MPTP-induced SN cell loss. IL-4 provided neuroprotection from toxicity mediated by activated microglia, and studies in ALS animal models suggest that Th2 lymphocytes as well as Tregs release IL-4 and modulate the pro-inflammatory M1 phenotype, suggesting that a similar mechanism may be relevant to PD. Several studies of the MPTP model suggest that increased Tregs mediate neuroprotection and Th17 lymphocytes promote neurotoxicity. Adoptive transfer of CD3-activated Tregs to MPTP-intoxicated mice provides greater than 90 % protection of the nigrostriatal system. The response is dose-dependent and parallels modulation of microglial responses and up-regulation of GDNF and TGF-β. Transplantation of Teffs provided no significant neuroprotective activities. The ratio of neuroprotective to cytotoxic T lymphocytes (Th2/Tregs to Th1/Th17) appears to be a relevant parameter in mediating neuroprotection versus neurotoxicity. Tregs were found to mediate neuroprotection possibly through suppression of microglial responses to aggregated and nitrated α-synuclein. In vitro, Tregs suppress microglial release of ROS induced by misfolded α-synuclein, whereas Teffs exacerbate microglial release of ROS. Thus, neuroprotection can be achieved through modulation of microglial oxidative stress and inflammation. Taken together, these results demonstrate that Tregs can suppress nitrated α-synuclein-induced cytotoxic neuroinflammation.

The cumulative data from in vitro and experimental studies of PD models suggest that both microglia and T lymphocytes can serve protective as well as cytotoxic functions. The combination of M2 monocyte/microglia and Tregs/Th2 lymphocytes are neuroprotective and actively suppress M1/Th1/Th17-mediated toxicity; the combination of M1 monocyte/microglia and Th1/Th17 lymphocytes are cytotoxic. However, the timing of protection and cytotoxicity remains to be determined.

Is the milieu protective early in the course of experimental models of PD as well as in PD patients, and toxic later in disease, as noted in models of ALS? The answers are not available. What is needed is a better understanding of how to limit populations of cells mediating cytotoxic immunomodulation and how to expand populations of cells mediating neuroprotective immunomodulation.

More critical is whether the experimental data are truly applicable to patients with PD, and whether neuroinflammation can mediate neurodegeneration in patients. Biomarkers specific for neuroinflammatory pathways that effectively monitor the evolution of disease and potential responses to immunomodulatory therapy would provide important support for the relevance of neuroinflammation to the pathogenesis of disease. As an approach, neuroimaging, as delineated in this book, may well function as a biomarker to monitor microglial activation, especially in concert with immune/inflammatory blood markers. Dr Thomas is to be congratulated for assembling these first-rate authors and chapters that detail the inflammatory responses and their contribution to neurodegeneration; novel pathways are defined that could potentially lead to meaningful therapies that promote effective neuroprotection.

Houston, TX, USA Stanley H. Appel, M.D.

Preface

Inflammation in Parkinson's disease (PD) has always been a subject of great interest. Chronic inflammation in the brain can be induced in a primate model even with a single injection of MPTP. Many genetic and environmental factors contribute to onset of PD. There is much to learn from recent developments in systems biology including study of proteomics and metabolomics in developing future biologic markers. Of great interest is whether inflammation is a co-contributor or an independent driving process for cell death in PD. Arguments can be made for both. However, PD is a heterogeneous disease. Based on familial risk factors for PD, one can hypothesize that PD can be due to pathways of pure apoptosis, inflammation, vascular risk factors, presence of oncogenes, and multifactorial etiology. One needs to review this carefully and perhaps develop biological markers based on familial risk factors, and these pathways need to be clarified further. There could be isolated subtype of PD, with predominant inflammatory pathology, and therapeutic targets can be developed in the future. The focus of this publication is to bring together basic laboratory advances as well as currently available clinical literature to support this concept of inflammation as being an important process in PD. In this maiden venture I have sought help from many of my learned colleagues, who have kindly agreed to work on bringing this volume together. I am hoping this volume will help students, researchers, and clinicians alike in developing novel concepts in research in the future.

Bedford, TX, USA — Madhavi Thomas, M.D.

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Parkinson's Disease: An Overview of Etiology, Clinical Manifestations, and Treatment

Arif Dalvi, Kelly E. Lyons, and Rajesh Pahwa

Parkinson syndrome is an umbrella term grouping together clinical syndromes that share the cardinal symptoms of bradykinesia, rigidity, and rest tremor. By far the largest occupant of this umbrella is idiopathic Parkinson's disease (PD), with degeneration of the nigrostriatal dopaminergic system and the presence of Lewy bodies in the substantia nigra as its pathological hallmarks [1].

A number of other neurodegenerative diseases share this space with distinct clinical features and pathological mechanisms. The combination of the cardinal features of Parkinson syndrome with additional manifestations such as ophthalmoplegia, dysautonomia, cortical and cerebellar signs, or dementia is referred to by the rubric parkinson-plus syndrome that includes disorders such as progressive supranuclear palsy (PSP), multiple system atrophy (MSA), corticobasal degeneration (CBD), and dementia with Lewy bodies (DLB). Other well-defined heredodegenerative diseases such as Wilson's disease and Huntington's disease can also mimic PD. Finally, structural lesions in the brain such as normal pressure hydrocephalus and small vessel ischemic disease can resemble PD [2].

Clinical Features

The diagnosis of PD is primarily based on clinical criteria. The United Kingdom Parkinson's Disease Society Brain Bank criteria are most often used, with positive diagnostic features including the presence of bradykinesia, and at least one of muscular rigidity, tremor at rest, or postural instability not explained by other etiologies.

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M. Thomas (ed.), *Inflammation in Parkinson's Disease: Scientific and Clinical Aspects*,
DOI 10.1007/978-3-319-08046-8_1,

Exclusionary criteria include history of strokes, repeated head injury, or definite encephalitis, oculogyric crisis, neuroleptic treatment at symptom onset, sustained remission, unilateral symptoms after 3 years, supranuclear gaze palsy, cerebellar signs, early, severe autonomic features or dementia, Babinski sign, cerebral tumor or hydrocephalus, or no response to high doses of levodopa. Supportive features include at least three of the following: unilateral onset, rest tremor, progression, persistent asymmetry with side of onset most affected, good response to levodopa, levodopa-induced dyskinesia, and clinical course of at least 10 years [3].

The clinical syndrome of PD is not limited to these cardinal symptoms. Other motor manifestations include postural instability, stooped posture, a shuffling, festinating or freezing gait, and dystonic posturing, particularly in the lower extremities. In addition, long-term treatment, particularly with levodopa is associated with the development of dyskinesia and a fluctuating response to medical treatment described as the "on–off" phenomenon [4].

There is an increasing recognition of non-motor symptoms in PD, some of which may precede the onset of motor symptoms by many decades. An altered sense of smell and constipation can occur in PD and have also been examined as potential predictors of the onset of PD. Depression and anxiety are common comorbid symptoms. Changes in the sleep–wake cycle and REM behavior disorder may also be seen. Dysautonomia usually becomes more prominent in the later stages of the disease, unlike in MSA, where it may be a presenting feature. Autonomic symptoms include orthostatic hypotension, urinary frequency and incontinence, delayed gastric emptying and sialorrhea, erectile dysfunction, and loss of libido [5]. Dementia is part of the natural history of PD, although, in contrast to DLB, it occurs relatively late in the course. A global dementia, such as is seen in Alzheimer's disease, is less commonly observed. Instead an executive dysfunction is seen with retrieval being affected more than memory and short-term memory loss more common than that of long-term memory. Long-standing PD may also be complicated by the development of hallucinations and psychosis [6].

Laboratory Studies

The diagnosis of PD remains a clinical diagnosis. However, judicious use of imaging and laboratory studies helps exclude diseases that may resemble PD but have a significantly different treatment and prognosis. In younger patients it is important to screen for Wilson's disease with serum ceruloplasmin, 24-h urine copper, and liver function studies. Imaging studies in the form of CT or MRI of the brain help rule out structural etiologies such as normal pressure hydrocephalus and vascular parkinsonism. Brain MRI also detects changes in the basal ganglia or thalamus in Wilson's disease and may reveal caudate atrophy in Huntington's disease [1].

Where diagnostic uncertainty exists, for example, between essential tremor (ET) and PD, the use of DaTscan SPECT imaging can be useful. Dopamine transporter (DaT) levels in the striatum are lower in parkinsonism due to loss of dopaminergic

cells but are normal in other etiologies of tremor such as ET. Labeling the dopamine transporter with ioflupane (a radioactive iodine-labeled cocaine derivative) and measuring the levels with SPECT imaging can help distinguish ET from parkinsonism including PD. It is important to note that DaTscan is unable to distinguish PD from other forms of parkinsonism [7].

Etiology of PD

In his seminal *An Essay on the Shaking Palsy* Sir James Parkinson considered "indulgence in spirituous liquors" and "long lying on the damp ground" as possible etiologies of PD [8]. Seventy years later Gower reported that about 15 % of his patients with PD had a family history [9]. A twin study on WWII veterans showed genetic factors in patients with typical or older age of onset played a lesser role, as similar concordance rates were observed in monozygotic and dizygotic twins. In contrast, in patients with an onset prior to age 50, there was a more substantial genetic contribution [10]. Following the discovery of the α-synuclein (SNCA) gene [11] and the subsequent discovery of a number of additional PARK genes the debate between supporters of genetic and environmental causes has been renewed. The assumptions of Parkinson regarding the etiology of PD have not held up, but a number of alternative environmental factors have been proposed. Current understanding of the etiology of PD points to a multifactorial disorder with gene–environment interactions leading to neuronal cell death (Fig. 1).

Selective Vulnerability of the Nigrostriatal Tract

The reason for selective vulnerability of the substantia nigra pars compacta (SNc) in the pathogenesis of PD is an area of active study. A number of hypotheses have been proposed as an explanation. Oxidative stress involving mitochondrial dysfunction may be more prominent in the SNc relative to other brain regions, primarily due to reactive oxygen species produced during dopamine storage and breakdown [12]. Models of endoplasmic reticulum stress show age-dependent selective vulnerability of dopaminergic neurons, also related to the oxidative by-products of dopamine metabolism. Dopamine metabolites, especially the monoamine oxidase (MAO) metabolite 3,4-dihydroxyphenylacetaldehyde (DOPAL), can trigger SNCA aggregation in SNc neurons [13].

Accumulation of SNCA in cultured human dopaminergic neurons results in apoptotic cell death. This mechanism requires endogenous dopamine production and is mediated by reactive oxygen species. In contrast, SNCA is neuroprotective in non-dopaminergic human cortical neurons. Thus, accumulation of soluble SNCA protein complexes can render endogenous dopamine toxic, suggesting a potential

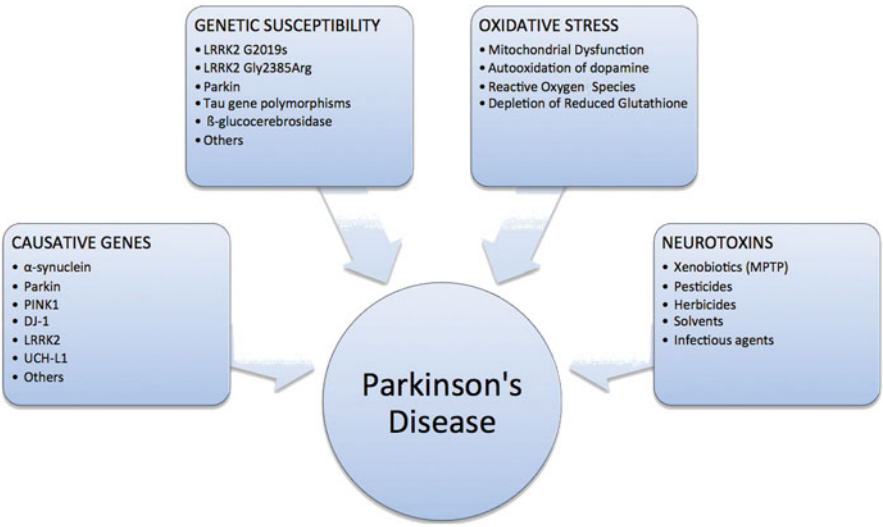

Fig. 1 The multifactorial etiology of Parkinson's disease. Causative genes and genetic susceptibility factors interact with environmental triggers and endogenous causes of oxidative stress. Misfolding and aggregation of proteins together with mitochondrial dysfunction provide the framework for neuronal cell death leading to Parkinson's disease. © Arif Dalvi 2007. Reprinted from Dalvi A, Walsh R. Etiology of Parkinson's disease. In: Simuni T, Pahwa R, editors. Parkinson's Disease. New York: Oxford University Press; 2009: 51–62, Figure 5.1. By permission of Oxford University Press, USA

mechanism for the selectivity of neuronal loss in PD [14]. Inflammatory mechanisms may also play a role, especially in the context of activated microglial cells. Patients with PD have selective degeneration of neurons in the SNc accompanied by microglial activation and a challenged immune system [15].

Environmental Factors

Environmental factors have always been considered to play a significant role in the etiology of PD. One of the most commonly identified is exposure to herbicides and pesticides. In a multifactorial model based on a study of rural populations, years of rural living and groundwater use were predictive of PD. Pesticide exposure was found to be a risk factor independent of rural living [16]. However, not all studies support rural living as a risk factor. In China drinking well water was associated with a reduced risk of PD, while living in proximity to rubber plants or drinking river water was associated with a higher risk [17]. In a large serial cross-sectional study of Medicare beneficiaries, two geographic belts with high predisposition to PD were found. This series of over 450,000 PD cases revealed a higher concentration of PD cases in the Midwest and Northeast regions. Prevalence in urban counties

was greater than in rural ones [18]. Epidemiologic studies investigating potential links between solvents and PD have yielded mostly null or weak associations [19]. However, a study of 99 twin pairs from the World War II Veterans Twin Cohort suggested possible etiologic relations with trichloroethylene (TCE) and other chlorinated solvents, although the sample size was small and dose–response gradients were not observed. It should be noted that TCE is the most common organic contaminant in groundwater [20]. Welding and manganese exposure have been suggested as risk factors for PD [21]. However, a meta-analysis that pooled data from 13 studies for welding and 3 studies for manganese exposure failed to support this hypothesis [22].

Genetic Factors

A first-degree relative of an affected individual is approximately twice as likely to develop PD compared to someone with no family history of PD [23]. While concordance rates in monozygotic and dizygotic twins are equal in late-onset PD, they are much higher in monozygotic (~100 %) than in dizygotic twins (~17 %) in early-onset PD, consistent with early-onset PD having a strong genetic determinant [24]. Several genes have been definitively linked with familial PD, along with other candidate genes whose association with PD is less established.

α-Synuclein

Three mutations of this gene as well as duplication and triplication of the gene region have been described in familial PD. The pathological role of SNCA in PD is also not definitively known, but SNCA is a major component of Lewy bodies observed in post-mortem studies of PD brains [12]. Autosomal-dominant inheritance of familial PD is observed with mutations of α-synuclein. The clinical course of affected individuals is similar to sporadic PD, but with an earlier mean age of onset, higher rate of dementia, and some neuropathological features not common in sporadic PD including more tau-positive extra-perikaryal spheroid-like and thread-like lesions and more marked neuronal loss. The physiological role of SNCA is unclear, but it may be involved in synaptic vesicle recycling particularly involving dopamine storage [25].

Parkin

This gene is the most common autosomal-recessive PD gene mutation, and a wide variety of parkin mutations have been found in familial PD. Parkin is mutated in ~50 % of autosomal-recessive early-onset PD and in ~70 % of juvenile PD with

onset less than 20 years. The clinical course of affected individuals is typically characterized by early onset, slow progression, and good response to dopamine [26]. The physiological role of parkin is thought to relate to its function as an ubiquitin ligase important in normal cellular protein degradation pathways. Deficiency in this function may underlie the pathology associated with mutant parkin, including possible disruption of microtubule and mitochondrial function, proteasomal degradation, and neuroprotection [27].

PINK1

Mutations of this gene are thought to result in loss of kinase function consistent with the observed autosomal-recessive inheritance pattern. The clinical course in affected individuals typically demonstrates disease onset at less than 50 years with otherwise mostly classical features of sporadic PD [28]. The physiological role of PINK1 is thought to involve regulation of the electron transport chain and maintenance of mitochondrial membrane potential, and the pathology resulting from mutations of PINK1 may relate to mitochondrial dysfunction in response to oxidative stress, possibly involving parkin [29].

DJ-1

Multiple types of DJ-1 mutations have been identified, and inheritance is autosomal-recessive. Affected individuals typically have age of onset around 20–40 years with mostly classical Parkinsonian symptoms and usually respond well to dopaminergic therapy. Focal dystonia and blepharospasm may present early in the course of the disease [30]. DJ-1 is thought to play a role as an antioxidant and sensor of oxidative stress, but may also be involved in protein degradation pathways and apoptotic signaling possibly in conjunction with parkin and PINK1. Furthermore, dysfunction of DJ-1 may affect these pathways in a manner that preferentially involves dopaminergic neurons [31].

LRRK2

LRRK2 mutations are found throughout this gene's functional domains with the most common mutant being relatively frequent in both familial autosomal-dominant PD (~4 %) and in sporadic PD (~1 %) [32]. In certain populations, such as North African Arabs and Ashkenazi Jews, the prevalence of LRRK2 mutations may account for up to 40 % of all PD cases [33, 34]. LRRK2 mutations result in parkinsonism very similar to classical PD, and penetrance of symptoms is very tightly linked with aging, also similar to idiopathic PD. Despite the relatively uniform

classic parkinsonism seen with LRRK2 mutations, the neuropathology is quite diverse suggesting that LRRK2 dysfunction may be important in the initiation of altered function of multiple cellular systems with a final common pathway resulting in dopaminergic cell death [35]. The physiologic role of LRRK2 is thought to involve its putative kinase and GTPase activity, and mutant forms have shown increased kinase activity consistent with a gain of function seen often in autosomal-dominant diseases [36]. PD-causing LRRK2 mutations deregulate the autophagy–lysosomal pathway. G2019S mutant LRRK2 can lead to abnormal accumulation of autophagic and lysosomal structures in primary cortical neurons and neuronal cell lines in culture [37].

UCH-L1

It is unclear if mutations of UCH-L1 identified in several families are truly involved in PD due to the failure of the mutation to segregate with disease in one family and the failure of other mutations to be identified despite extensive screening [38]. It is intriguing, however, that a polymorphism in this gene may protect against development of PD, possibly through alteration of interaction of UCH-L1 with SNCA [39]. UCH-L1 accumulation is likely to play a pathological role in inclusion formation in PD through a malfunction of the ubiquitin/proteasome system that leads to an inability to clear aggresomes [40].

Gene–Environment Interactions

The ultimate etiology of PD may be based on multiple factors including genetics, environmental exposures and aging-related apoptotic processes. The complex interaction between these factors may serve as an explanation for the heterogeneity observed in clinical presentations. Various animal models have attempted to examine the interplay of these interactions.

DJ-1 mutations have been associated with autosomal-recessive early-onset PD. In a DJ-1 knockout transgenic mouse model the susceptibility of nigrostriatal deficits was found to increase following exposure to MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine). In addition, in wild-type mice adenoviral-mediated overexpression of DJ-1 was found to block MPTP induced neuronal loss and neurodegeneration in the substantia nigra. Thus, DJ-1 may play a significant role in the protection of neurons against oxidative stress and environmental neurotoxins [41]. Lymphoblast cells derived from DJ-1 patients display aberrant mitochondrial morphology. These DJ-1-dependent mitochondrial defects contribute to oxidative stress-induced sensitivity to cell death. The aberrant mitochondrial phenotype can be rescued by the expression of Pink1 and Parkin, two PD-linked genes involved with mitochondrial function. Thus a complex interplay between genetic factors can lead to a differential response to oxidative stress produced by environmental toxins [42].

Infectious Etiologies for PD

In 1916–1927 an epidemic of an influenza-like illness ravaged Europe and North America. Mortality was up to 40 % in those affected, and most survivors developed parkinsonism over the next 10 years [43]. The specific agent causing this pandemic of encephalitis lethargica was never isolated. However, it drew attention to an infectious etiology as a contributor to PD. Of note, the possibility that an encephalitis lethargica syndrome is still prevalent has been raised with the suggested mechanism being autoimmunity against deep grey matter neurons [44].

Antibodies to the Epstein–Barr virus have shown cross reactivity with SNCA in the brains of patients with PD [45]. Although no evidence of ongoing viral infection in PD has been reported, immunohistochemistry shows reactive microglia and activated complement components suggestive of chronic inflammation occur in affected brain regions in PD [46]. The viral hypothesis has also been invoked to explain the observation of the higher incidence of PD in teachers, medical workers, loggers, and miners [47]. In monozygotic twins discordant for PD a significantly increased risk was noted in the twin working as a teacher or health care worker [24].

PD as a Prion-Like Disease

SNCA has been determined to be the major component of Lewy bodies, which are the pathological hallmark of PD [48]. Three missense mutations in the SNCA gene have been associated with autosomal-dominant PD, and genome-wide association studies have linked single-nucleotide polymorphisms in this gene to sporadic PD [49]. The spread of SNCA pathology in the brain has been implicated in the progression of PD and in the caudal to rostral spread of Lewy bodies in PD that forms the basis of Braak staging in PD [50]. This has led to the recognition that SNCA has attributes that are common to prion-like proteins, including multiple conformations and the ability to transfer from cell to cell. The physiological form of SNCA has an unstructured α-helical conformation that changes to oligomers and fibrils rich in β-sheets in the mutant pathological form [51]. In vivo studies have also documented the ability of SNCA to transfer and propagate from cell to cell [52]. Support for the prion-like spread of PD was furthered by observations in autopsied brains of individuals who underwent embryonic stem cell implants in the 1980–1990s. Of note SNCA pathology was found not only in the patient's own brain tissue but also in the grafted neurons. These grafted neurons were found to show SNCA and ubiquitin immunoreactivity and showed the typical morphology of Lewy bodies with a dense core and a lighter halo. Given that the grafted cells were only 10–15 years old, it was felt that an independent autonomic process could not be implicated but rather the possibility of spread through a prion-like mechanism was raised [53]. However, in contrast to prion diseases like Creutzfeldt–Jakob disease there is no evidence that misfolded SNCA can be transmitted from one individual to another; thus, it is best described as a prion-like protein.

Inflammation and PD

Inflammation has been increasingly studied as part of the pathophysiology of neurodegenerative diseases. In PD an increase in microglial activation has been shown in the substantia nigra cells that may be a marker of neurotoxicity [54]. Microglia have multiple roles, including immune surveillance and mediating immune responses to pathogens by secreting cytokines, chemokines, prostaglandins, reactive oxygen and nitrogen species, and growth factors. Some of these factors have neuroprotective effects, while others enhance oxidative stress and can trigger apoptosis. Chronic neuroinflammation may reduce the levels of neuroprotective factors, increasing the vulnerability to inflammation induced cell death of substantia nigra neurons [55].

In a rat model created by injecting an adeno-associated virus vector for SNCA into the substantia nigra the control arm showed a significant loss of tyrosine hydroxylase positive (TH+) neurons. However, in rats that were fed a diet rich in spirulina, a blue-green algae, a significantly greater number of TH + cells were preserved. A neuroprotective effect from reducing the inflammatory component associated with microglial association was hypothesized as the mechanism [56]. Telmisartan, an angiotensin I receptor blocker has been shown in animal models to inhibit the microglial inflammatory response and thereby reduce dopaminergic cell death. The neuroprotective effect is believed to be mediated, at least in part, by the activation of peroxisome proliferator-activated receptor gamma (PPAR-γ) [57]. Oral antidiabetic thiazolidinediones have also been shown to exert neuroprotective effects in models of PD. Their antidiabetic effect is due to activation of PPAR-γ, and this may also reduce inflammation and apoptosis, thereby leading to a neuroprotective effect [58]. Pioglitazone and retinoic acid were tested in a rotenone-induced model of PD in rats. Rotenone significantly reduced locomotor activity of the rats and also significantly reduced dopamine levels in the striatum and hippocampus. Pioglitazone, but not retinoic acid, significantly reversed the reduced striatal dopamine level [59]. Isradipine, a dihydropyridine calcium channel blocker (DiCCB), has also been shown to be neuroprotective in preclinical models of parkinsonism. It was suggested that with increasing age, dopaminergic neurons relied more on L-type voltage-gated calcium channels with a pore-forming Cav1.3 (caveolin.3) subunit, making them more vulnerable to toxin-induced injury. Neurons of younger animals used sodium-dependent channels. If Cav1.3 channels in older animals were blocked by isradipine, their neurons reverted to the juvenile form of the channels rendering them less prone to injury [60].

Oxidative Stress and PD

The discovery that MPTP is toxic to nigral dopaminergic cells led to research into the environmental causes of PD and oxidative stress as an underlying mechanism [61]. A higher incidence of PD had been reported in those living in rural areas, and

the observation that paraquat, a weed killer, had structural resemblance to MPTP lent further credence to this hypothesis [62]. Oxidative stress remains a key concept in understanding the pathophysiology of PD [63]. It is believed that free radical production resulting from the enzymatic oxidation of dopamine as well as exposure to external toxins that cause oxidative stress play a role in the causation of the disease and disease progression [64]. In contrast, the observation of a negative correlation between plasma urate levels and disease progression in PD may represent altered antioxidant activity through reduced glutathione levels or the antioxidant and metal complexing properties of urate [65].

Both neuronal and glial sources have been implicated in oxidative stress. The most likely contributor of oxidative stress is believed to be increased free radical formation from the mitochondria. Neurotoxicity of MPTP through its metabolite MPP^+ occurs through the inhibition of complex 1 in the electron transport chain [66]. Mutations in SNCA, parkin, PINK1, DJ-1, and possibly LRRK2 have been associated with altered mitochondrial function. Thus the mitochondria may represent a common target for both genetic and environmental etiologies of PD [67].

Mechanism of Action of PD Medications

Arvid Carlsson in 1957 discovered that depleting dopamine from the brains of rabbits by using reserpine caused them to become slow and rigid, similar to the symptoms of PD. He also found that injecting the rabbits with L-Dopa reversed these symptoms. This eventually led to the acceptance of levodopa as a medication for treating PD [68]. Levodopa is a dopamine precursor that unlike dopamine crosses the blood–brain barrier and is enzymatically converted into dopamine within the brain. This dopamine replacement offers significant symptomatic relief compared to untreated patients with PD. Cotzias was the first to use levodopa successfully in clinical practice. He countered the severe nausea experienced when using levodopa by starting with very small doses and gradually building up the dose. However, even in his initial series of patients dyskinesia was reported to be present and dose failures and motor fluctuations were also reported [69]. The addition of a dopa-decarboxylase inhibitor allowed considerably lower doses of levodopa to be used for the same clinical effect, allowing for reduced peripheral conversion of levodopa to dopamine and thus reducing nausea and other side effects thus cementing the role of levodopa as the mainstay of PD therapy [70]. However, the short half-life of levodopa is associated with long-term side effects including motor fluctuations and dyskinesia. Stimulating the dopamine receptors with long-acting dopamine agonists could potentially alleviate these side effects [71].

The pharmacotherapeutic basis of treatment of the motor symptoms is correction of the underlying dopamine deficit. The gold standard remains levodopa, which has the highest efficacy [72]. The bioavailability of levodopa is increased by combining it with a dopa decarboxylase inhibitor and/or with a catechol-O-methyltransferase (COMT) inhibitor [73]. Dopamine agonists may be used as an alternative and have

a significantly longer half-life but lower efficacy. In early stages the antiviral amantadine may provide adequate symptomatic benefit. Monoamine oxidase type B (MAO-B) inhibitors extend the duration of dopamine in the synaptic cleft and may also be used in early PD as monotherapy. Anticholinergics have been used based on the idea that there is a relative excess of acetylcholine, but their propensity to cause cognitive side effects limits their use [74].

Levodopa

Dopamine does not adequately cross the blood–brain barrier; hence, direct replacement is not possible. However, the dopamine precursor levodopa can cross the blood–brain barrier where it is enzymatically converted into dopamine. A significant amount of levodopa is metabolized in the periphery to dopamine. Carbidopa reduces this peripheral conversion, improving levodopa delivery to the brain and reducing peripheral side effects of dopamine such as nausea and orthostatic hypotension. The half-life of levodopa is increased from approximately 50 to 90 min by this combination. Thus, levodopa is rarely prescribed by itself but is used in the form of a combination tablet of carbidopa/levodopa (CD/LD) or Sinemet [75].

CD/LD is available in dosages of 10/100, 25/100, and 25/250 mg tablets. The initial target dose of CD/LD is generally one 25/100 mg tablet three times per day. It is advisable to initiate CD/LD slowly, starting with one-half of a 25/100 mg tablet twice a day for 1 week and then increasing by one-half tablet daily until symptoms are well controlled.

A controlled-release formulation of CD/LD (Sinemet-CR) is available in doses of 25/100 and 50/200 mg. This formulation is generally started with 25/100 mg/day and increased to 25/100 mg three times per day or 50/200 mg twice a day. Controlled-release preparations are not as well absorbed, and the bioavailability is 20–30 % lower than standard preparations [76]. CD/LD is also available in an orally disintegrating tablet (Parcopa). This formulation is available in the same strengths as immediate-release CD/LD and has similar bioavailability, safety, and efficacy. It is particularly useful in patients with swallowing difficulties [77].

Common acute adverse effects with CD/LD include nausea, vomiting, drowsiness, and orthostatic hypotension. Other side effects include diaphoresis, cardiac arrhythmias, and pedal edema. Cognitive side effects include confusion, vivid dreams, and hallucinations. The long-term use of CD/LD is associated with the development of dyskinesia and motor fluctuations, which are discussed below [78].

Dopamine Agonists

Pramipexole (Mirapex) and ropinirole (Requip) are the nonergot dopamine agonists in current use as oral agents. Older ergot derivatives such as bromocriptine and pergolide have long-term side effects such as cardiac valve damage and

retroperitoneal fibrosis and have fallen out of use. Apomorphine (Apokyn) is a post-synaptic nonergot dopamine agonist available as an injectable preparation mainly used as rescue therapy during "off" episodes. Rotigotine (Neupro) is a nonergot dopamine agonist formulated as a transdermal patch.

Unlike the direct replacement of dopamine by levodopa, dopamine agonists work by stimulation of the post-synaptic dopamine receptors. The oral agents have a significantly longer half-life than levodopa of around 6–8 h. This more continuous stimulation of the dopaminergic receptors may play a role in reducing the incidence of dyskinesia and motor fluctuations compared to levodopa [79]. However, while improvement in motor symptoms is based on their effect on D2 receptors in the basal ganglia, there is a relatively high affinity for D3 receptors as well, increasing the tendency to cause cognitive side effects such as excessive daytime somnolence (EDS), hallucinations, and compulsive behavior including gambling [80].

Pramipexole (Mirapex) is approved for use as monotherapy and adjunctive therapy in PD. Pramipexole acts on the D2, D3, and D4 dopamine receptors and has a half-life of 8–12 h. It reaches peak drug plasma concentration in approximately 2 h. It is excreted mostly unchanged in the urine. Pramipexole is initiated at 0.125 mg three times per day and increased over several weeks to a maximum dose of 1.5 mg three times per day [81]. An extended-release form of pramipexole (Mirapex ER) is also available that allows once-daily dosing [82].

Ropinirole (Requip) is approved for both monotherapy and adjunctive therapy in PD. It has affinity for the D2 dopamine receptors and no effect on the D1 or D5 dopaminergic receptors. The plasma half-life of ropinirole is approximately 6 h, with peak drug plasma concentrations occurring in 1–2 h. Ropinirole is initiated at 0.25 mg three times per day and increased over several weeks to a maximum dose of 8 mg three times per day [83]. An extended-release formulation, ropinirole (Requip XL), is also available, allowing once-daily dosing [84].

Rotigotine (Neupro) is a dopamine agonist that is available as a transdermal preparation and is approved both as monotherapy and as an adjunct to levodopa. Rotigotine for monotherapy is initiated at 2 mg/24 h and titrated weekly up to 6 mg/24 h [85]. When used as an adjunct to levodopa in patients with motor fluctuations, rotigotine may be started at 4 mg/24 h and titrated weekly up to 8 mg/24 h [86]. Rotigotine is generally well tolerated, with the most common adverse events being application-site reactions, gastrointestinal disturbances, somnolence, and headache. Application-site reactions are generally mild to moderate in severity. However, up to 3 % of patients had severe skin reactions [87].

Apomorphine (Apokyn) is approved for advanced PD as a rescue therapy for severe off periods and is available as a subcutaneous injection. It is a fast-acting, injectable dopamine agonist. It is rapidly absorbed in 10–60 min. However, the half-life of approximately 40 min results in an effect that lasts for only up to 90 min. It can be given every 2 h up to five times per day. A test dose of 2 mg (0.2 mL) is given in the physician's office, and the dose is titrated by 0.1-mL increments up to a maximum single dose of 0.6 mL [88]. Premedication with an anti-nausea medication, usually trimethobenzamide (Tigan), is required because apomorphine can cause severe nausea.

MAO Inhibitors

Metabolism of dopamine within dopaminergic terminals by MAO-B shortens the effect of dopamine. In addition, this metabolic pathway creates oxygen radicals including O_2^- and H_2O_2 that may potentially accelerate the death of dopaminergic neurons. MAO-B inhibitors are a treatment option for PD.

Selegiline (Eldepryl) is approved as an adjunct treatment to levodopa; however, it may also be used as monotherapy in early disease. The typical dose is 5 mg with breakfast and lunch. An orally disintegrating form of selegiline (Zelapar) is available in the strength of 1.25 mg. It is approved for use in advanced PD with motor fluctuations. The initial dose is 1.25 mg a day, which can be increased to 2.5 mg per day if clinically indicated. By the avoidance of first-pass metabolism, it results in higher concentrations of selegiline and lower concentrations of its metabolites compared with the 5-mg swallowed selegiline tablet [89].

Selegiline is generally well tolerated. The most common adverse effects include nausea, dizziness, insomnia, confusion, hallucinations, dry mouth, and orthostatic hypotension. It can lead to increased dyskinesia when used as an adjunct to levodopa therapy. As the dose of selegiline is increased, its selectivity to inhibit MAO-B is decreased, and inhibition of MAO-A can also occur; thus, it is important to not to increase the dose beyond 5 mg twice a day [90].

Rasagiline is an irreversible MAO-B inhibitor. It reaches peak plasma concentrations in approximately 1 h and has a half-life of approximately 3 h. However, since it irreversibly inhibits MAO-B, its therapeutic benefit is independent of its half-life. It is approved as monotherapy [91] in early disease at a dose of 1 mg/day, and in PD patients with motor fluctuations on levodopa starting at 0.5 mg/day, which can be increased to 1 mg/day [92].

Commonly observed adverse events with rasagiline monotherapy are arthralgia, depression, and gastrointestinal side effects. As an adjunct to levodopa, the common adverse effects included worsening of dyskinesia, weight loss, postural hypotension, arthralgia, gastrointestinal side effects, somnolence, and paresthesia. Unlike selegiline, rasagiline does not have amphetamine metabolites [93].

COMT Inhibitors

COMT inhibitors inhibit the action of catechol-O-methyl transferase, one of the metabolic pathways for the metabolism of levodopa. Tolcapone and entacapone are two COMT inhibitors used in the treatment of PD. The COMT inhibitor entacapone when given with CD/LD increases the area under the curve of levodopa by about 35 % and prolongs the half-life of levodopa to about 2.4 h while leaving the average peak levodopa plasma concentration unaffected, thus reducing the risk of peak-dose side effects [94].

Entacapone (Comtan) is approved for the management of motor fluctuations in PD. Its half-life is 0.4–0.7 h. It reduces the peripheral metabolism of levodopa, thereby increasing the half-life of levodopa to about 2.4 h. It is initiated at 200 mg with each dose of levodopa for a maximum of eight doses per day [95]. The common side effects of COMT-inhibitors are mostly related to increased dopaminergic stimulation. Dyskinesia, nausea, vomiting, and hallucinations are the most commonly seen dopaminergic adverse effects. These may be reduced by decreasing the levodopa dose. There is no known hepatotoxicity associated with entacapone and liver enzyme monitoring is not required. Diarrhea as an adverse effect usually begins at 6–12 weeks but can appear as early as 2 weeks after entacapone is started. If the diarrhea is bothersome, therapy must be discontinued. Urine discoloration is a harmless side effect that occurs in less than 10 % of patients [96].

The triple combination of carbidopa/levodopa/entacapone (Stalevo) is available in six different combinations: Stalevo 50 (carbidopa 12.5 mg/levodopa 50 mg/entacapone 200 mg), Stalevo 75 (carbidopa 18.75 mg/levodopa 75 mg/entacapone 200 mg), Stalevo 100 (carbidopa 25 mg/levodopa 100 mg/entacapone 200 mg), Stalevo 125 (carbidopa 31.25 mg/levodopa 125 mg/entacapone 200 mg), Stalevo 150 (carbidopa 37.5 mg/levodopa 150 mg/entacapone 200 mg), and Stalevo 200 (carbidopa 50 mg/levodopa 200 mg/entacapone 200 mg). This triple combination is indicated in PD patients as a substitute for immediate-release carbidopa/levodopa and entacapone previously administered separately [97].

Tolcapone (Tasmar) has a half-life of approximately 2–3 h with maximum plasma concentrations occurring in approximately 2 h. It is initiated at 100 mg three times a day and increased to 200 mg three times a day if needed [98]. Tolcapone should always be used with levodopa. Due to the risk of fatal hepatotoxicity, it may only be used in PD patients who have tried all other antiparkinsonian medications, and serum ALT and AST should be tested at baseline, every 2–4 weeks for the first 6 months, and then as clinically indicated. Tolcapone should be discontinued if the patient does not have a response or in the event of a two times increase in the upper limit of ALT and AST [99].

Anticholinergics and Amantadine

Anticholinergics were used in the treatment of PD before the discovery of levodopa. The basis for using these drugs is that in PD the nigral dopaminergic neurons that inhibit the GABAergic output from the striatum are lost. This allows cholinergic neurons in the striatum to exert an unopposed excitatory effect on these GABAergic neurons with resulting inhibition of the motor system. Anticholinergic drugs can reduce this effect. However, they are poorly tolerated by elderly patients due to their cognitive side effects. Other antimuscarinic side effects such as dry mouth, constipation, and reduced bladder outflow may also occur. Their use is mostly restricted to patients with tremor that is intractable to levodopa treatment [100].

Anticholinergics are generally well absorbed orally and usually require dosing two or three times a day. Anticholinergics most commonly used in the treatment of PD include trihexyphenidyl (Artane) and benztropine (Cogentin). Anticholinergics should be started at low doses and increased very slowly. Contraindications include narrow-angle glaucoma, tachycardia, prostate hypertrophy, gastrointestinal obstruction, and megacolon. Common side effects include blurring of vision, nausea, constipation, urinary retention, and dry mouth. Confusion, hallucinations, psychosis, and sedation may also occur. Central side effects occur more often in the elderly and in patients with impaired cognitive function [101].

Amantadine is a glutamate antagonist that increases release of dopamine at the nerve terminals and has anticholinergic properties. It may be used in early PD, but cognitive side effects may occur due to its anticholinergic properties. Due to its glutamate antagonist properties, it has a role as an anti-dyskinetic agent in later stages of PD [102]. Amantadine is well absorbed orally, with peak blood levels occurring in 2–4 h. It should be avoided in patients with renal failure. The usual dose is 200–300 mg/day in divided doses. Side effects of amantadine include dizziness, anxiety, impaired coordination, insomnia, and nervousness. Nausea and vomiting occur in 5–10 % of patients. In some patients, pedal edema and a type of skin rash called livedo reticularis can require discontinuation of therapy [103].

Management of Motor Fluctuations and Dyskinesia

While the immediate response to levodopa is often dramatic, the long-term use is limited by the development of motor fluctuations. The most common of these are an end-of-dose wearing off with a return of symptoms before the next dose is due, also called the wearing-off effect. The duration of response becomes increasingly shorter and patients who were previously well controlled on three to four doses a day may need a higher frequency of dosing. Some 40–50 % of patients on levodopa monotherapy will experience a degree of motor fluctuations at the 5-year mark [104]. Over time the fluctuations may occur at random with respect to the timing of medications described as the on–off effect. Dose-failures may occur, and the latency to clinical effect may be prolonged [105]. The addition of a COMT inhibitor or a MAO-B inhibitor can increase the duration of action of levodopa, thus allowing for a smoother therapeutic effect. Adding a dopamine agonist as an adjunct therapy may also reduce wearing off, as these drugs have a longer half-life than levodopa [106].

Dyskinesia is another limiting factor in the long-term use of levodopa. These occur in the form of involuntary choreiform movements, usually at the peak of the levodopa dose. However, other forms, including end-of-dose dyskinesia and diphasic dyskinesia, are also seen [107]. The incidence of dyskinesia in patients on CD/LD monotherapy was approximately 40 % at the 5-year mark compared with an incidence of approximately 10 % on ropinirole or pramipexole. Most dyskinesias represent a peak-dose response to levodopa, thus reducing individual doses, and

administering doses more frequently can help reduce dyskinesia. In patients who have dyskinesia, switching from sustained-release formulations of levodopa to regular formulations may reduce the duration of the dyskinesia after any given dose. Amantadine also has a role as an anti-dyskinetic agent [108].

Management of Cognitive and Psychiatric Symptoms

Anxiety and depression are common comorbid symptoms of PD and may even precede the onset of motor symptoms. A wide variety of selective serotonin uptake inhibitors (SSRIs) have been successfully used in the treatment of PD. These drugs must be used with caution when patients are on MAO-B inhibitors. Benzodiazepines may be used in the treatment of anxiety, but long-term use should be avoided if possible [109].

Dementia is a common finding in later stages of PD. Rivastigmine showed a beneficial effect in PD-associated dementia both as an oral preparation and as a transdermal patch. GI side effects are less common with the patch, but skin irritation may be a limiting factor in some cases [110]. PD is also associated with hallucinations, paranoid symptoms, and psychosis in later stages. The dopamine agonists are more likely to have these side effects because they have a relatively high affinity for D3 dopamine receptors present in the limbic system. Traditional neuroleptics such as haloperidol should be avoided because they can cause a marked worsening of motor symptoms of PD due to their dopamine antagonist effects. Of the novel neuroleptics, clozapine and quetiapine have been used with some degree of success, but clozapine requires frequent monitoring for agranulocytosis [111].

Treatment of Nonmotor Symptoms

While PD has been recognized as a movement disorder, its nonmotor symptoms have been generally less well recognized though documented in the literature. James Parkinson himself described sleep disorders, constipation, urinary incontinence, and delirium in his seminal essay. Nonmotor symptoms can occur at any stage of the disease including early PD and can even be a marker of the disease state prior to development of motor symptoms, the so-called premotor phase of the disease [112].

In addition to the cognitive and psychiatric symptoms discussed above, hyposmia, disturbances of sleep–wake cycle regulation, and features of autonomic dysfunction, including orthostatic hypotension, urogenital dysfunction, and constipation, are commonly seen [113]. There is no specific treatment for hyposmia. Sleep–wake cycle disorders in PD include insomnia, EDS, and REM sleep behavior disorder (RBD) [114]. Modafinil was shown to be helpful in treating EDS in a small clinical trial [115]. Attention to good sleep hygiene is important in

addressing insomnia. Quetiapine can be helpful in insomnia in the setting of PD; however, long-term use should be approached with caution [116]. Treatment options for RBD include benzodiazepines and melatonin. Clonazepam is the preferred benzodiazepine as it is long-acting and lasts through the night. It can also help reduce off-state dystonia that may occur through the night as the dopaminergic medications wear off. The mechanism of action of clonazepam may include controlling phasic locomotor activity at the brainstem level and modifying dream content in REM sleep [117].

Orthostatic hypotension when occurring soon after diagnosis raises suspicion of a parkinson-plus syndrome such as MSA. However, it is common and can have a significant adverse impact on the quality of life in the later stages of PD [118]. Management includes non-pharmacological measures such as increasing salt in the diet and pressure stockings. Fludrocortisone can also be helpful in this setting [119]. Midodrine has been shown to be helpful in neurogenic orthostatic hypotension and can also be helpful in the PD setting [120]. Botulinum toxin for sialorrhea, sildenafil for erectile dysfunction, and lubiprostone and probiotics for constipation are other suggestions for the management of these troubling non-motor symptoms. There is a great need for well-designed clinical trials to allow firm evidence-based recommendations for non-motor PD symptoms [121].

Neuroprotective Strategies

There is no drug with a proven neuroprotective effect in PD. Neuroprotective strategies are generally aimed at reducing oxidative stress or improving handling of free radicals through the use of antioxidant molecules. The DATATOP trial, one of the earliest studies of neuroprotection, compared selegiline with high-dose vitamin E in PD. The endpoint was the delay in the need for symptomatic treatment with levodopa. While vitamin E was no better than placebo, patients on selegiline required levodopa about 6 months later. Subsequent studies, however, indicated that this was due to a mild symptomatic benefit of selegiline rather than a true neuroprotective effect [72]. The ADAGIO trial used a delayed start design with placebo compared to patients started on rasagiline immediately or 9 months after the baseline visit. All groups were followed for a total of 18 months from the baseline visit. While the 1 mg dose of rasagiline met the hierarchical statistical criteria, the 2 mg dose failed to do so. Possible explanations for failure of the 2-mg doses that were proposed include a U-shaped dose–response effect and the presence of a "floor effect" on the rating scale used in the study, with patients with the mildest disease failing to show benefit. A post-hoc analysis did reveal a statistically significant difference in the most severely affected quartile even on the 2-mg dose [122]. Coenzyme Q10 was hypothesized to have a neuroprotective effect due to its role as an antioxidant and free radical scavenger. However, clinical trials have failed to support any neuroprotective role for Coenzyme Q10 in PD [123].

Surgical Treatment of Parkinson's Disease

With the advent of levodopa, the surgical treatment of PD receded into the background for some years. However, with time the limitations of levodopa treatment in the long-term, including motor fluctuations and dyskinesia, led to the recognition that surgical treatment could play a role in selected cases. Thalamotomy helped control intractable tremor, and pallidotomy helped reduce severe dyskinesia. The discovery that high-frequency stimulation of the surgical targets could control symptoms while offering a relative degree of reversibility in case of suboptimal target localization led to a resurgence of surgical treatment for PD. Deep brain stimulation (DBS) has become part of standard of care in selected patients in the later stages of PD [124].

The initial targets for DBS surgery were based on the experience of lesioning techniques of thalamotomy and pallidotomy. The corresponding targets included the ViM nucleus of the thalamus and the internal segment of the globus pallidus (GPi). The ViM was found to be an excellent target for control of tremor from PD; however, its impact on other features was limited. The GPi was found to be a good target in patients with troublesome dyskinesias [124]. The subthalamic nucleus (STN) was also explored as a surgical target and was found to be of benefit in control of overall symptoms of PD as well as allowing for a reduction in medication dosing to a somewhat greater extent than with GPi DBS. The current consensus appears to be that both GPi and STN are viable targets for overall control of PD symptoms including tremor. The choice of target is usually determined by institutional preferences. Alterative targets have included the pedunculopontine nucleus for patients with prominent freezing of gait. However, clinical experience is limited for alternative targets [125].

Appropriate patient selection is critical with respect to both the type of parkinsonism and duration and severity of the disease. Only patients with idiopathic PD are suitable candidates and those with parkinson-plus syndromes should be excluded. Appendicular symptoms such as tremor and dyskinesia improve more than axial symptoms such as gait and balance. In general, it is difficult to improve symptoms beyond the level seen in the patient's best "on-state" prior to surgery. There is approximately a 2 % risk of hemorrhage that can lead to greater disability or even death. Hence, surgery should only be offered to patients who have either significant motor fluctuations or specific symptoms such as tremor or dyskinesia that are intractable to medication adjustments. On the other hand, surgery should not be offered too late in the course of the disease when both physical debilitation and cognitive symptoms can play a limiting role preventing a successful outcome [125]. Alternative surgical therapies such as stem cell implantation, nerve growth factor infusion, and gene therapy are not part of current clinical practice, although research is ongoing both at the basic science level and in the form of early clinical trials [126].

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Neuropathology of Parkinson's Disease

Kurt A. Jellinger

Introduction

Parkinson's disease (PD) (OMIN 168600), or "brain stem type of Lewy body disease," is the most frequent neurodegenerative movement disorder in advanced age, affecting 1–3 % of the population over 65 years and an estimated seven to ten million people worldwide [1], which will considerably increase in the future [2]. It is characterized by progressive degeneration of the dopaminergic nigrostriatal system, responsible for the core motor symptoms, and by involvement of many other neuronal networks and extraneuronal organs. These lesions are associated with widespread deposition of phosphorylated α-synuclein (α-syn), the major protein marker and biological hallmark of PD and other synucleinopathies [3, 4], as well as intracytoplasmic Lewy bodies (LB), dystrophic neurites (LN) in cell processes, and other inclusions. Multifocal neurodegenerative lesions are affecting the central, peripheral, and autonomic nervous system and many other organs (e.g., adrenals, retina, heart, skin) [5–11]. Recent studies have confirmed the multiorgan distribution of α-syn and Lewy pathologies [5, 12–15]. There is early involvement of the peripheral autonomic system, spinal cord, and adrenal medulla in neurologically unimpaired subjects (incidental Lewy body disease, or ILBD) considered as initial or preclinical stage of PD [16, 17]. In sporadic PD, the pathologic process initially involves preganglionic neurons, plexuses, and nerves, particularly in the gastrointestinal system [18–23] and is suggested to advance caudo-rostrally from there along the neuraxis [15, 24, 25]. The resulting biochemical deficits cause the heterogeneous clinical picture of PD including the core features—tremor at rest, rigidity, akinesia (or bradykinesia), and postural instability (TRAP)—and often early presenting nonmotor deficits [26–31]. However, PD can present with a variety of additional clinical features and complications in advanced stages [32].

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M. Thomas (ed.), *Inflammation in Parkinson's Disease: Scientific and Clinical Aspects*,
DOI 10.1007/978-3-319-08046-8_2,

Various clinical phases and subtypes have been described [33–35], and the neuropathology underlying clinical variability in PD and other synucleinopathies has been reviewed [13, 36, 37].

Recent research has improved the clinical diagnostic criteria of PD [38–40] for which histopathological confirmation following new criteria using standardized methods of assessment of lesions by a semiquantitative grading system and immunohistochemical methods for the detection of α-syn/Lewy pathology are required [36, 41–43].

The etiology and pathogenesis of PD and other synucleinopathies are poorly understood, but the majority of PD cases may result from complex interactions between environmental factors and genetic background [44]. There is increasing evidence that the neurodegenerative process in PD is caused by a combination of complex mechanisms including dysregulation of mitochondrial bioenergetics and of calcium homeostasis, impaired protein turnover control, prion-like behavior of misfolded proteins, oxidative and nitritive stress, neuroinflammation and their complex interaction (for review, see [45, 46]).

This chapter will critically update the neuropathology of "sporadic" PD, formation and development of Lewy pathology including new guidelines, its relations with α-syn deposition, and their clinical relevance. Furthermore, it will discuss the spreading and grading of α-syn and its interaction with other pathologic proteins, clinicopathologic subtypes of PD, and major factors in the pathogenesis of PD. Several recent reviews of the neuropathology of sporadic PD [13, 47–49] and of genetic PD are available [50, 51].

Basic Neuropathology of PD

Degeneration of the Nigrostriatal System

The brain is usually grossly unremarkable or shows mild frontal atrophy and enlargement of the ventricles. There is no significant atrophy of the brain stem, which differs from progressive supranuclear palsy (PSP) and multisystem atrophy (MSA). The brain stem usually shows loss of the normally dark black pigment in the substantia nigra (SN) and locus coeruleus (LC).

Histopathology of PD is featured by widespread α-syn-immunoreactive deposits in neurons (Lewy bodies [LBs]) and dystrophic neurites (LNs) in nerve processes, as well as within the synaptic compartment, enriched in the presynaptic terminals [52, 53] throughout the CNS. It is associated with variable neuron loss in many subcortical nuclei, in particular the substantia nigra compacta (SNc), LC, nucleus basalis of Meynert (NBM), and dorsal motor nucleus of vagus (dmX), but also affecting many other neuronal systems. Severe depletion of melanized and dopaminergic neurons immunoreactive for tyrosine hydroxylase (TH), the key enzyme of dopamine synthesis, affects the A-9 group of SNc. It is most severe in the caudal and ventrolateral tier (about 98 %), projecting to the striatum, than in the matrix, and spreading along a caudorostral, lateromedial, and ventrodorsal progression [54].

This temporospatial disorder corresponds to a somatotopic pattern of dopaminergic terminal loss in the striatum that is more severe in the dorsal and caudal putamen, with later involvement of the ventral putamen and caudate nucleus [55].

Degeneration of the nigrostriatal system causes denervation of the striatum with dopamine loss, ranging from 44 to 98 % [56]. In earlier disease stages, dopamine depletion provokes an increased number of striatal dopaminergic neurons [57], which may be the result of a phenotypic shift and not neurogenesis [58]. Similar changes in the olfactory bulb could reflect a compensatory mechanism [59] that may be more efficient in younger PD patients [60], while MRI studies showed early volume changes in the striatum [61]. Higher nigrostriatal dopaminergic neuron loss occurs in early-onset than in late-onset PD [62]. Longitudinal PET studies identified dopamine dysfunction approximately 10 years before disease onset in older PD patients, which may be present for as long as 25 years in younger ones. Preclinical intervals thus vary depending on the age of onset. Younger PD patients are able to endure more damage to the dopaminergic system before the onset of motor symptoms.

The degree of area A-9 SNc cell loss and the resulting progression of striatal dopaminergic hypofunction (reduction of dopa uptake, TH and dopamine transporter [DAT] immunoreactivity), indicating the extent of loss of dopaminergic neurons and terminals in the striatum, are strongly correlated with disease duration and the severity of motor dysfunction [63–65]. Relative preservation of F-DOPA (fluorodopa) uptake in the anterior striatum may reflect a delay in pathologic involvement of the striatonigral projections, while loss of monoaminergic function in extranigral regions is delayed and occurs independently from striatonigral dysfunction in PD [64]. SN volume loss has been observed before basal forebrain degeneration in early PD [66]. Patients with end-stage PD showed a massive loss of SN neurons with significant atrophy of the remaining cells (20 % of controls) [67]. However, recent SPECT studies indicated that degeneration of the dopaminergic system is not total, even after many years of illness, the loss being more prominent in the putamen than in the caudate nucleus [68]. DAT immunoreactivity in the striatum is inversely correlated with the total α-syn burden but not with LB counts in the SN [69], which supports the concept of synaptic dysfunction or impairment of axonal transport of α-syn aggregation. Alterations in axonal transport proteins in sporadic and experimental PD have been confirmed [70].

Morphometric studies have shown a 35–40 % reduction in pigmented SN cells, with severe loss of DAT-immunoreactive neurons in older persons, with an estimated SN cell loss between 4.3 % and almost 10 % per decade [71, 72]. Stereologic studies of the human SN revealed a significant loss of pigmented (28.3 %) and TH + neurons (36.2 %) in older versus younger controls, with hypertrophy of cells in older subjects as a compensatory mechanism to allow normal motor function despite cell loss [72]. SN cell degeneration is preceded by loss of neurofilament proteins, neurofilament mRNA, neuronal TH and DAT immunoreactivity, and cyclooxygenase (COX) indicative of functional neuronal damage [73]. Neuronal loss is accompanied by extracellular release of neuromelanin with uptake by macrophages, rare neuronophagia, and astroglial reaction [74]. Microglial activation and corresponding dopaminergic terminal loss in the affected nigrostriatal system suggest that neuroinflammatory reactions contribute to the progressive

degenerative process [75–80]. α-Syn has an important role in the initiation and maintenance of inflammation in PD [81], which is a double-edged sword that is protective in the early stage but becomes detrimental with disease progression [76].

Lewy pathology first appears in the ventrolateral parts of the SN and spreads to the paranigral nucleus, to the medial part, and, finally, to the dorsal tier of the SNc [82]. The proportion of LB-bearing and α-syn-positive SN neurons does not correlate with disease duration and is apparently stable over time, with 3.6 % of the neurons being involved on average, suggesting that during the course of the disease, the destruction of LBs may be equal to their production and that they are destroyed together with the afflicted neurons. With LB-bearing neurons having an estimated life span of around 6.2 months (15.9 months for any type of α-syn inclusion), neuronal loss of 71 %, necessary for the manifestation of motor symptoms, would be reached after about 20 years [83], which is in line with estimated standard progression of the disease. At the time of motor symptom onset, the extent of striatal dopamine marker loss exceeds that of dopaminergic SN neurons. Of note, the previous concept that PD motor symptoms first appear which more than 50 % of dopaminergic SN neurons are lost [65] has recently been changed by the finding that at the time of first diagnosis of PD, only around 30 % of dopaminergic SN neurons, but 50–60 % of their axon terminals have been lost [84]. This is preceded by substantial loss of dopamine markers in the nigrostriatal terminals in early phases of PD, while melanin-containing SN neurons more than TH+ cells may persist for a longer time [85].

Neuronal Vulnerability

That multiple neuronal systems are affected by α-syn/Lewy pathology is largely undiscussed. How these multiple systems become involved over time and the reasons for only certain neurons within different brain regions becoming affected is still a matter of conjecture and has been discussed [15, 86].

The neurodegenerative lesions in PD show a selective vulnerability of midbrain neurons rich in neuromelanin located in the densely populated ventral tier of the SNc, which are rich in DAT but poor in glycolytic enzymes and calbindin, a calcium-binding protein, which has a neuroprotective role by buffering effects of Ca^{2+} influx into cells. All are projection neurons with a long, thin axon that is unmyelinized or poorly myelinated. The selective vulnerability of A-9 nigral neurons may be related to increased iron content, making them susceptible to oxidative stress [87–92]. In later stages of degeneration, SN neurons show a significant reduction in intracellular pigment, whereas those of normal morphologic appearance exhibit increased pigment density associated with higher concentrations of α-syn. No such changes have been observed in other melanin-containing neurons in the A-10 areas in early PD, which emphasizes the selectivity of early neuromelanin changes in A-9 neurons [93]. Iron regulates α-syn expression at the translational level [94] and promotes α-syn aggregation [95]. Overexpression of α-syn by cells increases iron (II) levels [96], leading to their selective loss [97]. Increased concentrations of α-syn around

pigment-associated lipid under oxidative conditions may trigger a cascade of events leading to intracellular aggregates of α-syn and dispersal of protective pigments to precipitate cell death [89, 93, 98].

SNc neurons in PD show reduced expression of brain-derived neurotrophic factor (BDNF) and reduced numbers of TrkB mRNA (a high-affinity BDNF-receptor) without a decrease in the remaining cells [99]. While biochemistry reveals no reduction of glial cell line-derived neurotrophic factor (GDNF) in the nigrostriatal regions in PD [100], immunohistochemistry showed a 20 % reduction of GDNF and loss of BDNF in both neurons and neuropil [101], but increased numbers of BDNF- and neurotrophin-3 (NT-3)-immunoreactive microglia around damaged neurons [102].

Selective increase of mitochondrial DNA deletions/rearrangements involves the SN but also other regions of the PD brain compared with age-matched controls, indicating that mitochondrial dysfunction is not limited to the SN [103]. This suggests that dopaminergic SN neurons are more dependent on mitochondrial energy metabolism and oxidative phosphorylation than other brain stem populations. Reduction of cerebral mitochondrial metabolism occurs early in PD, but whether it is a primary or secondary event remains to be elucidated [104].

Conversely, most dopaminergic neurons that resist degeneration in PD are located in the scantly populated dorsal tier of SNc and contain calbindin and glycolytic enzymes but are poor in DAT, therefore being involved only in terminal stages of PD [105, 106]. The A-10 group of dopaminergic neurons—ventral tegmental area, nucleus parabrachialis, and nucleus parabrachialis pigmentosus—projecting to the striatal matrix, thalamus, cortical, and limbic areas (mesocorticolimbic system), shows less severe involvement (40–50 % cell loss) [89], whereas the retrorubral A-8 region, which contains only a few dopaminergic but calbindin-rich neurons, and the central periventricular gray matter show little or no degeneration [107]. Cell depletion in these nuclei does not correlate with the duration of disease. The minor vulnerability of other populations, e.g., dopaminergic hypothalamic neurons [108], has now been confirmed by recent findings of increased hypocretin (orexin) cell loss in PD [109, 110].

Other neurons that remain unaffected by Lewy pathology such as the large Betz cells of the motor cortex, the large Meynert cells of the visual cortex, the relay nuclei of the thalamus and subthalamus, brain stem, and spinal neuronal groups (motor neurons of the medulla oblongata and dorsal column nuclei) have heavy myelinated axons [86], but whether such neurons have susceptibility factors [105, 111] remains to be determinated. Of note, the neuronal populations susceptible to PD are similar to those involved in DLB, with Lewy pathology more restricted to the amygdala in many patients with AD [36, 112]. Such different regional susceptibility to similar pathological processes needs to be answered.

Multiorgan Distribution of α-Synuclein and Lewy Pathologies

It has been known for many years that LBs in PD extend well beyond the SN [113]. α-Syn pathology is not restricted to specific dopaminergic brain stem nuclei but involves non-nigral nuclei [113–116], the olfactory bulb, and related olfactory

nuclei in the brain (amygdala, perirhinal cortex), suggesting that olfactory dysfunction frequently occurring in PD is related to involvement of the central olfactory pathways rather than from peripheral sensory nerve fibers [117, 118]. α-Syn pathology further involves parts of the spinal cord [19, 86, 119, 120] and multiple areas of the autonomic and peripheral nervous system, sympathetic and parasympathetic ganglia and plexuses, the intramural enteric nervous system, the skin, the retina, the submandibular gland, the cardiac nervous system, and other visceral organs [6–8, 10, 11, 22, 23, 117, 121–128]. The greatest densities and frequencies of α-syn pathology occur in the spinal cord, followed by the paraspinal sympathetic ganglia, vagus nerve, gastrointestinal tract, and endocrine organs, with negative involvement of the musculoskeletal system and major parts of sensory components of the nervous system including the sciatic nerve [36, 43, 129]. Despite early involvement of the enteric nervous system, there is no gastrointestinal myenteric or ganglion cell loss in PD [130]. Early involvement is seen in the peripheral autonomic system and adrenal medulla in neurologically unimpaired subjects (ILBD) [16, 17, 131, 132], as well as in cutaneous nerves [132a]. Diminished TH-immunoreactivity is seen in the cardiac conduction system and myocardium in both ILBD and PD [17, 125]. Early involvement of the olfactory system related with local inflammation leads to olfactory neuronal dysfunctions causing early olfactory impairment, α-syn aggregation, and spreading through the olfactory network to the brain that may contribute to PD initiation and progression [133]. Since α-syn internalized by neurons can be transported in neurons, but not by trans-synaptic transfer [134], affection of the autonomic nervous system and gastrointestinal tract before involvement of the CNS has forwarded the hypothesis of another possible route for spreading α-syn via the vagus nerve to the brain [6, 25].

Development of α-Synuclein/Lewy Pathology

Biochemical increase of phospho-α-syn precedes α-syn aggregation, which is followed by the formation of LBs and LNs [14, 135], but it does not necessarily correlate with Lewy pathology [135, 136], the latter showing an inconsistent relationship to clinical disease progression [137]. Loosely packed α-syn filaments as premature "pale neurites" are initiated at axon collaterals and extend centripetally into proximal segments [138]. These changes and the formation of "pale bodies," rounded areas of granular, pale-staining eosinophilic material displacing neuromelanin, in brain stem neurons precede the development of LBs [139]. The early intra-axonal aggregation of α-syn could damage the parental neurons by interfering with axonal transport [138, 140], but it is still unclear why some types of neurons seem more predisposed to developing axonal inclusions compared with perikaryal LBs.

Recent studies showed lower neuron densities in the SN before LB deposition in the nigrostriatal system, suggesting that neurodegeneration and cellular dysfunction precede Lewy pathology and supporting a dying-back mechanisms in neuronal α-syn pathology, in which dysfunction starts at the synapse and leads to axonopathy as the hallmark of presymptomatic and early-stage PD followed by neuronal

degeneration [70, 141]. More than 90 % of α-syn aggregates are present as small deposits in presynaptic terminals of the affected neurons. This is considered a key event in the pathogenesis of synucleinopathies, but the responsible mechanisms are poorly understood. Dysfunction of the soluble N-ethylmaleimide-sensitive factor activating protein receptor (SNARE) leading to accumulation of α-syn in nerve terminals is suggested to occur in an early stage of neurodegenerative changes in the nigrostriatal system [141a], which appears to be a downstream event associated with abnormal accumulation of α-syn.

A BAC (Bacterial Artificial Chromosome) mouse model, created with the most frequent disease-causing human mutant (LRRK2/R1441G/) recapitulating cardinal features of human PD, showed no loss of mesencephalic dopaminergic neurons, but diminished dopamine release and axonal pathology of nigrostriatal projections [142]. Another autosomal recessive rat model featured by overexpression of α-syn and neuronal loss in the SN, degeneration of the striatonigral dopaminergic pathway, and reduced dopamine in the striatum revealed neuronal degeneration of the "dying-back" type, providing a naturally occurring animal model for PD [143]. Enlarged vGlut1-positive nerve cells of SNAP-25 mice showed concomitant accumulation of α-syn and p-α-syn triggered by presynaptic dysfunction [143a]. Assessment of the relationship between TH immunoreactivity as marker of nigral dysfunction and LB burden in the SN of ILBD cases suggests that Lewy pathology is not the first sign of degeneration in vulnerable neurons in PD and ILBD [144]. This data and the demonstration of small α-syn aggregates in presynaptic terminals in PD and DLB suggest that synaptic dysfunction and axon degeneration, not nerve cell loss, may be the primary determinant of progression of the neurodegeneration in PD [85, 145]. Loss of neurons might be an epiphenomenon after the loss of synapses, defining PD as a "synaptopathy" [53, 145].

The presence or absence of abnormal immunostaining for α-syn cannot be interpreted as evidence that the cell suffers from or is free of dysfunction related to abnormal protein deposition [69]; immunostaining for α-syn in pigmented SN neurons could also reflect a successful response to proteolytic stress. Reduced TH immunoreactivity in neurons early associated with α-syn accumulation may represent a cytoprotective mechanism [146], decrease of dopamine synthesis causing a reduction of cytotoxic α-syn oligomers [147], but it can also be preserved in neurons with α-syn accumulation [46, 69]. Evidence for the formation of soluble pathologic forms of α-syn in the regions with progressive neuronal loss in PD is currently lacking, since normal levels in the cytosolic fraction and no correlations with nigral LB intensity have been found. No widespread extranigral α-syn accumulation as suggested by immunohistochemical reports has been confirmed by neurochemical methods demonstrating only mildly increased α-syn in putamen [148, 149]. However, PD brains show a significant increase in soluble and lipid-associated phospho-α-syn over the disease course, with progressive decrease of soluble α-syn but no corresponding decrease in α-syn mRNA levels [150].

The mechanism that causes conformational changes of α-syn includes phosphorylation at Ser 129 (promoting fibril formation in vitro [151], C-terminal truncation, and ubiquitination [152, 153]). Changes in properties of serine 129 phosphorylated α-syn occur with progression of Lewy pathology in human brains [153a]. In PD

brain, tissue transglutaminase (tTG)-induced cross-links have been identified in α-syn monomers, oligomers, and aggregates, suggesting an interaction between α-syn and tTg [154], and α-syn aggregation is promoted by increased calcium [155]. Reduction of proteasome activity may be involved as a cellular defense mechanism against dopaminergic neuronal death [156]. The presence of numerous α-syn deposits in younger-onset patients with long disease duration and LB distribution consistent with Braak staging [157] suggests that neurons harboring inclusions may remain viable in the tissue for a long time. On the other hand, serine 129 phosphorylation of α-syn as its dominant modification in LB disorders induces unfolded protein-mediated cell death [158]. Although the duration and severity of motor dysfunction and the corresponding decrease of dopamine, DAT, and vesicular monoamine transporter-2 (VMAT2) in the striatum are negatively correlated with total SN α-syn burden and neuronal loss [65, 159, 160], the latter shows no correlation with LB formation [69]. No relationship between LB stage and both clinical severity of PD (Hoehn and Yahr score) and age at death was found [137]. On the other hand, many studies have shown higher LB densities in PD patients with later disease onset and shorter disease duration [36, 43, 48, 161–163], consistent with a more rapid disease course with aging. This may be associated with an increased amount of normal cellular α-syn with age [164].

Structure and Molecular Components of Lewy Bodies

Lewy bodies occur in two types. *Classic LBs* are spherical cytoplasmic intraneuronal inclusions 8–30 ym in diameter with a hyaline eosinophilic core, concentric lamellar bands, and a narrow pale-stained halo, forming single or multiple inclusions (Fig. 1a). In some brain regions, such as the dmX, similar inclusions within neural processes are intraneuritic LBs. They can be detected in routine histological preparations and should be distinguished from LNs, which are not visible on routine histology. Ultrastructurally, LBs are non-membrane-bound, granulofilamentous structures composed of radially arranged 7–20 nm intermediate filaments associated with electron-dense granule material and vesicular structures, with the core showing densely packed filaments and dense granular material and the periphery having radially arranged 10 nm filaments (see Fig. 1b) [165]. *Cortical LBs* are eosinophilic, rounded, angular, or reniform structures without a halo (see Fig. 1c). They present invariable numbers in almost all cases of PD [157]. Ultrastructurally, they are poorly organized granulofibrillary structures with a feltlike arrangement composed of 7–27 nm wide filaments, mostly devoid of a central core [166, 167]. They are found in small nonpyramidal neurons in the lower cortical layers, with densest accumulation in the insular cortex, amygdala, and parahippocampal and cingulate gyri [168].

α-Syn as the best marker to decorate LBs and LNs has replaced that for ubiquitin as the preferred method for detecting these inclusions. Antibodies that preferentially recognized N-terminal epitopes (Syn 505, 506, and 514) selectively detect α-syn, consistent with the conformational changes associated with its polymerization into amyloid fibrils that form pathologic inclusions [169]. α-Syn adopts a three-

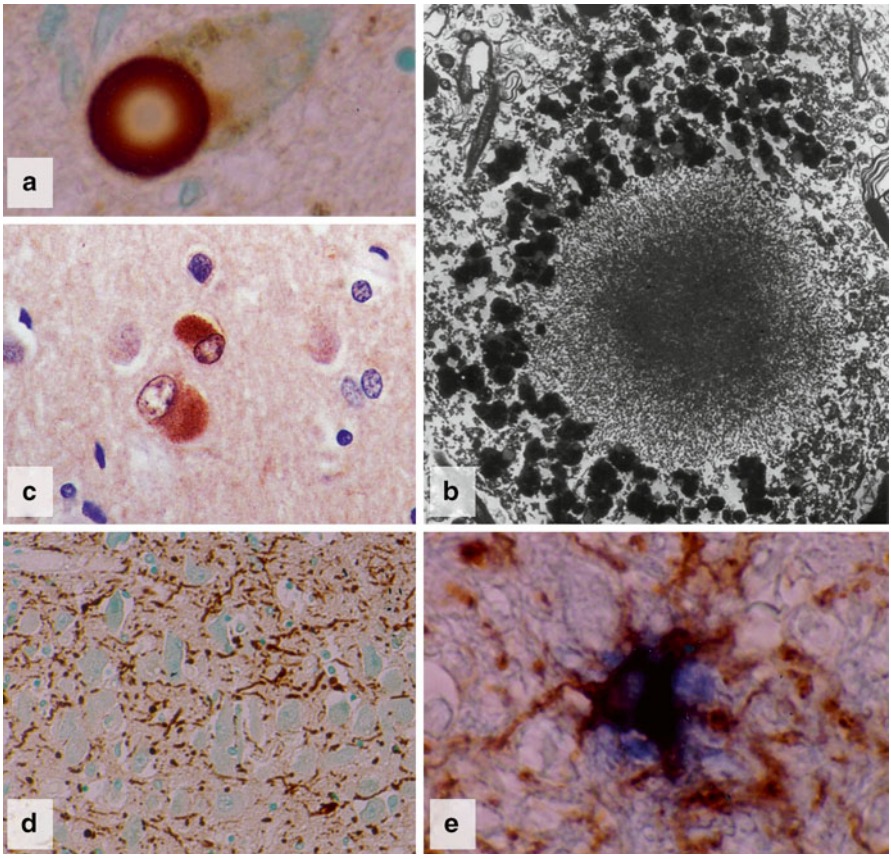

Fig. 1 (**a**) Lewy body (LB) in substantia nigra whose peripheral rim is stained with anti-α-synuclein × 300. (**b**) Electron microscopy of nigral LB showing a central electron-dense filamentous core with a loosely fibrillary rim (× 2.500). (**c**) Cortical Lewy bodies (anti-α-synuclein, × 150). (**d**) Dystrophic Lewy neurites in the hippocampal C2/3 region, anti-α-synuclein × 150. (**e**) Astrocytic inclusion in substantia nigra of Parkinson's disease brain labeled with anti-α-synuclein (*black*) and glial fibrillary acidic protein (GFAP; *gray*; × 900). Reprinted with permission from Jellinger KA. Neuropathology of sporadic Parkinson's disease: Evaluation and changes of concepts. Mov Disord 2012; 27: 8–30. Copyright © 2011 Movement Disorder Society

dimensional structure and undergoes N-terminal ubiquitination [170]. The mechanisms of its aggregation that may serve as a nidus for LB formation in vivo have not yet been fully elucidated. Mutant and wild-type α-syn interact with the mitochondrial complex IV enzyme and cytochrome C oxidase (COX), suggesting that α-syn aggregation may contribute to enhancing mitochondrial dysfunction, a key factor in the pathogenesis of PD [171].

Both classic and cortical LBs share immunochemical and biochemical characteristics, the major components being α-syn, ubiquitin, and phosphorylated ubiquitin associated with many other substances (see [14]). The molecular components can be divided into several groups: (1) structural elements of the LB fibril (α-syn, neurofila-

ments); (2) α-syn-binding proteins (microtubule-associated protein [MAP], synphilin-1, tau, 14-3.3 protein); (3) synphilin-1-binding proteins (α-syn, dorfin, parkin, etc.), (4) proteins implicated in the ubiquitin–proteasome system (UPS) (ubiquitin, proteasome subunits, and related enzymes and proteins); (5) proteins implicated in the autophagosome–lysosome system (NUB1, a synphilin-1-binding protein, glucocerebrosidase); (6) aggresome-related proteins (γ-tubulin); (7) proteins implicated in cellular responses (molecular chaperons, heat-shock proteins, etc); (8) molecules associated with protein phosphorylation and signal transduction (kinases and other enzymes or proteins); (9) cytoskeletal proteins (MAPs, neurofilament, tubulin); (10) mitochondria-related proteins (cytochrome C, COX); (11) cell cycle proteins (cyclin B, etc.); (12) cytosolic proteins passively diffused into LBs (amyloid precursor protein, calbindin, choline acetyltransferase [ChAT], synaptophysin, TH, VMT 2);, and (13) others (complement proteins, lipids, immunoglobulin).

Double-labeling revealed TH and ChAT co-localization with α-syn in cortical LBs, whereas brain stem LBs had intense TH and ChAT immunoreactivity in the core surrounded by a peripheral rim of α-syn [172]. Sequestration of ChAT and TH within LBs suggested that they may disrupt cholinergic and catecholaminergic transmitter production [168]. Demonstration of the autophagy adapter protein NBR1 in LBs [173, 174] and ubiquitin-1 (UBQLN1) in cytoplasmic and nuclear inclusions in several proteinopathies suggests that they are involved in their formation [175]. Co-localization of α-syn, synphilin, and parkin within LBs suggests that parkin plays a role in posttranslational modification of α-syn, which results in changes in protein size and structure-enhancing fibrillation and formation of LBs [176]. These modifications and alternative splicing have been suggested to trigger neurodegeneration in PD [152]. Pathogenic mutants of α-syn promoting toxic interactions between α-syn oligomers and lipids may interact with liposomal and ubiquitin/proteasome-mediated protein degradation or mitochondrial dysfunction [177]. LBs further contain 14-3-3 proteins that are involved in numerous signal transduction pathways and interact with α-syn and torsin A, a novel protein that may serve as a chaperon for misfolded proteins that require refielding or degradation [168].

Proteomic analysis of cortical LBs revealed 296 proteins related to multiple or unknown functions. In brain stem LBs, more than 90 proteins were identified [178], whereas another study identified 1,263 proteins in PD SN [179]. The proteome of LC in PD is of some relevance for pathogenesis [180].

The formation of LBs runs through several stages. Classic LBs show an initial intraneuronal appearance of dustlike particles related to neuromelanin or lipofuscin that are cross-linked with α-syn, with homogenous deposition of α-syn and ubiquitin in the center, with diffuse, pale, or fine granular cytoplasmic staining [153, 181]. Follows a stepwise condensation of ubiquitinated dense filamentous inclusions, forming "early LBs" that later develop into classical LBs, that are finally degraded to extraneuronal LBs after disappearance of the involved neuron. The development of cortical LBs has been divided into six stages [140]. They first show diffuse α-syn and ubiquitin labeling, whereas subcortical LBs have a distinct, central ubiquitin domain with α-syn occurring primarily in the periphery and ubiquitination being the later event. Initial granular accumulation of α-syn in the neuronal cytoplasm is followed by stepwise accumulation of dense filaments, spreading to dendrites, forma-

tion of LBs, and final degradation by astroglial processes. Glutathione peroxidase (GPX-1)-positive microglia, an important antioxidant enzyme, may be involved in neuroprotection of LB-containing neurons [182]. LBs are accompanied by coarse dystrophic neurites and also contain α-syn and ubiquitin as inclusions in axonal processes (see Fig. 1d), which, according to three-dimensional studies, may evolve into LBs, with ubiquitin at the core and neurofilaments at the outermost layer [183].

About 10 % of pigmented SN neurons contain abnormal α-syn aggregates, with diffuse cytoplasmic staining and less often pale bodies, while in the LC, 55 % of pigmented neurons contain α-syn aggregates; diffuse cytoplasmic staining is more frequent than pale bodies or LBs [146].

The absence of TH immunoreactivity suggests that many of the neuritic processes are not derived from dopaminergic neurons, although non-dopaminergic neurons partly expressing a similar phenotype are widely distributed throughout the brain [184]. α-Syn-immunoreactive glial cells (see Fig. 1e) are suggested to be responsive for the progression of PD degeneration and to play an important role in initiating early tissue response by causing recruitment of phagocytic microglia and attacking selected neurons in specific brain areas responsible for clinical symptoms [185]. Such glial inclusions are found in wide brain regions; they are also composed of abnormal filaments [186].

Although LBs are not specific to PD and may occur in a variety of conditions as a secondary pathology, a positive diagnosis of PD can usually be made by inspecting two unilateral sections from the mid-part of the SN and finding LBs. If no such inclusions are found, two further sections should be examined. If LBs are not seen in either the SN or LC, then the diagnosis of PD of the LB type could be excluded. In case of cell loss from the SN and LC in the absence of LBs, an alternative cause for parkinsonism should be pursued [17, 42].

Intranuclear inclusions, referred to as Marinesco bodies, are found in higher frequency in elderly individuals in the pigmented neurons of the SN and LC that contain LBs than in those without such inclusions, and their frequency appears to have an inverse relationship with striatal concentration of DAT and TG [187].

Pathobiological Role of Lewy Bodies

The significance of these insoluble proteinaceous cytoplasmic inclusions and their impact on promoting neurodegeneration or neuroprotection are poorly understood. Similar to other inclusions such as Pick bodies, neurofibrillary tangles, or Rosenthal fibers, LBs may represent end products or reactions to unknown neuronal degenerative processes [188]. Inhibition of complex I (reduced nicotinamide adenine dinucleotide ubiquinone oxidoreductase), a central factor in the pathogenesis of PD, causes aggregation of α-syn, which contributes to the impairment in protein handling and detoxification, whereas mitochondrial accumulation of α-syn may interact with complex I and interfere with its function [189], producing neuronal death due to mitochondrial energy deficit, oxidative stress, and impaired regulation of protein turnover [190, 191]. α-Syn mutants are prone to formation of oligomeric and

prefibrillar structures, being the consequence of abnormal membrane changes, alterations in vesicle traffic, and involvement of mitochondria or lysosomal membranes [171, 192, 193]. This may be a result of the action of substances produced during early phases of protein misfolding, induced by oligomeric species of α-syn and activated in nigral dopaminergic neurons in PD and experimental models [194, 195]. Recent evidence indicates that early oligomeric forms of α-syn and not the final protein aggregates are responsible for its toxicity [196]. Small intermediates termed "soluble oligomers" in the aggregation process might lead to synaptic dysfunction and neuronal death, whereas insoluble fibrillary aggregates may function as a reservoir for bioactive oligomers [197].

Oligomerization of α-syn at the initial stage of PD is well documented [198–200], and initiation of α-syn oligomers may induce protein aggregation, disrupt cellular function, and eventually lead to neuronal death [198, 201]. Although α-syn aggregates are related to neuronal loss and LBs are considered markers of an ongoing neuronal damage, they might even be harmless end products of sequestration of toxic molecules as a type of cellular protective mechanism rather than a cause of neuronal cell death [14, 188]. LBs and pale bodies are immunoreactive for autophagic adaptor proteins p62 and NBRI [153, 175, 202], which may sequester the soluble proteins containing oligomeric α-syn into inclusions. The UPS and autophagy–lysosomal pathway (ALP) render mutated or damaged proteins less toxic than their soluble forms. They are suggested to contribute to α-syn turnover, while alterations in these major proteolytic pathways may result in α-syn accumulation due to impaired clearance [203]. Conversely, increased α-syn protein burden promotes the generation of aberrant species that impair further UPS and ALP function, thus generating a bidirectional positive-feedback loop leading to neuronal death [201, 203]. This suggests that the ubiquitinated proteins in LBs may be a manifestation of a cytoprotective response designed to eliminate damaged cellular components and to delay the onset of neuronal degeneration. However, the ultimate mechanisms for the regulation of the machinery that handles toxic waste by segregating it into insoluble aggregates is still poorly understood.

Fragmentation of the Golgi apparatus, seen in 5 % of PD nigral neurons with LBs and 3 % in those without them but in 19 % of neurons containing pale bodies, suggests that the cytotoxicity of α-syn is reduced by the process of LB formation [204], whereas SN neurons showing DNA fragmentation have no somal LBs. Mitochondrial DNA deletion was highest in LB-positive neurons in PD brains, followed by LB-negative neurons and controls, suggesting increased mitochondrial damage in LB-positive neurons in PD [205].

Lewy Pathology Staging

There are three current major staging system in use for LB disorders, one for PD [24, 206], another one for DLB [207], and revised guidelines for LB disease [208].

Based on semiquantitative assessment of LBs in a large autopsy series, a staging of the chronological spread of Lewy pathology was proposed to designate the predictable sequence of lesions in the nervous system [6, 24, 206, 209] (Fig. 2).

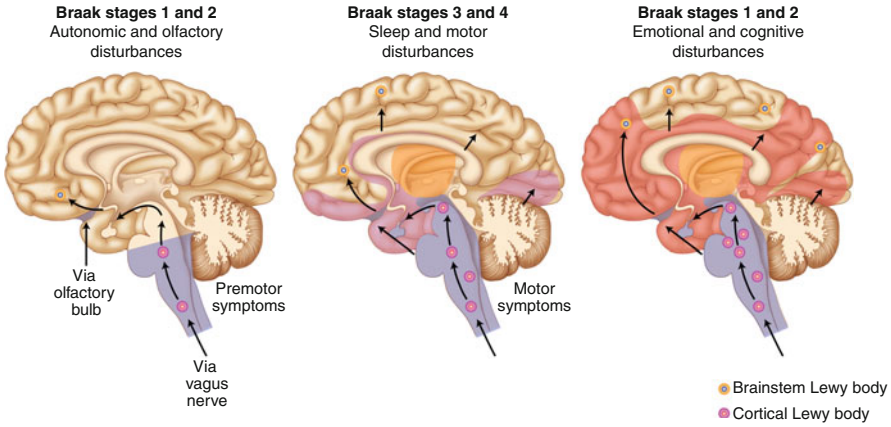

Fig. 2 Stylized representation of the Braak staging for Parkinson's disease showing the initiation sites in the medulla oblongata and olfactory bulb through to the later infiltration of Lewy pathology into the cortical regions

LB pathology is suggested to begin in the lower brain stem and involve the dmX, intermediate reticular zone, and anterior olfactory nucleus, with NBM and midbrain regions being preserved (stage 1). It extends to the caudal raphe nuclei, gigantocellular reticular nucleus, and coeruleus–subcoeruleus complex (stage 2). These initial stages are considered asymptomatic or presymptomatic and may explain the early nonmotor (autonomic and olfactory) symptoms that precede somatomotor dysfunctions [27, 31, 116, 210]. In stage 3, the LC, the central nucleus of the amygdala, and the posterolateral and posteromedial SNc are the focus of cytoskeletal changes and neuronal depletion, whereas the allocortex and isocortex are preserved. In stage 4, the anteromedial temporal limbic cortex, neocortex, and amygdala are affected. Stages 3 and 4 have been correlated with clinical motor symptoms. In terminal stages 5 and 6, the pathologic process reaches the neocortex, with the high-order sensory association cortex and prefrontal areas affected first, later progressing to the primary sensory and motor areas or involving the entire neocortex [86, 123, 209]. Metabolic and functional abnormalities already occur in brain regions in early stages of PD, which are not accompanied by Lewy pathology [16, 131, 190, 211].

Cases with severe Lewy pathology (stages 5 and 6) that show overlap or transition between PD and dementia with Lewy bodies (DLB) frequently are associated with cognitive impairment that may increase with neuropathologic stages [6].

The validity of the Braak staging scheme, which corresponds roughly to the classification of Lewy body disorders into three phenotypes—brain stem predominant, limbic/transitional, and diffuse neocortical [211]—has gained acceptance [212–215] but has also been a matter of vigorous debate [15, 42, 137, 217–220]. It often, but not consistently, shows acceptable correlations between morphological findings and clinical data, mainly in a subgroup with early onset and prolonged duration [157].

However, retrospective clinicopathologic studies have shown that at least 15 % of PD patients did not conform to this pattern [218, 219, 220]. Between 6.3 and 47 %

of all cases of autopsy-proven PD and 18 % of ILBD did not follow the predicted caudo-rostral spread of Lewy pathology [217, 219, 220]. In 7–8.3 % of PD cases, the dmX was not involved despite α-syn inclusions in the higher brain stem or even in cortical regions [216, 218, 219]. In contrast, in large samples, 49–55 % of individuals with widespread α-syn pathology lacked clinical symptoms or were unclassifiable [208, 219–221]. Although the Braak staging scheme is attractive, it should be remembered that it is not based on distribution of neuronal loss, but on distribution of LBs, and how it relates to progression of cell loss has not been rigorously studied. The numbers of LB cases show no age-related increase in incidence [208], suggesting that both prodromal PD and DLB are likely to occur in a continuum. While there will probably always be a number of outlying cases that do not conform to any staging scheme, the numbers with alternate distributions of LBs may be greater than expected for PD but may also reflect the different types of cohorts examined [15]. Thus, the proposed staging should be interpreted cautiously.

Incidental Lewy Body Disease

This term is used when LBs are found in the nervous system of individuals without clinically documented Parkinsonian symptoms. The distribution of LBs is similar to that in definite PD, with one or multiple brain areas involved and some sparing of LBs in the limbic or temporal cortex (average Braak PD stage 2.7), whereas in definite PD, more numerous LBs are found and the Braak stages significantly higher (average 4.4). Decreased TH immunoreactivity was shown in the striatum and epicardial nerve fibers compared with normal controls, but not to the same extent as in PD [17, 131]. Lewy pathology was also seen in the spinal cords of many ILBD cases [119]. Based on these and other findings, ILBD is considered as a precursor of PD, in which the lack of definite symptoms is due to subthreshold pathology. These studies support the concept that dopamine deficits begin preclinically, although the loss of dopaminergic nigral neurons is also common in DLB and differentiates LB disorders from AD [222, 223].

Initial diffuse Lewy pathology (stages 5 and 6) without clinical PD symptoms could represent a precursor to DLB, suggesting a dichotomy in the distribution of ILBD [132].

Lipoxidative damage and advanced glycation end products (AGEs) indicating oxidative damage in the cerebral cortex have been demonstrated in ILBD with none or only mild neurologic symptoms [190]. Single clinicopathological case reports suggested that REM sleep behavior disorder (RBD) may represent ILBD or an early clinical manifestation of PD [224], and that the lack of symptoms is due to subthreshold pathology [225]. The time interval between RBD and the onset of Parkinsonian symptoms ranged up to 50 years with a median of 25 years [226], while others reported that olfaction and color vision identify early-stage α-syn-mediated neurodegeneration at least 5 years before disease onset [227].

An explanation for the relatively high prevalence of asymptomatic pathologic phenotypes is threshold effect, that is, pathology in the absence of lethal comorbidities

would continue to accumulate until it reached the threshold for onset of clinical disease [17, 132].

Many studies have reported that between 5 and 24 % of clinically unremarkable elderly people showed abundant Lewy pathology [16, 18, 228–233], often not related to cognitive status [234]. The distribution pattern of LBs was similar to that seen in PD, but the pigmented SN neurons were relatively well preserved. Some elderly individuals have LBs confined to the olfactory bulb [129, 235] or the amygdala, the latter particularly true if associated with concurrent Alzheimer-type pathology [112]. Moreover, some neurologically unremarkable subjects have sparse but widespread Lewy pathology even involving the cortex [132, 236], which would seem to violate the theory of progression from the brain stem and perhaps fit better with a multicentric disease progress from the onset [42]. Clearly, the observed distribution of LBs is dependent on case selection [231].

New Guidelines for Lewy Pathology

The revised guidelines for the pathologic diagnosis of Lewy body diseases with semiquantitative assessment of LB density in the brain stem, limbic system, and five cortical regions [220] distinguish three phenotypes—brain stem predominant, limbic/transitional, and diffuse neocortical (Table 1)—taking into account

Table 1 Neuropathological staging of Lewy body disease[a]

Kosaka LBD stage	Braak PD stage	Anatomical distribution of Lewy bodies
		Enteric nervous system, peripheral autonomic system, anterior olfactory nucleus
Brain stem-predominant type	1	Medulla oblongata: dorsal IX/X motor nucleus, intermediate reticular zone; lower raphe nuclei; spinal cord
	2	Medulla oblongata and pontine tegmentum: caudal raphe nuclei, gigantocellular reticular nucleus, and coeruleus–subcoeruleus complex; olfactory bulb
	3	Midbrain: pathology of stage 2 plus midbrain lesions, substantia nigra compacta (!)
Transitional (limbic) type	4	Basal prosencephalon and mesocortex: stage 3 plus prosencephalic lesions, amygdala, intralaminar thalamus. Cortical involvement confined to the temporal mesocortex (transentorhinal region) and allocortex (CA2 sector)
Diffuse cortical type	5	Neocortex: stage 4 plus lesions in high-order sensory association areas and the prefrontal neocortex
	6	Advanced neocortex: stage 5 plus first-order sensory association areas of the neocortex and premotor areas; occasionally, mild changes in primary sensory areas and the primary motor field

[a]Metabolic and functional abnormalities already occur in brain regions at early stages of PD that are not accompanied by Lewy pathology

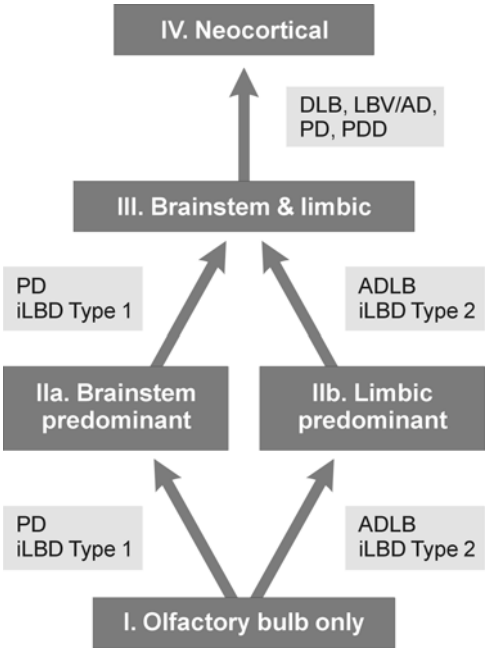

Fig. 3 Scheme of the hypothetic progression pathways and stages of Lewy body (LB) disorders. The pathway for Parkinson's disease (PD) is suggested to proceed through stage IIa (brain stem predominant), and those for dementia with Lewy bodies (DLB) and Alzheimer's disease (AD) with LBs probably pass through stage IIb. For incidental LB disease (iLBD), both pathways seem possible, whereas only PD/PD dementia (PDD), DLB, and the LB variant of AD (LBV/AD) progress to the neocortical stage. Reprinted with permission from Jellinger KA. Neuropathology of sporadic Parkinson's disease: Evaluation and changes of concepts. Mov Disord 2012; 27: 8–30. Copyright Â© 2011 Movement Disorder Society

Alzheimer-type pathology. However, the stage of AD-related lesions is usually not associated with the pattern of Lewy pathology [208]. A recently proposed unifying system correlates α-syn pathology with nigrostriatal degeneration, cognitive impairment, and motor dysfunction [129]. Whereas the old classification left 45–50 % of elderly individuals unclassified, all were classified into one of four stages (Fig. 3). Progression through these stages was accompanied by stepwise deterioration in terms of striatal TH concentration, SN pigmented cell loss, Mini-Mental State score, and the Unified Parkinson's disease Rating Scale. There were significant correlations between these measures and α-syn pathology. This is supported by an increase of phosphorylated α-syn restricted to the olfactory bulb and brain stem in early stages of Lewy pathology [5, 129]. This staging system would allow a better classification of Lewy disorders but it needs validation in a greater proportion of patients. However, despite several limitations, the brain pathology for most patients with typical PD can be predicted using Braak's scheme [15, 208]. The relationship between age and late progression of PD has been studied recently [215].

Interaction Between α-Syn and Other Proteins

Considerable overlap between synucleinopathies and other protein-misfolding diseases suggests interactions of pathological proteins engaging common downstream pathways [13, 49, 188, 237, 238]. The co-occurrence of α-syn, tau, and other proteins in various neurodegenerative disorders highlights the interface between these misfolded proteins, which may be co-aggregated in the same brain, in the same region, or even in the same cell in human brain and transgenic mice [239–241].

Accumulated α-syn (promoted by oxidative stress) has a stimulatory effect on tau phosphorylation by glycogen synthase kinase-3β (GSK-3β), a major kinase that hyperphosphorylates tau to produce pathologic forms [242], while the chaperone HSP70 may suppress α-syn-mediated tau phosphorylation in initial disease stages [243]. In MPTP models, α-syn has been shown to induce GSK-3β-catalyzed tau phosphorylation [244, 245].

Recent postmortem studies showed increased accumulation of tau protein, in particular tau phosphorylated at Ser 262 and 396/404, in the striata of PD patients related to increased GSK-3β activity [149]. Tauopathy in PD striata is restricted to dopaminergic neurons, whereas degeneration in the inferior frontal cortex, associated with increased α-syn deposition because of diminished proteasomal activity in the absence of oxidative stress and pGSK-3β activity, is not associated with tauopathy [149]. Thus, the restricted pattern of tauopathy in PD [246] differs from its generalized affection in AD [247]. Interaction with tubulin suggests that α-syn could be a microtubule-associated protein similar to tau. PD-associated risk factors, for example, environmental toxins and α-syn mutations, may also promote tau phosphorylation, causing microtubule instability, which leads to loss of dopaminergic neurons in PD [248]. Induction of intracellular tau aggregates is promoted by α-syn seeds [249].

There is a strong interaction between α-syn, tau, and β amyloid, particularly in their oligomeric forms, which might synergistically promote their mutual aggregation and vice versa, thereby amplifying neuronal damage [250–252]. Cross-seeding between dissimilar proteins that share β-sheet structures has been described, e.g., for tau and α-syn [253]. Modification of α-syn may induce both Lewy-like and tau pathologies [248], while tau enhances α-syn aggregation and toxicity and disrupts inclusion formation in cellular models [254]. Other links between tau and α-syn are suggested by their co-localization in both neurofibrillary tangles and LBs, especially in neuronal populations vulnerable for both aggregates [178, 235, 239]. LB formation may be triggered, at least in part, by AD pathology and vice versa [255].

Other studies have suggested that β-amyloid is more likely to promote the deposition of α-syn than tau [256], and Aβ is known to initiate hyperphosphorylation of tau [257]. Cortical α-syn load is associated with Aβ plaque burden in a subset of PD patients [258], and α-syn-induced synapse damage is enhanced by Aβ-42 [259]. PD and AD could be linked by progressive accumulation of phospho-tau, GSK-3β, and α-syn, and activation of caspase and caspase-cleft Δ-tau may represent a common way of abnormal intracellular accumulation of both α-syn and tau, promoted by Aβ

deposition, thus unifying the pathology of these diseases. Interaction of α-syn, tau, and Aβ may be a molecular mechanism in the overlapping pathology of several proteinopathies, possibly representing a complex continuum characterized by variable amounts of pathologic proteins generated by the same stimulus probably depending upon genetic and environmental factors [260, 261]. Despite documented co-localization of α-syn and tau in LBs and Aβ and phospho-tau in synaptic terminals in AD [262] and in triple transgenic mice [263], the basic mechanisms leading to the intimate association and synergism of these proteins, suggesting a dualism or triad of amyloidogenic neurodegeneration, await further clarification.

Pathophysiology of Clinical Subtypes of Parkinson's Disease

The basal ganglia consist of the caudate, putamen (striatum), and globus pallidus (GP) and are functionally connected to many brain stem nuclei, the basal forebrain, the cortex, and the cerebellum.

Current concepts about the organization of the basal ganglia emphasize the existence of "internal" mechanisms that modulate input/output activity and sustain normal execution of movements. In the normal basal ganglia network, the globus pallidus externus (GPe) emerges as a main regulatory station of output activity (Figs. 4 and 5a). Dopamine not only is an important modulator of basal ganglia function but also may modulate it at sites outside of the striatum, and changes in dopaminergic transmission at these sites may contribute to the symptoms of PD and other disorders [264]. In early stages of PD, overactivation in the basal ganglia is suggested to compensate the dopaminergic deficit in the striatal circuit. In PD, this filtering mechanism is deranged; dopamine depletion shifts the basal ganglia toward inhibiting cortically generated movements by increasing the gain in the GPe-subthalamic nucleus (STN)-GPi network and reducing the activity in "direct" cortico–putaminal–GPi projections (see Fig. 5b). GPi/SNr activity is controlled by the balance between a direct and indirect putaminal–pallidal GABAergic pathway; GPe, in turn, projects via another GABAergic pathway to STN that has a glutamatergic projection to GPi/SNr. Impairment of synaptic plasticity of striatal neurons, in particular medium spiny neurons, may account for the onset and progression of motor symptoms and contribute to the pathogenesis of dyskinesias (see Fig. 5c) [265, 266]. Anatomical connections between the basal ganglia and cerebellum suggest their association in movement disorders [267].

The major clinical subtypes of PD show specific morphological patterns of pathophysiological importance, with different involvement of the striatal and cerebello-thalamic-cortical pathways (Fig. 6) [268].

In the *rigid–akinetic type*, which occurs in about 50 % of all PD patients, the ventrolateral SN projecting to the dorsal putamen shows the brunt of degeneration (see Fig. 5b). There is a gradient loss of TH- and DAT-immunoreactive fibers from the dorsal to the ventral putamen, with prominent involvement of the striosomes projecting to the severely involved ventrolateral SNc. Consequently, nigral cell loss

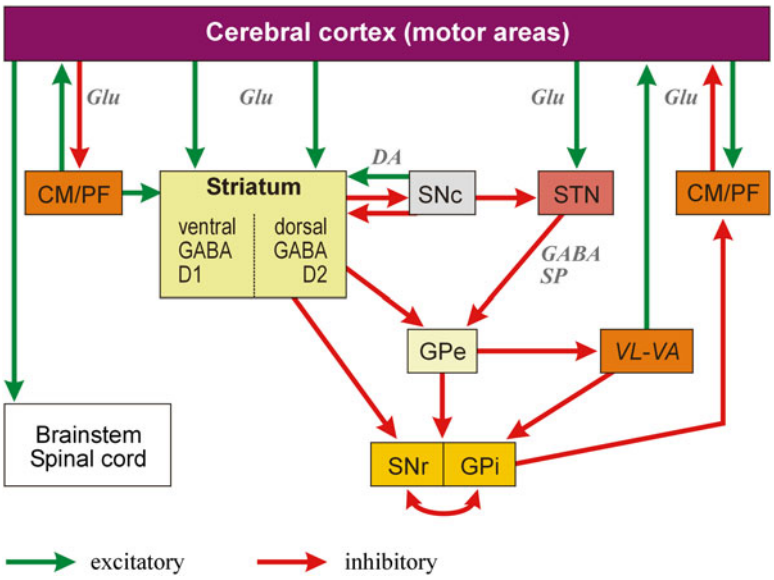

Fig. 4 New model of the basal ganglia. Cortical innervation to the basal ganglia is primarily via corticostriatal and corticosubthalamic projections. The primary basal ganglia projections back to the cortex originate in the GPi (and SNr) and pass through the ventral nuclei of the thalamus (VL-VA). "Internal" or "horizontal" circuits control basal ganglia excitability. These include substantia nigra pars compacta (SNc) dopaminergic projections to the striatum and other basal ganglia, centromedian–parafascicular (CM/Pf) thalamic projections to the striatum and subthalamic nucleus (STN), and reciprocal connections between the external globus pallidus (GPe) and the STN and striatum (Glu, glutamate; DA, dopamine; D1, D2, dopamine receptors; GABA, γ-aminobutyric acid; Enk, enkephalin). Modified with permission from Jellinger KA. Neuropathology of sporadic Parkinson's disease: Evaluation and changes of concepts. Mov Disord 2012; 27: 8–30. Copyright © 2011 Movement Disorder Society

correlates with dopamine loss in the posterior putamen and severity of akinesia–rigidity [65]. Dopamine loss in the GPe and globus pallidus internus (GPi) does not match the more severe dopamine loss in the putamen [56]. Preservation of the calbindin-positive somatostatin-rich matrix, which projects to the GABAergic neurons of the SNr and motor thalamus, suggests that the endings richest in DAT are most sensitive to degeneration. Dopaminergic denervation of the striatum causes severe loss of dendrites on type I medium spiny neurons (MSNs), the principal target of dopaminergic input from the SN [269], and loss of convergent nigrostriatal dopamine and corticostriate glutamate axon integrity. The majority of D1-expressing neurons comprise the "direct" pathway and project to the GPe/SNr, whereas D2-bearing neurons project to the GPe and are part of the "indirect" pathway [270, 271] (see Figs. 4 and 5a).

In early stages of PD, overactivation in the basal ganglia is suggested to compensate for the dopaminergic defect in the striatal motor circuit [272]. The increase in striatal neuronal activity with decreased excitation of D1-bearing

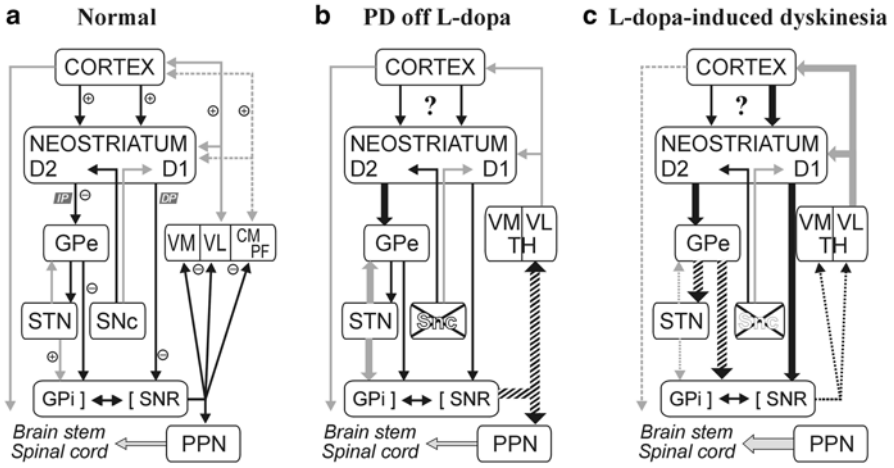

Fig. 5 Schematic diagram of the basal ganglia–thalamocortical circuitry under normal conditions and in hypokinetic and hyperkinetic movement disorders. The *width of lines* represents the relative change in activity versus normal. *Disrupted lines* represent altered patterns with an increase or decrease in neuronal activity. *Dashed arrow*, reduced activity; *solid arrow*, increased activity; D1 and D2, dopamine 1 and 2 receptor subtypes; GPe and GPi, external and internal segment of the globus pallidus; Normal, normal conditions; PPN, pedunculopontine nucleus; PD, Parkinson's disease; PSP, progressive supranuclear palsy; SNc and SNr, substantia nigra pars compacta and reticulata; STN, subthalamic nucleus; TH, thalamus; VM/VL, ventromedial/ventrolateral thalamic nuclei; CM, centromedian nucleus of the thalamus; PF, parafascicular nucleus of the thalamus. Modified with permission from Jellinger KA. Parkinson's disease. In: Dickson DW, Weller RO, editors. Neurodegeneration: The Molecular Pathology of Dementia and Movement Disorders, 2nd ed. Oxford: Blackwell Publishing Ltd.; 2011: pp. 194–223

neurons leads to reduced activity of the "direct" pathway, whereas reduced inhibition of D2-bearing striatal neurons results in decreased activity in striatopallidal (GPe) projections (see Fig. 5b). Later, this filtering mechanism is deranged, and dopamine depletion shifts the basal ganglia toward inhibiting cortically generated movements by increased gain in the indirect GPe–STN–GPi network and reduced activity in the "direct" cortico–putaminal–GPi circuit by dopamine D2 receptors and reduces the inhibition mediated by the direct pathway because of loss of D1 excitation in this pathway [265]. Neuritic changes, α-syn inclusions, and loss of dopaminergic neurons in the neostriatum increase with progression of PD [57, 273]. The essential pathophysiological feature of the rigid–akinetic PD is increased neuronal firing activity in the output nuclei (GPi and SNr) leading to excessive inhibition of thalamocortical and brain stem motor systems [274].

Impairment of synaptic plasticity of striatal MSNs accounts for the onset and progression of motor symptoms and contributes to development dyskinesias [265, 266]. Abundant α-syn pathology in the neostriatum [273, 275], dystrophic neurites in the caudate nucleus, and progressive loss of TH and DAT-immunoreactive nigrostriatal fibers suggest trans-synaptic degeneration as a substrate for the motor deficits and decreased efficacy of dopaminomimetic therapy in late stages of PD [276, 277].

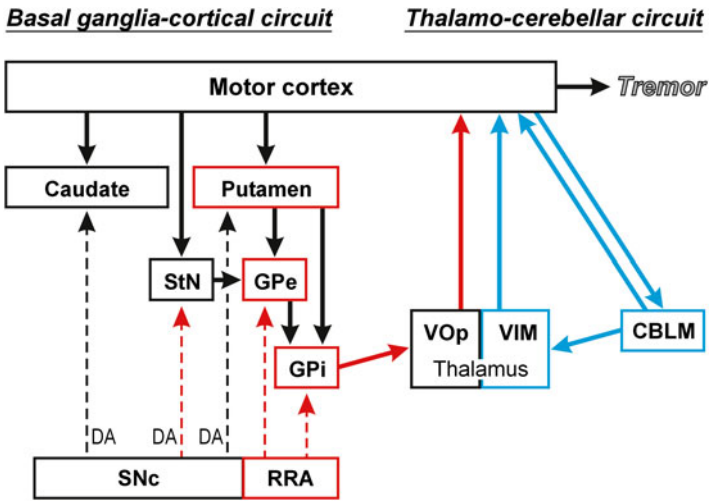

Fig. 6 A model of cerebral mechanisms underlying Parkinson's disease (PD) resting tremor. It emerges from the ventral intermediate nucleus of the thalamus (VIM)–motor cortex (MC)–cerebellum (CBLM) circuit (in *blue*), when triggered by transient pathological signals from the basal ganglia motor loop (in *red*). In tremor-dominant PD, the basal ganglia (globus pallidus internus [GPi], globus pallidus externus [GPe], and putamen) have increased connectivity with the VIM–MC–CBLM circuit through the MC (thick *red line*), and the basal ganglia are activated at critical times in the tremor cycle (onset/offset of tremor episodes). These alterations may be caused by loss of dopaminergic projections from retrorubral area 8 (RRA; in *red*) to the GPi and GPe. These alterations are different from the dopaminergic denervation of the striatum, associated with bradykinesia and rigidity (Vop, thalamic ventralis oralis posterior nucleus; DA, dopamine; SNc, substantia nigra pars compacta; StN, subthalamic nucleus). Reprinted with permission from Jellinger KA. Neuropathology of sporadic Parkinson's disease: Evaluation and changes of concepts. Mov Disord 2012; 27: 8–30. Copyright Â© 2011 Movement Disorder Society

Reduced dopaminergic input to the putamen causes increased activity of the GABAergic indirect efferent loop via SNr and GPi to the ventrolateral thalamus projecting to the cortex. Excessive excitatory glutamatergic drive from the SN and GPi/SNr leads to an akinetic-rigid syndrome through reduced cortical activation (see Fig. 5b). Recent studies suggest that rigidity is associated with widespread changes in the brain, as opposed to a single discrete locus [278].

The increased GABAergic activity is reduced by L-dopa treatment and disappears in the course of the disease, and N-methyl-D-aspartate (NMDA) receptors and glutamatergic synapses may be degenerated, favoring drug resistance and motor complication. Hyperstimulation of dopaminergic receptors may account for the development of motor fluctuations and dyskinesias after L-dopa treatment (see Fig. 5c). Relative preservation of the striatal matrix and its efferents enables restitution of the dopaminergic transmission by L-dopa substitution and maintained the function of the "motor loop," but progressive degeneration with transgression to non-dopaminergic systems in later stages causes loss of postsynaptic D2 and muscarinic cholinergic receptors in the striatum. This uncoupling of receptor

systems is considered a major cause of drug resistance of motor symptoms and adverse L-dopa effects (dyskinesias, fluctuation, etc.) via loss of synaptic depotentiation [266, 279]. Sprouting of dopaminergic terminals and decreased DAT may contribute to increased dopamine release/turnover and increased dopamine sensitivity of striatal cholinergic neurons, predisposing to motor complications and L-dopa-induced dyskinesia as the disease progresses [280, 281]. Recent data have shown a differential role of pre- versus postsynaptic mechanisms, dopamine receptor subtypes, glutamate receptors, and non-dopaminergic transmitter systems in the pathophysiology of dopamine-induced dyskinesias [282, 283]. Clinicopathological studies revealed that the non-tremor-dominant subgroup had a significantly higher mean grading of cortical LBs than all other subgroups and more cortical Aβ-plaque load than early-onset and tremor-dominant groups. These data confirm the link between bradykinetic onset, cognitive decline, and LB deposition in the neocortex [37].

The *tremor-dominant type of PD* occurs in about 25 % of patients and shows less severe cell loss (mean 69 %) in the lateral than the medial SNc, but damage to the retrorubral A-8 field, which is usually preserved in rigid–akinetic PD [13, 284]. It projects to the matrix of the dorsolateral striatum and ventrolateral thalamus and influences striatal efflux via the SNc and thalamus to the prefrontal cortex. In contrast to akinetic-rigid PD, dopamine levels in the ventral internal pallidum were normal in PD with predominant tremor, suggesting functional disequilibrium between GABAergic and dopaminergic influences in favor of dopamine in the caudoventral parts of the GPi, which may contribute to rest tremor [56]. Functional neuroimaging in patients with resting tremor suggested increased activity of the ventral intermediate thalamus and dysfunction of cerebellar connections [285]. Morphometric studies demonstrated volume reduction in the cerebellum of these patients, demonstrating involvement of the cerebello-thalamo-cortical circuit [286]. Deficits in cerebellar functions in PD with decreased excitability of the cerebello-thalamo-cortical pathway may be involved in the generation of postural tremor, whereas resting and postural tremors in PD are mediated by different neuronal pathways [287]. Rest tremor is produced by the pathological interaction between the basal ganglia and the cerebellar-thalamo-cortical circuit only in the presence of striatopallidal dopaminergic dysfunction (see Fig. 6) [288, 289], which has been confirmed by recent FDG-PET studies [290]. This has considerable implications for stereotactic treatment of tremor (deep stimulation of the ventral intermediate thalamus) [291, 292].

The current model provides a reasonable explanation for the origin of akinetic features in PD and suggests that rigidity and tremor are generated by basal ganglia downstream mechanisms struggling to compensate for PD akinesia. The stronger association between akinesia and tremor as opposed to the more independent nature of PD tremor indicates that both symptoms are generated by different mechanisms [293]. Recent FR-CIT SPECT studies confirmed the models for a reduced dopaminergic projection to the dorsal putamen in akinetic-rigid PD as well as the lateral putamen and caudate nucleus in tremor-dominant patients in vivo [294].

Neuropathology of Cognitive Changes in Parkinson's Disease

Cognitive deficits are common in PD, but the range of clinical deficits and their structural and molecular/biochemical backgrounds are variable [295]. Cognitive impairment (without dementia) may precede the onset of dementia, with a delay of up to 20 years; it was observed in 19 % of untreated PD patients [296]. PD patients have an increased risk of developing mild cognitive impairment (MCI), the frequency of which is 21–62 %, with a mean of 25.8 % [297, 298], and with a shorter progression to dementia. Its cumulative prevalence is between 48 and 78 %, with a mean of 75 % after survival for more than 10 years [297] and of 83 % after 20 years [299]. Parkinson's disease dementia (PDD) has a 4–6 times increased lifetime incidence rate compared with age-matched controls [298].

The putative brain changes underlying dementia in PD have not yet been explored in sufficient detail to permit a consensus definition. Although some studies indicate LB-type pathology in cortical and limbic structures as the main histological substrate of PDD, it now appears that even widespread α-syn lesions often cannot reliably account for the presence of dementia with frequently encountered concomitant AD-type changes, suggesting a synergism of multiple pathogenic mechanisms [300]. A recent clinicopathologic study confirmed that a combination of Lewy- and Alzheimer-type pathologies is a robust correlate of dementia in PD [301]. A novel classification of LB disorders based upon unbiased statistical methods suggesting pathologic heterogeneity of dementia in PD awaits further validation [302]. In conclusion, the heterogeneity of both motor/nonmotor and neuropsychological symptoms in PD and related LB disorders may emerge from a variety of morphological and neurochemical deficiencies, which need further elucidation and validation.

Mild Cognitive Impairment in Parkinson's Disease

MCI, representing the earliest clinical features of cognitive dysfunction, according to current criteria includes the amnestic and nonamnestic phenotypes (aMCI and naMCI, respectively), which may involve single or multiple domains. Its prevalence ranges from 3.2 to 23.4 %, with an average of 15–18 % for individuals aged 70 years or older. Classification, diagnostic biological markers, outcome, and neuropathology have been reviewed, showing considerable variability among populations [303]. The frequency of MCI in PD (PD-MCI) varies considerably, between 21 and 62 % [304, 305]. Characterization of PD-MCI has been reported recently [306]. Two studies on the neuropathology of PD-MCI showed variable lesions with brain stem, brain stem-limbic, and rare neocortical LB lesions, low Braak AD stages, amyloid but only rare neuritic plaques in the cerebral cortex, and mild CAA, with some cases having an additional mild lacunar state in the basal ganglia or cerebral infarcts

[307, 308]. naMCI cases showed a significantly shorter disease duration than aMCI cases of variable LB stages, lower AD Braak scores (mean 2.1 vs. 2.7), less frequent neuritic cortical plaques but similar cortical amyloid load, as well as similar rare concomitant cerebrovascular lesions. These data in PD-MCI suggest heterogeneous cognitive presentation [309] and heterogeneous neuropathology similar to that found in MCI cases without PD [310, 311]. Recent evidence strengthens the importance of amyloid imaging in vivo as a tool in the diagnosis and prognosis of patients with MCI [312, 313].

Parkinson's Disease and Dementia (PDD)

The morphological substrate of cognitive impairment in LB disorders has been a matter of considerable controversy. The morphologic changes underlying dementia in PD can be classified into three main types: (1) Cognitive impairment may be primarily a result of involvement of subcortical and brain stem structures, (2) an extension of LB pathology into limbic or higher cortical association areas is accepted by others as a plausible substrate for the dementia in LB disorders, and (3) another proposition is that concurrent AD-type pathology underlies the cognitive decline in PD. In general, one of these lesions may not completely explain the development of PDD, whereas at least two or all three conditions may frequently concur [300, 314]. CNS lesions contributing to the cognitive impairment in PD are dysfunctions of the subcorticocortical networks as a result of neuronal loss and atrophy in the amygdala and limbic areas [315], cholinergic deficits in the cortex and thalamus associated with neuronal loss in the NBM (nucleus basalis of Meynert) and decreased striatal dopaminergic function, decreased nicotinic acetylcholine receptors [204, 316], and degeneration of the medial SN and nuclei of other ascending pathways, causing dysfunction of the striato-subfrontal and mesocorticolimbic loops. The cognitive deficits in early PD are associated with impaired nigrostriatal dopaminergic function, which results in abnormal processing in the corticobasal ganglia circuit with reduced prefrontal and parietal metabolism, whereas mesocortical dopaminergic transmission initially appears to be preserved [317]. There is significant subcortical degeneration in PD, with neuronal loss and LBs in the NBM, although this may precede the onset of dementia because of cortical cholinergic denervation [318] and α-syn pathology in the NBM is high in both demented and nondemented PD patients. Severe pathology also involves the noradrenergic LC [319] and the serotonergic dorsal raphe nucleus [318] and the ventral tegmental area [320]. The loss of LC innervation in PD subjects as measured by TH immunoreactivity appears not to lead to LC neuronal loss because TH-IR in AD was robust despite similar loss of LC neurons. These data suggest a differential response of the noradrenergic system in PD compared with AD in response to loss of LC neurons [321].

Dementia with Lewy Bodies and Parkinson's Disease Dementia

DLB and PDD are considered to be parts of an α-syn-associated disease spectrum. DLB, a progressive disorder associated with some core clinical neuropsychiatric features, is considered to be the second most common neurodegenerative dementia syndrome in the elderly [207]. An arbitrary cutoff is used: If dementia develops first or within 1 year of PD diagnosis, then DLB is diagnosed, while if PD develops first and dementia later, this suggests PDD. Distinction between both disorders may be difficult [322]. Their pathological hallmarks are α-syn/Lewy pathology or a variable mixture of Lewy and AD pathologies, which may interact synergistically [301]. Both cortical and subcortical α-syn lesions have been suggested to be predominant [323], whereas for others, AD lesions were more important, the Aβ load being similar to that in AD [173, 174]. Occipital and posterior parietotemporal hypometabolism is a distinguishing feature of DLB that is independent of amyloid pathology [324]. According to revised guidelines, the severity and extent of α-syn are scored semiquantitatively in specific brain regions [207, 325]. Neurofibrillary tangles, being frequent in both DLB and PDD, are often restricted to limbic regions, while extensive tau pathology may be absent. Between 10 and 50 % of PDD cases had enough AD lesions to attain the pathological diagnosis of definite AD. A recent clinicopathologic study identified three subgroups of PDD: (1) predominantly synucleinopathy (Braak LB stages 5–6; 38 %); (2) synucleinopathy with b-amyloid deposition but minimal or no tau pathology (59 %); and (3) synucleinopathy and AD with considerable neocortical tauopathy (Braak tau stages 5–6; 3 %). Patients in group II had significantly shorter survival than those with pure synucleinopathy. Reduced cortical cholinergic innervation in both DLB and PDD is similar and more severe than in AD, while synaptic loss in DLB is similar to that in AD [326]. Elevated APOE ε4 frequency in DLB and PDD cases, in which the overall brain neuritic plaque burden was lower, indicates that ε4 may contribute to neurodegeneration through mechanisms unrelated to amyloid processing [327].

Brains of DLB patients with the first appearance of dementia showed significantly more severe Aβ scores (4–5) than those without apparent dementia, whose scores were lower (0–1), suggesting that Aβ deposits may contribute to the timing of the onset of dementia related to that of parkinsonism in DLB [328]. The presence of Aβ in DLB but not in PD and their great sensitivity to differentiate DLB from PDD with 100 % negative predictive value was confirmed recently [36, 43, 329].

Other differences between PDD and DLB concern neurochemistry findings. Nigrostriatal dopamine changes occur in both disorders, but the severity and distribution of changes differ, with more marked nigral cell loss in PDD. There is postsynaptic dopamine upregulation in PD but not in DLB, which may be related to the increased risk for neuroleptic sensibility reaction in DLB [330]. DLB shows significantly higher frontal 5-HT1A receptor binding compared with PDD [331]. DLB patients with early dementia (less than 10 years after onset of PD) had morphological and neurochemical changes similar to those with DLB, whereas PD patients

with later-onset dementia had less cortical pathology (LBs, Aβ plaques, NFTs), but more severe cholinergic deficit in the temporal cortex [161].

Some studies have demonstrated that the number of LBs in the frontal cortex or in the limbic cortex is a better predictor of dementia in PD than is AD pathology [332–335]. Cognitive impairment is often correlated with the density of LNs and neuritic degeneration in the hippocampus and periamygdaloid cortex, which causes disruption of the limbic loop and "disconnection" from key areas, as described in AD [336], and is a major basis for the dementia and visual hallucinations [337]. The density of both limbic LBs and neuritic plaques correlated well with the severity of the dementia, although hippocampal atrophy and cell loss are not necessarily involved in the memory impairment in PD [338]. Increasing cognitive decline with increasing pathologic LB stages secondary to progression of α-syn pathology [123] was not confirmed by others [217, 218, 301, 339–341]. PD patients without dementia may have AD pathology largely restricted to the limbic system (neuritic Braak stage <4), whereas patients with PDD often have severe AD lesions, with or without neocortical atrophy [318]. PDD patients showed significantly more severe AD pathology (neuritic Braak stages, cortical amyloid plaque load, and generalized CAA) than nondemented ones [342] which was in accordance with previous postmortem studies [343, 344] and in vivo neuroimaging studies showing increase amyloid load in human brain [312, 313, 345–347].

The association between cognitive impairment, moderate LB scores, and AD lesions suggests an influence of AD-related pathology on the progression of neurodegeneration and on cognitive decline in PD, whereas it appears to be largely independent of coexisting mild vascular pathology, except in cases with severe CVLs related to CAA and AD pathology [342]. DLB is usually associated with only mild cerebrovascular lesions, showing an inverse relationship with the severity of Lewy pathology [348].

Despite many similarities between DLB and PDD, there are some morphological differences, in particular, higher amyloid plaque load in the striatum, usually absent in nondemented PD [36, 43, 329, 342] and more severe α-syn load in hippocampal CA 2/3 areas [13]. DLB cases had more severe Aβ load than PDD, but no differences in neuritic and α-syn scores, while others reported higher Aβ load in cortical and subcortical areas [301].

Based on available data, both DLB and PDD show heterogeneous pathology and neurochemistry, which depend on the different lesion patterns, supporting the hypothesis that these α-syn-related disorders and AD share a common underlying molecular pathogenesis.

Etiology and Pathogenesis of PD

The etiology of PD is poorly understood, but a molecular interaction between genetic factors and environmental risk factors appears reasonable [349, 350]; multiple etiologies are more likely than a single factor [45, 351]. The impact of various gene mutations, such as α-syn (SNCA), LRRK2, Parkin, PINK 1 and DJ1 on

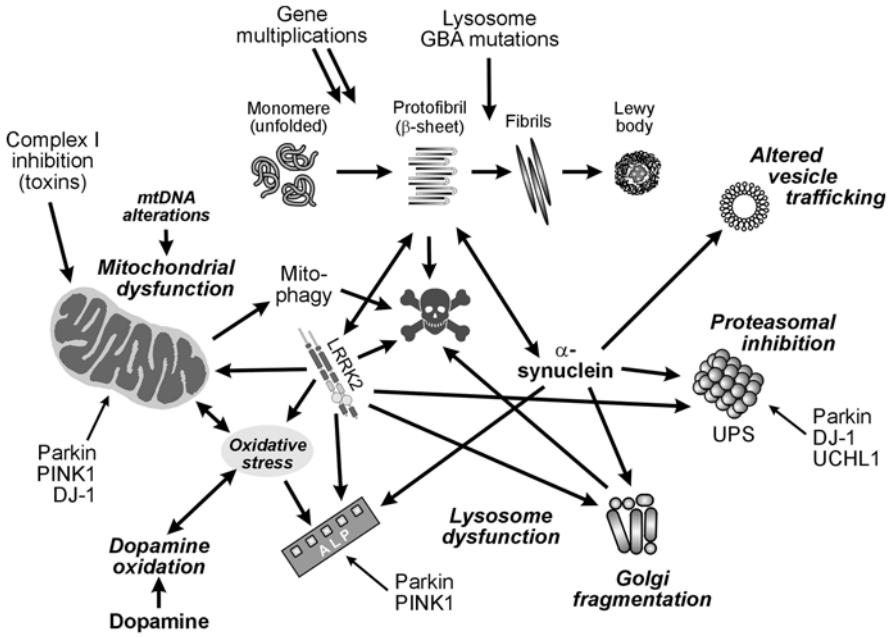

Fig. 7 Simplified scheme of major factors underlying PD pathogenesis. Modified with kind permission from Springer Science+Business Media: Jellinger KA. The role of α-synuclein in neurodegeneration – An update. Translational Neurosci 2012; 3(2)

mitochondrial maintenance, synaptic homeostasis, autophagy, axonal transport, protein regulating systems, oxidative damage, and metabolic functions is well documented (Fig. 7) (see [46, 352]). Genetic susceptibility may be determined in part through impaired metabolism of free radicals or complex I activity, which in turn may be the product of nuclear or mitochondrial genomic deficits [191, 353–355], while environmental interactions include exogenous compounds with uptake and conversion similar to MPTP, cyanide, carbon disulfide, or endogenously generated neurotoxins, such as rotenone or tetrahydroisoquinoline, which are involved in reactive oxidative species and disruption of calcium homeostasis [356]. Recent studies in individuals with POLG mutations showed a selective deficit of the respiratory chain complex I in SNc due to motochondrial DNA deletion, which was or was not associated with clinical parkinsonism [357]. Disturbed calcium homeostasis and abnormal cortical metabolism due to the convergence of multiple deficits have been observed in early stages of PD [190, 358].

A growing body of evidence has been collected regarding the pathogenesis of PD, which has been related to a complex cascade of multiple noxious factors, protein mishandling, in particular misfolded α-syn and its oligomers, perturbation of protein degradation systems, such as the UPS [359], formation of free radicals, oxidative, nitritive, and proteolytic stress [360], production of reactive oxidative species and of advanced glycation products [97], mitochondrial dysfunctions [97, 103, 171, 352, 361–366], impaired bioenergetics [191, 350, 367], lipid peroxidation,

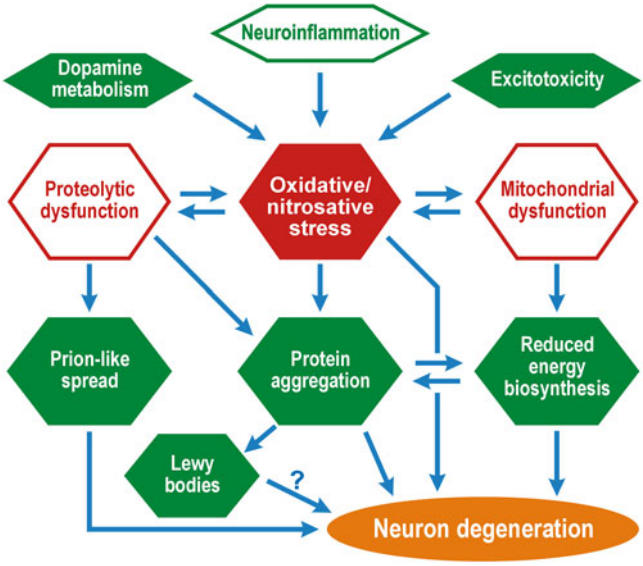

Fig. 8 Common pathways underlying PD pathogenesis. Schematic impairment by α-synuclein and gene mutations enhancing α-synuclein misfolding, fibril formation, and Golgi fractionation; impairing proteasome and mitochondrial functions, altering vesicle traffic and translation. Reprinted from Oxidative and nitrosative stress in Parkinson's disease, 1792/8, Tsang HK, Chung KK, Biochimica et Biophysica Acta (BBA) – Molecular Basis of Disease, 643–650, Copyright 2009, with permission from Elsevier

nuclear RNA deficits, protein–iron and neuromelanin–iron interactions [87, 89, 91, 367, 368], transcriptional α-syn dysregulation [152], disorders of calcium homeostasis, prion-like behavior of misfolded proteins [200, 369, 370], excitotoxicity from increased glutamatergic input, neuroinflammation [45, 79, 133], and interaction between these and other noxious factors (see [46]). The demonstration that α-syn is degraded by both proteasome and autophagy pathways, and the fact that mutated α-syn inhibits the autophagy–lysosome and the autophagy pathways that maintain intracellular proteostasis support its role as the essential trigger for neurodegeneration in PD [104, 359]. Conversely, boosting chaperone-mediated autophagy in vivo mitigates α-syn-induced neurodegeneration [371] (Fig. 8). The complex relationship between autophagy and oxidative stress in PD has been reviewed recently [360, 372], macroautophagy of mitochondria (mitophagy) is thought to be a significant contributor to many different forms of PD [373]. Misfolded α-syn may contribute to oxidative stress through a pathway that induces microglial activation as well as antioxidant responses [81]. Based on the new body of information, the following pathogenic scenario of PD emerges: In dopaminergic neurons, increased calcium conductance and the ensuing greater production of reactive oxygen species lead to higher levels of damaged mitochondria. In normal individuals, Parkin and PINK eliminate damaged mitochondria by macroautophagy allowing neurons to retain a normal pool of healthy mitochondria. In contrast, in PD a loss of Parkin function leads to a progressive buildup of damaged mitochondrial

and a defect in Parkin/Pink1 turnover. Thus, over time, the burden caused by mitochondrial dysfunction will reach a pathological threshold, provoking neuronal dysfunction and ultimately cell death [76]. The major components inducing neuronal loss are (1) presynaptic and/or axonal α-syn aggregation, synaptic and axon degeneration, (2) mitochondrial dysfunction, (3) environmental oxidative stress, (4) neuroinflammation, and other noxious factors. Despite speculations as to how aberrant protein activity might lead to neurodegeneration, it is not certain that α-syn aggregation is the primary cause or an epiphenomenon in the pathogenetic process of PD.

To this and other molecular factors, the contribution of other mechanisms must be added, among which complex cell interactions due to a prion-like cell-to-cell spread of α-syn are documented [369, 370, 374, 375]. Worsening of the clinical features over time, the temporal evolution of neuropathology in PD, and postmortem observation of α-syn pathology within embryonic grafted cells in the striata of PD patients implicated that the spread of α-syn (or its toxic truncated forms) from cell to cell is the main mechanism underlying disease progression in PD and other synucleinopathies [133, 376]. This suggests that pathology could spread from one cell to another and that α-syn can be transferred from an affected neuron to a previously healthy neuron by a "prion-like" process [369, 370, 374]. Interestingly, converging preclinical evidence indicates the ability of α-syn to spread throughout the brain through neuronal networks [377]. Extracellular α-syn may also play a role in disease propagation and could further trigger neuroinflammation through microglia activation [378–380]. Recent evidence suggests that a trans-synaptic or transcellular spreading of α-syn may be a more likely explanation for the propagation of the disease than other mechanisms, such as oxidative stress, neuroinflammation, or loss of neurotrophic transport [370]. Cell-to-cell transfer of α-syn can explain the formation of LBs and LNs but may not account for the full spectrum of PD and especially not for the involvement of multiple non-dopaminergic neuronal populations, although a correlation exists between the degree of neuronal degeneration in the dopaminergic SNc and the pedunculopontine nucleus, which sends cholinergic projections directly to nigral dopaminergic neurons. However, the exact mechanisms underlying the prion-like transfer/spread and the relative contributions of soluble and oligomeric species of α-syn or of other pathogenic proteins in the propagation of PD require further elucidation. Of note, recent studies found no evidence to support concerns that proteins underlying PD (or AD) transmit disease to humans.

Animal Models of Parkinson's Disease

Experimental models suggested to shed light on the neuropathobiology of PD come from essentially five sources: pharmacological (e.g., reserpine), toxic (e.g., MPTP [the pyridine toxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine]) [381], rotenone and paraquat [357, 382–384], transgenic [142, 143, 385, 386], and cellular [387]. During recent years, a myriad of different models carrying mutations similar to those found in humans in *Drosophila melanogaster* [388, 389], *Caenorhabditis*

elegans [390], rodents [143], and mammalians [391] have been developed to study the pathogenesis of this disease. Although some genetic models reproduced the key features of PD, they did not succeed in reproducing both the pathology and progressive degenerating process in human PD [392–395]. The sole model of DLB reported is an α-syn transgenic mouse [396].

Viral PD models comprising α-syn and LRRK-2-based overexpression or mimicking PARKIN loss of function by overexpression of PARKIN substrates [397], viral-vector-mediated α-syn lesion as a chronic and progressive model [398], and other recent genetic models may provide valuable insights into PD mechanisms in order to contribute to the development of therapeutic targets. The relevance of both pathogenic and etiologic models and their limits for new therapeutic studies have been reviewed recently [393, 399, 400].

Conclusion and Future Directions

Although PD remains as much an enigma as when James Parkinson first described its clinical features, the current knowledge of this devastating disease, its diagnosis, pathogenesis, and development continues to evolve and be challenged by future scientific discovery. Severe damage to the dopaminergic striatonigral system and many non-dopaminergic neuronal systems together with widespread occurrence of α-syn-positive LBs and dystrophic neurites are characteristic pathological findings, Lewy bodies representing its morphological hallmark. However, damage is not restricted to these structures, and PD and other synucleinopathies are now established multiorgan disorders, the etiology and pathogenesis of which are far from being fully elucidated. Recent evidence suggests that the heterogeneous mechanisms leading to sporadic PD are linked and that this progressive disorder is not the result of a single causative factor but is rather multifactorial, integrating the effects of genetic and environmental factors and a complicated cascade of molecular events to work in concert on many cellular systems to induce progressive degeneration in vulnerable neuronal populations. Among the most challenging problems are (1) the mechanisms of selective vulnerability in PD, (2) the factors leading to cell death in the SN and other important neuronal circuits, (3) the pathogenic role of α-syn aggregations and LBs, (4) the interaction of α-syn with other co-deposited proteins, including tau and β-amyloid, (5) the role of inflammation in the pathogenesis of PD, and (6) the better knowledge of the prion-like behavior of proteins and their impact on disease progression. Further research on the dysfunction of protein surveillance identified by the susceptibility genes, the interplay of the degenerative process with α-syn and Lewy pathology, essential pathogenic factors in the development of neurodegeneration, and the nature of triggers that unmask the disease process will be needed to enable the development of reliable biomarkers for early diagnosis, optimal animal models, and disease-modifying treatments of this hitherto incurable movement disorder.

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Role of the Innate and Adaptive Immune System in the Pathogenesis of PD

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Introduction

In our current understanding of PD, there is a dearth of disease-modifying therapies, comprehension of disease pathogenesis, and relevant biomarkers [1]. Inflammation has been increasingly recognized as an important process in disease pathogenesis. The role of inflammation needs to be clarified, particularly in relation to etiology and disease progression. Immunomodulatory therapeutics may be able to delay or attenuate disease, while functional profiling of peripheral immune cells could provide insight into biologic markers of disease [2]. The vast majority of research in PD has focused on neuronal and glial dysfunction independent of contribution of immune-related factors. In this chapter, we review the latest evidence for the role of inflammation and immunity in promoting disease susceptibility and progression.

Immune-Relevant Parkinson's Disease Pathology

Chronic neuroinflammation, Lewy body inclusions, and loss of dopamine-producing (DA) neurons in the substantia nigra parts compacta (SNpc) of the midbrain characterize PD pathology [3, 4]. The clinical diagnosis of PD and other Lewy-body diseases is confirmed postmortem by the histologic presence of Lewy bodies [4]. Intraneuronal aggregates of various proteins such as α-synuclein, tau, and ubiquitin make up Lewy bodies. The Braak hypothesis puts forth a comprehensive pattern for the spread of Lewy body pathology in PD. This hypothesis proposes that Lewy bodies initially form in the olfactory bulb or the gastrointestinal tract (areas that are constantly exposed to the environment). With disease progression, it is proposed that pathological hallmarks then appear in the vagus or olfactory nerve and

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M. Thomas (ed.), *Inflammation in Parkinson's Disease: Scientific and Clinical Aspects*,
DOI 10.1007/978-3-319-08046-8_3,

subsequently in the brainstem. Neurodegeneration of greater than 70 % of DA neurons occurs before onset of clinical symptoms. In addition, significant Lewy body pathology and neuroinflammation is present in that region. Pathology also occurs in the forebrain and cortical regions in the final stages of the disease [5]. This hypothesis is attractive for two reasons. First, it can explain the presence and timing of both nonmotor and motor symptoms. Second, it takes into account the multisystemic nature of the disease. Inflammation and the immune system would fit well into this hypothesis as a mediator of the spread of disease pathology.

One of the foremost challenges in the field is to identify the at-risk population for PD in order to understand early pathologic changes. Screening protocols using nonmotor symptom-based mechanisms are underway at various centers to identify people at risk for PD. Prospective study of such at-risk individuals can lead to further understanding the Braak hypothesis [5]. Furthermore, assessment of the inflammatory status of at-risk patients could serve to pinpoint the critical period in which immunomodulation could alter disease onset and progression. Understanding the neuroinflammatory changes that occur early in the progression of PD will also clarify the immune system's contribution to disease. The chronic neuroinflammatory hallmarks of PD such as microglial activation and immune cell infiltration will be discussed in detail later in the chapter.

Potential Inflammatory Etiology of Sporadic Parkinson's Disease

At clinical presentation, over 70 % of DA neurons in the SNpc have undergone degeneration. The severity of pathology at the time of presentation has precluded researchers from identifying the precise etiology of PD [6]. Despite this hurdle, there is general agreement in the field that both genetic and environmental factors contribute. Genome-wide association studies (GWAS) have identified multiple genetic polymorphisms that are associated with increased risk for sporadic PD. These identified genetic loci are thought to contribute to disease susceptibility. Examples of such loci include genes for α-synuclein, glucocerebrosidase, and tau. These polymorphisms could contribute to risk for disease in the context of disease-promoting environmental factors. Thus, specific environmental exposures combined with genetic background could promote pathologic process underlying PD.

Epidemiological studies and case reports have helped identify environmental exposures that promote the development of PD. In this way, we now understand that environmental exposures also modify the risk for PD. Indeed, the identification of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) was critical in recognizing a link between environmental exposures and the hallmark of PD pathology, the loss of DA neurons in the SNpc [7]. Before this discovery, no mechanisms were known that would selectively destroy cells of the nigrostriatal pathway. MPTP is metabolized into the cytotoxic MPP+ in DA neurons leading to neuronal death [7, 8]. MPTP has become routinely used in animal models of PD. The contribution of the immune system to these models will be discussed later in the chapter.

Unlike MPTP, the mechanisms through which other environmental exposures modulate risk of PD are unclear. The risk for PD is increased by pesticide/organophosphate exposure and head trauma while it is decreased by moderate amounts of cigarette use and caffeine consumption [9–11]. In terms of inflammation-related exposures, positive associations of PD with influenza, toxoplasmosis, and autoimmunity have been reported, while non-steroidal anti-inflammatory drugs decrease risk for PD [12–20]. In summary, genetic factors, as well as environmental factors, clearly contribute to the development of PD; however, neither is believed to be independently sufficient to cause sporadic disease. Thus, the complex interplay between genetic and environmental factors predisposes people to the development of sporadic PD. Determining the relative contributions from genes and environment and the pathologic processes such as inflammation which link them is important for understanding PD etiology. The immune system may be the nexus between genetics and environment.

The immune system interacts constantly with our environment. It must tolerate certain exposures or antigens while it responds to and eliminates others. Similar to other biological systems, abnormal immune function can lead to disease. An inadequate response to an infection leads to microbial invasion of the bloodstream, while hyper-reactivity to a harmless antigen can cause allergy or autoimmunity. The immune system is composed of both innate and adaptive immune cells. Innate immune cells respond to general patterns of antigens in the environment. These innate cells become activated in response to recognizing these antigen patterns, after which they recruit and activate cells of the adaptive immune system. The adaptive immune system actually develops a specific, tailored response to the antigens that activated the innate immune cells. Adaptive immune cells are uniquely suited to modulate inflammation in the context of certain environmental exposures and neuronal dysfunction. These adaptive immune cells can be activated by specific environmental exposures that cause these cells to become toxic to specific neuronal populations. The Braak hypothesis suggests that mucosal sites (nasal and intestinal) may be the initial focus of PD pathology. Furthermore, many complex immunoregulatory processes occur at mucosal surfaces that are constantly exposed to antigens. Thus, the immune system is uniquely poised to determine the body's response to various environmental exposures in predisposing to or protecting against PD. The immune system's capacity to play a role in propagating neuronal dysfunction and pathology within the central nervous system (CNS) could explain the systemic, progressive nature of PD. The contributions from genetic background, neuronal dysfunction, environmental exposure, and immunologic memory to immune function and risk for PD will make it difficult to contextualize its role in pathology. A survey of our current knowledge of the role of the immune system in PD is presented below.

Evidence for Inflammation in PD

There is a complex interplay between inflammation and neuronal dysfunction. Inflammation can induce neuronal death and neuronal death can induce inflammation. Mechanisms of neuronal dysfunction such as proteasome-mediated protein

degradation, mitochondrial dysfunction, and oxidative stress have been implicated in PD pathogenesis [21]. The mechanisms by which neuronal dysfunction is caused by inflammation have also been well established. Specifically in PD, midbrain DA neurons are very sensitive to cytokines such as TNF and IFN-γ [22–25]. Often, these cytokines are produced by microglia, CNS-resident immune cells. The SN's enhanced sensitivity to inflammatory stimuli is likely explained by the high density of microglia in this region [26]. Lipopolysaccharide (LPS), a bacterial endotoxin, is an activating ligand for TLR4 receptors on microglia. Because it can trigger the production of pro-inflammatory factors, a number of different LPS regimens have been used to model inflammatory signaling and its relationship to PD [27].

Abundant evidence in humans also demonstrates a role of chronic neuroinflammation in PD. CSF and postmortem nigrostriatal brain regions of individuals with PD relative have been reported to have elevated levels of cytokines (including IL-1β, TGF-β, IFN-γ, and IL-6) relative to those in age-matched healthy controls [24, 28–30] as well as complement proteins in extraneuronal Lewy bodies [31]. Interestingly, several studies have reported elevated levels of cytokines in the peripheral blood of PD patients relative to healthy controls, and these often correlate to Hoehn and Yahr staging [32] or nonmotor symptoms like depression [33]. Taken together, this evidence indicates that an active inflammatory process is ongoing in the CNS of PD patients that may also be reflected in the peripheral circulation.

An important challenge facing the field is the need for early diagnosis because this limitation impacts not only our ability to intervene with disease-modifying therapies in a timely manner but also our ability to definitely establish whether inflammation is a cause or consequence of the pathophysiology of PD. Nevertheless, thanks to cutting-edge neuroimaging technology, neuroinflammation is now recognized to be definitively present in the PD brain and has also been reproducibly detected in most animal models of PD [34]. LPS models are useful to test the extent to which inflammation alone can initiate neuronal dysfunction and nigral cell death. On the other hand, direct toxin models may better reflect how neuronal death induces inflammation, which then propagates further neuronal injury [35]. In many of these PD models, inflammation is often present before detectable neuronal dysfunction or death occurs, and in neurotoxin animal models such as MPTP and 6-hydroxydopamine (6-OHDA), anti-inflammatory immunosuppressive drugs like dexamethasone can attenuate DA neuron loss [36]. Viral overexpression of human α-synuclein in the rat or mouse SN induces inflammation and microglial activation that is followed months later by progressive death of TH+neurons [37, 38]. Importantly, inflammation is also observed in various transgenic mouse models of α-synuclein overexpression. Multiple models of transgenic mutant and wild-type human α-synuclein overexpression display chronic microgliosis [39–42]. Transgenic overexpression of wild-type human α-synuclein under the direction of the Thy1 promoter results in microglial activation and striatal TNF expression at 1 month of age, progressing to the SN at 5–6 months of age and persisting up to 14 months of age [43].

In summary, neurotoxin models (MPTP, 6-OHDA) directly damage DA neurons thereby initiating an inflammatory response, whereas in LPS and α-synuclein overexpression models, inflammatory responses appear to precipitate neuronal dysfunction and death. It has yet to be determined which of these two pathogenic processes predominates in PD since, at the present time, we are only able to diagnose the disease upon clinical presentation of motor symptoms in an already advanced stage. Investigation of inflammation in the context of multiple animal models remains extremely important because it will allow investigators to clarify the relationship between neuronal dysfunction, neuroinflammation, and the contribution of the peripheral immune system in disease risk and progression.

Peripheral inflammation is typically accompanied by elevated levels of pro-inflammatory cytokines that can alter the permeability of the BBB allowing increased influx of innate and adaptive immune cells from the periphery. These can in turn promote inflammation and propagate a feed-forward cycle to accelerate dopamine neuron loss and acceleration of PD motor symptoms [25]. PET imaging has enabled researchers to visualize inflammation in vivo, and evidence for increased blood brain permeability has been revealed through uptake by the molecular efflux pump P-glycoprotein and increased albumin in the CSF of PD patients [44, 45]. Possible mechanisms for this increased BBB permeability include cerebral capillary basement membrane thickening and collagen accumulation [46], as well as increased proliferation of blood vessels or endothelial cells [47]. Upregulation of ICAM-1, an important adhesion molecule for immune cell diapedesis or entry into brain parenchyma, has been reported in MPTP-induced rodent and nonhuman primate PD models [48, 49] and in blood vessels near the SN in postmortem brain tissue from PD patients [49]. Functionally, inflammatory responses that enhance BBB permeability may create permissive conditions for greater influx of peripheral immune cells that further propagate inflammation and efflux of an immunogen from the CNS to lymphoid organs where adaptive immunity can be engaged and maintained. In fact, the selective loss of specific neuronal populations while others are spared suggests a role for adaptive immunity in this process. Understanding how adaptive immune cells are exposed to neuronal antigens and are then recruited to the CNS in PD will be important for developing a coherent model of PD pathogenesis and will aid in the development of therapeutic approaches to promote neuroprotective immune responses.

Overall, because DA neurons are especially susceptible to damage from inflammatory stimuli, the role of neuroinflammation as a key pathogenic process in PD has become more than a curiosity and is now at center-stage. However, it is important to recognize that until earlier diagnosis or biomarkers are established, it will be rather difficult to unequivocally establish whether inflammation precedes neuronal injury by a significant extent and has a causal role in the process. Nevertheless, because the brains of PD patients with motor symptoms display chronic neuroinflammation, it may be possible to target inflammatory mediators that can be shown to actively contribute to disease progression and achieve disease modification.

Microglial Activation and MHC in PD

Some of the earliest evidence for inflammation in PD is from identification of chronic microglial activation in postmortem brains [50, 51]. Microglia are CNS-resident immune cells that play an important role in immune surveillance and tissue repair [52–54]. They are critical for recruitment of peripheral immune cells and thus amplify inflammatory signals in the CNS. The inflammatory signals that activate microglia originate from normal cross-talk between neurons and glia, neuronal dysfunction, or external insults (including trauma, pathogens, toxicants, etc.). Among the confirmed signals that activate microglia are bacterial and viral products, α-synuclein, complement proteins, antibodies, and cytokines [55–58]. Neuronal death may trigger microglial activation through loss of inhibitory CD200:CD200R signaling, ligation of microglial receptors that recognize factors released during apoptosis, release of α-synuclein, and/or through binding of complement or antibodies bound to neurons [56, 58]. In response to these stimuli, microglia produce cytokines [58–60], reactive oxygen species [61, 62], prostanoids that have immunomodulatory functions [63], and chemokines that recruit peripheral immune cells [64, 65]. While it has become increasingly clear that cytokines such as TNF have important roles in modulating synaptic plasticity [66], neuronal death can occur in response to cytokines through ligation of receptors with "death domains" such as TNFRs and Fas, reactive oxygen species, and phagocytosis of opsonized neurons [58, 64]. Activated microglia and other cells with antigen-presentation functions (B lymphocytes, macrophages, monocytes, dendritic cells) will express Major Histocompatibility Class II (MHC-II) molecules that present endocytosed or lysosomal peptides to CD4+ T lymphocytes. MHC-II genes are commonly known for their use in tissue typing for organ donation. MHC-II molecules are α/β heterodimers that require binding of a peptide approximately 8–10 amino acids in length to be stably expressed on the surface of activated antigen-presenting cells. The majority of these peptides are derived from the processing of endocytosed proteins or from the typical protein constituents of endolysosomes. Recognition of a specific peptide:MHC-II complex by a cognate CD4+ T cell through its unique receptor will activate the T cell and allow it to perform its effector functions: proliferation, cytokine secretion, assisting B cells to differentiate into plasma cells that produce higher affinity antibodies (through a process called hyperaffinity maturation), and allow for proper activation and maintenance of cytotoxic CD8+ T cells [67–70].

Microglial expression of MHC-II molecules has been reported in the states of chronic neuroinflammation, including neurodegenerative diseases [71]. In the healthy CNS, microglial MHC-II expression is difficult to detect. In PD brains, HLA-DR + microglia are found throughout the nigrostriatal tract and other parts of the CNS, including the hippocampus, entorhinal cortex, and cingulated cortex [50, 51, 72]. Positive correlations between disease duration and the microglial/macrophage activation marker CD68 and between MHC-II expression and amount of α-synuclein deposition in the SN of postmortem human brain sections have been observed [34, 55]. These activated microglia are associated with damaged neurons and Lewy body pathology [72]. In vivo PET imaging of PD patients reveals that

while the SN region of the midbrain has the highest level of microglia activation, other brain regions including cortical areas also display increased inflammatory signal. Interestingly, microglial activation does not increase linearly with the number of years after diagnosis, suggesting that neuroinflammation may start early in the disease and is maintained throughout the duration of the disease [73]. PET studies have also correlated microglial activation in the human midbrain with increased motor score as measured by the Unified Parkinson's Disease Rating Scale (UPDRS) [74]. After accidental exposure to MPTP in a synthetic heroine generated with faulty chemistry, microglia activation in the brains of drug addicts persisted for decades after initial exposure [75]. Similar observations have been made in nonhuman primates exposed to acute or chronic MPTP dosing [76–78]. In summary, microglial activation is prevalent in PD and MPTP-induced parkinsonism and occurs early in the degenerative process, raising the interesting possibility that some inflammatory processes and microglial effector functions may play a prominent role in promoting degeneration and neuronal loss. Microglial effector functions are likely to engage adaptive immunity in this context and together accelerate the progressive neurodegenerative process.

Particularly relevant for adaptive immunity, common single nucleotide polymorphisms (SNPs) in the MHC-II locus have been associated with increased risk for late-onset PD. Specifically, people homozygous for a risk-conferring SNP at rs3129882 in the first intron of HLA-DRA have a 1.7-fold higher risk for PD than in individuals homozygous for the non-risk-conferring allele [79, 80]. Other SNPs in the MHC-II locus associated with PD have also been reported in the HLA-DRB1 and -DRB5 genes. Another study has shown an association with the HLA-DQB1*06 allele in a German cohort [81–85]. The upregulation of MHC-II mRNA (HLA-DRA, HLA-DPA1, HLA-DQA1) and increased expression of HLA-DR on monocytes in cerebrospinal fluid (CSF) has been reported [86, 87]. Additional studies will be needed to assess whether any of these SNPs have functional consequences on MHC-II expression. Nevertheless, associations between late-onset PD and common variants in the MHC-II locus suggest that certain antigens or patterns of MHC-II expression may increase the risk for development of PD perhaps in response to some environmental trigger or pathogen. The quality of the interaction between CD4+ T cell receptors and peptide:MHC-II impacts CD4+ T cell expansion and differentiation [68, 69]. Thus, MHC-II expression in immune cells in the brain could modulate the overall quality of the neuroinflammatory response. Individuals with a certain MHC-II expression pattern or haplotype could more readily propagate inflammation initiated by neuronal dysfunction or death and in turn, such a mechanism may act to promote and/or exacerbate PD progression [88]. The evidence for involvement of T cells, B cells, and antibodies in promoting inflammation in PD is discussed below.

Given that gene–environment interactions are strongly suspected to underlie the etiology of late-onset PD, certain antigens or environmental exposures could synergize with a person's MHC-II genome to put them at risk for developing PD. In this manner, it could be said that the immune system plays a role in pathogenesis. Peptides presented on MHC-II would then activate T cells to influence neuronal

function through secretion of cytokines with known cytotoxic effects on vulnerable neuronal populations. This idea is supported by the association between PD and autoimmune diseases, such as bullous pemphigoid, systemic lupus erythematosus, and Sjögren's syndrome [12, 13, 15, 16]. In addition, gut inflammation is hypothesized to play a role in the development of sporadic PD. Given the extensive neuronal network of the GI tract, its constant interaction with the environment, and its extensive, unique immunological repertoire, the GI tract represents a potential initiation site of PD pathogenesis. Overrepresentation of *CARD15* mutations associated with Crohn's disease and *Helicobacter pylori* seropositivity before age 75 in people with sporadic PD supports this hypothesis [89, 90]. Additionally, a GWAS study identified associations between polymorphisms in the *LRRK2* gene (which encodes a MAP kinase kinase) and Crohn's disease, a type of inflammatory bowel disease. Interestingly, several mutations in the *LRRK2* gene give rise to an autosomal dominant form of PD (see below).

Also implicated in PD are immune responses to infections such as influenza, toxoplasmosis, and Epstein-Barr virus (EBV) [14, 17, 91]. In support of the idea that immune responses to certain viruses may predispose an individual to PD are the numerous cases of post-encephalitic parkinsonism after the 1918 Spanish Flu pandemic and a cross-reactive antibody between EBV and α-synuclein. Japanese encephalitis virus (JEV) has also induced a post-encephalitic parkinsonism with similar neuropathologic features and locomotor dysfunction as seen in people with sporadic PD [92]. JEV infection in rats results in hypokinesia attributable to depletion of catecholamines in the CNS [93]. Lastly, intranasal infection of mice with the H5N1 influenza A/VN/1203/04 strain induced α-synuclein phosphorylation and aggregation in the enteric nervous system [94] and resulted in viral replication in the gut, microglial activation, and increased proinflammatory cytokine and chemokine expression in the CNS lasting 90 days after viral clearance [95]; a transient decrease in tyrosine hydroxylase (TH) expression in the SN was also observed, suggesting that dopaminergic neurons displayed transient neurotoxicity and dysfunction. Because this specific strain of H5N1 was not detected in the brain, these studies provide compelling evidence that an immune response initiated solely by inflammation can induce PD-related neurologic dysfunction. In these examples, MHC expression would play a critical role in determining which antigens are presented to the adaptive immune system. Activated adaptive immune cells would then propagate the inflammatory reaction. The use of animal models of PD such as the human α-synuclein-overexpressing adenoassociated virus (AAV-Syn) model [38] that more directly engages adaptive immunity could help address the validity of these associations. Other neurotoxin models employing MPTP or 6-hydroxydopamine (6-OHDA) may be less likely to engage adaptive immune mechanisms because they are mainly driven by neuronal toxicity; but the role of adaptive immune cells in these neurotoxin models has also been investigated [48]. Clarifying whether the immune system acts as an etiologic factor in PD or simply propagates inflammation initiated by neuronal dysfunction will be an important part of determining PD pathogenesis and disease progression. A more clear and in-depth understanding of the immune system's role in PD and other neurodegenerative disorders will certainly help accelerate development of immunomodulatory strategies to delay or attenuate this progressive disease.

T Lymphocytes in PD

Naïve and memory T cells perform homeostatic surveillance in the CNS [96, 97], and as such these lymphocytes may be involved in both initiating and propagating steps of PD pathogenesis. In both PD patients and animal models, infiltration of T cells into the SN has been demonstrated [48, 50, 51]. In conjunction with HLA-DR expression on microglia, T-cell infiltration has been shown in postmortem brain sections of PD patients [48]. This evidence suggests potential direct interaction with antigen-presenting functions of microglia [50, 51]. Furthermore, levels of B2 microglobulin, a protein required for stability of MHC-I molecules, are increased in the striatum of PD patients [28]. MHC-I molecules activate CD8+ T cells by presenting cytosolic peptides processed through the proteasome pathway. Unlike MHC-II, MHC-I molecules are ubiquitously expressed on all cells (including neurons) except erythrocytes. Upon engagement of peptide:MHC-I, CD8+ T cells can directly kill cells by engagement of death receptors via Fas or TNF or by direct lysis through release of granzymes and perforin [98]. In addition to CD4+ T-cell-mediated toxicity, direct neuronal injury may be caused by CD8+ T cells that are reactive against neuronal antigens presented on MHC-I molecules [99]. Thus, T-cell-mediated neurotoxicity may be driven by direct cell lysis, engagement of cell death receptors, and cytokine secretion through recognition of peptide:MHC molecules.

Several studies have analyzed the composition of T-cell subsets in the peripheral blood of PD patients and have shed light on how adaptive immune responses are altered in this disease. PD patients are reported to have decreased overall numbers of lymphocytes without a change in frequency [100, 101]. Compared to people with other neurologic diseases, individuals with PD have increased memory T cells but decreased naïve T cells [86]. Memory T cells respond faster and with greater magnitude than activated naïve T cells [102]. A more activated, cytotoxic T-cell response is suggested by decreased CD4+:CD8+ ratios and a shift to more IFNγ− versus IL-4-producing T cells in PD patients [100, 101, 103]. Within the CD4+ T cell compartment, PD patients have been reported to have an increase in CD45RO + T cells, which signal an activated and/or memory T-cell population [104]. CD45RO + expression also positively correlates with UPDRS motor score [104]. Naïve CD4+ T cells have increased Fas expression, which may explain why their frequency is decreased [100]. Fas expression is normally upregulated after T-cell activation and ligation by FasL induces apoptosis. This process enables T lymphocytes to maintain immune privilege or initiate contraction after an immune response [105]. Increased memory and activated CD4+ T cells in conjunction with a relative increase in CD8+ T cells could suggest an active inflammatory process. Conflicting reports of changes in relative frequencies of CD4 + CD25+ T cells in the peripheral blood of PD patients exist, but without further characterization of this subpopulation, the functional significance of this is unclear [100, 101, 103, 106]. CD25 is increased on activated or memory T cells, as well as regulatory T (T_{reg}) cells. CD4 + CD25 + CD127- regulatory T cells from PD patients have been reported to have less suppressive capacity compared to cells from healthy controls [104]. The relative number of effector to

regulatory T-cell responses is thought to regulate immune responses [107]. Decreased effectiveness of regulatory T cells could promote a chronic neuroinflammatory state or allow for breaking of tolerance to neuronal antigens that would lead to abnormal immune responses against CNS proteins. A more clear understanding of why these regulatory T cells have reduced suppressive capacity could also provide insight into PD pathogenesis and disease progression.

Although some information about the changes in relative frequency of T-cell subsets in patients with PD relative to age-matched healthy controls is emerging, nothing is known about the identity of CNS antigens to which activated and memory T cells are responding in people with PD. One study looked at whether specific T-cell receptor genes are preferentially expressed in PD patients and reported that CD8+ T cells have a lower frequency of Vβ8 expressing cells [108]. The use of deep sequencing techniques to identify patterns of T-cell receptor usage in PD patients could provide evidence for response to specific antigens and aid in identification of those antigens whose patterns could also be potentially useful biomarkers of disease risk or progression. T-cell biomarkers have been investigated and some candidate proteins have been identified within these cells such as β-fibrinogen and transaldolase [109]. The pathogenic relevance of these changes remains to be established.

Other overall pathogenic changes in peripheral blood lymphocytes from PD patients have been reported. Lymphocytes from PD patients display an increased incidence of micronuclei, single-strand DNA breaks, and oxidized purine bases [110]. Interestingly, levodopa treatment seems to reduce this DNA damage in peripheral blood lymphocytes [111]. Markers of apoptosis, caspase-3 activation, and Cu/Zn superoxide dismutase activity are increased in lymphocytes from individuals with PD [112]. This DNA damage and increased level of apoptosis could be representative of a systemic, pathogenic process involving oxidative stress, specific immune responses, and/or intrinsic genetic factors. The use of animal models to assess the contribution of lymphocytes will inform how these in vivo changes in immune cell subsets may contribute to the disease etiology and progression in humans.

The contribution of T cells to PD-like pathology in animal models has been assessed but mainly in neurotoxin models. CD8+ T cells are detectable in greater numbers compared to CD4+ T cells in the acute MPTP neurotoxin model. In this model, these lymphocytes have increased expression of LFA-1, an integrin that binds endothelial adhesion molecules to allow for diapedesis across the BBB [113]. DA neuron loss and behavioral deficits caused by both chronic and acute MPTP administration are attenuated in RAG2 knockout mice that lack both T and B cells [48, 114]. Mice in which there is a global loss of αβ-T cells through knockout of the T cell receptor β-chain, as well as mice lacking CD4 T cells (*CD4−/−*), are also protected in the MPTP model [48]. The loss of CD8 T cells was not protective. Reconstitution of *Rag1−/−* mice with FasL-mutant splenocytes attenuated DA neuron cell death, while reconstitution with *IFNγ−/−* splenocytes did not. These findings suggest that Fas–FasL interactions involving CD4+ T cells play an important role in promoting MPTP-induced neurodegeneration. As discussed above, cytotoxic T cells use FasL to induce apoptosis in target cells but Fas receptor is also important in T-cell homeostasis and contraction. Impaired FasL–Fas interactions result in

unchecked T-cell activation and proliferation. An extreme example manifests itself in autoimmune lymphoproliferative syndrome caused by unchecked T-cell proliferation [115]. Dysregulation of this pathway may also leave chronically activated macrophages or microglia unchecked allowing for propagation of the inflammatory response. Fas–FasL signaling may also indirectly contribute to neuronal damage by inducing Fas-induced apoptosis-resistant astrocytes to produce proinflammatory cytokines that damage neurons [116]. In the intranigral AAV-human-α-synuclein overexpression mouse model, B- and T-lymphocyte infiltration in the SN persists after peak of microglial activation, suggesting that microglia in the SN engage adaptive immune cells to propagate inflammation in this model of PD [38].

Nitrated α-synuclein draining from the CNS into cervical lymph nodes may represent one molecular mechanism by which adaptive immune engagement occurs after MPTP-induced neuronal damage. Robust T-cell responses were initiated when mice were immunized with the C-terminus of the nitrated α-synuclein. Transfer of T cells from immunized mice into MPTP-treated mice enhanced neuroinflammation to a slight but significant degree [117]. T cells from mice immunized with nitrated α-synuclein produced mostly IL-17 and TNF after ex vivo restimulation. Adoptive transfer of Th17-polarized T cells from nitrated α-synuclein-immunized mice into wild-type mice greatly exacerbated MPTP-induced neurodegeneration. Unexpectedly, neurodegeneration was not exacerbated by adoptive transfer of T cells polarized to Th1 (IFNγ-producing) or Th2 (IL-4-producing) phenotypes. Yet, adoptive transfer of ex vivo differentiated regulatory T cells into MPTP-induced mice attenuated neurodegeneration [65]. The novelty of this study is that it demonstrates that modulation of the adaptive immune response can indeed determine the outcome of neurodegeneration in a model of PD.

Together, this evidence supports a model in which T-cell responses to modified α-synuclein or other modified antigens from DA neurons initiate an adaptive immune response that further propagates neuronal death. These responses would differ based on the context of antigen presentation. Various etiologies could allow for the adaptive immune system to be exposed to modified α-synuclein (i.e., toxins) or increased escape of CNS antigen (i.e., head trauma). These mechanisms would break normal tolerance mechanisms by presenting self-antigens that are cross-reactive with cognate peptide:MHC of existing memory T cells. These antigens could also activate naive T cells by presenting self-antigens for which T cells are not normally negatively selected or tolerized against in the context of an inflammatory response. Additional studies to identify specific antigens to which T cells respond in animal models of PD and in humans with PD will be critical to our understanding of the role of the adaptive immune system in pathogenesis and progression of PD.

Antibodies and B Lymphocytes in PD

B lymphocytes are the key mediators of humoral immunity in that their secreted immunoglobulins can have effects at sites far from the actual site of secretion [118]. Unique antibodies, as well as increased antibody levels against CNS proteins, are

present in people with PD [119, 120]. However, infiltration of antibody-producing B lymphocytes into the CNS is not a common histopathological postmortem finding in brains of PD patients [48, 50, 51]. While infiltration of B lymphocytes is present under some inflammatory conditions, the aggregation of antibodies at a specific site is enough to promote inflammation without the presence of actual B cells. Antibodies can precipitate inflammatory reactions through activation of the complement system or effector cells through surface immunoglobulin receptors [118]. A decrease in the number of peripheral blood B cells in PD patients has been reported but the functional significance of this finding needs to be contextualized [100, 101]. For instance, reduced peripheral blood B cell counts in other autoimmune or inflammatory diseases are due to decreased circulating memory B cells. Thus, the reduction in B cell number may be related to active inflammation and cellular activation [121–123]. Additional research is needed to understand the role for B cells in PD beyond their antibody-secreting functions which are discussed below.

Antibodies against DA neurons that are not present in healthy people have been reported in PD patients [119, 120, 124, 125]. At present, the lack of a universal antibody or "PD"-specific antigen refutes an etiologic role for B cells and antibodies but does not rule out a pathogenic or protective role in modulating inflammation in PD. Alternatively, it is entirely possible that a universal antibody or antigen in PD will not be found because no single antigen is required for disease induction. On the other hand, it may not be possible to identify such an antigen because antibody production is typically assessed during the late stages of disease. In neurohistological evaluation of postmortem PD brains, immunoglobulins have been reported to colocalize with pigmented DA neurons in close proximity to FcγR-positive microglia [126]. The number of IgG-immunoreactive neurons positively correlated with the number of MHC-II-positive microglia, suggesting a link between antibody-mediated destruction of neurons and antigen presentation. Further supporting this link, these microglia contained intracellular pigmented granules, suggesting their participation in antibody-mediated phagocytosis of pigmented DA neurons. A pathogenic role for anti-neuronal antibodies in PD is further supported by studies in which immunoglobulins from PD patients induced neurotoxicity in a rat model when injected into the SN resulting in a loss of TH+neurons while sparing acetylcholinesterase-positive neurons in the medial septal region [127]. The injected immunoglobulins induced perivascular inflammation, microgliosis, and loss of TH+neurons in the SN in an Fcγ receptor-dependent fashion [128]. Similarly, Fcγ receptor knockout mice are resistant to chronic but not acute MPTP administration [114]. One caveat to these results is that the deletion of Fcγ receptors in mouse models impacts numerous cell types (monocytes, microglia, neutrophils, basophils, mast cells, NK cells) and reduces the ability to respond to cellular-bound IgG through multiple mechanisms. These mice are also resistant to neurodegeneration and microglial activation induced by AAV-induced intranigral expression of human wild-type α-synuclein [129]. The AAV-α-synuclein model also leads to significant IgG deposition, suggesting that humoral immunity and/or breakdown of the BBB is playing a role in the degenerative process [38]. Together, these findings strongly suggest that the humoral arm of the immune system can play a role in chronic neuroinflammation and demise of DA neurons.

Given the chronic inflammatory nature of PD, humoral immunity could play an important role in the progression of PD while T-cell immunity may be more important for disease onset. Intuitively, this idea makes rational sense because CD4+ T-cell activation is essential for potent engagement of humoral immunity. In contrast, antibodies may also play a protective role. The use of a mouse antibody against α-synuclein demonstrates that microglia can take up antibody-bound α-synuclein in vivo and prevent neuron-to-astrocyte transmission of α-synuclein [130]. It is unknown whether this phenomenon can happen in the PD brain, but it raises the distinct possibility that certain sets of antibodies could play a pathogenic versus a protective role. Lastly, pathogenic antibodies could promote inflammatory reactions or propagate α-synuclein spread through uptake by Fc receptor-expressing cells, while other antibodies may be protective by aiding in clearance of α-synuclein aggregates.

A handful of studies have investigated the extent to which antibodies could be used as biomarkers for PD. One study found decreased levels of anti-α-synuclein antibodies in PD patients but not in controls or patients with other neurodegenerative conditions with sensitivity and specificity of 85 and 25 %, respectively. These antibodies did not correlate with age, duration of disease, or Hoehn and Yahr staging [119]. It should be noted that while the presence of α-synuclein antibodies may hint at pathologic mechanisms, it is unclear whether they could be used alone for diagnostic purposes. Another study demonstrated the ability to use ten autoantibody biomarkers to differentiate PD from normal aging, Alzheimer's disease, breast cancer, and multiple sclerosis with accuracies of over 85 % [120]. Yet other studies reported negative findings in that the frequency of anti-α-synuclein antibodies in patients with sporadic PD is not significantly different from those in healthy controls [125]. However, around 90 % of people with familial PD have these antibodies. The authors suggest that the antibodies in familial PD may represent more pathogenic epitopes rather than incidental ones resulting from secondary tissue damage. Additional research aimed at detecting antibodies at earlier stages of disease will likely provide more insight into the role of antibodies in PD pathogenesis. Antibodies generated in the earliest stages of PD are more likely to be related to pathogenic epitopes and less likely to be incidental (i.e., from the release of CNS antigens) as inflammation becomes exacerbated during the progressive neuronal damage in the disease.

Genetic Mutations that Cause PD and Adaptive Immunity

Many of the genetic causes of familial PD have been identified [131, 132]. Hereditary forms of PD, caused by specific gene mutations, only comprise 5–10 % of PD cases [4, 133]. These genetic forms of PD typically have an earlier age of onset and provide insight into the potential role of ubiquitously expressed proteins in neuronal and immune dysfunction in sporadic PD [133]. Yet, it is generally believed that genetic and sporadic forms of the disease are likely to share common mechanisms. Therefore, the study of these genetic mutations has provided insight

into how perturbation of certain pathways may contribute to the development of sporadic PD. Despite the fact that most of these PD-linked genes have been shown to be expressed in immune cells and may contribute to nonautonomous cell death of neurons, the majority of research efforts to date have investigated the pathogenic roles of these mutations in neurons only. Yet, it is important to recognize that dysfunction of these proteins in both neurons and immune cells would allow for synergy of inflammatory and neuronal death mechanisms that promote the development and/or progression of PD.

The *SNCA* gene, which encodes α-synuclein, was the first mutation linked to a familial form of PD [134]. Since then, a gene triplication in SNCA has also been linked to a familial form of PD [134], and polymorphisms in the promoter region of SNCA that drive increased expression of α-synuclein have also been identified as genetic risk loci [135]. But while α-synuclein was once believed to be primarily expressed in neurons, it should be noted that the *SNCA* gene and therefore the α-synuclein protein are also expressed in a wide variety of immune cells including T cells, B cells, NK cells, microglia, and monocytes [136–139] as are most of the genes implicated in familial forms of PD including parkin and DJ-1. In microglia, α-synuclein has been shown to have a homeostatic role. Microglia from mice lacking *SNCA* have a more activated phenotype in terms of morphology and cytokine secretion in addition to decreased phagocytic ability [139, 140]. Consistent with the idea that it may have some regulatory role in inflammatory responses, the level of α-synuclein protein increases after LPS stimulation of monocytes and lymphocytes [141]. Autophagy plays a critical role in maintenance, activation, and proliferation of T cells [142], and individuals with the autoimmune disorder systemic lupus erythematosus CD4+ T cells express five times higher levels of α-synuclein that correlate with higher basal levels of autophagy [143]. Therefore, changes in immune cell levels of α-synuclein could modulate cellular function via mechanisms such as altered autophagy. This last point may be especially relevant as altered number or frequency of T and B cells have been reported in PD patients. In mice expressing human wild-type α-synuclein on the Thy1 promoter, frequencies of CD4 and CD8 T cells are increased in the peripheral blood [43]. α-Synuclein is also overexpressed in the T cells of these mice; thus the role of this protein in immune cells versus neurons will need to be evaluated in greater depth. It is unknown whether the inflammatory phenotype is attributed to primary deficits in immune cells overexpressing α-synuclein versus to secondary activation of immune cells as a result of overexpression of α-synuclein in neurons and neuronal dysfunction. The critical point here is that the functional significance of mutations or polymorphisms in α-synuclein in immune cells has been understudied. In addition, perturbations in the levels of α-synuclein by various stimuli, such as LPS, in the context of genetic polymorphisms inherent in immune gene loci could lead to immune dysfunction in those individuals that may synergize with and exacerbate neuronal dysfunction. Consistent with these observations, signs of synergy between inflammation and α-synuclein-induced degeneration have been observed in numerous cell culture and animal model studies as discussed previously. This synergy is likely to have important consequences in the brains of humans affected with PD.

Mutations in *LRRK2* (*PARK8*), a gene encoding a protein kinase with an MAPK-kinase–kinase, GTPase, and ankyrin domains, cause an autosomal dominant form of PD with a clinical presentation and age of onset similar to sporadic PD [144]. The incomplete penetrance (estimated at 33–67 %) of these mutations also suggests a strong environmental and/or immune component that modifies disease onset [145, 146]. Genetic variants in this gene have also been associated with increased susceptibility for sporadic PD [83, 147]. Early studies analyzing LRRK2 function and expression in neurons demonstrated that its expression is low in the SN and greater in areas of the brain that receive dopamine signals [148–151]. *LRRK2*−/− mice are more susceptible to dextran sodium sulfate-induced colitis [152]. In vitro and targeted in vivo overexpression of mutant LRRK2 induces neurotoxicity in a kinase- and GTPase-dependent manner [153–157]. However, germ-line mutant LRRK2 transgenic mice do not completely replicate PD-like phenotypes or show spontaneous DA neuron degeneration [158–161]. However, these mouse models do show behavioral alterations and impaired dopamine release in the nigrostriatal system. Indeed, perturbations in LRRK2 function alone may not be sufficient to induce PD-like pathology, but may require a second insult such as inflammation.

Interestingly, LRRK2 expression occurs in many immune cells: dendritic cells, monocytes, microglia, B cells, and T cells [162, 163]; yet the role of LRRK2 in various immune cell populations has not received much attention in recent years. The level of expression in many immune cell subsets is significantly greater than in neuronal populations [164]. Mouse bone marrow-derived macrophages (BMDMs) significantly upregulate LRRK2 mRNA expression after stimulation with LPS and lentiviral particles. BMDMs from R144C-LRRK2 mutant mice have reduced LC3-II expression, a marker of autophagy [162]. LRRK2 expression is consistently upregulated in immune cells after IFNγ stimulation and activation of the NF-κB, a key pathway important for production of microbicidal reactive oxygen species during phagocytosis of pathogens during intracellular infection [164]. In microglia, LRRK2 expression can be detected upon in vivo activation with LPS and is upregulated by ex vivo LPS treatment [165]. Furthermore, LRRK2 inhibition or knockdown in microglia attenuated LPS-induced inflammatory signaling as measured by cytokine secretion, microglial chemotaxis, and morphologic remodeling. Specific LRRK2 SNPs have been shown to associate with leprosy and Crohn's disease [166, 167]. In fact, increased LRRK2 expression is found in the epithelium of Crohn's disease patients in many cell types including macrophages, dendritic cells, and B lymphocytes [164]. Of direct relevance to adaptive immunity, LRRK2 inhibits the nuclear translocation of NFAT, a master regulator of transcriptional activation in T cells [152]. Overall, it is clear that LRRK2 is likely to be involved in regulating the activation status and effector functions in multiple kinds of immune cells. Therefore, the role of pathogenic *LRRK2* mutations in specific LRRK2 domains is worthy of investigation within the context of the immune system and its role in predisposing an individual for age-related degeneration.

The *Parkin* gene encodes a multidomain protein that contains E3 ubiquitin ligase activity that plays a role in the regulation of numerous cellular activities including proteasomal degradation and ubiquitin-mediated signaling; and loss of function mutations and deletions in the *Parkin* (*PARK2*) gene cause autosomal recessive

forms of juvenile PD [168]. Although the physiologic substrates of parkin are unknown [169], the potential roles for this protein include a role in preventing protein aggregation and/or promoting mitophagy [170–172]. Parkin has also been suggested to function as a transcription factor that regulates p53 expression [173] independent of its ligase activity. Parkin knockout animals do not have loss of DA neurons in the SN and do not have increased vulnerability to MPTP, yet they do show nonmotor behavioral changes related to changes in the nigrostriatal pathway [174, 175]. This phenotype suggests compensatory changes in the global Parkin knockout animal that prevent death of DA neurons or that a second insult is required in conjunction with lack of Parkin expression. Consistent with this idea, Parkin knockout mice display increased vulnerability to inflammation-related degeneration. Specifically, chronic peripheral low-dose intraperitoneal injections of LPS in Parkin knockout mice trigger fine-motor deficits and loss of DA neurons in the SN [176]. In addition, in microglia, neurons, and peripheral macrophages, LPS and TNF dose-dependently reduce *Parkin* expression due to the presence of an NF-κB response element in the *Parkin* promoter [177]. Therefore, chronic inflammation may downregulate Parkin and phenocopy loss of function *Parkin* mutations. Interestingly, Parkin has also been implicated as a susceptibility locus for leprosy and typhoid fever [178, 179] and more recently was demonstrated to be critical for host defense against *Mycobacterium tuberculosis* [180]. In summary, current evidence implicates a protective role for *Parkin* in inflammation-induced degeneration, and inflammatory pathways negatively regulate Parkin expression. Therefore, the role of Parkin in inflammation and immune cells merits a more thorough evaluation as it may represent an important point of convergence for the functional consequences of inflammatory stress and its effects on PD pathogenesis and progression.

Identification of the normal physiological role of proteins wherein pathogenic mutations cause genetic forms of PD will be critical for a clear mechanistic understanding of PD pathogenesis. It is clear that in the last decade, most of the research focus on the role of these proteins has been neuron-centric despite the fact that most PD-linked genes are expressed in many other cell types. Immune cells in particular are poised to interact with the environment and respond in a coordinated manner. In genetic forms of PD, dysfunction of these proteins may render neurons more vulnerable and promote their dysfunction. In sporadic PD, environmental exposures may alter the expression of these genes in immune cells which could then indirectly modify neuronal function and promote the development of PD. Therefore, further research of this normal and pathogenic role of these proteins in immune cells is well-justified and is expected to advance our understanding of nonautonomous cell processes that contribute to degeneration in PD.

Immunomodulatory Therapies

Given the chronic, progressive nature of PD and the large amount of neurodegeneration that has already occurred before clinical presentation of motor symptoms, it is highly unlikely that immunomodulatory therapies will have a significant impact in

altering the course of PD. Therefore, efforts to enable early diagnosis in premotor stages and timely treatment will be necessary to significantly impact disease. Nevertheless, because intense investigation into biomarkers of early disease is in progress, it is critical that we continue identifying immunomodulatory therapies that could be translated to the clinic. To this end, we will review a handful of therapies that have targeted immune pathways in animal models of PD and shown some therapeutic benefit.

Modulation of the T-cell repertoire to suppress T effector and boost regulatory T-cell function is one such example. Copaxone, also known as glatiramer acetate, is a random polymer of amino acids from myelin basic protein used in the treatment of multiple sclerosis. This mixture is thought to mimic the antigenic properties of myelin basic protein but alters the T-cell response to a more anti-inflammatory phenotype [181]. Adoptive transfer of T cells from a copaxone-immunized animal afforded protection against neurodegeneration in an MPTP mouse model of PD [182], suggesting that T-cell skewing to dampen the adaptive immune response could potentially have a disease-modifying effect. The adoptively transferred T cells accumulate in the SN, suppress microglial activation, and are correlated with an increase in neurotrophic factors [183]. Consistent with this mechanism, adoptive transfer of T_{regs} also attenuated neurodegeneration in an MPTP mouse model of PD [65]. Decreased effectiveness of regulatory T cells in their suppressive capacities and changes in the T-cell compartment suggest that correcting phenotypic T-cell changes in PD patients may provide a beneficial disease-modifying effect [104]. To this end, Gendelman and colleagues are initiating a clinical trial to assess the efficacy of Sargramostim, a granulocyte–monocyte colony-stimulating factor analog thought to promote the development of T_{regs}, in the treatment of PD. If successful, this trial could demonstrate the usefulness of immunomodulatory therapies in slowing down progression of PD.

The use of a dominant-negative inhibitor of TNF (DN-TNF) in PD animal models suggests that anti-TNF biological agents may also be of clinical value to delay or attenuate nigral DA neuron degeneration if administered in a timely fashion. Specifically, administration of soluble DN-TNF peptides whose mechanism of action renders them selective for the soluble form of TNF [184] via osmotic pump or lentiviral overexpression in the SN shows protection in 6-OHDA models [185, 186]. Even delayed lentiviral overexpression of DN-TNF in the SN can be protective against progressive DA neuron loss [187]. Therefore, if the idea is to dampen overall levels of peripheral or central inflammation, anti-TNF biologics may be of therapeutic value in PD.

Passive immunization in animal models with anti-α-synuclein antibodies have also been investigated as a potential mechanism for clearing accumulations of α-synuclein. In mice expressing human α-synuclein under the platelet-derived growth factor β promoter, administration of an anti-α-synuclein antibody improved behavioral performance and promoted degradation of accumulated α-synuclein [188, 189]. Clearance of α-synuclein is thought to occur via uptake through Fc-receptor-expressing cells such as microglia. These observations will need to be replicated using other human α-synuclein overexpression models independently to

confirm the promising potential of synuclein immunotherapy. While it is unclear if removal or prevention of α-synuclein-containing Lewy body formation is required to ameliorate PD progression, it is an important question that requires investigation in order to determine whether such therapies should be translated to the clinic.

Lastly, in light of the possibility that adaptive immune cells may contribute to the pathogenesis and/or progression of PD as highlighted in this review, several other strategies to modulate T-cell function may merit investigation in preclinical animal models of PD. Specifically, immunosuppressive therapies that block T-cell proliferation may be useful in modifying progression or onset of PD. Such therapeutics could include corticosteroids, colchicine, or peripheral T-cell-depleting antibodies such as muromonab-CD3. Colchicine blocks mitosis and thereby prevents proliferation of rapidly dividing cells such as activated immune cells; although medicinal chemistry to modify this drug may be needed as is has significant gastrointestinal side effects. In a rat model of the pesticide rotenone wherein microglial activation is believed to be critical for nigral degeneration [190], colchicine showed benefit in preventing neurodegeneration [191]. Muromonab initially activates CD3+ T cells but then these activated cells die; this antibody is currently used to prevent transplant rejection [192].

A second avenue for immunomodulation is the use of drugs that block T-cell activation or recruitment. Abatacept and belatacept block the interaction between CD28 on T cells and CD80/CD86 molecules on antigen-presenting cells [192, 193]. These drugs have been used in transplant rejection and rheumatoid arthritis with great success [193, 194]. Given the documented changes in activated and memory T-cell subsets in people with PD, these drugs could have therapeutic success in limiting such T-cell populations. The antibody against the α4 integrin, natalizumab, has been successful in the treatment of multiple sclerosis because it blocks the recruitment of T cells to sites of inflammation [195]. Therefore, it may be possible to dampen neuroinflammation and lessen degeneration in PD by blocking activation and recruitment of T cells to sites where vulnerable neuronal populations reside.

Ex vivo modification of autologous T cells to make them produce anti-inflammatory molecules or have them target effector T cells that are pathogenic in PD might also be another useful therapeutic approach. This approach would require identification of pathogenic T-cell populations or the relevant antigens to which pathogenic T cells are responding in PD. Such technologies are currently being explored in harnessing immune responses against cancer [196].

Therapies to target humoral immunity do exist and have shown effectiveness in autoimmune diseases where antibodies are known to play a pathogenic role. Rituximab is an example of a B-cell depleting antibody that has shown some effectiveness in SLE [197]. Intravenous immunoglobulin (IVIG) has been effective in treating diseases that are mediated by pathogenic antibody responses such as erythroblastosis fetalis, autoimmune thrombocytopenia, and chronic inflammatory demyelinating polyneuropathy. IVIG is thought to block proinflammatory antibody-mediated responses through many pathways including modulating Fc-receptor expression, expansion of T_{regs}, and blocking activating Fc receptors [198]. IVIG is generally well tolerated with less than 5 % of people experiencing headache, fever,

or nausea. In a recent Phase III trial by Baxter, IVIG did not show efficacy in the treatment of mild to moderate Alzheimer's disease; however, since the underlying pathogenic mechanisms in PD are different, the lack of success in AD may not reflect on its potential in PD. It should be noted that this study demonstrated that IVIG was safe in elderly persons with a neurodegenerative disease. Thus, IVIG could be a safe immunomodulatory therapy potentially useful in PD.

As with any immunomodulatory therapy, immunosuppression or off-target effects are of great concern, especially in an elderly population that is at increased susceptibility to infections. But as learned in the transplant field, timing of these and any other therapeutic intervention is also critical [192]. The use of these therapeutics at the time when inflammation is beginning or is primed could have significant positive impact on the course of disease while having minimal effects much later in the course of the disease. Additional research in preclinical models of disease should enhance our understanding of the role of the adaptive immune system in PD pathogenesis and progression and will inform which immunomodulatory therapies are most suitable for advancing to clinical trials in PD.

Conclusion

The adaptive immune system normally responds to specific pathogens or antigens and has various effector mechanisms to clear them, i.e., direct cytotoxicity, cytokine production, and antibody production. The adaptive immune system must receive the appropriate signals from innate immune cells to be activated in order to respond to specific antigens recognized by uniquely specific receptors. T cells recognize peptides presented on MHC molecules while B cells recognize antigens through their cell surface receptors. Abundant evidence exists for the role of inflammation in human PD as well as in animal models of the disease. Environmental and genetic factors synergize to create susceptibility to PD. It remains to be seen whether these factors create susceptibility primarily through inducing neuronal dysfunction or through direct activation of the immune system that then propagates neuronal dysfunction. Regardless, engagement of the adaptive immune system can further precipitate neuronal dysfunction in a specific manner, i.e., from antigens derived from DA neurons. Modification of these antigens by inflammation and environmental factors may allow for an adaptive immune response in bypassing normal tolerance mechanisms. Nitrated α-synuclein is one potential antigen in this case. From the initiation site of this inflammation, a chronic adaptive immune reaction could spread neuronal damage and dysfunction to other CNS pathways implicated in sporadic PD. The adaptive immune system may prove to be the missing link between environment, genetics, and the spread of pathology put forth by the Braak hypothesis. Further investigations aimed at delineating the role of the adaptive immune system in PD pathogenesis and progression and the specific peptides that are being presented to T cells is prerequisite for development of suitable immunomodulatory therapies that can delay onset or modify the course of the disease.

In this review, the evidence for activation of the adaptive immune system in human PD has been presented, as well as our present understanding of how the effector functions of adaptive immune cells damage DA neurons in animal models of PD. Immune cell expression and functions of proteins encoded by genes wherein mutations give rise to familial forms of PD have also been presented. Taken together, exciting clues for a newly recognized role of the adaptive immune system exist but much work remains to be done in this area. Closer and more in-depth examination of this important topic may provide a better understanding of PD as well as an opportunity to engage in paradigm-shifting research to identify immune-related biomarkers of PD and disease-modifying therapies to prevent, arrest, or at least delay the onset of this debilitating disease.

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"Good" and "Bad" Microglia in Parkinson's Disease: An Understanding of Homeostatic Mechanisms in Immunomodulation

Yu Tang and Weidong Le

Parkinson's Disease and Neuroinflammation

Parkinson's disease (PD) is the second most common neurodegenerative disease, manifesting with motor and non-motor symptoms, and the incidence increases markedly with age [1]. It is pathologically characterized by the intracellular inclusions containing α-synuclein called Lewy bodies (LBs) and the progressive loss of dopaminergic (DA) neurons in the substantia nigra (SN) of the midbrain [2]. It is now widely accepted that PD pathogenesis is driven by combined genetic and environmental factors. As the vast majority of PD cases are sporadic (>95 %), environmental factors that interact with common susceptibility genes are most likely to influence the onset of most cases of sporadic PD.

PD is also a chronic inflammation disease, with extensive neuroinflammation and "reactive microgliosis" defined by the microglial response secondary to direct neuronal lesions [3, 4]. The involvement of neuroinflammation in DA neuronal death in PD can be briefly accounted for by the following evidence: (1) prominent reactive microgliosis in the SN of patients and animal models of PD is revealed in postmortem and imaging analysis; (2) DNA polymorphisms in

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M. Thomas (ed.), *Inflammation in Parkinson's Disease: Scientific and Clinical Aspects*,
DOI 10.1007/978-3-319-08046-8_4,

several inflammatory cytokines are studied as risk factors for PD, whereas deficiency of pro-inflammatory factors can produce protective effects; (3) experimental evidence shows that the suppression of inflammatory processes mitigates neuronal impairment in both in vitro and in vivo models; (4) long-term use of non-steroidal anti-inflammatory drugs (NSAIDs) in PD manifests a possible beneficial effect [5]. Although neuroinflammation may not typically represent an initiating factor, current scientific evidence demonstrates that sustained inflammatory responses involving microglia and astrocytes definitely contribute to progression of PD.

Microglia Involvement in PD Pathogenesis

The main executors of neuroinflammation, microglia, are the resident innate immune cells in the central nervous system (CNS), providing the first line of defense of the innate immune system whenever injury or disease occurs [6]. Microglia density varies in brain regions of both adult human and mice, with the highest concentrations being found in the hippocampus, olfactory telencephalon, basal ganglia, and SN [7, 8]. Thus, DA neurons in the SN might be particularly vulnerable to inflammatory insults compared with other regions, owing to the co-localization with a large increased number of microglia in the SN [7, 9].

In the early stage of PD, positron emission tomography (PET) imaging shows an increase of microglial activation, and the level of activation is inversely correlated with density of DA terminals, whereas it positively correlates with motor impairment [10]. Postmortem analysis of PD patients also reveals activated microglia presenting upregulated surface molecules and increased accumulation of inflammatory mediators in the SN [11, 12]. In addition, increased levels of cytokines in the cerebrospinal fluid (CSF) in PD have also been reported [4].

"Good" Microglia and "Bad" Microglia

Although microglial activation has been described extensively in PD, its impact on disease pathogenesis remains controversial. Until recent years, neuroinflammation was thought to be a double-edged sword. For a long time, we considered microglial activation as a monophasic process that contributes to neuronal death. Microglia rapidly sense a wide range of stimuli and produce a series of pro-inflammatory cytokines, numerous toxins, including reactive oxygen species (ROS), free radicals, proteases, and chemokines to destroy infectious organisms and infected neurons [13]. These inflammatory mediators together with the neuron debris, in turn, induce more widespread damage to neighboring neurons (reactive microgliosis).

Consequently, a vicious cycle between injured neurons and uncontrolled inflammation inevitably occurs [13] (Fig. 1).

In 2005, Heppner et al. [14] generated CD11b-HSVTK transgenic mice, in which microglial release of nitrite, pro-inflammatory cytokines, and chemokines was specifically abolished. Interestingly, this "microglial paralysis" was able to repress the development of experimental autoimmune encephalomyelitis (EAE), a mouse model of multiple sclerosis (MS), and repress EAE-associated inflammatory infiltrates [14]. This finding suggests that microglial activation might be detrimental to development of MS, and this also provides insights into the therapy of other immune-mediated CNS diseases, including PD, by targeting microglia.

It is important to understand the complexity of microglial activation in the healthy brain. Under physiological conditions, microglia exhibit a quiescent phenotype surveying the microenvironment and secrete anti-inflammatory and neurotrophic factors that are seemingly not toxic, but protective [15]. The primary objective of microglial activation is to engulf invading viruses, bacteria, or other foreign materials and promote clearance of toxic proteins and cell debris in the injury site through phagocytosis [5, 16, 17]. However, pro-inflammatory cytokines released upon microglial activation are also reported to inhibit microglial phagocytosis that can be relieved by co-incubation with anti-inflammatory cytokines or cyclooxygenase (COX) inhibitors, suggesting that microglia in different states of activation behave differently while removing pathogens or dead cells [18].

In addition to eliminating harmful stimuli, inflammation is also critical for repairing damaged tissue. Glial-derived neurotrophic factor (GDNF) has been reported to promote neuronal survival and rescue injured DA neurons [19, 20]. Microglia also secrete multiple anti-inflammatory cytokines and promote gene expression related to tissue recovery and regeneration. Moreover, microglia facilitate repair by directing the migration of stem cells to the site of inflammation and injury and instruct neurogenesis in the neurogenic niches in various areas [21, 22]. Therefore, without neuroinflammation, removal of offending pathogens and recovery from CNS injuries become impossible.

Regulatory Mechanisms of "Good" and "Bad" Microglia

Microglia exist in two differentiated states: "bad," or neurotoxic, microglia and "good," or neurorestorative, microglia. These two yin and yang states are interchangeable depending on the milieu of the affected region. "Bad" microglia are not bad as described, which is also necessary and crucial for host defense. Both "good" and "bad" microglia are required for keeping the homeostasis of the CNS.

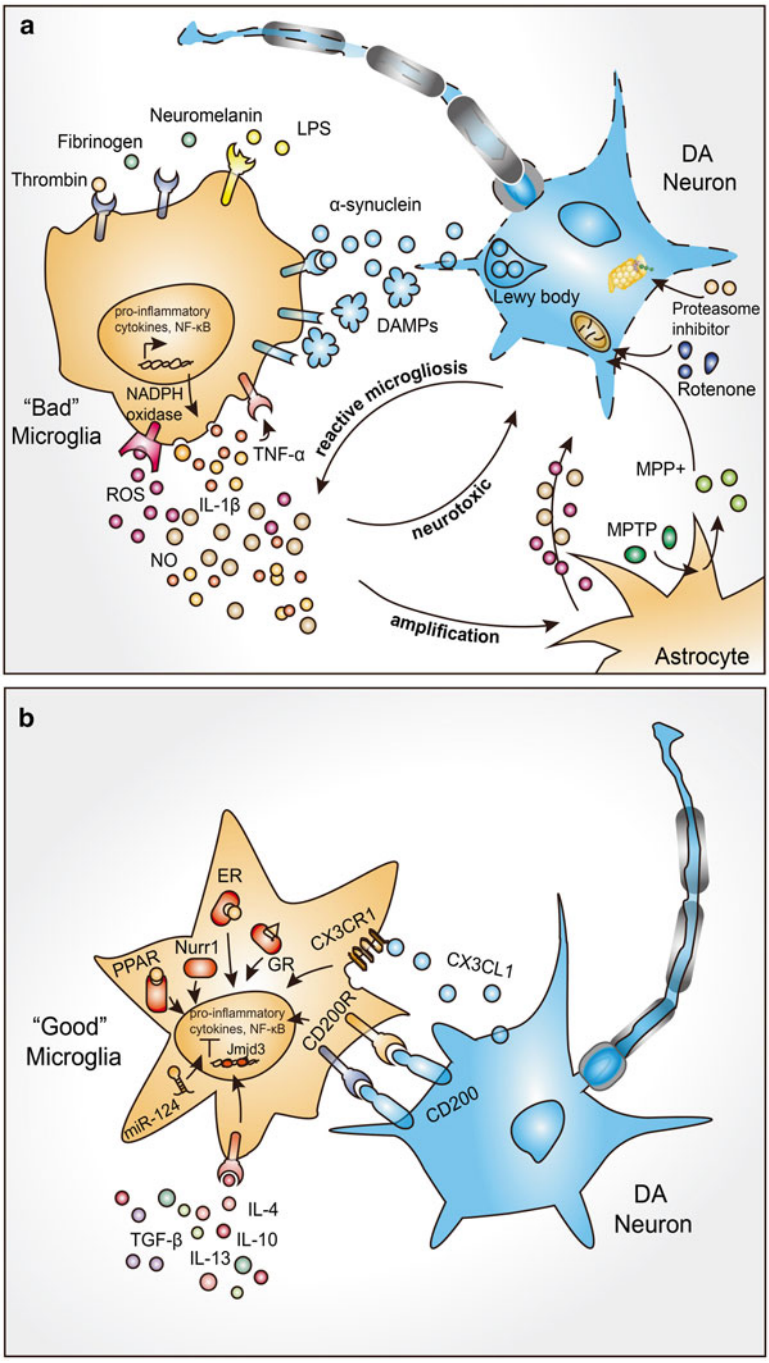

Fig. 1 Illustration of involvement of "bad" microglia and "good" microglia in PD. (**a**) Illustration of the vicious cycle between "bad" microglia and neurons. Microglia can rapidly sense a wide range of stimuli including released endogenous proteins such as α-synuclein, damaged cell debris,

Inducers and Effectors of "Bad" Microglia

Inducers of "Bad" Microglia

Activation of "bad" microglia produces a variety of pro-inflammatory factors, chemokines, and cytokines. There are different outcomes in microglia corresponding to different inducers. Briefly, these inducers are involved in aggregation of proteins such as α-synuclein [23, 24], matrix metalloproteinase-3 (MMP-3) [25], neuromelanin [26, 27], substance P [28], fibrinogen [29], thrombin [30], and various pro-inflammatory cytokines, as well as a variety of environmental cues (Table 1).

α-Synuclein Aggregates

α-Synuclein as a pathological gene alters neuropathological behaviors in sporadic PD and in most forms of familial PD, and it is initially thought to function mostly as an intracellular component. It is interesting to find out that the release of aggregated α-synuclein (the major component of LBs) from neurons into the extracellular space can induce microglial activation [23, 24]. α-Synuclein, when it is nitratized and oxidized, is more prone to induce microglial activation [31, 32]. Extracellular α-synuclein aggregates are sensed and internalized by microglia, followed by activation of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, production of ROS, and release of pro-inflammatory cytokines [23].

Interestingly, Rojanathammanee et al. [33] have found that overexpression of mutant α-synuclein solely in microglia was able to change microglia into a form of

Fig. 1 (continued) and cytosolic components (DAMPs, etc.) and a variety of environmental cues. All these pathways eventually converge into the common effectors including NADPH oxidase, ROS, superoxide, and multiple pro-inflammatory cytokines that are neurotoxic to neurons. The inflammatory mediators together with the neuron debris, in turn, induce more widespread damage to neighboring neurons (reactive microgliosis). Those inducers of microglia are deleterious through direct stimulation of microglia (e.g., α-synuclein, DAMPs, LPS, etc.) or indirectly revoke reactive microgliosis after neuronal lesions (e.g., MPTP, rotenone, etc.). Additionally, secreted inflammatory mediators can also act on astrocytes to induce secondary inflammatory responses and amply neurotoxic effects. Consequently, a vicious cycle between injured neurons and uncontrolled inflammation inevitably occurs. (**b**) Illustration of mechanisms or pathways in "good" microglia. The mechanisms employed by "good" microglia are mainly involving (1) transrepression pathways through multiple receptors such as GRs, ERs, Nurr1, and PPARs; (2) anti-inflammatory cytokines (IL-4, IL-10, IL-13, TGF-β); (3) neuron-microglia cross-talk (CX3CL1–CX3CR1 and CD200–CD200R pairing); (4) microRNAs (e.g., miR-124); and (5) epigenetic modifications (e.g., Jmjd3). All those mechanisms render "good" microglia immunosuppressive or quiescent by repressing pro-inflammatory cytokines, NF-κB, and related genes, whereas enhancing the expression of repair genes, trophic genes, and anti-inflammatory genes. The general effects of "good" microglia are neuroprotective, immunosuppressive, promoting tissue rebuilding, and possibly neurogenesis

Table 1 Inducers and effectors of "bad" microglia

Major inducers and effectors	Direct or indirect on microglial activation	Major references
Proteins		
α-Synuclein	Direct	[23, 24, 31–33]
Neuromelanin	Direct	[26, 27]
MMP-3	Direct	[25]
Substance P	Direct	[28]
Fibrinogen	Direct	[29]
Thrombin	Direct	[30]
Environmental toxins		
LPS	Direct	[34, 35, 60–64]
MPTP	Indirect	[43–55, 65]
Rotenone	Indirect	[38, 39]
Paraquat	Direct	[40–42]
Pesticides	Indirect	[36, 37]
Proteasome inhibitors	Indirect	[56, 57]
Heavy metals	Indirect	[58, 59]
Effectors		
NADPH oxidase	–	[23, 28, 38, 40, 43, 44, 68–70]
ROS and superoxide	–	[13, 23, 25, 27, 31, 43, 44]
Pro-inflammatory cytokines	Direct	[12, 50–53, 67–75]

reactive phenotype characterized by elevated levels of pro-inflammatory cytokines including tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6), as well as increased nitric oxide (NO) production and reactive nitrogen species (RNS) secretion. Their study shows that microglial activation in transgenic mice with *α-synuclein* mutation does not cause neurodegeneration. Of note, other components in LBs or cytosol proteins and membrane breakdown products released from dying neurons also have the potential to trigger microglial activation [5].

Environmental Factors

Apart from genetic factors, environmental factors are strongly implicated in PD development. The cumulative influence of environmental exposures on microglial activation has been reported. Among those, lipopolysaccharide (LPS) [34, 35], pesticides [36, 37], rotenone [38, 39], paraquat [40–42], 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) [43–55], proteasome inhibitors [56, 57], and heavy metals [58, 59] have been implicated in PD progression. Generally, the environment is implicated as a source for compounds that are both deleterious through direct stimulation of microglia (e.g., LPS, etc.) or indirectly revoke reactive microgliosis after neuronal lesions (e.g., MPTP, rotenone, etc.) (see Table 1).

LPS

Bacterial LPS, the polysaccharide component of the cell walls of gram-negative bacteria, is used to model microglia-induced loss of DA neurons in rodents. Various studies have demonstrated that LPS is neurotoxic to neurons only in the presence of microglia and inhibition of microglial activation has been shown to be neuroprotective [60–62]. Embryonic exposure to LPS leads to long-term microglial activation persistent into adulthood and progressive neuron loss [34, 63]. After chronic infusion into the SN, or a single systemic injection, LPS is capable of triggering a chronic inflammatory process and induces a delayed, progressive degeneration of DA neurons in rodents [35, 64]. Moreover, LPS-induced inflammation can also synergize with mutations in *α-synuclein* and *Parkin* that are associated with familial PD to potentiate the loss of DA neurons in animal models [32].

MPTP

Although not directly sensed by microglia, neurotoxin widely used in PD animal models, MPTP is known to induce neuronal degeneration that provokes reactive microgliosis, which exacerbates neurotoxicity. MPTP is metabolized to 1-methyl-4-phenylpyridinium (MPP^+) by glial cells, which is then taken up by DA neurons via the dopamine transporter (DAT), leading to mitochondrial damage and neuronal cell death [1]. Reactive microgliosis is then initiated and a series of pro-inflammatory cytokines are released.

Mounting evidence in animal model experiments shows that MPTP toxicity is markedly reduced in mutant mice deficient in inflammatory mediators including NADPH oxidase [43, 44], myeloperoxidase [45], COX-2 [46, 48], inducible nitric oxide (iNOS) [50], interferon-γ (IFN-γ) [51], and TNF-α [52, 53]. Studies have also applied anti-inflammatory drugs in the MPTP-models, in an attempt to discover or confirm any drugs that may alleviate DA neuron death and repress microglial activation [44, 54, 55, 65]. Overall, these studies indicate that MPTP-treated animals are good models for studying immunopathogenesis of PD.

Effectors of "Bad" Microglia

Microglia have evolved to express multiple, diverse membrane receptors, also termed pattern recognition receptors (PRRs), which are generally constitutively expressed to identify and bind pathogen-associated molecular patterns (PAMPs) associated with microbial pathogens, as well as damage-associated molecular patterns (DAMPs) associated with cell components released during cell damage [66]. There is increasing evidence to indicate that protein aggregates or neurotoxins could interact with multiple PRRs, including toll-like receptors (TLRs), scavenger receptors (SRs), macrophage antigen complex 1 (MAC1) receptor or through receptor

complexes [13]. Although different combinations of receptors might be involved in the recognition of toxic and dangerous inducers, there are common deleterious downstream effectors that both induce neuronal death and amplify ongoing microglial activation. These effectors are NADPH oxidase, pro-inflammatory factors, ROS, superoxide, and so forth (see Table 1).

NADPH Oxidase

NADPH oxidase is a membrane-bound enzyme that catalyzes the production of superoxide free radicals from oxygen and a robust source of extracellular ROS and pro-inflammatory signaling in microglia [67]. NADPH oxidase is activated by various stimuli, including bacterial PAMPs, inflammatory mediators, and multiple neurotoxins, which are associated with PD and neuronal damage. It is found to be activated in the brains of patients with PD and the catalytic subunit (gp91) is upregulated [43]. The crucial role of NADPH oxidase in mediating inflammation-related neurotoxicity has been extensively studied. Genetic ablation of NADPH oxidase attenuates the neurodegeneration induced by diverse challenges both in vivo and in vitro, including LPS [68], rotenone [38], paraquat [40], MPTP [43, 44, 69], substance P [28], and α-synuclein aggregates [23, 70]. It appears that NADPH oxidase is at the very center of the downstream pathway and is the major source of effectors including pro-inflammatory factors, NO, ROS, and other factors in activated microglia in PD. Thus, NADPH oxidase could be a potential therapeutic target for PD [13].

Pro-inflammatory Factors

Pro-inflammatory factors are important aspects of elucidating pathogenesis of PD. After exposure to stimuli such as LPS or other inducers, microglia produce various pro-inflammatory mediators including TNF-α, IL-1β, and iNOS and thereby modulate the progression of neuronal cell death in PD [12, 67]. Microglia are the major sources of pro-inflammatory cytokines, which were then transferred to stimulate astrocytes and amplify inflammatory responses, leading to the extensive death of DA neurons [71]. Therefore, the combination of factors that are produced by activated microglia and astrocytes promote neurotoxicity.

Pro-inflammatory cytokines are extremely critical for neurodegeneration. Genetic analyses show that the DNA polymorphism of several pro-inflammatory cytokines including IL-1α, IL-1β, IL-6, and iNOS are a risk factor for PD [72–74]. Mangano et al. [41] have reported that knockout of IFN-γ prevents the paraquat-induced microglial activation and expression of key NADPH oxidase subunits, meanwhile reduces the time-dependent changes in pro-inflammatory enzymes iNOS, IL-1β, TNF-α, and signaling factors such as NF-κB. Similarly, IFN-γ-deficient mice also display protection against MPTP (in vivo) or rotenone (in vitro)-induced DA neuron loss [51], whereas overexpression of IFN-γ is able to induce progressive nigrostriatal degeneration [75]. Of note, these secreted inflammatory mediators should be considered as new inducers of “bad” microglia.

ROS and Superoxide

Activated microglia also release superoxide free radicals and extracellular ROS, which are mainly produced by the help of NADPH oxidase [67]. Excessive production of superoxide and its downstream products are highly reactive and can damage proteins, lipids, nucleotides, leading to cell dysfunction and death. Extracellular α-synuclein or neuromelanin is phagocytosed by microglia and leads to activation of NADPH oxidase, ROS production, and NF-κB regulated genes [23, 27, 31]. Lack of NADPH oxidase in mice makes them resistant to the toxic effects by α-synuclein or active recombinant MMP-3 exposure with reduced production of ROS and superoxide [23, 25]. In the MPTP-injected mice, MMP-3 deficiency attenuates microglial activation and superoxide generation, which contributes to neuron survival [25].

Regulation of "Good" Microglia

Regulatory mechanisms in microglia attenuate excessive inflammatory response to bad microglia and promote recovery and repair. Briefly, the mechanisms in "good" microglia that may be relevant to PD pathology are involved in Table 2. Eventually, these mechanisms of "good" microglia converge into counteraction with "bad" microglia by repressing pro-inflammatory cytokines, NF-κB, and other factors (Fig. 1).

Table 2 Mechanisms or pathways in "good" microglia

Mechanisms	Major references
Counter-regulation or transrepression pathways	
GRs	[76–79]
ERs	[81–84]
Nurr1	[71, 89]
PPARs	[90–96]
Anti-inflammatory factors	
IL-4	[96–100, 120]
IL-10	[98]
TGF-β	[98, 101, 120, 121]
G-CSF	[102–105]
Neuron-microglia cross-talk	
CX3CL1–CX3CR1	[107–110, 121]
CD200–CD200R	[113–115]
MicroRNA	
miR-124	[116]
Epigenetics	
Jmjd3	[123]

Counter-Regulation or Transrepression Pathways

A spectrum of endogenous protective receptors that mediated transrepression to counter-regulate pro-inflammation in the brain have been identified, these are glucocorticoid receptors, estrogen receptors, NR4A2, peroxisome proliferator-activated receptors, and others as shown (see Table 2).

Glucocorticoid Receptors

Glucocorticoids (GCs) predominantly are released during stressful states to maintain homeostasis and are the most efficient endocrine molecules that exert anti-inflammatory and immunosuppressive effects via glucocorticoid receptors (GRs). Upon binding, GRs translocate into the nucleus and regulate gene expression, resulting in suppression of NF-κB and downregulation of a wide variety of pro-inflammatory cytokines and immune mediators [76, 77]. In PD patients and MPTP-intoxicated mice, GR level is found to be decreased in the SN, suggesting the potential role of GRs in PD pathogenesis [78]. Various studies have showed that absence of GRs augments microglial reactivity and leads to persistent microglial activation; GR-deficient microglia produce higher nitrite level that precedes the loss of DA neurons and are resistant to the inhibitory effects of GCs [78, 79]. Furthermore, GR-deficient microglia also express higher level of pro-inflammatory genes (e.g., TNF-α) with a concomitant downregulation of anti-inflammatory genes (e.g., IL-1R2) [78]. GRs play an important role in suppression of microglial activity and deregulation of this leads to inflammation-mediated neuronal injury.

Estrogen Receptors

PD is more prevalent in men than in women and studies suggest a differential response to dopaminergic therapy between men and women raising the possibility of involvement of estrogen receptors (ERs) [80]. The protective effects of estrogen on the onset and severity of PD symptoms have been extensively evaluated. Numerous studies have showed that estrogens act through ERα or ERβ to suppress the production of several pro-inflammatory mediators including iNOS, TNF-α, as well as other secretory products induced by LPS in vitro and render neuroprotection to cultured neurons [81–83]. Notably, these protective effects could be blocked by treatment of ER antagonists, suggesting ER as a critical mediator of anti-inflammatory function of promoting neuron survival [82, 83]. Similarly, Tripanichkul et al. [84] have also reported that estrogen could decrease microglial activation and attenuate DA neuron loss in the MPTP intoxicated male mice in vivo. It is important to explore the role of estrogen in microglial activation and specific mechanism in pathophysiology of PD.

NR4A2

NR4A2 (Nurr1) belongs to the orphan nuclear receptors NR4A subfamily and is required for differentiation and maintenance of midbrain DA neurons [85, 86]. Reduced Nurr1 expression causes increased vulnerability of DA neurons to MPTP-induced injury in mice, and mutation in the gene has been reported in familial PD [87, 88]. Nurr1 is expressed both in neuronal and microglial cells. In recent years, Nurr1 has attracted increasing attention to its effect on neuroinflammation by mechanism of transrepression. After exposure to LPS express of Nurr1 gene expression increases, and it has been shown that nuclear translocation of Nurr1 occurs in the in vitro model [89]. In 2008, Saijo et al. [71] have demonstrated a Nurr1-mediated transrepression mechanism in LPS-induced PD model. In their study, Nurr1 is able to inhibit the expression of pro-inflammatory neurotoxic mediators and NF-κB targeted genes in microglia. Reduced Nurr1 expression initiates exaggerated inflammatory response in microglia in addition to astrocyte activation, leading to the extensive death of DA neurons [71]. Based on this evidence Nurr1 could be a potential therapeutic target.

Peroxisome Proliferator-Activated Receptors

Peroxisome proliferator-activated receptors (PPARs) are members of the steroid hormone nuclear receptor superfamily, which have been extensively studied in the regulation of genes involved in lipid metabolism, energy homeostasis, and macrophage differentiation. Numerous studies have shown that PPARs play a very important role in the regulation of brain inflammation through mechanisms involving transrepression pathways. Activation of PPARs inhibits the synthesis of NO, TNF-α, COX-2, and chemokines in microglia [90, 91]. One of the major natural PPAR-γ agonists, 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 (15d-PGJ_2), contributes to inhibition of LPS-induced microglial activation [90]. Similarly, synthetic agonists of PPAR-γ including ciglitazone, troglitazone, and pioglitazone could also reduce LPS-induced microglial activation and inhibit the excessive production of cytotoxic molecules, thereby preventing neuronal death [91, 92]. In addition, pioglitazone also attenuates DA neuron death in the MPTP model of PD, by blocking NF-κB and iNOS activation [93–95]. Interestingly, rosiglitazone, another agonist of PPAR-γ, possesses anti-inflammatory properties by increasing IL-4 expression [96], suggesting a cross-talk between counter-regulation and anti-inflammatory factors in the mechanisms of "good" microglia.

Anti-inflammatory Factors

IL-4, IL-13, IL-10, and transforming growth factor-β (TGF-β) are major anti-inflammatory cytokines that antagonize pro-inflammatory responses and promote the expression of genes involved in tissue reconstruction and repair. IL-4 is a

well-described immune regulatory cytokine capable of suppressing inflammation. Its interaction with microglial IL-4 receptors suppresses superoxide production and NO release; reduces the production of pro-inflammatory cytokines such as IL-6, IL-8, and TNF-α; and alleviates LPS-induced neuron injury both in vitro and in vivo [97–100]. TGF-β is a pleiotropic cytokine with diverse functions including angiogenesis and increased extracellular matrix deposition and also plays a critical role in minimizing microglial responses and thus avoiding exacerbation of brain damage [101]. It is important to note that anti-inflammatory cytokines (IL-4, IL-10, and TGF-β) affect the production of inflammatory mediators in LPS-activated co-cultures of microglial cells differentially [98]. Granulocyte-colony stimulating factor (G-CSF) is known to stimulate the proliferation of hematopoietic cells and has been reported to modulate the immune system by downregulating pro-inflammatory cytokines [102]. Two studies have shown that G-CSF administration provides neuroprotection against MPP^+-induced cell death in vitro and MPTP-induced DA neuron loss in vivo [103, 104]. Moreover, G-CSF could also enhance the recovery of nigrostriatal system from MPTP toxicity by modulating microglial responses [105].

Neuron-Microglia Cross-Talk

Fractalkine (CX3CL1) and Fractalkine Receptor (CX3CR1)

Microglia are the major CNS cells that express CX3CR1. CX3CL1, the exclusive ligand for CX3CR1, is synthesized as a transmembrane glycoprotein highly expressed in neurons [106]. CX3CL1 is cleaved from neuronal membranes in response to neurotoxic insults and subsequently attracts reactive immune cells as microglia cells by binding CX3CR1 [107]. Previous studies have supported that the interaction between CX3CL1 and CX3CR1 plays an important role in preserving microglia survival and attenuates neurodegeneration. For example, treatment of microglia with CX3CL1 maintains microglia survival and prevents from Fas-mediated apoptosis [108]. CX3CL1 also suppresses the production of NO, IL-6, and TNF-α from activated microglia upon LPS and IFN-γ insult that significantly suppresses neuronal death in vitro [109]. Importantly, Cardona et al. [110] have showed that CX3CR1 deficiency in vivo leads to enhanced microglial activation and exaggerates neuronal damage in response to systemic LPS treatment or augments DA neuron loss in the SN following MPTP administration, suggesting CX3CR1 signaling is critical to protect against microglial neurotoxicity.

CD200 and CD200R

CD200 receptor (CD200R), an inhibitory receptor present on microglia, actively maintains microglia in a quiescent state through its interaction with CD200, a member of the immunoglobulin superfamily expressed on the neuronal membrane

surface [111, 112]. CD200–CD200R has been identified as one of the critical pathways in attenuating microglial activation. Microglia in CD200-deficient mice exhibit more characteristics of activation, which are less ramified with shorter processes. Moreover, this increased microglial activation is accompanied by enhanced expression of TNF-α and iNOS, demonstrating a loss of the neuronal inhibitory signal for microglial response [113]. Blocking CD200–CD200R engagement dramatically exacerbates DA neuron death in a primary neuron/microglia co-culture system [114]. Furthermore, deficits in the CD200–CD200R system also enhance microglial activation with elevated TNF-α and IL-6 production and thus exacerbate DA neuron loss in a 6-hydroxydopamine (6-OHDA)-induced rat model of PD [115]. Therefore, in the healthy brain, microglial activation is tightly restricted by signaling from neurons, whereas the progressive loss of DA neurons in the SN of PD patients might accelerate reactive microgliosis due to the lack of neuronal signals.

MicroRNA

Apart from CD200–CD200R, microRNAs have also been reported to promote microglia quiescence. Ponomarev et al. [116] published evidence that brain-specific miR-124 acts as a novel modulator of microglia and macrophage activation. There is downregulation of miR-124 in activated microglia and highly activated peripheral macrophages. Knockout modeling of miR-124 in microglia and macrophages results in activation of these cells both in vitro and in vivo. The administration of miR-124 before or after disease onset causes systemic deactivation and suppression of EAE [116]. This study offers insight into inhibition of microglial overactivation in PD.

Activation States of Microglia

The complexity of microglial activation states or phenotypes has been attracting increasing attention in recent years. As noted above, microglia exhibit a deactivated phenotype in the healthy brain helping in maintenance of tissue homeostasis through communication with other cells, analogous to homeostatic roles of "alternatively activated" macrophages in other tissues. Actually, increasing studies have reported that microglia, similar to peripheral macrophages, possess states of "classical activation," "alternative activation," and "acquired deactivation" [117, 118].

Classical activation is associated with the production and release of proinflammatory cytokines, proteases, superoxide anion, NO, and ROS/RNS that recapitulate the effects of "bad" microglia. Microglia in this state are also termed "M1 microglia" according to the studies on macrophages, whereas the term "M2 microglia" is used to include the states of both alternative activation and acquired deactivation that reflect the effects of "good" microglia [118]. Alternative activation is limited to microglia treated with IL-4 or IL-13 and is primarily associated with M2

genes that promote anti-inflammation, tissue repair, and extracellular matrix reconstruction, such as Arginase 1 (Arg1), Mannose receptor (CD206), Found in inflammatory zone 1 (FIZZ1), and Chitinase-3-Like-3 (Ym1) [117]. Acquired deactivation is another state to alleviate acute inflammation and is induced primarily by uptake of apoptotic cells or the insult of anti-inflammatory cytokines IL-10 and/or TGF-β [117, 118]. All three states could be transited into each other in different context that may contribute to pathogenic forms of inflammation in neurodegenerative diseases including PD.

However, molecular mechanisms that regulate M2 microglia are poorly understood and require further investigation. Nevertheless, the mechanisms of "good" microglia aforementioned can also converge into the elucidation of M2 microglia. For instance, it is reported that IL-4 production is essential for the maintenance of Ym1 expression and alternative activation in both microglia cells and peripheral infiltrating macrophages, thereby suppressing EAE clinical symptoms [119]. Treatment with the PPAR-γ agonist rosiglitazone increases IL-4 expression and attenuates the LPS-induced increase in IL-1β concentration in wild-type microglia while not $IL4^{-/-}$ microglia, suggesting that the anti-inflammatory actions of rosiglitazone are mediated by its ability to increase IL-4 expression [96]. More interestingly, since miR-124 is required for microglia quiescence, transfection of miR-124 results in downregulation of markers and cytokines associated with M1 microglia such as CD86 and iNOS, whereas cytokines and markers associated with M2 microglia including TGF-β, Arg1, and FIZZ1 are upregulated [116]. This is also consistent with the notion that quiescent microglia show properties of alternative activated microglia [116, 119].

It might be interesting to determine whether other molecules involving in the regulation of "good" microglia such as GRs, CX3CR1, ERs, and Nurr1 contribute to alternative activation of microglia.

Conclusion and Future Perspectives

In summary, "bad" microglia can be induced by endogenous proteins such as α-synuclein and a variety of environmental cues that eventually converge into the effectors including pro-inflammatory cytokines, ROS and superoxide, and especially NADPH oxidase (see Fig. 1). The function of "good" microglia is mainly involving transrepression pathways through multiple receptors such as GRs, ERs, Nurr1, and PPARs, anti-inflammatory cytokines, neuron-microglia cross-talk, and microRNAs that counteract with "bad" microglia by repressing pro-inflammatory cytokines, NF-κB, and other factors (see Fig. 1).

Notably, those mechanisms attributed by "bad" or "good" microglia are not in parallel pathways but in cross-talk with each other to amplify their effects. Activation of "bad" microglia produces a variety of pro-inflammatory factors, chemokines, and cytokines, which could then be involved as new inducers of "bad" microglia. This self-amplifying nature of neuroinflammation, together with secondary inflammatory

responses mediated by astrocytes, partially underlies uncontrolled inflammation in PD [71]. Similarly, the various mechanisms that contributed to the resolution of inflammation by "good" microglia are not in parallel. Upon activation, they can promote expression of genes in other pathways and orchestrate each other against the pro-inflammation. This is already demonstrated by multiple studies on (1) IL-4 and TGF-β cross-talk [120]; (2) TGF-β and CX3CR1 cross-talk [121]; (3) IL-4 and PPARs cross-talk [96].

Depending on host environment microglial cells have dual responses and exist in differential states of activation. In most cases, these responses are self-limiting and well regulated to keep the balance between the pro-inflammation/injury and the resolution of inflammation/repair. However, it seems that the beneficial effects in PD are apparently either inadequate or ineffective due to the complex mechanisms involved. Because neural tissues have a restricted cell renewal and regenerative capacity, the CNS is extremely vulnerable to uncontrolled inflammatory processes. Deficits in generating those beneficial effects in response to CNS injuries might compromise important defense mechanisms of inflammation (e.g., the regulation of "good" microglia). In pathogenesis of PD there is a persistent stimulus resulting from the formation of endogenous factors especially α-synuclein aggregates or environmental factors (e.g., the inducers of "bad" microglia) that contribute to an excessive pro-inflammation. Thus, the persistence of pro-inflammation or a failure of normal protective mechanisms might lead to uncontrolled and sustained inflammation in the CNS. Uncontrolled inflammation acts either as an initiator or as a secondary reaction that drives the chronic and progressive neurodegenerative process in PD where detrimental effects of inflammation overwhelm beneficial effects.

Therefore, it becomes critical and urgent to investigate what determines the progressive nature of neuroinflammation in PD, i.e., a full understanding of the mechanisms of both "bad" microglia and "good" microglia. Focusing solely on "bad" microglia would not be sufficient or effective for PD therapy. Use of NSAIDs has shown mixed results with decreased risk of PD, but the same studies showed no benefit in Alzheimer's disease (AD) [13]. A major issue is whether inhibition of pro-inflammatory responses will be a safe and effective method for reversing or slowing the course of disease. Targeting any one of a large number of pro-inflammatory factors released from overactivated microglia is less efficacious.

A more detailed understanding of the transition of "bad/good" microglia or "M1/M2" microglia will be helpful in defining the molecular events and pathways to halt the progress of neuroinflammatory injury by switching the microglia states from M1 to M2 in an optimal therapeutic window, the concept of which has been demonstrated in the treatment of experimental autoimmune neuritis (EAN) disease [122]. More importantly, in a recent study of our lab, Tang et al. [123] have demonstrated that the histone H3K27me3 demethylase Jmjd3 is essential for M2 microglial activation. Suppression of Jmjd3 in microglia leads to defective M2 responses and augments M1 pro-inflammation that eventually accelerates DA neuron death [123], suggesting that switching microglia phenotypes is possible by epigenetic modification and any other mechanisms involved in this switch might be needed considering the complexity of microglia states. Moreover, it is important to monitor

the microglial activation throughout the chronic progression of the disease that would present the dynamics of microglia states and give an indication of when to begin the treatment capable of altering disease progression.

Originally, activation of "bad" microglia is necessary and crucial for host defense. Targeting on "bad" microglia too early would not produce beneficial effects. Transrepression mechanisms are employed by "good" microglia to facilitate anti-inflammation and tissue reconstruction; however, abnormal prolongation of the same immunosuppressive and repair mechanisms is also associated with chronic disease with long-term "escape" of persistent parasites or pathogens from immune-mediated killing [124]. Further research is crucial to our understanding of mechanisms of microglial activation and regulatory homeostasis of microglial-mediated inflammatory processes.

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The Role of Astrocytes in Parkinson's Disease

Claire Stevens and Glenda Halliday

Astrocytes: Structure and Function in the Healthy Brain

Two main types of astrocytes tile the brain in a contiguous and non-overlapping manner: protoplasmic astrocytes in gray matter, which wrap neuronal cell bodies and synapses (estimates suggest a single human astrocyte wraps more than 270,000 synapses [1]), and fibrous astrocytes in white matter, which wrap nodes of Ranvier and oligodendroglia [2]. Once considered to be only passive support cells for neurons, astrocytes are now known to have an active regulatory role in the brain. They form part of the basic functional element, the neurovascular unit, which is comprised of astrocytes, neurons (which are contacted by astrocyte processes), and blood vessels (which are contacted by astrocyte end feet) [3]. Due to their strategic position, astrocytes are able to directly influence neuronal electrical excitability, neurogenesis, synaptogenesis and plasticity, synaptic transmission, ion homeostasis, neurotransmitter uptake, production of neurotrophic factors, glial scar formation, blood flow regulation, and maintenance of the blood–brain barrier (reviewed in [2]). Their function is achieved via the calcium-dependent release of chemical transmitters, or gliotransmitters such as glutamate, adenosine triphosphate, and gamma-aminobutyric acid, and also by their removal through uptake of potentially toxic neurotransmitters that are released into the extracellular space during neuronal activity (reviewed in [2]). A number of neurodegenerative conditions including Alzheimer's disease and epilepsy show abnormalities in calcium signaling, highlighting the importance of astrocytes in normal brain functioning [4].

Astrocytic release of chemicals, including prostaglandins and nitric oxide, controls blood flow by restricting or expanding the diameter of blood vessels [5, 6]. In addition, astrocyte end feet in contact with blood vessels contain the water channel protein aquaporin-4 [7]. Aquaporin-4 is critical to the regulation of water movement

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M. Thomas (ed.), *Inflammation in Parkinson's Disease: Scientific and Clinical Aspects*,
DOI 10.1007/978-3-319-08046-8_5,

in the CNS [7, 8] and also maintains ion homeostasis through the transport of ions such as K^+ [9]. Astrocytes can regulate neural stem/progenitor cell differentiation and proliferation [10] by the secretion of factors through Wnt signaling [11] and the release of the cytokines interleukin 1-β and interleukin-6 [12]. It is also becoming increasingly recognized that astrocytes are actively involved in the formation and elimination of synapses and synaptic transmission [13]. Astrocyte-derived factors are important in synaptogenesis [14, 15] and include thrombospondins [16–18] and cholesterol bound to apolipoprotein-E-containing particles [19], while astrocytes and the complement cascade factor C1q appear to have a role in synaptic pruning [20]. Finally, astrocytes are also important in synaptic transmission through physical contact with neurons and the release of substances including glutamate and acetylcholine-binding protein [21–23].

Although the CNS was once considered to be an immune-privileged site, it is now recognized that both innate (nonspecific) and adaptive (antigen-specific response) immune responses occur in the CNS [24]. While research in the field of CNS immunity has concentrated on the role of the resident immune cells microglia, it is becoming increasingly recognized that astrocytes are also active players in the CNS immune response and are implicated in the initiation and development of immune-mediated mechanisms in a number of brain diseases [25]. Astrocytes release an extensive array of both pro- and anti-inflammatory cytokines and chemokines and may also be targets for these inflammatory mediators [25]. They express pattern-recognition receptors including toll-like receptors and complement factors, receptors, and inhibitors, which are critical components of innate immunity [25]. These receptors recognize pathogens and markers of cells damage, and dampening of their activation has been implicated in a number of CNS diseases including meningitis, encephalitis, Alzheimer's disease, multiple sclerosis, and prion disease [25].

Typically, astrocytes respond to brain tissue changes (whether it be due to injury, infection, or disease) by undergoing astrogliosis, a process involving the upregulation of the intermediate filament protein glial fibrillary acidic protein (GFAP), cell body enlargement, and proliferation [2] (Fig. 1). Astrogliosis has a graded response with progressive morphological enlargement that may eventually culminate in the formation of a glial scar (for review see [2]) (see Fig. 1). Interestingly, this process may have both protective and harmful effects. Protective effects include the release of neuroprotective substances, containment of inflammation or infection in the affected area, and repair of damaged tissue [2]. However, there is increasing evidence that astrocytes contribute to the progression of CNS disorders through the loss of their normal protective roles or a gain of abnormal function, resulting in a range of detrimental effects including the release of toxic substances and proinflammatory cytokines and subsequent exacerbation of inflammatory processes (for review see [2]).

The importance of astrocytes in the brain and their capacity to have both a neuroprotective and neurodegenerative role are further illustrated in mouse models with altered astrocyte protein function (reviewed in [26]). After lesioning of the entorhinal cortex the reactive astrocytes in mice lacking the intermediate filaments GFAP and vimentin show a reduction in process hypertrophy but significantly improved

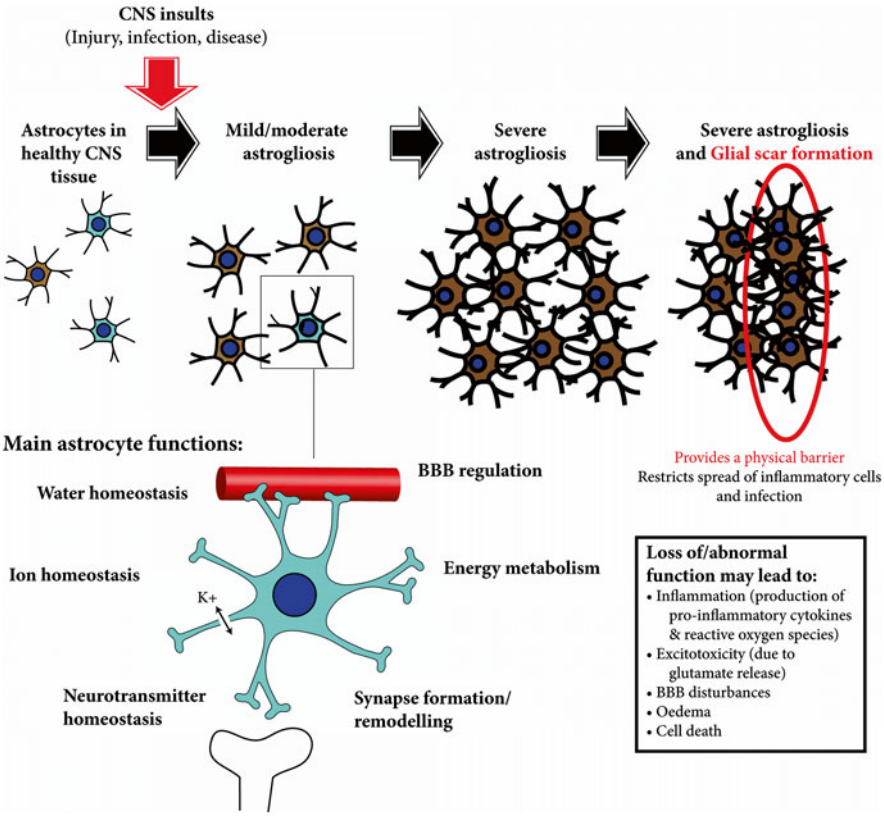

Fig. 1 Schematic representation of the main functions of astrocytes, their response to CNS insults, and the consequences of disrupted or abnormal astrocyte function. If astrocytes sense a disturbance in brain tissue, they undergo a graded process known as astrogliosis, which involves the upregulation of the intermediate filament protein glial fibrillary acid protein (*brown*), cell body enlargement, and proliferation, and may eventually culminate in the formation of a glial scar (*at right*). *BBB* blood–brain barrier, *CNS* central nervous system

synaptic regeneration [27]. Similarly, after unilateral hemisection of the spinal cord, mice deficient in these filaments show improved axonal sprouting and functional recovery [28], suggesting that reactive astrocytes actually impair neuroregeneration. Furthermore, the survival and integration of grafted neurons and neural progenitor cells is dramatically improved in GFAP and vimentin knockout mouse models [29, 30]. In contrast, mice overexpressing the human GFAP gene show abnormal astrocyte morphology, increased stress proteins, and the formation of filaments identical to Rosenthal fibers (hallmark pathology of the neurodegenerative disease Alexander disease) [31]. Similarly, mice lacking the astrocytic glutamate transporter GLT-1 show hippocampal neuron loss, severe and eventually lethal seizures, and an increased susceptibility to acute cortical injury, most likely due to raised levels of glutamate [32]. It is clear from the extensive array of functions outlined,

and the mouse models illustrating the consequences of altered astrocyte proteins, that the role of these glial cells in the brain is highly complex and not yet fully understood. The typical response of astrocytes in common neurodegenerative diseases will be outlined in the following section.

Astrocytes: Typical Response in Neurodegenerative Diseases

Neurodegenerative diseases are characterized by the selective loss of neurons in vulnerable areas of the nervous system, resulting in clinical symptoms such as movement disorders and dementia [33]. The presence of abnormal protein aggregates in neurons and glial cells is a hallmark feature of many neurodegenerative diseases, which are collectively termed proteinopathies. Proteinopathies may be further divided on the basis of the major protein constituent of these inclusions. Tauopathies, including Alzheimer's disease, corticobasal degeneration, and progressive supranuclear palsy, are characterized by tau-positive inclusions [34]. Similarly, TDP-43 proteinopathies are characterized by inclusions composed of transactive response DNA-binding protein (TDP-43) and include the disorders amyotrophic lateral sclerosis and a subgroup of frontotemporal lobar degeneration with TDP-43 inclusions (FTLD-TDP-43) [35]. The focus of this chapter is Parkinson's disease, which is one of a group of disorders also including multiple system atrophy that are characterized by the deposition of α-synuclein protein and collectively referred to as synucleinopathies [36]. These disorders, regardless of whether they are characterized by tau-, TDP-43-, or α-synuclein-positive inclusions, show astrocytic gliosis in regions associated with degeneration, suggesting a typical astrocyte response to disease, with the exception of Parkinson's disease (see "Astrocytes: Role in Parkinson's Disease").

Alzheimer's disease is the most common cause of dementia and is characterized by two hallmark pathologies, tau-positive neurofibrillary tangles and β-amyloid (Aβ)-positive plaques [37]. Astrogliosis is a pathological feature of Alzheimer's disease, with activated astrocytes commonly observed in close proximity to hallmark Aβ plaque pathology (Fig. 2a) in postmortem brain tissue from patients with Alzheimer's disease [38–41]. There is also evidence that astrocytes may contribute to Aβ-induced neurotoxicity through altered glucose metabolism [42], by releasing proinflammatory substances and mediating neuronal tau phosphorylation [43]. In addition, a number of studies suggest that astrocytes, but not microglia, may be involved in the uptake of Aβ protein [44, 45]. Corticobasal degeneration and progressive supranuclear palsy, which clinically display variable disturbances in gait, balance, vision, and cognition, are also pathologically characterized by up-regulated astrocytes (see Fig. 2b, c) that correlate with the severity of neurodegeneration and disease stage [46]. Interestingly, in all three diseases some astrocytes accumulate abnormal tau protein [46–50]; however, the type of inclusion varies between diseases. GFAP-positive thorn-shaped astrocytes with cytoplasmic tau staining are observed in brain regions with up-regulated fibrous astrocytes in all

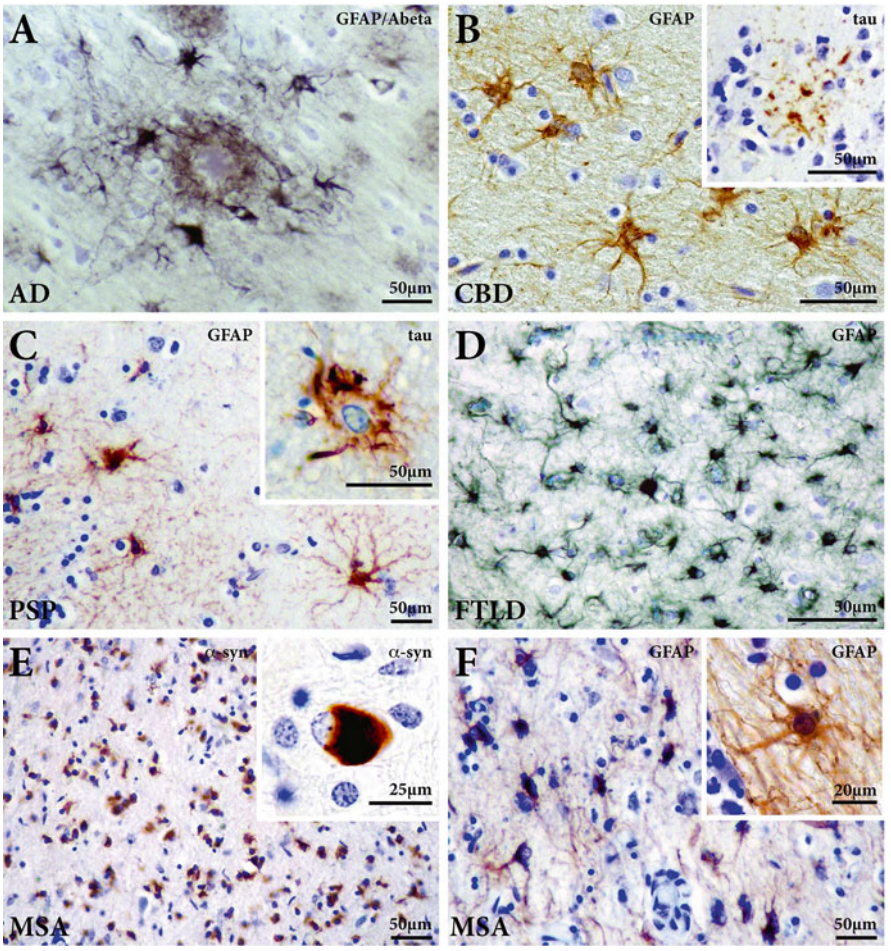

Fig. 2 Photomicrographs showing the typical astrocyte response in common neurodegenerative disorders. *α-syn* α-synuclein, *GFAP* glial fibrillary acidic protein. (**a**) Reactive GFAP-positive astrocytes around an Aβ plaque in Alzheimer's disease (AD) (double-labeling immunoperoxidase with GFAP in *black* and Aβ in *brown* and with cresyl violet counterstain). (**b**) Reactive GFAP-positive astrocytes in corticobasal degeneration (CBD) (immunoperoxidase with cresyl violet counterstain). Inset of tau-immunopositive astrocytic plaque in CBD (immunoperoxidase with cresyl violet counterstain). (**c**) Reactive GFAP-positive astrocytes in progressive supranuclear palsy (PSP) (immunoperoxidase with cresyl violet counterstain). Inset of tau-immunopositive tufted astrocyte in PSP (immunoperoxidase with cresyl violet counterstain). (**d**) Reactive GFAP-positive astrocytes in frontotemporal lobar degeneration (FTLD) (enhanced immunoperoxidase with cresyl violet counterstain). (**e**) Unlike in Parkinson's disease (see Fig. 3), α-synuclein does not accumulate in astrocytes in multiple system atrophy (MSA), but forms inclusions in oligodendroglia known as glial cytoplasmic inclusions (α-synuclein immunohistochemistry with cresyl violet counterstain). (**f**) Reactive GFAP-positive astrocytes in MSA (immunoperoxidase with cresyl violet counterstain)

three diseases [49]. However, in progressive supranuclear palsy gray matter protoplasmic astrocytes show accumulations of cytoplasmic tau termed tufts of abnormal fibers, while astrocytic plaques with tau-positive processes characterize corticobasal degeneration [49]. To assess the effect of tau pathology in astrocytes, transgenic mouse models have been created that express human tau protein under the control of the GFAP promoter. These mice show considerable accumulation of abnormal tau in astrocytes, blood–brain barrier disruption, and neuron degeneration [51], as well as loss of neuromuscular strength and altered expression of glutamate transporters [52].

Of the TDP-43 proteinopathies, amyotrophic lateral sclerosis or motor neuron disease is characterized by the progressive degeneration of motor neurons resulting in muscle weakness, atrophy, and spasticity [53], while FTLD-TDP-43 is a heterogeneous disorder characterized by language and/or behavior dysfunction [54]. In postmortem human brain tissue, reactive astrocytes are observed in gray and white matter cortical regions and the spinal cord of patients with amyotrophic lateral sclerosis [55–57] and throughout the cortex in patients with FTLD-TDP-43 [58] (see Fig. 2d). Interestingly, human astrocytes expressing causal mutations for amyotrophic lateral sclerosis in the enzyme Cu/Zn-superoxide dismutase (SOD1) confer toxicity to motor neurons through the generation of reactive oxygen species [59], suggesting that astrocytes may actively participate in the neurodegeneration of this disorder.

Finally, multiple system atrophy, which is a clinically and pathologically heterogeneous disease, is characterized by changes in executive and autonomic functions and usually a predominance of either parkinsonian symptoms (similar to Parkinson's disease) or cerebellar signs [60]. α-Synuclein-positive inclusions in oligodendroglia termed glial cytoplasmic inclusions are the hallmark pathology of multiple system atrophy (see Fig. 2e), but α-synuclein does not accumulate in either fibrous or protoplasmic astrocytes [46]. However, a typical astrocyte response is observed in this disease (see Fig. 2f), involving the upregulation of GFAP protein, enlarged cell bodies, and distorted processes of white matter fibrous astrocytes, changes that correlate with measures of neurodegeneration in this disease [46].

Although only discussed here briefly, astrocytes are clearly implicated throughout the progression of these common neurodegenerative diseases. Although these disorders are characterized by the accumulation of different proteins in variable cell types, they each exhibit a typical reactive astrocyte response to disease (see Fig. 2). In contrast, there is evidence that this response is very different in Parkinson's disease, and this will be discussed in the following section.

Astrocytes: Role in Parkinson's Disease

The role of astrocytes in Parkinson's disease is not well understood, mainly due to the limited number of studies that have been performed in the human disease and the discrepancies observed between these studies and those performed in animal

models of the disease. As will be discussed in detail below, current evidence suggests that astrocytes are involved in the earliest stages of Parkinson's disease, where they accumulate α-synuclein in their cytoplasm, but do not undergo substantial reactive astrogliosis, making Parkinson's disease unique amongst most brain disorders, including other parkinsonian conditions (see "Astrocytes: Typical Response in Neurodegenerative Diseases").

The Role of Astrocytes in Animal Models of Parkinson's Disease

The majority of toxin-induced, inflammatory and genetic animal models of Parkinson's disease display varying degrees of astrocyte activation [61, 62]. However, there are two key issues that need to be considered with regard to the astrocyte response in animal models: (1) the accuracy of the model at recapitulating the pathological features of Parkinson's disease and (2) whether the results reflect those reported from studies using postmortem tissue from patients with Parkinson's disease. Nonetheless, assessing astrocytes in animal models of Parkinson's disease can provide important information, particularly relating to the timing of astrocyte involvement in Parkinson's disease that can be difficult to determine in human patients.

In humans there are two essential features required for a pathological diagnosis of Parkinson's disease, the severe loss of pigmented neurons in the substantia nigra and the presence of α-synuclein-positive inclusions in neuronal perikarya and axons known as Lewy bodies and Lewy neurites, respectively [63]. Common animal models of Parkinson's disease (such as those generated by the neurotoxins 1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), 6-Hydroxydopamine (6-OHDA), and rotenone and genetic models that overexpress human α-synuclein or express common mutations in the α-synuclein gene such as A53T) pathologically show a loss of nigral neurons; however only the rotenone-induced and α-synuclein genetic models also show cytoplasmic inclusion bodies resembling Lewy bodies [64, 65], potentially making these more accurate models of the pathological process occurring in Parkinson's disease.

While a number of different neurotoxins may be used to generate mouse models of Parkinson's disease (MPTP, 6-OHDA, rotenone), they all show dopaminergic cell death that is associated with oxidative stress and inflammation including, in most models, severe astrocyte and microglial activation and consequent production of toxic inflammatory mediators that contribute to cell death [62]. The treatment of astrocytes from these models with inhibitors for the various proinflammatory molecules secreted results in the downregulation of their expression and associated cell damage [62], suggesting that astrocyte upregulation is an important contributor to the pathogenesis of Parkinson's disease. However, it also important to consider whether such an acute insult resulting in the rapid activation of both microglia and astrocytes truly reflects the chronic nature of the human disease which develops over years, if not decades. In line with this, MPTP use in humans, while inducing a clinical

phenotype similar to Parkinson's disease (levodopa-responsive parkinsonism) together with the loss of dopaminergic neurons in the substantia nigra, is associated with a significant glial response involving both astrocytes and microglia that is observed in patients with drug use ranging from 3 to 16 years [66]. However, many of these cases do not have substantial Lewy body formation [66], similar to the toxin-induced animal models and different from classic Parkinson's disease. In this aspect, it is interesting to note the effect different rotenone administration methods have on the astrocyte response in mice. A single unilateral infusion of rotenone in the medial forebrain bundle resulted in the loss of tyrosine hydroxylase neurons in the substantia nigra and fibers in the striatum and was accompanied by a significant glial reaction involving both astrocytes and microglia [67]. In contrast, mice undergoing chronic rotenone infusion every day for up to 4 weeks also showed selective degeneration of dopaminergic neurons, α-synuclein aggregates, severe microglial activation but only minimal if any upregulation of astrocytes [68], reflecting pathological observations in postmortem tissue from patients with Parkinson's disease (see the following section).

Since the discovery of mutations in the gene encoding α-synuclein, a number of mouse models have been developed that overexpress human α-synuclein or express common mutations in the α-synuclein gene [61]. Importantly, these mice (unlike the majority of toxin-induced models) show the abnormal accumulation of insoluble α-synuclein aggregates in neuronal cell bodies and processes, recapitulating the human Lewy pathology [61]. Astrogliosis is consistently observed in these models, but appears to occur early in the disease process, when there is minimal neuronal α-synuclein deposition and no significant neuronal loss [61, 69]. As will be discussed in more detail in the following section, studies in postmortem tissue from patients with Parkinson's disease reveal the accumulation of α-synuclein inclusions in gray matter protoplasmic astrocytes in Parkinson's disease [46, 70]. The suggestion is that astrocytes can take up altered α-synuclein released from axon terminals [70], and direct experiments confirm the transfer of α-synuclein from neurons to astrocytes [71]. It has been suggested that astrocytes may be neuroprotective in the earliest stages of Parkinson's disease, but over time with increasing exposure to toxic α-synuclein aggregates may lose their protective effect or might be aberrantly activated [61]. Supporting this, retracted astrocyte processes have been observed in postmortem brain tissue from patients with Parkinson's disease [72], and proliferating nonreactive astrocytes with sparse and significantly smaller processes have been identified in neurotoxin-induced models [73, 74]. A study in mice expressing A53T mutant α-synuclein directly addressed the question of the pathological consequences of selective astrocytic accumulation of α-synuclein and found that astrocytic α-synuclein initiates noncell autonomous killing of neurons, producing a rapidly progressive paralysis [75]. In these mice, there was increasing presymptomatic and symptomatic accumulation of α-synuclein aggregates and astrocyte dysregulation of the blood–brain barrier and glutamate transporters [75]. Similar accumulation of α-synuclein aggregates in astrocytes has been observed in transgenic mice with nonselective α-synuclein expression where astrocytes produced proinflammatory cytokines and chemokines [71]. These astrocytic changes led

directly to microglial activation in the midbrain, brainstem, and spinal cord, where a significant loss of dopaminergic and motor neurons was observed [75]. Suppression of this subsequent microglial activity extended mice survival, suggesting that the excess microglial responses elicited by α-synuclein-containing astrocytes directly contribute to the degeneration of neurons [75].

Assessment of Astrocytes in Parkinson's Disease

As discussed in the preceding section, the typical response of astrocytes to tissue changes involves GFAP upregulation. Only a small number of studies have assessed the astrocyte response in brain tissue from patients with Parkinson's disease. While a variable increase in GFAP in select regions has been reported [76], most studies observe only minimal, if any, astrocyte activation [46, 77], in direct contrast to the severe astrogliosis observed in the majority of rodent models of Parkinson's disease (see preceding section). Song et al. assessed the number of GFAP-immunopositive cells in different parkinsonian conditions (Parkinson's disease, multiple system atrophy, corticobasal disease, and progressive supranuclear palsy) with classic reactive astrocytes reported in the gray matter regions examined (substantia nigra and putamen) in all diseases except Parkinson's disease [46]. Gray matter protoplasmic astrocytes did not display any morphological changes in Parkinson's disease (Fig. 3); however the astrocytes were abnormal with approximately 45 % displaying immunoreactivity for α-synuclein (see Fig. 3), with no obvious changes in white matter fibrous astrocytes [46]. The distribution of α-synuclein-immunoreactive protoplasmic astrocytes not only parallels the distribution of Lewy bodies but also occurs more broadly in regions without Lewy bodies (striatum, dorsal thalamus) where terminals are likely to be dysfunctional [70].

Protoplasmic astrocytes normally envelope all neurons and synapses, and astrocytes continue to do so around healthy neurons in Parkinson's disease [72]. However, in patients with Parkinson's disease, these protective protoplasmic astrocytic processes withdraw from around damaged neurons and are replaced by phagocytic microglia [72]. Astrocytes in other neurodegenerative conditions express growth factor receptors in response to microglial-secreted brain-derived growth factors with the downstream consequences of the perpetuation of neurodegeneration [78]. In contrast, in Alzheimer's disease protoplasmic astrocytes themselves produce brain-derived growth factors following their contact with β-amyloid, and the astrocytic production of this growth factor has been shown to rescue neurons from β-amyloid toxicity [79]. Although speculative, the abnormal accumulation of α-synuclein in protoplasmic astrocytes may impact on their ability to produce brain-derived growth factors in the same manner (note this abnormality has been recently described for astrocytes abnormally accumulating huntingtin [80]). This could assist with explaining the marked difference in the reactivity of the protoplasmic astrocytes in Parkinson's disease versus similar neurodegenerative conditions and the loss of their primary protective function in this disease.

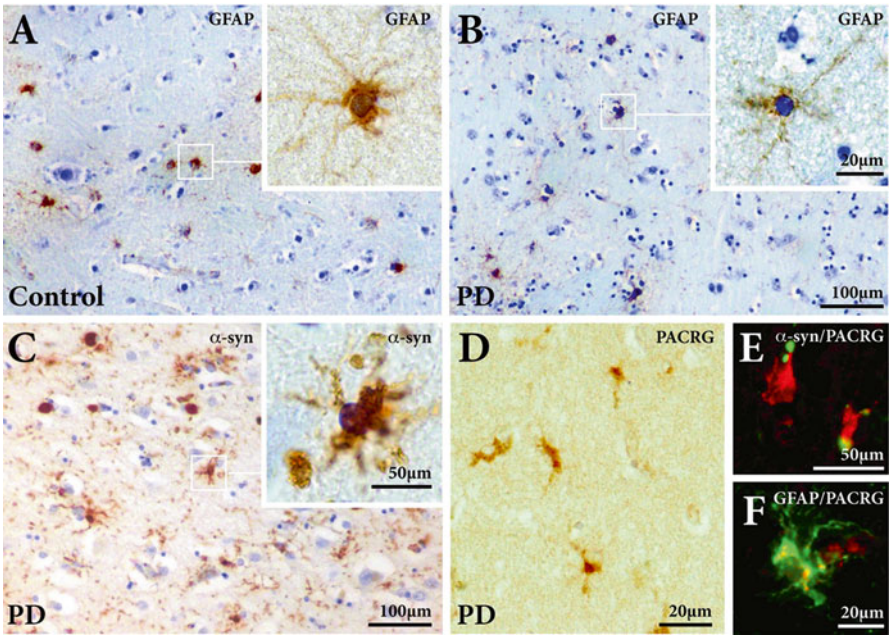

Fig. 3 Photomicrographs showing the unique astrocyte response in Parkinson's disease (PD). *α-syn* α-synuclein, *GFAP* glial fibrillary acidic protein, *PACRG* parkin-coregulated gene protein. (**a**) GFAP-immunoreactive protoplasmic astrocytes in healthy brain tissue (immunoperoxidase with cresyl violet counterstain). (**b**) GFAP-immunoreactive protoplasmic astrocytes in PD show minimal, if any, upregulation of GFAP compared to controls (**a**) (immunoperoxidase with cresyl violet counterstain). *Scale bars* in (**b**) are equivalent for (**a**). (**c**, **d**) Protoplasmic astrocytes in PD accumulate α-synuclein in the cytoplasm (**c**) (immunoperoxidase with cresyl violet counterstain) and show constitutive, but not increased expression of PACRG (**d**) (immunoperoxidase). (**e**, **f**) Double-labeling immunofluorescence shows the co-localization of α-synuclein (*green*) with PACRG (*red*) in the same astrocytes in patients with PD, with PACRG-immunopositive protoplasmic astrocytes (*red*) also expressing GFAP (*green, overlap seen as yellow*)

In addition to these observed structural alterations in astrocytes, identifying any changes in other constituent proteins could assist with understanding the role of protoplasmic astrocytes in the etiology of Parkinson's disease. Increased levels of S100B, an astrocytic calcium-binding protein shown to cause neuronal death directly or through microglial activation [81–83], have been found in the substantia nigra and cerebrospinal fluid of patients with Parkinson's disease and in MPTP mice [84]. S100B knockout mice show reduced numbers of activated microglia and expression of molecules including the proinflammatory cytokine tumor necrosis factor-α [84]. Furthermore, in the human brain, protoplasmic astrocytes express most of the recessive gene products involved in Parkinson's disease (PINK-1, parkin, and DJ-1) [85–88], and their production is usually increased in association with astrogliosis in disease states [46, 85, 86, 88, 89]. In particular, protoplasmic but not fibrous astrocytes constitutively express *parkin-coregulated gene* (PACRG)

(see Fig. 3), which has the same promoter as the *parkin gene* and is upregulated with parkin in neurodegenerative conditions other than Parkinson's disease [46]. Genetic alterations in the *parkin-coregulated gene* have also been identified in early-onset recessive cases of Parkinson's disease [90]. It is of interest that these proteins are not up-regulated in astrocytes in Parkinson's disease [46, 85, 86] providing further evidence to suggest a dramatically different response to the α-synuclein protein accumulation and degeneration that occurs in this disease.

All astrocytes from parkin knockout mice exhibit damaged mitochondria (disintegration and reduction of mitochondrial cristae, mitochondrial enlargement, and formation of protrusions or even disruption of the outer membrane) from 3 months of age, although some even have damage at 16 days [91]. Similarly, astrocytes from PINK-1 knockout mice have mitochondrial defects (decreased mitochondrial mass and membrane potential, increased levels of intracellular reactive oxygen species, decreased glucose-uptake capacity, and decreased ATP production) [92]. Of interest, PINK-1-deficient astrocytes are incapable of wound healing [92], similar to astrocytes in the brain of patients with Parkinson's disease, and DJ-1-deficient astrocytes have a neurotoxic effect by enhancing brain inflammation [93–95]. DJ-1 knockout astrocytes show increased expression of proinflammatory substances [95] including cyclooxygenase-2 and interleukin-6, have reduced capacity to support neuronal cells resulting in neuronal death or damage [93], and are less able to mount a protective response against oxidative stress induced by the neurotoxin 6-OHDA [94], effects that could be due to defective mitochondria. Of interest, similar astrocytic mitochondrial defects are also observed in transgenic mice where α-synuclein is overexpressed only in neurons as well as in transgenic mice where α-synuclein is expressed in astrocytes and other glia [92]. The uptake of α-synuclein into human astrocytes impairs their mitochondria leading to cell degeneration and death [96]. Furthermore, mitochondrial uncoupling proteins (UCP) (which dissociate ATP synthesis from oxygen consumption in mitochondria and suppress free-radical production) are reduced in astrocytes resulting in endoplasmic reticulum stress and neuroinflammation [97], and changes in UCP proteins are associated with the causal mutations for Parkinson's disease in both DJ-1 [98] and LRRK2 [99]. While speculative, it appears that the loss of function of these Parkinson's disease-associated proteins in astrocytes precipitates early mitochondrial defects that are likely to have significant consequences for both normal astrocytic and neuronal functions.

The recessive gene products constitutively expressed by astrocytes (PINK-1, parkin, and DJ-1) are also found in Lewy bodies [85, 86, 100–102], providing additional evidence for a role of astrocytes in Parkinson's disease. Furthermore, astrocyte-concentrating proteins such as Pael-R (substrate for Parkin) [102], DnaJ/Hsp40 homologue, subfamily B, member 6 (DnaJB6) [103], and nonselenium glutathione peroxidase [104] are found in the cores of Lewy bodies, suggesting astrocyte involvement at the earliest disease stages. Interestingly, a number of these proteins are also found to be up-regulated in astrocytes in patients with Parkinson's disease. DnaJB6, a molecular chaperone with an important role in the maintenance of normal cellular functions, has been shown to be up-regulated in astrocytes in

both the substantia nigra and frontal cortex [103], possibly in an attempt to prevent or suppress α-synuclein accumulation in a manner similar to that described for huntingtin in Huntington's disease [105]. Similarly, the metal-binding proteins metallothioneins, which are associated with neuroprotection, show a variable increase in substantia nigra astrocytes [106], possibly in response to mitochondrial oxidative stress [107]. Finally, increased expression of the antioxidant enzyme non-selenium glutathione peroxidase is observed predominantly in the unaffected white matter astrocytes but also in gray matter astrocytes in the frontal cortex and cingulate cortex of patients with Parkinson's disease [104]. In contrast, no significant immunoreactivity for this enzyme is observed in substantia nigra astrocytes [104], and reduced levels of glutathione (an enzyme that protects against protein oxidation) are reported in the substantia nigra of patients with Parkinson's disease [108], again supporting the evidence that protoplasmic astrocytes are unable to maintain their normal protective functions in regions highly affected by degeneration in Parkinson's disease.

Of major interest is the recent study examining how L-DOPA, the most commonly used dopamine replacement therapy for Parkinson's disease [109], enters the brain, particularly as astrocyte end feet surround brain blood vessels [110]. This study showed that L-DOPA accumulates mainly in astrocyte cell bodies, astrocytic end feet surrounding blood vessels, and pericytes and that the pattern of monoamine oxidase type B staining (enzyme that catabolizes L-DOPA to dopamine) in pericytes and astrocytic end feet was similar to that of L-DOPA [110]. The study concluded that astrocytes control L-DOPA uptake and metabolism and, therefore, play a key role in regulating brain dopamine levels during dopamine-associated diseases [110]. If this is applicable to human astrocytes, then any astrocytic dysregulation, as discussed above, would have consequences for the long-term success of dopamine replacement therapies, and dysfunction of astrocytes in Parkinson's disease could underlie difficulties with dopamine medication use over time.

Conclusion

The astrocyte response in Parkinson's disease is clearly unique from most neurodegenerative conditions, including other parkinsonian disorders. Instead of a typical reactive astrogliosis, protoplasmic astrocytes in Parkinson's disease accumulate α-synuclein, withdraw their processes from around damaged neurons, and show altered expression of constituent proteins, suggesting that they have reduced neuronal support and protection capabilities, particularly in regions associated with significant degeneration. In contrast, the majority of work in rodent models of Parkinson's disease has reported severe astrogliosis occurring in the brain. This combined with the link between dopamine replacement medication and astrocytes highlights the need for a greater understanding of the role of these glial cells in the human disease.

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Proinflammatory Chemical Signaling: Cytokines

Kumi Nagamoto-Combs and Colin K. Combs

Microglial Activation in Parkinson's Disease

For several decades, reactive microglia have been known to be associated with the histological changes that occur in Parkinson's disease brains. Stemming from an initial report of HLA-DR immunoreactive microglia in the substantia nigra of Parkinson's disease patient brains [1], multiple subsequent studies performed from autopsy brains have verified with a range of immunomarkers that microgliosis is a characteristic of diseased brains in not only the pars compacta region of the nigra but also the striatum, hippocampus, and various cortical areas [1–6]. Although microglial numbers are reportedly higher in the nigra than other brain regions [1, 7, 8] and some age-associated nigral microgliosis has been reported [9], it is generally assumed that some form of disease-relevant stimulus is still involved in driving microglia to acquire a particular reactive phenotype that contributes to neuron loss during disease.

Unfortunately, histologic data from autopsy brains does not allow for temporal assessment of microglial activation during disease course. Therefore, in spite of the convincing histologic evidence of microgliosis, a lingering question is whether or not microglial-mediated inflammatory changes contribute or simply respond to neuron death. It is clear that the degree of reactive microgliosis is increased proportionally to the degree of dopaminergic cell loss in the nigra. However, their activation state appears to be independent of the amount of Lewy bodies present [4]. In vivo imaging of microglia during disease using the positron emission tomography

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M. Thomas (ed.), *Inflammation in Parkinson's Disease: Scientific and Clinical Aspects*,
DOI 10.1007/978-3-319-08046-8_6,

marker, [^{11}C](R)-PK11195, has demonstrated in a small study of 18 Parkinson's disease patients and 11 healthy, age-matched controls that levels of microglial activation in diseased brains were elevated in the basal ganglia, cerebral cortex, and brainstem compared to controls [10]. Longitudinal comparison in eight of these Parkinson's disease patients indicated that levels of reactive microgliosis remained stable over a 2-year period with no correlation with clinical severity [10]. The authors concluded from their findings that microglial activation may occur early in disease and simply remain, perhaps with altered phenotypes, for years [10]. On the other hand, additional in vivo imaging using [^{11}C](R)-PK11195 in ten early-stage Parkinson's disease patients and ten healthy, age-matched controls with a 4-year follow-up for four patients in each group demonstrated increased midbrain microgliosis in diseased brains that spread throughout the brain in correlation with loss of dopaminergic transporter density in the putamen and increased motor symptom severity [11]. As stated prior, it appears that the precise role of microglial activation in the ongoing process of dopaminergic cell loss in Parkinson's disease is still unclear.

Nevertheless, data from a variety of models of disease continue to support the notion that microgliosis may be something more than a reaction to cell death. The 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) toxin-induced model of Parkinson's disease provides support for a prolonged microglial-mediated inflammatory process during disease. Microgliosis is apparently maintained for years based upon data derived from MPTP-treated rhesus monkeys whose brains displayed reactive, persistent microgliosis resulting from drug exposure 5–14 years prior to death [12]. This correlates precisely with human data derived from patients with MPTP-induced Parkinsonism 3–16 years before death who also demonstrated robust microgliosis in their nigras [13].

This human and monkey data demonstrating the ability of the nigra to maintain prolonged microglial activation has been supplemented by data from rodent studies which provide mechanistic support for an active role of inflammatory changes in cell death. For example, several studies demonstrate that microgliosis correlates with ongoing dopaminergic cell loss using the rat 6-hydroxydopamine (6-OHDA) toxin injection paradigm [14, 15]. An additional in vivo rodent model of Parkinson's disease involving the use of intranigral injection of bacterial endotoxin, lipopolysaccharide (LPS), has also supported this idea. This paradigm similarly demonstrates microgliosis occurring with and potentially preceding dopaminergic cell death [16]. Moreover, the LPS-stimulated, microglial-associated cell death appears unique to dopaminergic neurons in the nigra with cholinergic and serotoninergic neurons unaffected [17]. MPTP injection into rodents also results in dramatic striatonigral microgliosis potentially preceding and certainly correlating with loss of dopaminergic cells [18–24]. Collectively, histologic findings from human brain and temporal analysis from rodent studies continue to support the idea that microglial activation and the associated inflammatory changes are a part of the disease process.

Peripheral Immune Cell Changes in Parkinson's Disease

Although abundant data supporting microglial activation during neuron loss have been generated from both human and animal models of disease, there are also a significant number of findings that report altered peripheral immune cell behavior during disease. This suggests that some communication exists between the peripheral immune system as well as the possibility that disease involves a more general alteration of immune function. For example, a report by Ton et al. examining patients from the Cardiovascular Health Study determined that the risk of prevalent Parkinson's disease was significantly higher with elevated IL-6 levels and white blood cell counts in women and men, respectively [25]. However, over a 13-year follow-up, the risk of incident disease did not correlate with higher levels of interleukin-6 (IL-6), fibrinogen, tumor necrosis factor-alpha (TNF-α), C-reactive protein, albumin, or white blood cell counts [25]. Based upon these data from 60 prevalent and 154 incident disease cases, it appears that peripheral inflammatory biomarkers are elevated during disease with unclear value for pre-diagnostic or progression assessments. However, a nested case-control study including 84 incident Parkinson's disease patients and 165 health age-matched controls prospectively examined blood for inflammatory markers on average 4.3 years before diagnosis to reveal that higher pre-diagnostic levels of IL-6 but not C-reactive protein, fibrinogen, or TNF-α receptor 1 or 2 (TNFR1 or TNFR2) were significantly associated with greater risk of disease after controlling for caffeine intake, age, smoking, and creatine and uric acid levels [26]. Another study of incident versus prevalent disease from Wong and colleagues compared 61 cases of Parkinson's disease and matched controls to demonstrate that high fibrinogen levels correlated significantly with both prevalent and incident disease in men after adjusting for age, smoking, and low-density lipoprotein cholesterol [27]. Elevated levels of interleukin-2 (IL-2) but not IL-6, cortisol, adrenocorticotropic hormone (ACTH), or interleukin-1β (IL-1β) have been reported in the serum of 21 non-drug-treated Parkinson's disease patients versus healthy, age-matched controls [28]. A similar study of cytokine serum concentrations from 78 idiopathic Parkinson's disease patients versus healthy controls aged 30–90 years demonstrated elevated TNF-α and IL-6 in disease patients with elevated TNF-α correlating with abnormal postural and psychomotor performance [29]. Hofmann et al. also quantified IL-6 levels from serum of 23 Parkinson's disease patients versus age-matched, healthy controls to demonstrate that IL-6 levels were not significantly elevated compared to controls but levels did increase with disease severity [30]. Comparison of 20 Parkinson's disease patients and 22 age-matched healthy controls by Bessler et al. found that isolated peripheral blood mononuclear cells had impaired ability to secrete IL-2 compared to controls [31]. Similar findings were reported by Kluter et al., who also found that mitogenically stimulated peripheral blood mononuclear cells from Parkinson's disease patients had impaired IL-2 secretion compared to cells isolated from controls [32]. Another cytokine comparison by Rentzos and colleagues found elevated serum RANTES (Regulated on Activation, Normal T Cell Expressed and Secreted) when comparing

41 Parkinson's disease patients to 19 matched controls that significantly correlated with the Unified Parkinson's Disease Rating Scale (UPDRS III) score of patients [33]. A subsequent study of 41 Parkinson's disease patients compared to 19 healthy age-matched controls demonstrated elevated IL-10 concentrations in serum of diseased individuals [34]. Quantitation of multiple cytokines, TNF-α, IL-1β, IL-1α, IL-6, and IFN-γ, secreted upon LPS stimulation of peripheral blood mononuclear cells demonstrated the Parkinson's disease patient cells secreted significantly lower amounts compared to both healthy age-matched controls and patients with cerebrovascular disease that paralleled disease progression [35]. Lindqvist and colleagues also measured select inflammatory markers from the blood of 86 Parkinson's disease and 40 healthy control patients to correlate with changes in fatigue, anxiety/depression, and sleep patterns [36]. They observed that elevated levels of soluble IL-2 receptor (sIL-2R) and TNF-α in the blood of Parkinson's disease patients significantly correlated with more severe symptoms of depression and anxiety [36]. In fact, after controlling for effects of age, gender, motor symptoms, and medications, sIL-2R increases were predictive of depression/anxiety scores [36]. Collectively, these data suggest that serum concentrations of a range of cytokines differ between Parkinson's disease patients and controls. However, outcome variability and differences in study design contribute to uncertainty in identifying a particular biomarker cytokine that is predictive of either disease risk or progression.

Beyond simply looking for a biomarker through quantifying cytokine differences in serum, a multitude of studies suggest that peripheral immune cells from Parkinson's disease patients have different phenotypes when compared to controls. For instance, mononuclear cells isolated from peripheral blood of Parkinson's disease patients demonstrate reduced immunoglobulin secretion basally and with mitogen stimulation [37]. Saunders and colleagues concluded that immune changes in 113 Parkinson's disease patients versus 96 age-matched caregiver controls correlated with UPDRS III severity but not disease duration by demonstrating increased effector/memory T cells with increased $CD45RO^+$ and FAS^+ $CD4^+$ T cells and decreased $CD31^+$ $\alpha4\beta7^+$ $CD4^+$ T cells in disease patients [38]. Fiszer et al. compared lymphocyte subpopulations from blood of Parkinson's disease patients versus age-matched patients with other neurologic disease or tension headache to find that Parkinson's disease patients had decreased $CD4^+$ $CD45RA^+$ cells as well as increased gamma delta+ T cells [39, 40]. Comparison of peripheral blood lymphocyte subsets from 32 drug-treated and 32 non-drug-treated Parkinson's disease patients with 38 healthy age-matched controls also found overall decreased T cells ($CD3^+$) and B cells ($CD19^+$) in both treated and untreated disease [41]. In particular, the number of naïve $CD4^+$ ($CD4^+$ $CD45RA^+$) helper (T_h) cells but not $CD8^+$ cells was decreased by disease [41]. However, the number of activated $CD4^+$ $CD25^+$ T cells was increased with disease although the number and percentage of lymphocyte subsets did not correlate with disease duration or severity as assessed by the UPDRS [41]. Chiba and colleagues also reported elevated numbers of activated peripheral T lymphocytes isolated from blood of Parkinson's disease patients compared to controls [42]. A study of 40 idiopathic Parkinson's disease patients compared to

22 age-matched healthy controls and 33 patients with mild cerebrovascular disease demonstrated that Parkinson's disease patients had increased $CD4^+$ $CD8dull^+$ T cells in peripheral blood compared to either of the other conditions similar to what is sometimes seen postinfection [43]. Baba and colleagues compared peripheral blood from 33 sporadic Parkinson's disease patients and 34 healthy age-matched controls to again find alteration of lymphocyte subsets. Parkinson's disease patients had significantly lower $CD4^+$ T cells and increased $CD8^+$ T cells demonstrating an increased $CD8^+$ suppressor/cytotoxic T cell to $CD4^+$ T cell ratio [44]. In addition T cells had an increased interferon gamma (IFN-γ) to IL-4 production ratio demonstrating a skew towards at T_h1 phenotype [44]. Although many of the studies have profiled changes in lymphocytes, other peripheral blood immune cells clearly are altered with disease as well. For instance, Luo et al. demonstrated impaired ability of monocyte-derived macrophages from Parkinson's disease patients to upregulate expression of CD200R upon stimulation compared to young as well as age-matched controls [45]. Moreover, impairment of induced CD200R expression correlated inversely with the age of onset of disease and ability of the cells to secrete TNF-α [45]. Although the evidence is not conclusive and somewhat variable, the trend certainly supports the idea that peripheral immune cells, particularly lymphocytes, have altered behavior during disease.

Further evidence of the possibility of peripheral immune cell behavior contributing to disease progression or neuron loss has been provided again, by rodent studies. Villaran et al. used a rat model of ulcerative colitis induced by dextran sulfate to produce elevations in levels of several serum as well as nigral cytokines including TNF-α, IL-1β, and IL-6 that correlated with increased permeability of the blood–brain barrier [46]. Moreover, ulcerative colitis potentiated the increase in nigral cytokine levels and the decrease in tyrosine hydroxylase-immunoreactive neurons induced by LPS injection [46]. This exacerbation of LPS-dependent change was attenuated by depleting peripheral macrophage from the ulcerative colitis animals by pretreating with clodronate encapsulated in liposomes [46]. This data suggested not only that peripheral immune cells and cytokine levels influence brain cytokine levels but they also directly contribute to nigral inflammatory degeneration. Pott Godoy et al. reported similar findings focusing on IL-1β-mediated loss of dopaminergic cells in the nigra. By injecting an adenoviral vector responsible for driving human IL-1β expression into the striatum of rats, they demonstrated not only decreased tyrosine hydroxylase-immunoreactive cell numbers in the nigra by 21 days but also potentiation of this loss if IL-1β was simultaneously overexpressed in the periphery [47]. This observation was consistent with prior work by this group demonstrating that peripheral IL-1β overexpression via adenoviral vector also potentiated 6-OHDA-induced decrease in nigral tyrosine hydroxylase cell numbers but had no effect on its own [48]. This suggests that peripheral inflammation, at least via IL-1β alone, was not sufficient to produce nigral neuron loss but instead exacerbates a brain challenge. Moreover, it appears that chronic rather than transient or acute exposure to IL-1β was required since at least 21 days of overexpression was required for significant changes to occur [47]. In addition, prior studies

with single acute injections of TNF-α, IFN-γ, or IL-1β into the substantia nigra of rats produced no toxicity at least for 7 days [49]. Additional rodent studies have also lent significant support to the notion that elevated peripheral circulating cytokine levels or peripheral inflammatory stimuli may contribute to brain inflammation. For example, in a recent mouse study Skelly and colleagues compared brain effects following intraperitoneal injection of LPS, IL-1β, TNF-α, and IL-6. Systemic stimulation with LPS, IL-1β, and TNF-α but not IL-6 was sufficient to increase IL-1β and TNF-α mRNA in the hippocampus by 2 h of stimulation with only LPS able to increase IFN-β mRNA [50]. Although data from the substantia nigra was not reported, these changes in brain cytokine mRNA correlated with increased neuronal c-Fos immunoreactivity in the central nucleus of the amygdala [50]. This data again demonstrates that the peripheral cytokine changes clearly affect brain physiology consistent with the possibility that they may also exert neurotoxic effects. Finally, Engler and colleagues performed an elegant study in which they demonstrated that intrastriatal injection of 6-OHDA into rats lead to a transient increase in peripheral white blood cell counts that included T cells, B cells, and neutrophils during the periods of active dopaminergic degeneration [51]. In addition, LPS stimulation of whole blood cultures demonstrated impaired production of TNF-α, IL-6, and IL-1β from the 6-OHDA-injected animals although plasma levels of cytokines were not different from control rats [51]. On the other hand, hypoactivity of the dopaminergic system due to 6-OHDA injection superimposed upon a peripheral proinflammatory stimulus, intraperitoneal LPS injection, led to decreased numbers of peripheral white blood cells and a heightened proinflammatory response following intraperitoneal LPS injection [51]. 6-OHDA-injected mice had significantly higher plasma cytokine concentrations of IL-1β, TNF-α, and IL-6 following LPS injection [51]. These data suggest that inflammatory neurodegenerative changes in the substantia nigra directly influence the phenotype of circulating peripheral immune cells, and loss of dopaminergic cells exacerbates the peripheral response to immune challenge.

As reviewed above, it is important to keep in mind that numerous conflicting findings exist regarding the cytokine and immune cell changes during disease both in the periphery and in the brain. Moreover, animal models designed to allow more mechanistic dissection of the disease process have not yet provided conclusive evidence of inflammatory change driving or responding to dopaminergic degeneration during disease. Finally, it is also clear that the immune responses of mice and humans, particularly following peripheral LPS challenge, are characterized by unique genomic expression profile changes leading some investigators to conclude that mice poorly mimic human immune responses in general [52]. In an effort to determine whether a particular mechanistic contribution of inflammatory changes can be extracted from the extensive existing literature, we will focus on the immune cell-secreted inflammatory signaling molecules already mentioned, cytokines, to determine whether they are more than potential biomarkers of disease and perhaps represent molecular targets for therapeutic intervention.

Cytokines in Parkinson's Disease

In spite of the fact that the specific cause(s) and temporal phenotypes of microglia and peripheral immune cells during disease remain unclear, it is well known that either of these cell types rely upon secretion of a plethora of low molecular weight signaling molecules, termed cytokines, to act via both paracrine and autocrine cellular mechanisms. These critical signaling proteins not only regulate and coordinate the behavior of peripheral immune cells and brain microglia but also a multitude of cells throughout the brain and body. We will review, sequentially, evidence of particular cytokine changes reported in Parkinson's disease patients in either the brain or periphery for comparison to animal models of disease in an effort to determine whether particular changes can be mechanistically linked with disease progression or neuron loss. The focus of the remaining part of the chapter will be restricted to TNF-α and IL-1β not necessarily because they are the most important cytokines for disease processes but instead based upon the fact that they appear to be the more intensely studied with respect to Parkinson's disease.

Tumor Necrosis Factor-Alpha (TNF-α)

TNF-α was originally described as a lymphocyte-secreted toxin [53–56] and then later included as a macrophage-secreted protein with an ability to contribute to necrosis of tumors [57, 58]. Although it was identified outside of the brain, it is clear that it can be expressed by multiple cell types in the brain, particularly microglia [59–61], with receptor binding throughout the brain as assessed by radioiodinated TNF binding [62]. It is an approximately 17 kDa pleiotropic cytokine capable of stimulating a variety of cell types with a diverse set of consequences [63, 64]. Its effects on target cells occur via binding to a trimerized form of two distinct receptors, CD120a (p55, TNFRI) and CD120b (p75, TNFRII) [65]. Both neurons and glia have been reported to express these receptors [62, 66]. The cytoplasmic domain of TNFRI contains a death domain (DD) sequence of 60–80 amino acids responsible for recruiting the adaptor protein TRADD and leading to transduction of diverse signaling pathways including activation of IKK kinases and NFκB or the adapter protein Fas-associating protein with a death domain (FADD) and subsequent caspase activation [67–71]. Although TNFRII lacks the DD sequence, it contains a region of 78 amino acids responsible for recruiting the adaptor protein, TRAF2, also allowing subsequent activation of NFκB [72]. The extracellular portion of both TNF receptors contains four cysteine-rich domains termed, CRDs. TNF-α binds to CRD2 and 3, while CRD1 contains a self-association domain required for homotypic receptor trimerization in order for the receptors to be able to bind the TNF-α trimer [73, 74]. This has been termed the pre-ligand assembly domain (PLAD) since it is necessary for receptor trimerization independent of ligand binding. The PLAD

sequence differs significantly between TNFRI and TNFRII, allowing the design of competing peptides that will attenuate trimerization of one and not the other. Indeed, these have already been synthesized and used in vitro and in vivo to selectively attenuate TNFRI versus TNFRII-mediated cell death and inflammation in rodents [75].

TNF-α in Parkinson's Disease

Perhaps due to its widely held role as a master regulatory cytokine, characterization of changes in TNF-α related biology, as compared to other cytokines, is best described in Parkinson's disease. Increased glial TNF-α immunoreactivity has been reported in the substantia nigra of Parkinson's disease brains compared to controls with both displaying robust neuronal immunoreactivity for TNFR1 [76]. Indeed, subsequent study has demonstrated robust TNF-α immunoreactivity localizing to particularly, microglia, in the Parkinson's disease brain [4]. Mogi and colleagues also demonstrated elevated TNFR1 levels in the substantia nigra of Parkinson's disease patients compared to controls suggesting a receptor subtype-specific change is involved in disease [77]. A microarray study from the substantia nigra of Parkinson's disease patients compared to controls again verified elevated TNF-α levels with disease [78]. Collectively, there appears significant evidence of elevated TNF-α levels in the brains of Parkinson's disease patients.

As already mentioned, abundant peripheral changes in TNF-α biology have also been documented during disease. For instance, serum TNF-α levels measured from 78 idiopathic Parkinson's disease patients compared to 140 health controls demonstrated elevated levels of the cytokine in Parkinson's disease patients with abnormal postural and psychomotor function compared to controls [29]. Mogi et al. reported similar data from both striatum and cerebrospinal fluid showing significantly increased TNF-α levels in Parkinsonian patients compared to controls [79]. An additional study by Scalzo and colleagues examined serum levels of soluble TNFRI and soluble TNFRII from 46 Parkinson's disease patients compared to 23 healthy age-matched controls to find that diseased patients had significantly higher levels of soluble TNFRI indicating again that changes in the cytokine as well as its receptors occur in the periphery during disease [80]. A comparison of peripheral blood mononuclear cells from 40 Parkinson's disease and 40 healthy, age-matched controls found that basal as well as LPS-stimulated TNF-α release (as well as numerous other cytokines) was elevated from Parkinson's disease patient cells [81]. This is consistent with data from Bongioanni et al. who demonstrated that peripheral blood T cells from Parkinson's disease patients had elevated TNF-α binding and therefore higher receptor expression compared to control cells [82]. A study of 60 idiopathic Parkinson's disease patients compared to 24 healthy controls again demonstrated significantly higher serum TNF-α levels with disease [83]. As mentioned above, Lindqvist and colleagues correlated increased non-motor symptoms such as depression and fatigue in Parkinson's disease patients with this elevation in serum TNF-α [36]. Similar data from Menza et al. also demonstrated that elevated serum TNF-α levels in Parkinson's disease patients with depression significantly correlated with deficits in cognition and depression [84].

TNF-α Polymorphisms in Parkinson's Disease

These changes in measured levels of TNF-α correlate well with single-nucleotide polymorphism analysis from Parkinson's disease patients. A comparison of 289 idiopathic Parkinson's disease patients and 269 matched controls examined a single-nucleotide polymorphism in the TNF-α promoter (G-308A) to find a 1.14-fold increased risk for heterozygous carriers and a 2.49-fold increased risk for homozygous carriers [85]. This is consistent with evidence that the polymorphism at −308 in the TNF-α promoter is associated with high TNF-α production [86]. A subsequent study by Bialecka et al. found that the T-308A polymorphism was significantly more frequent in early-onset Parkinson's disease patients than controls in a study comparing 102 early-onset patients to 214 late-onset patients [87]. Kruger and colleagues also found 1.56-fold relative increased risk in heterozygous carriers [88], while Ross et al. [89] as well as Passarelli et al. [90] failed to find such an association for the TNF-α-308 polymorphism. Nishimura and colleagues reported data from additional promoter polymorphism analysis studying 172 varying-age onset Parkinson's disease patients compared to 157 healthy controls to demonstrate that carriers of the T-1031C polymorphism, associated with elevated TNF-α levels, significantly correlated with early-onset PD [91]. Similar conclusions were reached by Wu et al. who analyzed 369 Parkinson's disease patients and 326 controls to find that individuals homozygous for the −1031 polymorphism had elevated risk of disease [92].

TNF-α in Animal Models of Parkinson's Disease and Neurodegeneration

In an effort to identify a mechanism by which TNF-α contributes to dopaminergic cell death, a number of animal model studies have been performed. Many of these mechanistic dissections have relied upon transgenic mouse studies that are deficient in either TNFRI or TNFRII. A study by Sriram et al. demonstrated increased TNF-α levels in the striatum of 1-methyl-4-phenyl-1,2,3,4-tetrahydropyridine (MPTP) mice prior to gliosis and a decrease in striatal dopamine and tyrosine hydroxylase levels [93]. However, animals deficient in both high- and low-affinity TNF-α receptors (TNFRI and TNFRII, respectively) were protected against this decrease [93]. A similar study by Rousselet and colleagues demonstrated that TNFRI/RII double-knockout mice, but not mice lacking only one receptor, had lower striatal dopamine levels basally and lower dopamine levels after MPTP injection compared to wild-type littermates correlating with increased turnover in the knockout animals [94]. In addition, the degree of nigral neuron loss was comparable in the TNFRI/RII double-knockout mice compared to single receptor deletion and wild-type mice [94]. This study concluded that TNF-α does not have a direct role in cell death in this particular mouse MPTP toxicity paradigm. Similar findings were reported by

Leng et al., who demonstrated that MPTP treatment also produced no difference in the toxin-induced decrease in striatal dopamine levels or nigral dopamine transporter immunoreactive neuron numbers between TNFRI knockout, TNFRII knockout, or wild-type mice [95]. Likely based upon experimental design differences, data from these toxin-based models of disease have not yet provided a cohesive argument for a particular TNF-α receptor in mediating neuron death or protection in Parkinson's disease.

Other studies have focused on TNF-α itself rather than its receptors in an effort to validate a role for the cytokine in Parkinson's disease-related neuron death. Using mice deficient in TNF-α, Ferger et al. demonstrated significant protection against MPTP-induced decreases in striatal dopamine levels and tyrosine hydroxylase immunoreactivity with no effect on toxin-induced changes in nigral tyrosine hydroxylase or dopamine transporter immunoreactivities [96]. McCoy and colleagues demonstrated that neutralizing secreted TNF-α with a dominant negative TNF-α inhibitor protected nigral neuron death in rats in response to both LPS infusion and 6-hydroxydopamine (6-OHDA) injection [97]. Consistent with the notion that chronic cytokine elevation is needed to produce dopaminergic loss, De Lella Ezcurra and colleagues demonstrated that adenoviral expression of high levels of TNF-α in the rat substantia nigra was sufficient to produce macrophage infiltration by day 7 and significant decrease in tyrosine hydroxylase-immunoreactive cell numbers by 14 days of overexpression [98]. However, a subsequent study by Chertoff et al. demonstrated that it is the level of TNF-α overexpression in the substantia nigra that dictates the effects of the cytokine [99]. In this study, low levels of adenoviral-mediated nigral TNF-α expression protected murine dopaminergic neurons from 6-OHDA-mediated toxicity, while overexpression again mediated neuron loss [99]. Therefore, in these studies focusing on the cytokine rather than any particular receptor, there may still be some dichotomy of behavior with respect to a role in protection or death. Nevertheless, the majority of data seems to indicate across several different paradigms that TNF-α can stimulate dopaminergic neuron death.

To better appreciate this dichotomous role that TNF-α appears capable of having in the brain, it is useful to remember, as already mentioned, that TNFRI and TNFRII have unique signaling responses, at least in nonneuronal cells, in which the two receptors are able to help mediate distinct cellular phenotypes. More importantly, a conflicting body of literature has demonstrated that neuronal stimulation via either TNF-α receptor mediates protection or death across a range of experimental paradigms well beyond the field of Parkinson's disease research. In fact, even using the same toxin can produce differing results with regard to a role for TNF-α in protection or not depending upon the particular experimental design [100, 101]. To add to the complexity of TNF-α biology in the brain, in several experimental systems, TNF-α stimulation mediates its toxicity in the context of a proinflammatory milieu suggesting that its effects are often superimposed upon responses initiated by a plethora of additional glial secreted stimuli [102–104]. For example, expression and secretion of TNF-α, particularly by microglia, has been observed in several neurotoxic paradigms including multiple sclerosis, Alzheimer's disease, stroke/ischemia, traumatically injured, and epileptic brains and implicated in mechanisms of neuron loss [105–111].

Some explanation for the varying effects of the cytokine on neuron behavior stems from the fact TNF-α not only operates through two different receptors on neurons but these receptors can also utilize a variety of signal transduction pathways to mediate phenotype changes. The more classic caspase and NFκB transduction responses have been mentioned already, but a growing body of literature demonstrates additional TNF-α-mediated responses that can control death or survival. For example, the potential neuroprotective or neurotoxic effects of TNF-α stimulation have been examined in the context of its ability to regulate synaptic scaling and ischemic tolerance. For several years now, glial secreted TNF-α has been implicated in the neuronal phenomenon of synaptic scaling, which describes the ability of TNF-α to stimulate increased cell surface localization of glutamatergic AMPA receptors to strengthen synapses [112, 113]. It appears that the particular localization of the receptors to synaptic or extrasynaptic compartments and the particular subunit composition of the receptors allow TNF-α to serve as more than a synaptic modulator capable of potentiating excitotoxicity or attenuating it as seen during ischemic preconditioning. For instance, to support a protective role of the cytokine, it has been shown that low-level TNF-α stimulation of neuron or organotypic slice cultures not only attenuates the intracellular calcium response of neurons to subsequent excitatory stimulation but also increases protein levels of excitatory amino acid transporter 3 (EAAT3) to increase glutamate uptake [114, 115]. More specifically, transient (15 min) murine hippocampal slice treatment with low-concentration (6 nM) TNF-α stimulates a rapid increase in neuronal cell surface localization of both GluR1 and GluR2 of the AMPA receptor [116]. Similar findings were observed by Rainey-Smith and colleagues using murine motor neuron cultures to demonstrate that 15-min stimulation with 10 ng/mL TNF-α resulted in increased GluR1 and GluR2 cell surface localization offering protection from subsequent calcium excitotoxicity [117]. This effect appears to be mediated by the high-affinity receptor, since TNFRI but not TNFRII knockout neurons are resistant to the effects of TNF-α to increase cell surface AMPA receptor subunit localization [118].

On the other hand, TNF-α stimulation can augment intracellular calcium signaling to contribute to cell death in other paradigms. Indeed, TNF-α can potentiate excitotoxic death via altering AMPA or NMDA excitatory neurotransmitter receptor activities [119–121]. A similar experiment using rat hippocampal neuron cultures demonstrated that TNF-α stimulation for 15 min was sufficient to increase GluR2-lacking AMPA receptor localization in the extrasynaptic compartment as well as GluR2-containing AMPA receptors but with a delayed time course that resulted in potentiation of excitotoxic death [122]. This role in potentiation of cell death was supported by in vivo data using a soluble TNF-α receptor in spinal cord injury.

To summarize the vast field of TNF-α-related neuron death and cite only a few of the many studies it appears that data from receptor-specific knockout mouse in vivo studies support a role for the high-affinity receptor, TNFRI, rather than the low-affinity receptor, TNFRII, in mediating TNF-α-stimulated neuron death [118, 123]. This mechanism has been linked to caspase-mediated apoptotic events based upon the understanding that TNF-α binding to TNFRI has the ability to stimulate caspase and/or NFκB activity. However, these signaling pathways have been almost exclusively characterized in nonneuronal cells, and it is largely assumed that these

responses are also employed by neurons. There is mounting evidence that TNF-α receptors utilize atypical signaling pathways in neurons. In fact, Jarosinski et al. demonstrated in 2001 that TNF-α stimulation of primary neurons is unable to activate NFκB [124]. A more recent study indicates that TNF-α stimulation does increase NFκB activity but to a degree that is less than even constitutive activation in other cells. Moreover, transcription of many NFκB-responsive genes is not increased by stimulation in neurons again suggesting that typical TNF-α-stimulated signaling responses described in nonneuronal cells may not be relevant in neurons [125]. This paradoxical behavior of TNF-α in neurons was even the topic of a review by Massa et al. [126]. Adding even more complexity to the field is the fact that gene array analysis from the brains of TNFRI and TNFRII receptor knockout mice displays significant basal brain expression differences across a range of different protein classes compared to each other as well as wild-type mice, indicating clear phenotype changes simply from loss of receptor expression that are likely developmental changes [127, 128]. This suggests, as always, that caution must be applied when interpreting data derived from the use of transgenic models such as the TNFRI or TNFRII knockout mice when attempting mechanistic predictions for the contribution of TNF-α in Parkinson's disease.

Evidence of Particular TNF-α Signaling in Parkinson's Disease

Although a mechanism of particular TNF-α receptor-mediated death remains unclear, Hartmann and colleagues have demonstrated decreased FADD immunoreactivity in dopaminergic neurons within the substantia nigra pars compacta of Parkinson's disease brains, suggesting that a FADD-caspase-mediated mechanism of death might occur [129]. This is supported by work from Mogi and colleagues, which demonstrated elevated caspase 1 and caspase 3 activity, based on fluoropeptide substrate cleavage, in the substantia nigra of Parkinson's disease patients compared to controls [77]. Similarly, elevated caspase 3 and BAX immunoreactivity has been reported from neuromelanin-containing neurons from the Parkinson's disease brains compared to controls [130]. An additional piece of evidence supporting a possible role for TNFRI-mediated cell death is based upon the observation by Hunot and colleagues who compared 5 idiopathic Parkinson's disease patients and 7 matched controls to find a very significant increase in neuromelanin-containing neuronal nuclear NFκB immunoreactivity in diseased brains at least correlating with a proposed consequence of TNFRI-mediated signaling [131]. Rodent data seems to support this idea of apoptotic death as a component of the disease process. For instance, it is clear that caspase-3 activity is required for cell death and microgliosis in the mouse MPTP injection model as determined by comparison between wild-type and caspase 3 knockout mice [132]. These data provide some support for the notion that an apoptotic process may be one of the mechanisms of death during disease. However, based upon the scarcity of apoptotic death-related evidence reported from human disease brains, it is clear that other death mechanisms are likely involved [133] (Fig. 1).

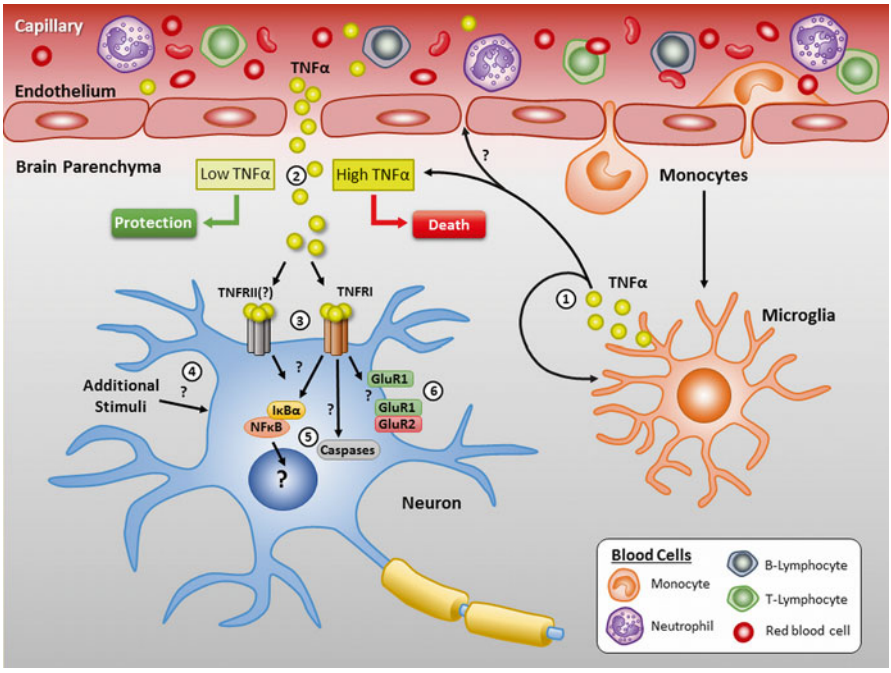

Fig. 1 Hypothesized signaling mechanism of TNF-α in Parkinson's disease. Following cleavage from its precursor form, secreted TNF-α can be released from numerous immune cells in the periphery as well as from microglia as has been reported from Parkinson's disease brains (1). It may act in an autocrine fashion to manipulate microglial phenotype or stimulate neurons where high concentrations of TNF-α are reportedly toxic to dopaminergic neurons, while low concentrations offer protective stimulation via interacting with a trimerized TNFR on the cell surface (2). Although it is not clear whether dopaminergic neurons express the low-affinity receptor, TNFRII, neurons in diseased brains have increased high-affinity receptor, TNFRI, immunoreactivity (3). Perhaps acting alone or with additional stimuli (4), TNF-α stimulation through interaction with one or both receptors may lead to the initiation of particular signaling events that are responsible for DA-neuron death or protection. Based upon signaling mechanisms defined in other cell types, TNF-α stimulation may initiate activation of NFκB-mediated transcriptional changes as well as caspase-mediated death in the dopaminergic neurons (5). In addition, based upon mechanisms defined in non-dopaminergic neurons, it is possible that TNF-α stimulates altered localization and subunit composition of AMPA receptors (GluR1/R2) to contribute to potentiate or attenuate excitotoxic death of the cells (6)

Interleukin 1-Beta (IL-1β)

Interleukin-1β (IL-1β) is one of the 11 interleukin-1 cytokine family members identified to date [134, 135] and shares 26 % sequence homology with its structurally and functionally similar but genetically distinct acidic relative, IL-1α [136–140]. Biologically active, mature form of 17-kDa IL-1β molecule is synthesized via proteolysis of its 31-kDa precursor by caspase-1, an inflammasome-associated cysteine

protease [141]. Both IL-1α and β exert a variety of biological functions through interaction with the cell surface IL-1 receptor (IL-1R), targeting the receptor at a picomolar range [142]. Another IL-1 family protein, IL-1 receptor agonist (IL-1ra), also binds to IL-1R with comparable affinity to that of IL-1α and IL-1β [143]. As the name implies, this endogenous antagonist binds to IL-1R in a competitive manner without eliciting downstream signaling, thus decreasing the activities of IL-1α and β [144–146].

IL-1R belongs to the IL-1R/Toll-like receptor (TLR) superfamily that shares a similar intracellular signaling sequence termed Toll-IL-1 receptor (TIR) domain [147]. There are two types of IL-1R, type 1 (IL-1RI) and type 2 (IL-1RII) receptors, and their functions differ in that the IL-1RI transduces downstream signaling upon binding of IL-1α or β, while IL-1RII sequesters the available ligands, thus reducing IL-1RI activation [148]. Today, nine additional members of IL-1RI-like receptors have been discovered, all of which have a conserved extracellular domain resembling immunoglobulin as well as the TIR domain [147, 149]. In the current signaling pathway model established by numerous in vitro studies in nonneuronal cells, the association of IL-1β to IL-1RI recruits another IL-1RI-like protein designated as the IL-1 receptor accessory protein (IL-1RAcP) [150]. This ligand-initiated dimerization of IL-1 receptor proteins is required for IL-1β signaling via IL-1R [151] and essential for the following interaction of the complex with myeloid differentiation factor 88 (MyD88) [152, 153]. MyD88 serves as an adaptor protein to facilitate the recruitment of first IL-1 receptor-associated kinase 4 (IRAK4) and then IRAK1, after which the latter enzyme becomes phosphorylated by the former and by itself [147, 154, 155]. The phosphorylation of the IRAK1 prompts the kinase to interact with tumor necrosis factor receptor-associated factor 6 (TRAF6), leading to the ubiquitination of TRAF6 that proceeds the activation of transforming growth factor-β-activated protein kinase (TAK1) [156, 157]. This IL-1R pathway diverges to activate at least two distinct sets of transcription factors, NFκB and AP-1/c-Jun, via the activation of the inhibitor of NFκB (IκB) kinase (IKK) and mitogen-activated protein kinase kinases (MKKs), respectively [156, 157].

Since IL-1β was initially identified as a pyrogen in the 1940s, additional biological functions and characteristics were discovered following its purification and cloning as a recombinant protein [158]. IL-1β is produced and secreted primarily by the cells of myeloid origin including macrophages and dendritic cells, and its function as a proinflammatory mediator has been extensively documented in a variety of fields [158]. It is now well acknowledged that IL-1β is one of the major immune response mediators and it is identical to the factors previously referred to as endogenous pyrogen, leukocytic endogenous mediator [159, 160], lymphocyte-activating factor [160–162], hemopoietin-1 [163], mononuclear cell factor [164], proteolysis-inducing factor [165], catabolin [166], and osteoclast-activating factor [167]. Elevated levels of IL-1β are widely reported in the peripheral blood and tissues in inflammatory conditions such as bacterial infections, injury, leukemia, and autoimmune diseases [168, 169].

In the central nervous system, IL-1β is expressed and released by microglia. Moreover, IL-1R localization inferred by radioiodinated IL-1α binding has validated

abundant neuronal receptor expression [170]. As in the periphery, increased expression of this cytokine is associated with central inflammatory conditions including Parkinson's disease [171–173], Alzheimer's disease [174], stroke/ischemia [175–180], traumatic brain and spinal cord injury [181–183], excitotoxicity [184, 185], and HIV-1 infection [186]. Therefore, IL-1β is thought to be one of the major cytokines utilized by activated microglia to mediate inflammatory responses [169].

IL-1β in Parkinson's Disease

In the mid-1990s, the involvement of IL-1β in Parkinson's disease was investigated in a few correlative studies with human patients. A pioneering study by Mogi et al. [171] quantified for the first time the levels of IL-1β and other cytokines in the brain tissues of Parkinson's disease patients using sandwich enzyme-linked immunosorbent assay (ELISA). They reported that the level of the cytokine was significantly increased by 3.5-fold in the striatum, but not in the cerebral cortex, of the Parkinson's disease patients when compared to their age- and gender-matched control brains [171]. IL-1β concentrations were also measured in the cerebrospinal fluid in subsequent studies by the same group and others. Blum-Degen and colleagues compared levels of IL-1β to additional cytokines, IL-2 and IL-6, in the cerebrospinal fluid versus plasma of 22 Parkinson's disease patients, 12 controls and 11 sporadic Alzheimer's disease patients to find no differences in IL-2 but elevated IL-6 and IL-1β in the cerebrospinal fluid but not plasma of both Parkinson's and Alzheimer's disease patients [172]. This observation was partly supported by another study in which IL-1β was measured in the ventricular cerebrospinal fluid of Parkinson's disease patients [173]. Results from this study showed a significant increase in IL-1β levels in patients diagnosed with juvenile Parkinsonism (age 39–55 years; mean age 48 years). However, the small increase in IL-1β levels observed in "classical" Parkinson's disease patient population (age 54–77 years; mean age 68 years) did not reach a statistically significant value [173]. These reported elevations in extracellular cytokine correlate well with increased glial IL-1β immunoreactivity reported in Parkinson's disease substantia nigra [187].

IL-1β Polymorphisms in Parkinson's Disease

Studying polymorphisms of the IL-1β gene (*IL-1B*) has provided some additional support for a role of IL-1β in the pathogenesis of Parkinson's disease. Single-nucleotide polymorphisms within the promoter region of *IL-1B* at position −511 have been repeatedly reported in population analyses, suggesting its association with increased risk [188–192] or earlier age at onset [193, 194] of Parkinson's disease. However, the results from these studies are inconsistent and even conflicting, perhaps due to multiple variables such as ethnicity, age groups, and genders of

the subjects. Supporting this notion, a meta-analysis of eleven published studies during 2000–2008 from 2,803 PD patients and 2,593 controls showed no significant association of *IL-1B* polymorphism at −511 with the risk of Parkinson's disease [195]. Additional extensive population studies are warranted in order to evaluate the involvement of IL-1β at the genomic level.

IL-1β in Animal Models of Parkinson's Disease and Neurodegeneration

In accordance with the observations in human patients, upregulation of IL-1-β has also been reported in the brains of animal models of Parkinson's disease. Daily intraperitoneal injections of MPTP for 13 days resulted in over a 20-fold increase in IL-1β levels in the striatum of MPTP-treated mice compared to control animals [196]. Similarly, maximum of a 16-fold increase was detected 30 days after a single intrastriatal injection of 6-OHDA [197].

A significant literature derived from the rodent studies suggests that this increase in IL-1β is actually neuroprotective. For example, the increase in IL-1β in Parkinson's disease brain might be a compensatory mechanism activated by the nigral cell death whereby the cytokine functions in combination with TNF-α to elicit neurotrophic effects via stimulation of astrocyte proliferation [171, 198]. Moreover, findings from an animal model study demonstrate that pretreatment with 20 ng IL-1β via stereotaxic infusion prevented dopaminergic cell bodies within the substantia nigra from degenerating after the subsequent 6-OHDA challenge [199]. A few in vitro studies reported similar neuroprotective actions of IL-1β, demonstrating that pretreatment with IL-1β increases the neural growth and survival of rat superior cervical ganglia explants [200], and IL-1β stimulation of a human microglial cell line culture results in production and secretion of nerve growth factor (NGF) synergistically with TNF-α [201].

On the contrary, several lines of evidence suggest neurotoxic actions of IL-1β in various animal models. Koprich et al. [202] reported that increasing the level of IL-1β (approximately twofold, to 13.5 pg/mg substantia nigra protein) by preadministration of a nontoxic dose of LPS in the substantia nigra rendered the dopaminergic neurons more susceptible to a subsequent 6-OHDA treatment, indicating a role of IL-1β opposite from the neuroprotective function proposed earlier by Saura et al. [199]. In the MPTP animal model, inhibiting the synthesis of biologically active, mature IL-1β with a dominant negative form of caspase-1 abolished MPTP-mediated dopaminergic loss [203]. Furthermore, continuous adenovirus-driven IL-1β expression alone resulted in progressive dopaminergic neuron death accompanied by T cell infiltration into the substantia nigra and Parkinson's disease-like motor impairments in the rat [47, 204]. Conversely, pretreatment with recombinant IL-1ra was found to be neuroprotective, corroborating the involvement of IL-1β activity in neurodegeneration. Inhibition of LPS-induced IL-1β activity by administration of IL-1ra prior to a 6-OHDA challenge protected the dopaminergic cells [202].

Similar results with IL-1ra have also been documented with hippocampal neurons with ischemia [205] and excitotoxic insults [206], suggesting that counteracting IL-1β activity with IL-1ra is beneficial in minimizing neurodegeneration.

Interestingly, a review by Allen et al. [207] has pointed out that the IL-1β-mediated neuronal damages detected in these animal models might be a combined outcome occurring with administration of an inflammatory stimulus (e.g., 6-OHDA, MPTP, LPS). Such observations argue against the idea that IL-1β alone is sufficient for dopaminergic cell death in Parkinson's disease, but rather multiple factors are involved in the pathogenesis of the disease [208]. In this view, IL-1β significantly exacerbates neuronal damage when the nervous system has been previously exposed to or "primed" by inflammatory stimuli such as infections, neurotoxins, and trauma [209–211], or by "sterile inflammation" instigated by secretion of endogenous molecules due to aging or cell death [211]. Inversely, repeated or prolonged inflammation from previous infections or injury may contribute to the "priming" of microglia, therefore triggering the disease with aging and/or additional exposures to pathogens and toxins.

Evidence of Particular IL-1β Signaling in Parkinson's Disease

In spite of the elevations in brain IL-1β protein levels and increased glial immunoreactivity reported from Parkinson's disease brains and its mouse models, it is not clear what particular signaling response the cytokine may be eliciting on neurons and/or glia at this point. Indeed, reports of evidence for IL-1R expression are also lacking in the striatum and substantia nigra. As already mentioned, the potentially dual neuroprotective and neurotoxic actions of IL-1β further increase the complexity of its potential effects in Parkinson's disease. Based upon the lack of particular signaling dissection of the IL-1R in dopaminergic neurons, it is even difficult to hypothesize how the cytokine might be stimulating the cells. Nevertheless, as stated in the TNF-α discussion, some evidence of possible IL-1R-mediated signaling in neurons during disease may be suggested by data from Hunot and colleagues who found significantly increased nuclear NFκB immunoreactivity in neuromelanin-containing neurons of Parkinson's disease brains compared to controls [131] (Fig. 2).

Clearly, the mechanistic implications of any particular cytokine contributing to dopaminergic cell death or loss during Parkinson's disease remain unclear. We have attempted to review the literature related to at least two of the cytokines reported to be elevated with disease. It is likely that neither TNF-α nor IL-1β would be acting alone during the prolonged changes occurring in a diseased brain. Moreover, there are many remaining cytokines/chemokines that could change or have already been reported to change during disease that undoubtedly also affect dopaminergic neurons. Perhaps future efforts quantifying the efficacy of receptor-selective antagonists for each cytokine of interest that is tested in the most disease-relevant animal model will offer insight into whether or not manipulation of cytokine biology offers a valuable therapeutic strategy for Parkinson's disease.

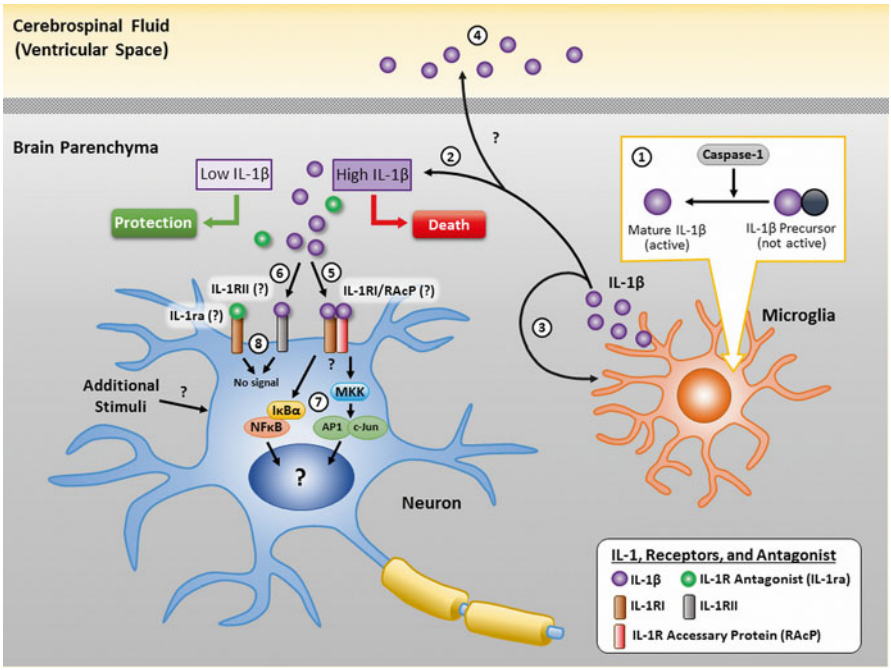

Fig. 2 Hypothesized signaling mechanism of IL-1β in Parkinson's disease. Upon inflammatory stimulation, microglia produce mature, biologically active IL-1β from a larger precursor molecule via activation of caspase-1 (1). Increased glial immunoreactivity in Parkinson's disease brains suggests that microglia may release IL-1β into the extracellular space where it could act as a paracrine (2) or autocrine (3) stimuli. IL-1β could also be released into the cerebrospinal fluid (4), where increased levels may be detected in Parkinson's disease patients. High levels of IL-1β are reportedly toxic to dopaminergic neurons, while low levels are toxic. Paracrine IL-1β may interact with IL-1RI (5) or IL-1RII (6), although only limited evidence exists for the presence of cell surface IL-1 receptors on dopaminergic neurons. Based upon mechanisms defined in non-dopaminergic cells, it is postulated that the association of IL-1β with IL-1RI prompts dimerization of the receptor-ligand complex with IL-1RAcP to initiate downstream intracellular signaling, ultimately leading to the activation NFκB and AP-1/c-Jun transcription factors via the IκB and MKK pathways, respectively (7). In contrast, the binding of IL-1β to IL-1RII or IL-1ra to IL1-RI does not lead to signal transduction (8)

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Cell Culture Models of Inflammation in Parkinson's Disease

Patrick Flood

Introduction

Parkinson's disease (PD) is a progressive degenerative disorder of the central nervous system (CNS) that leads to impairment of motor skills and speech, as well as other functions. The etiology of PD is primarily characterized by the chronic loss of dopamine production by a subset of neurons (DA neurons) within the substantia nigra (SN) and striatum. While it is postulated that a multitude of different factors, including genetic, environmental, and intrinsic mechanisms, can ultimately cause or contribute to dopaminergic neurodegeneration in PD, it is now clear that the progressive nature of PD is characterized or accompanied by chronic inflammation-induced neurodegeneration of dopamine-producing neurons [1–4]. The levels of a number of proinflammatory mediators, including TNF-α, IL-1β, IL-6, eicosanoids, as well as reactive oxygen species (ROS), are elevated in the brains and peripheral blood mononuclear cells (PBMCs) of patients with PD [5, 6]. Nitrite in the cerebrospinal fluid and increased expression of inducible nitric oxide synthase (iNOS) within the SN have been found in PD patients [7]. All of these findings lend strong support to the association of inflammation and PD. Many of these inflammatory mediators have been demonstrated to have strong neurotoxic effects on DA neurons [2, 8–10]. This evidence, and those detailed in other chapters within this book, all point to the microglial cell as central to the initiation, execution, and regulation of the inflammatory responses within the CNS leading to dopaminergic degeneration [2, 11, 12].

In order to study the role of inflammation, particularly microglial-mediated inflammation, on the production of dopamine by DA neurons, investigators have developed a number of in vitro cell culture models that measure the effects of inflammatory cells and mediators on the viability and functional dopamine production by

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M. Thomas (ed.), *Inflammation in Parkinson's Disease: Scientific and Clinical Aspects*,
DOI 10.1007/978-3-319-08046-8_7,

DA neurons in vitro. These models include the use of DA-neuronal cell lines, primary DA-producing cells, as well as the generation of primary cultures of mesencephalic neuron-glial cultures, to measure the effects of inflammatory cells and mediators on the viability, function, and dopamine production of DA neurons. Each culture system has its own unique advantages in studying the molecular and cellular mechanisms associated with DA neurodegeneration, and in measuring the effects of different inflammatory triggers, anti-inflammatory cytokines, cells, and therapeutics, as well as the role of genetic, molecular, and cellular abnormalities in DA neurons that initiate or contribute to the chronic inflammatory-mediated degeneration as seen in PD. In this chapter, we will detail several different in vitro cell culture models that are currently being used to measure the inflammatory-based etiology of PD.

Use of Cell Lines in Inflammatory Cell Culture Systems

Dopamine-Producing Cells Lines

One of the major in vitro approaches used by investigators seeking to investigate the mechanisms of dopamine loss during progressive is the use of dopamine-producing cell lines. These cell lines are either continuously growing cell lines developed from in vitro cultures or immortalized by cellular fusion or isolated from patients or animals with neuroblastomas that produce dopamine and express markers indicative of dopaminergic neurons. The most commonly used cell lines currently being studied include the human cell line BG01V2 [13], the murine cell line MN9D [14], or the rat cell lines PC-12 [15] and 1RB3AN27 [16]. A number of neuroblastomas cell lines have been isolated that express markers of DA neurons, as well as a high production of dopamine, which include the human cell lines SK-N-SH, SK-N-MC, and SK-N-BE [17], a subclone of the SK-N-SH cell line SH-SY5Y [18], and the murine cell line CATH.a [19]. A list of these cell lines, along with their derivation date and number of references utilizing these cells since 2005 (along with the number of times used for in vitro assays as a model for the study of PD), are shown in Table 1.

The BG01V2 human dopamine-producing cell line was derived from the embryonic stem cell line BG01 by co-culturing these hESC with the PA6 mouse stromal cell line as previously described [13]. These cells express Pax5, Otx2, and Msx1, as well as tyrosine hydroxylase (TH) and the dopamine transporter (DAT), strongly suggesting they arise from dopaminergic neurons. Although it appears this cell line has significant potential for investigating the function of DA neurons, it appears that neuroblastoma cell lines are more commonly used to study the biology and sensitivity of DA neurons to inflammatory signals. These neuroblastoma cell lines include the most commonly studied SK-N-SH, SK-N-MC, and SK-N-BE (1) and (2) neuroblastoma lines [17]. The SK-N-SH, MC, and BE neuroblastoma cell lines are pheochromocytoma cell lines that were isolated from distinct neuroblastoma patients and have been extensively used to study neuronal biology both for their

Table 1 Cell lines regularly used in in vitro models of Parkinson's disease

Dopamine-producing cell lines	Species	Reference	Year of origin	References since 2000
BG01V2	Human	[13]	2008	1 (1)
MN9D	Murine	[14]	1991	120 (80)
PC-12	Rat	[15]	1976	8,645 (39)
1RB3AN27	Rat	[16]	1996	7 (6)
DA-neuroblastoma				
SH-SY5Y	Human	[18]	1978	13,301 (686)
SK-N-MC	Human	[17]	1973	390 (32)
SK-N-SH	Human	[17]	1973	751 (54)
SK-N-BE 1/2	Human	[17]	1973	200 (11)
CATH.a	Murine	[19]	1993	105 (20)
Neuro2A (N2A)	Murine	[20]	1970	468 (15)
Microglial cell lines				
CHME-5	Human	[21]	1995	16 (1)
N9	Human	[22]	1989	36 (13)
HAPI	Rat	[23]	2001	103 (4)
BV-2	Murine	[24]	1990	454 (29)
Macrophage/monocyte cell lines				
THP-1	Human	[25]	1980	4,704 (17)
RAW 264.7	Murine	[26]	1977	3,763 (4)

high production of dopamine and also for their distinct genetic makeup, biological properties, and marker expression. A subclone of the SK-N-SH cell line, SH-SY5Y [18], has been extensively used to study neuronal differentiation, including ability to differentiate into functionally distinct cells when stimulated with a number of different stimuli, including retinoic acid, TPA, cAMP, and neurological growth factors NGF and BDNF [27]. The SH-SY5Y cultures include both adherent and cells that grow in suspension, leading to potentially different results depending on the state of the cells being investigated. In addition, even closed populations of SH-SY5Y cells may contain two distinct phenotypes: neuroblast-like cells and epithelial-like cells [28]. These two phenotypes may best be reflected in the "N" and "S" types of SH-SY5Y by [29]. This may also lead to a dichotomy of results, as it has been reported that the "N" cells, which exhibit neuroblast-like morphology, produce dopamine and express TH, while the "S" cells exhibit epithelial-like counterpart cells that lacked these enzymatic activities [28]. Despite these characteristics, these cell lines have been extensively studied for the biological characteristics of DA neurons and can easily be used in cell cultures to measure the effects of inflammatory mediators and cells.

Rodent in vitro models of PD are also used extensively to study the role of inflammation in dopaminergic neurotoxicity. Like the human in vitro models, a number of rodent cell lines have been developed that allow investigators to measure the effects of inflammatory mediators and cells on the biology of dopamine-producing neurons. A cell line that has been extensively studied as a target for inflammatory mediators is the rat pheochromocytoma PC12 cell line [15]. This line was derived from a

transplantable rat adrenal pheochromocytoma, and it differentiates into a neuronal phenotype upon exposure to NGF when plated on collagen IV-coated culture flasks. These cells produce large amounts of dopamine and DAT upon differentiation and are sensitive to a number of DA-specific neurotoxins that have been implicated in the pathogenesis of PD, including MPP^+ and 6-OHDA. Another rat cell line that serves as an in vitro model for DA neurons is 1RB3AN27 [16], an SV-40 immortalized cell line derived from fetal rat mesencephalic cells that produces a high level of dopamine in culture. Treatment of 1RB3AN27 with a cAMP-stimulating agent increases levels of TH and DAT, these cells express the neuron marker nestin but not GFAP, and these cells are likewise sensitive to 6-OHDA [30]. In contrast, the most popular murine DA-producing cell lines used in in vitro cultures are the CATH.a (Central Adrenergic TH-expressing) and N2A (Neuro2A) cell lines. The CATH.a was derived from a brain cell culture of TH-positive tumors in transgenic mice carrying the SV40 T antigen oncogene under the transcriptional control of the rat TH gene [19]. The CATH.a line expresses the SV-40 T antigen, as well as displays neuronal properties such as neurofilaments and synaptophysin. CATH.a cells are sensitive to the neurotoxic effects of MPTP and 6-OHDA, as well as to ROS-induced apoptosis. The N2A cell line is a mouse neural crest-derived cell line [20] that can be induced to produce high levels of TH and dopamine upon stimulation with dibutyryl cAMP [31]. N2A cells have been extensively used because they are both sensitive to apoptosis in the presence of inflammatory mediators TNFα and ROS, and they themselves express mRNA for the inflammatory mediators IL-1β, IL-6, TNF-α, NF-κB, and iNOS when stimulated with LPS [32].

Microglial Cell Lines

The first postmortem report of high levels of activated inflammatory cells within the SN of PD patients came over two decades ago [33], and these cells were identified as activated microglial cells. Since that time, the microglial cells have been the major focus of study as the source of inflammatory damage that leads to chronic DA neurodegeneration. Products of microglial cells, including TNF-α, IL-1β, and IL-6, have been found in high concentrations within the SN of PD patients [34, 35] as well as inflammatory mediators such as PGE2, NO, and ROS [36–40]. This indicates that the cell that is predominantly implicated in the pathology of PD is the microglial cell, and most of the in vitro cultures used as models to study inflammation in PD utilize the microglial cell. However, unlike the previously described DA-neuronal cell lines used in vitro, a microglial cell line must be able to be activated by inflammogens in order to be useful in studying the pathogenesis of inflammation on dopaminergic neurons. In addition, because of the similarities in phenotype and function between microglial cells and systemic macrophages, many in vitro cultures utilized to study inflammation and PD utilize macrophage/monocyte cell lines rather than microglial cell lines to generate inflammatory mediators and measure the effects of these mediators and/or cells on dopamine production, apoptosis, and phenotype on dopamine-producing cells.

Table 1 also includes a list of the most common microglia and macrophage cell lines used in in vitro cultures to measure the effects of inflammation on DA-neuronal activity. In vitro cell cultures investigating the role of innate immunity in PD have utilized both microglial cell lines and macrophage/monocyte cell lines to study the effects of inflammatory activation on the production of dopamine, the viability of DA neurons, and the function of these neurons following exposure to various inflammatory mediators. The most commonly used microglial cell lines for the study of human inflammation-induced DA neurodegeneration is the CHME-5 and the N9 microglial cell lines. The CHME-5 line was generated from human fetal microglia by transfection with the large T antigen of simian virus 40 [21]. This line expresses TLR2, TLR4, and TLR9 and is responsive to activation signals by producing ROS, as well as other inflammatory mediators such as TNF-α, IL-6, and MCP-1 in response to activation [41]. N9 is a human microglial cell line derived from primary mouse embryo brain cells using new recombinants (termed 3RV) carrying the v-myc or v-mil oncogenes of the avian retrovirus MH2 [22, 42]. It is capable of producing a proinflammatory response upon activation through the production of TNF-α, IL-1β, IL-6, NO, and ROS [22, 43]. Both of these cell lines have been used to study the role of microglia in DA-neuronal cell death in in vitro models of PD. A more commonly used human cell line to study DA neurodegeneration is THP-1. THP-1 is a human leukemic cell line (THP-1) cultured from the blood of a boy with acute monocytic leukemia [25]. While it is considered a monocyte line rather than a fully differentiated macrophage, this cell line exhibits properties similar to the human monocyte-derived macrophages in that activation of THP-1 with LPS or TNF-α leads to a rapid response and production of proinflammatory mediators IL-1β, TNF-α, and IL-6, as well as the production of NO and ROS species [25].

A number of rodent microglial or macrophage cell lines have been developed and utilized to measure the effect of innate immune cells on DA neurotoxicity. HAPI (highly aggressively proliferating immortalized) were isolated from a population enriched for microglial cells, and stain for the microglial markers B4 and OX-42, but do not express A2B5 or GFAP. HAPI cells are capable of phagocytosis, and when stimulated with the inflammogen, LPS produces measureable amount of the proinflammatory cytokines IL-1β, IL-6, and TNF-α, as well as the mounting of an oxidative stress response which produces NO and ROS. HAPI cells have been used to study inflammatory mediator production, cell signaling mechanisms, and sensitivity to therapeutic intervention using in vitro models of neurodegeneration. The most widely used microglial cell line of murine origin is the BV-2 cell line. The immortalized mouse microglial cell line BV-2 was developed in the laboratory of Dr. Blasi at the University of Perugia, Italy [24]. BV-2 was generated by infecting primary microglial cell cultures with a v-raf/v-myc oncogene carrying retrovirus (J2) [24]. BV-2 cells express phagocytic ability and secrete proinflammatory cytokines TNF-α, IL-1β, and IL-6, as well as NO and ROS, upon stimulation with LPS. Phenotypically BV-2 expresses the MAC1 and MAC2 antigens, but not GFAP or galactocerebroside (GC). The BV-2 cell line is the most widely studied cell line of confirmed microglial origin and has been used to study the role of microglia in a number of different chronic neurodegenerative conditions, including PD. In addition to microglial cell

lines, PD investigators have also used murine macrophage cell lines to determine the response of innate immune cells in PD. The most commonly used macrophage cell line is RAW264.7. The murine cell line RAW264.7 was established from an ascites of a tumor induced in a male mouse by intraperitoneal injection of Abelson Leukaemia Virus (A-MuLV) [26]. It is a well-established model for macrophage activation and is likely the most well-studied macrophage line currently being used to study the role of innate immunity in inflammation.

Use of Primary Mesencephalic Cultures in Inflammatory Cell Culture Systems

While the existence of pure cell lines of DA neurons and microglia/macrophages can facilitate the study of inflammation in PD, it is important to determine the biological functions of primary cells to validate and extend the findings into a system that more resembles the cellular makeup of the SN and striatum. Mesencephalic-glia cultures provide an excellent model of the cellular and architectural makeup of the midbrain, both in the distribution of the cell types expressed within the SN and also in the fact that these cells represent primary cell types whose responses to inflammatory signals or mediators are most likely to reflect those of resident cells of the brain. Mesencephalic cultures are composed primarily of four major cell types: astrocytes, neurons, microglia, and DA neurons. These cultures not only provide a relatively accurate ratio of cells to those found in the intact midbrain but also allow investigators to individually analyze the role of each major cell type in the inflammatory response by selectively depleting an individual cell type based on their unique phenotypic markers or by enriching for certain cell types by differential processing and cellular growth characteristics. These cultures include neuron-glial cultures, mixed-glial cultures, enriched microglia cultures, and reconstituted neuron-microglial cultures. Each system can serve to answer specific questions regarding the role or contribution of each cell type, the molecular mechanisms of cellular responses, and the interactions between microglia-neurons, microglia-astrocytes, and microglia themselves or the interaction between all cell types in the response of DA neurons to neurotoxins, inflammogens, or inflammatory mediators. A brief description of each is given below.

Primary Mesencephalic Neuron-Glial Cultures

Primary mesencephalic neuron-glial cultures are composed of all cells normally found within the SN. The distribution of cells is approximately 50 % $GFAP^+$ cells (astrocytes), 40 % $NeuN^+$ cells (neurons), and 10 % OX-42/MAC-1^+ cells (microglia). Within the 40 % $NeuN^+$ cells population, about 1–2 % are positive for tyrosine hydroxylase (TH), a unique marker for DA-producing neurons within the SN. This model is created by taking rat dams at gestational day 13–15, or mouse dams

at gestational day 13–14, removing the brain area, and isolating the mesencephalic region from the rest of the brain [44]. The timing of embryo harvest, as well as the delicacy of the surgery, is critical to success, so this is a procedure that may take a great deal of trial and error before achieving the desired number and proper ratio of cells within your cultures. A highly detailed procedure for proper preparation of these cultures can be found in Chapter 21 of *Microglia, Methods and Protocols* [44]. These cells are then seeded in flasks or tissue culture plates and can be utilized within 7–10 days of seeding for analysis. Once prepared, it is important to verify by immunohistochemistry the cellular composition of your culture using antibodies to GFAP, NeuN, and OX-42/MAC-1. The critical cell for measuring inflammatory responses in these cultures is the microglia, so cultures with more than 10 % OX-42/MAC-1$^+$ cells may produce highly variable results. While cumbersome, this model most closely resembles the in vivo situation within the SN, and the major advantage of using this type of culture model is for evaluating the effects of neurotoxins, inflammogens, or immunotherapeutics in DA-neuron viability and function and to measure the effects of blocking antibodies, gene silencers, or additional protein molecules on DA-neuronal activity.

Mixed-Glial Cultures

These cultures are prepared in similar to primary mesencephalic neuron-glial cultures except that they are plated with a different medium of growth, require exchange rather than supplementation of the medium, and are maintained in culture under different conditions which favors the outgrowth of astrocytes and microglia but not neurons. The final concentration of mixed-glial cultures should be 80 % GFAP$^+$ cells and 20 %OX-42/MAC-1$^+$ cells.

Enriched Microglial Cultures

Enriched microglial cultures are obtained from the mixed-glial cultures by gently shaking the culture flasks containing 14-day-old mixed-glial cultures and collecting the cellular suspension [44]. This technique relies on the fact that only microglial cells will be released from attachment under these conditions. Suspension cells can then be collected and replated and routinely result in >95 % OX-42/MAC-1$^+$ cells.

Reconstituted Neuron-Microglial Co-cultures

Neuron-glial cultures can be rendered devoid of glial cells by treatment with ara-C (arabinofuranosyl cytidine) and LME (leucine methyl ester) at a final concentration of 5–15 μM [44]. Ara-C and LME are routinely used to remove astrocytes and

microglia, respectively, from culture systems. After treatment, cells are extensively washed and then reconstituted with enriched microglial cells to determine the role or contribution of astrocytes to the in vitro inflammatory response.

Another general method for generating in vitro cultures as a model for PD has also been described [45]. This technique involves culturing postnatal SN cells and allows for the generation of mixed and chimeric neuron/glial cultures of postnatal SN cells, independent of other monoaminergic nuclei in the ventral midbrain. This approach can be used for the construction of total SNpc cultures, astrocyte cultures, or hybrid neuron-glial cultures. The advantage of this technique is that it can be performed on adult mice, thereby reducing the number of brains needed for dissection, allowing phenotypic analysis on genetically altered mice prior to harvesting, as well as the tissue(s) used in this procedure include only the specific cells lost in PD rather than a large number of unaffected cells. In addition, these cultures can be used to measure the effects of inflammogens, neurotoxins, and other potential initiators of inflammation leading to DA neurotoxicity.

Measurement of DA Neurotoxicity

Cell cultures that use DA-producing cell lines or transformed neuroblastoma cells as a model for PD can be an effective way of measuring the effects of inflammatory cells or mediators on the viability and functionality of DA neurons in vitro. This approach has been used extensively to measure the effects of inflammogens such as LPS and neurotoxins such as MPP^+ or to measure the direct effects of inflammatory mediators such as TNF-α [46, 47], IL-1β [48, 49], IL-6 [50, 51], NO [52, 53], ROS [54, 55], IFNγ [56, 57], and PGE2 [58, 59] or to measure the effect of activated microglial cells lines such as BV-2 [60, 61] or N9 [62] on DA-producing neuronal cell lines. Certainly one of the biggest advantages to using DA-producing neuronal cell lines or neuroblastoma cells, and conversely using microglial or macrophage cell lines to mimic inflammation, is the ability to molecularly manipulate these lines to alter the production or response of these lines using genetic constructs and cell signaling inhibitors.

Mesencephalic cultures have also been used extensively to study the effects of inflammogens or neurotoxins on primary DA-producing neurons. These studies show that while most inflammogens have little to no direct effect on DA-producing neurons in the absence of microglial cells [12], a number of inflammatory mediators, such as TNF-α, NO, and ROS, have strong effects on both the viability and functionality of DA neurons. The most common inflammogen used in these cultures to activate microglia is LPS, but it has been used also for measuring the inflammatory potential of alpha-synuclein, MPP^+, or other antigens proposed to be involved in PD activation. More importantly, these cultures are often generated from rodents which have been genetically manipulated to overexpress a certain protein, to express mutants of these proteins, or from animals that have had a genetic knockout of a gene of interest in the inflammatory, neuron, or astrocyte lineage. These approaches

have been used to study the potential role of alpha-synuclein, LRRK2, DJ-1, Parkin, and PINK1 in DA neurodegeneration [63].

The in vitro cultures used to measure inflammatory effects on DA neurons take several experimental designs. The most common is simply adding the agent of investigation directly into cultures and measuring the effects of this agent on DA viability and/or dopamine production. In neuronal cell cultures, cellular viability can be measured using vital stains, an MTT or LDH assay [64], or by measuring apoptosis using a TUNEL (TdT-mediated dUTP nick-end labeling) assay, a Comet Assay [65], and/or by measurement of cleaved caspase-3 by Western blot [66]. This is much more difficult in mesencephalic cultures; therefore, viability of DA neurons is determined by measuring the number of cells that stain positive in immunohistochemistry for tyrosine hydroxylase (TH^+), a unique marker for DA neurons. This assay simply compares the number of TH^+ neurons in control to the experimental group and is usually accompanied by an assay to measure dopamine production.

In vitro assays to measure dopamine production are generally the most definitive tests that identify alterations in DA-neuronal biology that most closely resemble PD. These tests usually involve the differential uptake of tritriated (3H)-dopamine by DA neurons [67]. This assay relies on the observation that DA neurons will uptake radioactive dopamine, while other cells without the DAT protein will not uptake this dopamine, and allows for investigators to measure the functionality of DA neurons by the amount they take up compared to other cells within the culture system. This is especially useful for the mesencephalic cultures, as the number of TH^+ neurons is often too low to accurately count, and measurement of DA uptake provides a good quantitative measure of cell functionality. Other methods are also available to measure dopamine production in vitro, including electroanalytical methods [68], plasmon resonance biosensors [69], nanofibers [70], chromatography, and various flow injection systems [71]. However, these other methods require significant time, volume, and equipment commitment for accurate measurement and do not lend themselves well to multiple sample analysis.

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Clinical Aspects of Inflammation in Parkinson's Disease

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There is consensus that inflammation is present in Parkinson's disease (PD). However, there is debate on whether the increase in inflammatory cells and the relevant markers of inflammation are a cause or consequence of PD pathogenesis. Further, there is debate regarding relative importance of inflammation as opposed to other possible mechanisms, such as mitochondrial dysfunction, oxidative stress, autophagy, and mitophagy. It is unclear whether the increased inflammation seen in PD is protective or contributory to cell death. Much of the preclinical evidence has been presented in earlier chapters. In this chapter we present translational and clinical evidence looking at the relationship between inflammation and PD.

Biological Markers

The World Health Organization has defined biomarker as "any substance, structure or process that can be measured in the body or its products and influence or predict the incidence or outcome of the disease." There are different classes of biomarkers, some are predictive and some are surrogate. Surrogate biomarkers must show consistency, accuracy and these are best used for measurement of changes in a biologic system. Clinical endpoints show changes in morbidity, mortality, and are more relevant to clinical research. Validation of these markers is an important step in clinical research. While we present evidence of various studies, none of these are widely accepted to be validated inflammatory biomarkers in current clinical practice in PD [1].

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M. Thomas (ed.), *Inflammation in Parkinson's Disease: Scientific and Clinical Aspects*,
DOI 10.1007/978-3-319-08046-8_8,

In the central nervous system (CNS) particularly in immune-mediated neurodegeneration, three mechanisms need to be reviewed: (1) Systemic response to neurodegeneration, which includes activation and transfer of immune molecules from the periphery to CNS, via the blood–brain barrier, propagation of secondary factors, and diffusion via circumventricular organs. (2) CNS autonomous mechanisms intrinsic to the neurons are immune responses within the extra-neuronal brain tissue and systemic responses to neurodegeneration. Within the neurons, cellular responses include important pathways that activate caspases. (3) Outside the neurons, there are responses initiated by glial cells [2].

T-Cell Markers

Many investigators looked specifically at peripheral T-cell-mediated response in patients with PD. In a cardiovascular health cohort, specific biomarkers including albumin, WBC count, fibrinogen, CRP (C-reactive protein), and IL-6 were measured in those who had PD. In women higher levels of IL-6 seem to correlate with higher risk of prevalent PD, while in men elevated total WBC count seemed to indicate higher risk of prevalent PD, while no biomarker could be identified for incident PD [3]. A more specific approach looking at chronic induction of T-cell stimulation as a peripheral response was taken by Saunders et al. [4]. They studied changes in phenotype of CD4 T cells and correlated with UPDRS to identify those markers that are associated with increased severity of disease. In $CD4^+$ T cells, specific patterns were noted in T Reg (regulatory T cells) and Tem (memory T cells). Higher UPDRS scores were associated with increased $CD45R0^+$ and Fas^+ CD4 T cells and decreased $\alpha4\beta7^+$ and $CD31^+$ $CD4^+$ T cells. This demonstration of predominant expression of memory T cells in association with higher UPDRS scores suggests a chronic inflammatory state [4]. When compared to patients with other neurological diseases, there is a higher expression of HLA-DR in CSF monocytes. There is increase in memory T-cell subset and decrease in naïve T cells in the peripheral blood. There is no change in suppressor T cells ($CD8^+$ and $CD11b^+$), supporting once again the theory of chronic immune state in PD [5]. There are also changes in hematopoietic stem cells in PD patients. Interactions between CCR2 and other interleukins can lead to further clues as to the exact mechanisms or pathways of peripheral response. In a colony-forming cell assay study from blood samples, upregulation of monocyte precursors CFU-GM and CFU-M has been shown to occur. Also, there is increase in CCR2 (chemokine receptor 2) expression but decrease in percentage of $CCR2^+$ cells overall. CCR2 and fractalkine receptor (CX3CR1) are important in the recruitment of peripheral monocytes in response to inflammation [6]. Expression of TNF-α from peripheral T cells was studied in patients with PD versus healthy controls, and patients with PD had a higher number of T-cell TNF-α receptors, and this is due to peripheral response to chronic inflammation [7]. Elevated $OKT\ 10^+$ cells and proportions of $IL2\ receptor^+$ and $HLA\ DR^+$ cells have been shown in PD, suggesting activation of peripheral T lymphocytes and increased adenosine deaminase activity [8].

In PD $\gamma\delta^+$ T cells are elevated in CSF and peripheral system of patients with PD compared to other neurologic diseases. While this finding is relevant, the key role of these activated T cells in PD is not established [9]. Circulating $CD4^+$ or $CD8^+$ when they are concomitantly expressed in the same cell represents immaturity. Activation of these cells as measured by CD4 bright$^+$ CD8 dull$^+$ lymphocytes occurs in PD [10]. Similar findings have been reported in other immunological disorders of the nervous system [11, 12].

Many changes occur in T-cell subtypes in PD. In a study with 88 patients, looking at peripheral T-cell subtypes, there were no changes in cell counts of sampled cells labeled for $CD14^+$ (monocytes), $CD8^+$ (cytotoxic T cells), $CD56^+$ (natural killer cells), TCR$\gamma\delta^+$, or $CD4^+$ $CD8^+$ cells in patients with PD. A decrease in TCR$\alpha\beta^+$, $CD4^+$ (T helpers), and $CD19^+$ (B) cells was noted in PD compared to controls. A reduction in $CD4^+$ T-helper and $CD19^+$ B cells in PD patients was noted, and there was variability with the stage of disease [13]. Specific subtyping of B-cell activity using microarray analysis is interesting, but the evidence is not conclusive [14].

Peripheral blood mononuclear cells (PBMC) are easy to study due to the fact that they are an integral part of the cytokine network in vivo. Several discrete cytokines are secreted by them when activated using mitogens in a cell culture system. Production of IL-2 and the mitogen response were significantly lower in PD patients, whereas the secretion of IL-1β, IL-6, and TNF-α was significantly enhanced. Secretion of IL-1β and IL-2 was not affected by levodopa, demonstrating a pure effect on the cytokines due to the disease pathology [15]. TNF-a, IL-1a, IL-1b, and IL-6 production by unstimulated PBMC and PBMC stimulated with mitogens LPS or Con A was significantly lower in PD than those in normal controls. IFN-c production by LPS-stimulated PBMC in PD was significantly lower than that in normal controls [16].

Immune responsiveness of PBMC in a whole-blood assay by quantification of IL-2, soluble IL-2 receptor (sIL-2R), IFN-γ, and IL-6 in T-lymphocyte subsets of patients with idiopathic PD showed selective IL-2 deficiency [17]. TNF-α, IL-1a, IL-1b, and IL-6 production by unstimulated PBMC and PBMC stimulated with mitogens was lower in PD than in normal controls [18]. In a separate study, the cytokine-producing capacity of whole-blood cultures was determined in de novo patients with idiopathic PD before and after treatment with amantadine [19].

Cytokine Markers

Multiple studies have found that CSF cytokines are increased in PD patients as compared to controls. TNF-α is a potent cytokine produced primarily by activated macrophages and elicits various biological and cytotoxic effects, including hemorrhagic necrosis, endotoxic shock, and inflammatory, proliferative, and antiviral responses, in addition to immunoregulatory effects. The first evidence of measurement of TNF-α was provided by Mogi et al. [22] in 1994. They measured TNF-α

both in the CSF and brain. Elevated levels of this cytokine were found in patients with PD versus controls [20].

IFN-γ has been shown to initiate cell death in in vitro mixed mouse microglia/midbrain neuron cultures. Mice deficient in IFN-γ are protected from rotenone-induced cell death in vivo [21]. IFN-γ-stimulated human monocytes/macrophages secrete neopterin during activation. IFN-γ induces indoleamine 2, 3-dioxygenase, which breaks down l-tryptophan to kynurenine. Elevated kynurenine/tryptophan ratio (kyn/trp-ratio) reflects induction of monocyte macrophage system via IFN-γ. Neopterin concentrations and kyn/trp-ratios were increased both in the serum and CSF of PD patients, while serum tryptophan was lower [22, 23]. IL-6 is upregulated in order to protect or regenerate neurons in PD [24]. IL-1 to 3, IL-2, IL-4, and IL-6 epidermal growth factor (EGF) and transforming growth factor (TGF)-α were measured in ventricular CSF, which has higher concentration of cytokines than lumbar CSF. It is important to note that there was upregulation of IL-6 in PD patients. The concentrations of IL-l, IL-2, IL-4, and TGF-α in IL-6 were higher in PD than those from control patients [25]. Similar studies reported elevations of IL-6 in Alzheimer's and PD [26]. Nitric oxide released by active microglia is triggered by pro-inflammatory cytokines to signal apoptosis in neurons. Qureshi et al. [27] found that nitrite, a product of nitric oxide synthase (NOS), is increased in the CSF of PD patients compared to controls. Some authors reported decreased nitrate, which reflects decreased nitric oxide production in neurodegenerative disorders; this could be a methodological issue [28]. Attempts have been made to identify antibodies to dopaminergic neurons in CSF in patients with PD, confirming immune activation in CSF of patients with PD [29]. Some investigators specifically looked into viral infectious etiologies and antibody response in PD [30–32].

Increased serum IL-2, IL-10, IL-4, IL-6, TNF-α, and INF-γ concentrations were measured by cytokine assay in patients with PD and atypical PD compared to controls. However, IL-10 and IL-6 elevations were more specific to PD. This indicates peripheral immune activation in PD [33]. Elevated serum TNF-α was reported in PD [34]. Elevated serum sTNF-α Receptor I was reported in PD [35]. Colonic inflammatory pathology on biopsy has been reported in those patients who had GI Lewy body pathology [36]. C-type natriuretic peptide (CNP) is a member of the natriuretic peptide family; its amino-terminal pro-peptide (NT-pro CNP) is easy to measure in serum. NT-pro CNP localizes to many areas in the CNS. NT-pro CNP, a pro-inflammatory marker, has been found to be elevated in serum in PD patients, in addition to TNF-α indicating presence of inflammation in CNS [37]. Elevation of IL-6 is correlated with gait and balance dysfunction in one study, suggesting perhaps a variation in cytokines in subtypes of PD or association with advanced motor impairment [38]. In PD patients serum IL-6 levels do not seem to vary with levodopa usage, but higher levels of this cytokine are observed in patients with severe disease [39]. Despite a small sample size in a nested case-controlled study, of 84 patients who developed PD average 4.3 years after sample was collected, there is some evidence that IL-6 is elevated, but the other markers of inflammation were not elevated [40]. Serum IL-10 and IL-12 are also elevated, but the data need careful interpretation due to small sample size [41].

Activated microglia produce alpha- and beta-chemokines, in response to immune stimuli. Many chemokine receptors, CXCR2, CXCR3, CXCR4, CCR3, and CCR5, are associated with microglial activation in addition to RANTES. This specific activation in periphery is important to be looked at in PD. RANTES is important for activation of T lymphocytes and monocytes. One of the early studies identified increase in activity of RANTES in serum in PD compared to controls. However measurement of serum chemokines is difficult to interpret as valid biomarker [42].

Macrophage migration inhibitory factor (MIF) is a pro-inflammatory cytokine. There is elevation of MIF in serum of PD patients. The elevation in these levels does not correlate with severity of disease or MIF transcripts in blood cells implying possible origin from microglia rather than peripheral cells [43]. Other systemic inflammatory markers such as high-sensitivity C-reactive protein (hs-CRP) have been measured in PD [44, 45]. Studies have shown elevated levels of oxidized LDL (oxi-LDL), hs-CRP, and intracellular adhesion molecule (ICAM) in PD patients compared to controls [46]. Validation of these is important.

Lindqvist et al. [47, 48] studied CSF and serum inflammatory markers as a panel including CRP, IL-6, TNF-α, and IL-2 receptor in PD. Based on this evidence, there may be specific marker elevation particularly in relation to non-motor symptoms in PD such as fatigue, mood disorder, and sleep disorder as identified by them. However, before such subtype analysis for markers, all of these have to reach statistical significance. Perhaps a study of a larger number of patients would be more informative.

Epstein–Barr virus (EBV) has been studied in many autoimmune neurologic conditions. Cross-reactivity of EBV antibodies to alpha-synuclein has been demonstrated in Lewy bodies and glial cytoplasmic inclusions in PD and MSA [49]. EBV DNA by PCR was detected in CSF in a patient with postencephalitic parkinsonism and MRI brain changes [50].

Han et al. [51] used protein microarray to find ten autoantibodies that showed the highest differential expression between control ($N=40$) and PD ($N=29$) serum samples. These autoantibodies could differentiate PD from control sera with a sensitivity of 93.1 % and specificity of 100 %. The samples were from patients with early- or late-onset PD. The autoantibodies were against intercellular adhesion molecule 4 (ICAM4), pentatricopeptide repeat domain 2 (PTCD2), FERM domain containing 8 (FRMD8), recombinant human CTLA-4/Fc, myotilin (MYOT), hematopoietic SH2 domain containing (HSH2D), fibronectin 1 (FN1), tripartite motif-containing 21 (TRIM21), elongation factor 1-alpha, poly(A)-binding protein, and cytoplasmic 3 (PABPC3). Further studies are needed to establish such markers in the future as a panel.

In studies specifically focused on autoantibodies, in patients with PD there is elevation of antineuronal antibodies, anti-brain lysate antibodies, and anti-ds-DNA antibodies. Specific subtype or characterization of clinical phenotype and correlation is needed [52]. Specific immune response generated by release of neuromelanin from dying dopamine neurons into extra-neuronal space is a humoral response. Stimulation of the pro-inflammatory nuclear factor NF-κB in glial cells by extra-neuronal NM in vitro does occur. In sera of patients with PD there is elevation of antimelanin antibody [53]. When α-synuclein is released, it can cross the

blood–brain barrier. Monomeric and oligomeric forms of α-synuclein are found in CSF and sera of PD patients. Various methods including ELISA and immunoblot were used in experiments to identify antibodies against α-synuclein in serum in PD patients. Elevation of antibodies to α-synuclein and its amyloid fibrillar fragments was demonstrated in serum of PD patients compared to controls [54]. In a separate study, individuals with autoantibodies for α-synuclein in their sera were found to be at increased risk for developing familial PD [55]. Other investigators have found no specific correlation between naturally occurring autoantibodies to α-synuclein in PD. This result could be due to variation in the methods of assay [56]. Anti-GM1 ganglioside antibodies have been shown to be increased in tremor-dominant PD patients [57].

Genome-Wide Association Studies

Inquiry into identification of genetics of inflammation in PD reveals involvement of several pathways and innate and adaptive immune system involvement. Genome-wide association studies show activation of leukocyte/lymphocyte activity and cytokine-mediated signaling pathways [58]. Individuals homozygous at TNF locus (−308AA) are more susceptible to PD, particularly as identified in early-onset PD [59, 60]. Of importance is the study by Wahner et al. [58] in which they studied IL-1β-511 and TNF-α-308 variant alleles together in PD. There was a threefold (OR, 2.92; 95 % CI, 1.66–5.16) increased risk of PD in carriers of a homozygous variant genotype for either or both polymorphisms compared to those carrying a homozygous wild-type genotype for both polymorphisms. Heterozygous genotype for either or both polymorphisms carried a 1.45-fold (OR, 1.45; 95 % CI, 0.97–2.16) risk, suggesting a gene-dosing effect ($p < 0.001$ for trend) [58]. Nishimura et al. [59] also found that the frequency of the TNF-1031C allele was significantly higher in early-onset PD in a Japanese population. TNF-1031C carriers homozygous for IL-1beta-511T had significantly earlier disease onset than noncarriers. IL-1beta appears to be neuroprotective, while TNF appears to increase the risk of developing PD. Consistent with a role of TNF in PD, individuals with both the TNFRSF1-609- and TNFRSF1^{+} 36-polymorphisms showed higher risk of developing PD in a German population [60].

A study of 122 Japanese PD patients found that those homozygous for IL-1beta-511* 2 showed significantly earlier disease onset, and IL-1 beta has possible neuroprotective effect on dopaminergic neurons [61]. There was no difference in the genotype or allele frequency of IL-1B-511 between PD patients and controls in Japanese patients [62]. In contrast, Schulte et al. did not find an association between IL-1β-511 polymorphism and PD. T-511 allele probably has higher frequency in the Japanese population. With regard to IL-1α-889 polymorphism a trend toward an increase of the T allele in PD was observed [63]. In the Turkish population IL-1α-889, IL-1Ra VNTR, and IL-1β +3953 polymorphisms are not associated with PD. IL-1β (−511) is associated with PD. There is protective effect of the

IL-1β-511 T allele in the development of PD [64]. Zhou et al. [65] have shown that presence of T allele of IL-1α (C-899T) is associated with reduced risk for PD. T allele of IL-1α (C-899T) has a role in modifying expression of IL-1α levels in the brain in PD, exerting a protective action thereby improving survival, regeneration, and differentiation of dopaminergic neurons. IL-1alpha-889C allele has been shown to have association with later-onset PD [66].

Cytokine gene polymorphisms such as L-17A rs8193036 and IL-10 1082G/A may be associated with higher risk for PD with cognitive impairment in the Han Chinese population [67]. IL-10 promoter -819T/C polymorphism carries a greater risk of PD in women and is associated with early-onset PD in the Han Chinese population. [68]. Polymorphisms in the genes of IFN-γ, IFN-γR2, IL-10, ICAM-1, and PAF-AH do not seem to be specific to PD. The G1082A polymorphism in the IL-10 gene promoter is related to the age at onset of PD. Based on linear regression analysis, there is significantly earlier onset of PD with more A-alleles and delayed age of onset of PD with two G-alleles compared with individuals having two A-alleles [69]. Gene polymorphisms in IL-18, a pro-inflammatory cytokine, revealed a higher risk for PD with two copies of the IL-18 −607 C alleles in a Han Chinese population [70]. In an Irish population, IL-8 (CXCL8, a chemokine) polymorphism in A-251T is associated with higher risk for PD [71]. NF-κB-mediated inflammation plays a role in the pathogenesis of PD. NOD1 (a product of CARD4 gene) and NOD2 (encoded by CARD15) are NOD (nucleotide-binding oligomerization domain) proteins. They mediate the activation of NF-κB and recognize bacterial proteins. Brain tissue is shown to have CARD15, and the action is astrocyte mediated in the setting of inflammation. Polymorphisms in CARD 15 gene are associated with higher risk of PD [72]. In another study, three SNPs in NOD 2 gene did not correlate with risk of PD [73].

The HLA-DR cell receptor is composed of an alpha- and a beta-chain coded by the HLA-DRA and HLA-DRB genes, respectively. Class II HLA-DR antigens are expressed by brain microglia and interact with T-cell receptors. Ahmed et al. [74] showed association between PD and rs660895 in the HLA-DRB1 locus compared to controls. HLA-DRB1*03 was more common in PD [75]. The International Parkinson's Disease Consortium (2011) found that the DRB5 polymorphism chr6:32588205(G) was lower in PD [76]. The HLA-DRA polymorphism rs3129882(G) was found to reduce the risk of developing PD in an Irish population, but differential effect was noted in patients from the Han Chinese and US population [77–79].

Clinical Epidemiological Studies

Traumatic brain injury can activate microglia. In 28 % cases, there were reactive microglia in patients with survival between 1 and 18 years post trauma. In the acute phase of traumatic brain injury, there are occasional microglia with amoeboid pathology; in the subacute stage there is increased activity of cr3/43 and CD

68-reactive cells, which are activated microglia. In the long-term survival group 28 % cases show extensive regions populated by amoeboid microglia [80]. The prevalence of PD in Thai boxers was 0.71, and 0.98 % in boxers older than 50 years. Incidence of PD has been studied in boxers. In a large study by Lolekha et al. [81] there is no significant difference in incidence of PD among boxers and general population. From the end of boxing career to development of PD symptoms, the time lag was 23–43 years (35.6 ± 7.9 years) [81]. There is threefold increase in risk for PD in a univariate analysis (OR, 3.0; 95 % CI, 1.2–7.6; $p = 0.020$) in a study of 93 twin pairs, suggesting a greater risk for PD in closed head injuries [82]. Other studies also indicate a higher risk of PD in patients with head trauma [83].

Paraquat causes neurodegeneration via activation of microglia via dose-dependent induction of extracellular O_2 [84]. Higher incidence of PD with agricultural and other uses of pesticides such as Rotenone and Paraquat was reported in many studies [85–90]. In 110 PD cases compared to 358 controls, PD is 2.5 times more often in Rotenone and Paraquat exposure, with an odds ratio of 2.5 with 95 % CI [85]. Other studies have also shown higher risk of PD with pesticide exposure with an odds ratio 1.99 with 95 % CI [1.12–3.21], and various pesticides were studied [86]. Kamel et al. [87] showed an association between pesticides, and PD exposure and odds ration varied between 1.4 and 4.5, depending on the degree of exposure. In East Texas Firestone et al. showed increased incidence of PD with pesticides and well-water consumption [88]. Rotenone, chlorpyrifos, Paraquat, and other compounds have been associated with increased incidence of PD [89]. Plasma manganese and serum iron were found to be significantly higher in PD patients than age-matched controls in the Chinese population study [90]. Occupational exposure to copper or manganese for over 20 years has been shown to increase the risk of developing PD by over twofold [91]. Increased risk is seen with dual combinations of lead, iron, and copper [92].

Braak et al. [93] hypothesized that the stereotypic topographic expansion pattern of lesions associated with PD may be due to a pathogen capable of passing the mucosal barrier of the gastrointestinal tract and entering the central nervous system by traveling along the fibers of the postganglionic enteric neurons [93]. This increased intestinal permeability has been demonstrated in a very elegant study by Forsyth et al. [94]. Evidence for hyperpermeability of the intestinal mucosa is based on increased staining for alpha-synuclein, nitrotyrosine, *Escherichia coli*, and LPS-binding protein (LBP) in addition to elevated serum levels of LBP [94]. This correlation is critical for our understanding of external pathogens influencing development of PD and also the action of cytokines both peripherally and in the CNS. There is a higher risk for PD in patients with *Helicobacter pylori* and GI symptoms. The risk varies with severity of GI symptoms. Risk is higher in those with proton pump inhibitor use (23 %) and gastritis (22 %) [95].

Occurrence of multiple CNS infections ≥5 years prior to onset of PD has been shown to carry a higher risk for PD compared to single CNS infection [96]. A large-scale epidemiologic study has corroborated a role of inflammation in PD. Based on recorded deaths in England and Wales ($N = 76{,}000$) from 1950 to 1992, individuals born around 1900 appear to have had twice the probability of dying from PD

than people born around 1920 and five times the probability of dying from PD than people born around 1930 [97]. Martyn hypothesizes that the reason for the increase in PD deaths in individuals born around 1900 was due to the encephalitis lethargica, which swept the world in 1918 and 1919. In a case-control study, individuals who experienced croup, which is a common respiratory infection affecting young children, or diphtheria, which was a relatively rare condition in the first half of the twentieth century, in childhood were at greater risk of developing PD ($N=172$) compared to controls ($N=343$) [98].

Albumin was measured in cerebrospinal fluid and serum samples obtained from 73 non-demented subjects with idiopathic Parkinson's disease and 47 age-matched control subjects. The albumin ratio (AR) was calculated to assess blood–cerebrospinal fluid and blood–brain barrier function. Analysis of CSF/serum albumin ratio in 73 PD patients compared to controls shows a higher level in PD patients compared to controls. An increase of high molecular proteins in the CSF indicates blood–CSF barrier dysfunction, and the resulting BBB impairment is maintained due to possible failure of choroidal Na-K ATPase and continuing inflammation [99]. [11C]-verapamil is normally extruded from the brain by P-glycoprotein, so increased uptake would indicate a defect in the blood–brain barrier. In one PET study, advanced PD patients had increased [11C]-verapamil uptake in the frontal white matter regions compared to controls indicating a compromised blood–brain barrier [100]. On the other hand, de novo PD patients showed lower uptake in the midbrain and frontal regions compared to controls, indicating an intact blood–brain barrier. In another study, [11C]-verapamil showed elevated uptake in the midbrain of PD patients [101]. ATP-binding cassette, subfamily B, member 1 (ABCB1) gene regulates transport of endogenous molecules and exogenous toxins encoding the protein P-glycoprotein (P-gp). Single-nucleotide polymorphisms have been identified in ABCB1 gene in PD, indicating problems in BBB [102]. MDR1 is another gene that codes for P-glycoprotein. MDR1-1236C allele was found to be associated with PD. This study also found that the MDR1-1236C-2677G haplotype was associated with PD again indicating abnormalities in BBB [103]. Consistent with these results, Lee et al. [104] found that the MDR1-1236C, 2677G, and 3435C alleles significantly increased the risk of developing PD in a Chinese population (PD = 206, Controls = 224) [104]. These mutations may allow inflammatory cells and cytokines into the brain that are normally filtered out.

Data on Anti-inflammatory Agents in Clinical Literature

In a meta-analysis of 14 observational studies, exposure to NSAIDs or aspirin was reviewed in PD. With nonaspirin NSAIDs there is reduced risk of PD (13 %), and with ibuprofen alone, there was greater reduction in risk for PD (27 %). Important data show benefit of anti-inflammatory agents [104]. Ibuprofen use has once again been shown to decrease risk of PD in a prospective study by Gao et al. [105]. Multivariate relative risk for PD in this study is 0.62 (95 % confidence interval (CI)).

Those who used ibuprofen regularly had approximately 30 % lower PD risk compared to nonusers [105]. Another meta-analysis showed protective effect with regular use of ibuprofen, with 15 % reduction of PD risk but not aspirin [106]. In a US population recruited from subjects from the Neuro Genetics Research Consortium, smoking, caffeine, and the use of NSAIDs were all reviewed, and the combined effect of all three agents reduced the risk of PD significantly. Smoking ever in a lifetime was associated with 23 % reduction in risk, while current smokers carried 55 % reduction in risk. High coffee consumption had 25 % risk reduction. Subjects who smoked, drank high level of coffee, and used NSAIDs had 62 % lower risk [107]. Wahner et al. [108] found that nonaspirin-NSAID users showed a two-fold reduction in odds ratio for having PD. This effect was even greater for individuals who reported using nonaspirin NSAIDs for over 2 years. In contrast, Hernán et al. [109] found that nonaspirin-NSAID use increased the risk of developing PD in females and reduced the risk in males (PD = 1,258, Controls = 6,638). Further, they found that aspirin use increased the risk of PD [110]. Health Professionals Follow-up Study and the Nurses' Health Study looked at 142,902 men and women without PD, stroke, or cancer at baseline and followed up with them from 1986–2000 to 1980–1998, respectively. Both questionnaires documented NSAID use and PD diagnosis every 2 years. Chen et al. [111] found that the use of nonaspirin NSAIDs reduced the risk of PD (N = 415) in this study population by twofold [111]. In a retrospective study, PD patients taking fibrates or statins showed a mean age of disease onset that was delayed by almost 9 years compared with controls [112]. Further, individuals taking statins showed a significantly smaller increase in levodopa-equivalent daily dose over 2 years. Minocycline has been shown to have anti-inflammatory properties. In a phase II placebo-controlled double-blind clinical trial, minocycline could not be rejected as futile based on the DATATOP futility threshold (Minocycline = 66, Placebo = 67) [113].

Conclusion and Future Directions

Inflammation is clearly present in PD patients based on cytological, biomarker, and comorbidity studies. Further, genome-wide association studies have uncovered multiple polymorphisms in genes involved in innate as well as acquired immunity. Epidemiological studies have also indicated that inflammation could result in PD. Based on review of current evidence, it is difficult to conclude presence of one specific biological marker for PD or a panel thereof. At this time, because of the lack of surrogate markers, be it imaging, serum, or CSF markers, and poor understanding of dynamics of the apoptotic and inflammatory pathways, it becomes difficult to postulate therapeutic options. Therapeutic targets can include anti-inflammatory agents, that work in periphery, or specific agents targeting cytokines. Further investigations are needed in the future to develop appropriate therapeutic agents that can cross the blood–brain barrier.

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PET Imaging in Neuroinflammation

David J. Brooks

Introduction

Microglia are cells derived from monocyte lineage and constitute around 15 % of the brain's white cell population. Normally they are in a resting state with extended ramified processes monitoring the brain milieu and making contact with neighbouring neurones and astrocytes [1, 2]. They are protected from antigens in the plasma by the blood–brain barrier and form the natural immune defence of the brain. Exposure to plasma proteins such as fibrinogen following blood–brain barrier disruption, or to intrinsic excitotoxic agents such as raised glutamate, nitric oxide, or cytokines, or any change in brain milieu causes them to become activated taking on amoeboid or rod-like morphology. When activated the microglia express MHC class 1 and 2 antigens and release cytokines such as TNF-α, IL1-β, and IL-6 and growth factors such as TGF-β1. They become phagocytic and can strip and remodel synapses.

It is now considered that activated microglia exist as at least two primary phenotypes: the M1 phenotype is associated with the release of cidal cytokines so promoting cell damage while the M2 phenotype is associated with phagocytosis of dead tissue, synaptic stripping and remodelling, and growth factor release so promoting neurogenesis [3]. These two phenotypes and others may well be interconvertible and predominate at different disease phases. After an acute focal brain injury activated microglia act locally to wall off, remove dead tissue, and remodel connections. In chronic neurodegenerative diseases they may act to release cytokines where disease is locally active but remodel downstream connections in the brainstem and thalamus.

The 18 kDa translocator protein (TSPO) is expressed at low levels in normal brain but, if microglial cells become activated, it can be detected in their outer mitochondrial

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M. Thomas (ed.), *Inflammation in Parkinson's Disease: Scientific and Clinical Aspects*,
DOI 10.1007/978-3-319-08046-8_9,

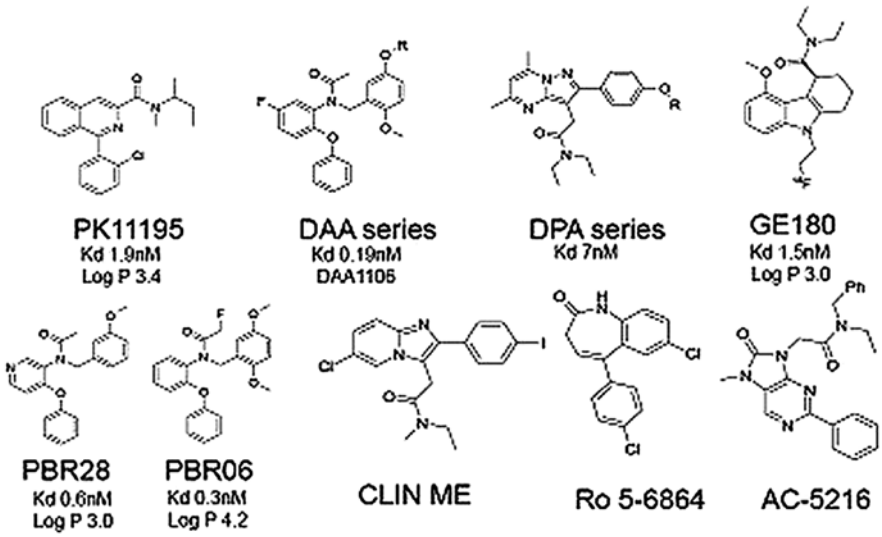

Fig. 1 Structures of PET and SPECT TSPO imaging agents

membranes. The TSPO serves a number of functions including transport of cholesterol, anions, and porphyrin, maintenance of mitochondrial membrane potential, regulation of cell apoptosis and proliferation, and immunomodulation [4]. It is present in many peripheral organs including the liver, spleen, adrenals, and myocardium [5]. TSPO was previously known as the peripheral benzodiazepine receptor (PBR) as it expresses a binding site for benzodiazepines and isoquinolines that is distinct from $GABA_A$ sites. This has enabled in vivo detection of the presence of TSPO in brain with the isoquinoline marker ^{11}C-PK11195 PET. A number of newer PET biomarkers are now available to image TSPO expression, and these include the phenoxyarylacetamides ^{11}C/^{18}F-DAA1106, ^{11}C-PBR28, ^{18}F-PBR06, and ^{18}F-PBR111, ^{18}F-FEPPA, pyrazolo-[1,5-a]-pyrimidines such as ^{11}C-DPA713 and ^{18}F-DPA714, the vinca alkaloid ^{11}C-vinpocetine, and the imidazo-[1,2a]-pyridines ^{11}C/^{18}F-CLINDE (Fig. 1) [6, 7]. While the advent of TSPO PET agents has enabled the distribution of activated microglia in the brain to be imaged in life, TSPO ligands bind to both M1 and M2 phenotypes. TSPO PET, therefore, provides a measure of total activated microglia load without providing information about the specific functional roles of these cells in different diseases and brain areas.

Imaging TSPO with PET

The radioligand that was first developed for imaging TSPO in the brain and that has seen the greatest use is the R enantiomer of the isoquinoline ^{11}C-(*R*)-PK11115. The rat unilateral facial nerve crush model results in an axonopathy, and activated

microglia can be seen in the denervated ipsilateral facial nucleus. Autoradiography studies of this model have shown that ^{3}H-PK11195 binds selectively to the activated microglia rather than astrocytes [8]. These activated microglia play a role in remodelling connections to restore facial muscle function. The human equivalent of this rodent facial nerve crush model is Bell's palsy where the facial nerve becomes inflamed as it passes through the auditory canal and the local oedema results in axon compression. ^{11}C-PK11115 PET studies have demonstrated tracer uptake in the facial nerve nucleus ipsilateral to the paralysed facial muscles where connections are being remodelled [5]. Acquired limb amputations following trauma often result in a phantom limb phenomenon where the absent limb still feels as if it is present. This sensation is unpleasant, and the limb may feel as if it is compressed into the remaining stump. In these subjects one can detect thalamic inflammation contralateral to the missing limb with ^{11}C-PK11115 PET [9]. These PET studies, therefore, reveal that central microglial activation can be detected after disconnection of brain nuclei following peripheral nerve injuries. These microglia are likely to be playing a role in the remodelling of connections though it remains to be determined whether the cells express the M2 phenotype.

In brain disorders TSPO imaging reveals microglial activation both at the site of local disease activity and also due to the effects of downstream disconnection. Endothelial cells also express TSPO, and so ^{11}C-PK11115 binding can be seen in the lateral and sagittal venous sinuses. This can be problematic as the vascular ^{11}C-PK11115 signal may spill over into adjacent brain tissue—particularly the cerebellum—due to the relatively low 5-mm spatial resolution of commercial PET cameras. The result is artefactually high signals from cerebellum unless a correction is performed.

Quantitative modelling of TSPO PET can be problematic as there is no single brain region that can be guaranteed to provide a tissue reference for non-specific tracer uptake. One option is to use an arterial plasma input function to calculate brain tracer binding but ^{11}C-PK11115 sticks to plastic tubing and it can be difficult to obtain accurate wash-in and washout time activity curves (TACs) allowing measurement of peak height, delay, and dispersion. For these reasons, cluster analysis has been used to define a collection of grey matter voxels in each subject's brain that have a time activity uptake and washout curve that behaves similarly to a population of normal grey matter reference TAC in healthy controls [10]. This reference cluster can then be used to define levels of non-specific ^{11}C-PK11115 uptake in brain regions where retention is occurring in patients. At the same time signal due to vascular tracer binding, which peaks rapidly, is separated from brain parenchymal signal. Ligand binding potentials (BPs) can then be computed using a standard simple reference tissue model (SRTM) with brain-specific and non-specific compartments or standard graphical modelling approaches.

^{11}C-PK11115 PET suffers from the problem of a relatively high non-specific signal and rapid brain washout. Recently, new higher-affinity tracers have been developed with lower non-specific signal and a longer brain retention with the aim of providing more sensitive detection of microglial activation. However, binding of these new tracers has been found to be influenced to varying extents by the polymorphism of the TSPO gene being expressed by individuals; this is a minor issue

with ^{11}C-PK11115 PET [11]. An Ala147Thr substitution at the rs6971 polymorphism of both TSPO genes results in homozygous subjects becoming low-affinity binders of the newer TSPO ligands and giving no specific PET signal. Heterozygotes for the Ala147Thr substitution at the rs6971 polymorphism are mixed affinity binders. In Caucasian populations around 60 % of individuals are high-, 10 % low-, and 30 % mixed affinity binders for the newer TSPO ligands. ^{11}C-PBR28 shows a 50-fold difference in affinity for TSPO between high (Kd 4nM)- and low (Kd 200nM)-affinity binders. The PET tracers ^{11}C-DAA1106, ^{11}C-DPA713, and ^{18}F-PBR111 show 4–5-fold differences in affinity for TSPO between high and low binders. Mixed affinity binders express high- and low-affinity TSPO binding sites in equal proportions. In order to use these newer TSPO PET markers subjects need to be stratified into high-, mixed, and low-affinity binders by prior genetic screening and their findings compared with appropriate control populations.

Imaging Inflammation in Parkinsonian Syndromes

Parkinson's Disease

Parkinson's disease (PD) is the second most common neurodegenerative disorder after dementia, affecting 1 % of the over sixties. It manifests as asymmetrical limb bradykinesia, rigidity, and tremor, and in 90 % of pathologically proven cases, these symptoms were responsive to levodopa. The neuronal pathology is characterized by Lewy body inclusions, which contain aggregated alpha synuclein and neurofilaments.

While the dopamine neurones of the substantia nigra compacta are primarily targeted by the Lewy body pathology, the disease process is thought to start in the medullary dorsal nucleus of the vagus at stage 1 and to then ascend the brainstem to the cortex in later stages [12]. In stage 2 the noradrenergic locus ceruleus, cholinergic pedunculopontine nucleus, and serotonergic median raphe in the pons become involved while the dopamine neurones of the substantia nigra compacta in the midbrain become targeted in stage 3 along with the cholinergic nucleus basalis. The limbic cortex and cingulate are involved at stage 4 and the association and primary neocortex in stages 5 and 6.

At postmortem microglial activation accompanies the Lewy body pathology in subcortical and cortical affected regions [13, 14]. Aggregated alpha synuclein fibrils are known to stimulate microglial activation in cell culture, but the in vivo relationship between brain inflammation and disease progression remains uncertain. The exact role of microglial activation in the degenerative process in PD is currently poorly understood. When humans and monkeys are acutely exposed to the nigral toxin MPTP, an inflammatory response results which can persist for years associated with progressively deteriorating parkinsonism [15, 16].

In an initial PET study it was reported that ten early cases of PD showed increased midbrain uptake of ^{11}C-PK11195, the levels of which correlated both with motor disability rated with the motor subscore of the Unified Parkinson's Disease Rating Scale (UPDRS) and with reductions in putamen dopamine transporter (DAT) binding measured with ^{11}C-CFT PET [17]. The authors suggested that their study provided evidence for the involvement of microglia in the loss of dopaminergic function that characterizes PD.

Subsequent ^{11}C-PK11195 PET studies, however, have failed to replicate a correlation between midbrain ^{11}C-PK11195 signals and loss of dopaminergic function in PD or indeed to consistently detect activated microglia in PD substantia nigra. In a series involving 18 more advanced PD cases, increased ^{11}C-PK11195 binding was detected in the striatum, thalamus, pons, and frontal and cingulate cortex compared to 11 age-matched controls. In this study the level and extent of regional microglial activation did not correlate with either clinical severity or striatal ^{18}F-dopa uptake. Over a 2-year follow-up period, despite ongoing clinical deterioration and loss of putamen ^{18}F-dopa storage, levels of ^{11}C-PK11195 uptake were reported to remain static in eight of these 18 PD patients [18]. These workers suggested that microglial activation is present at onset of PD and may act to drive the progression of locomotor disability.

At postmortem end-stage PD cases show widespread microglial activation which accompanies the Lewy body pathology in association cortical and subcortical regions. PET, however, allows patients with early disease to be investigated. ^{11}C-PK11195 PET is able to demonstrate the presence of cortical microglial activation in association cortical regions of early non-demented cases [18]. This suggests that cases that would be rated as Hoehn and Yahr stage 1 or 2 based on clinical criteria may well already be Braak stage 5 or 6 pathologically.

Eighty percent of PD patients will eventually develop dementia if they survive their condition for 20 years [19]. The relationship between microglia activation and cognitive status in PD has now been investigated. An inverse correlation between levels of cortical microglia activation and scores on the mini-mental state examination (MMSE) has been reported [20]. In non-demented PD cases the presence of raised levels of cortical ^{11}C-PK11195 binding correlates with an impaired performance on tests of verbal memory and visual perception [21]. These findings confirm that microglial activation is an early phenomenon in PD and suggest that it may be responsible for driving the cortical dysfunction that leads to later dementia.

Over a 2-year follow-up period, despite on-going clinical deterioration and loss of putamen ^{18}F-dopa storage, levels of cortical and subcortical ^{11}C-PK11195 uptake have been reported to remain relatively static in PD patients [18]. However, although the density of activated microglia may remain stable, these cells are still expressing cytokines at postmortem. Whether interventions should be developed to suppress microglial activity in PD is currently under debate. Clearly if they are releasing cytokines and potentially driving disease progression, switching them off would be a rational strategy; however, they also remodel connections and release growth factors, so development of drugs selectively blocking the M1 cidal or promoting the M2 restorative phenotype would be advantageous.

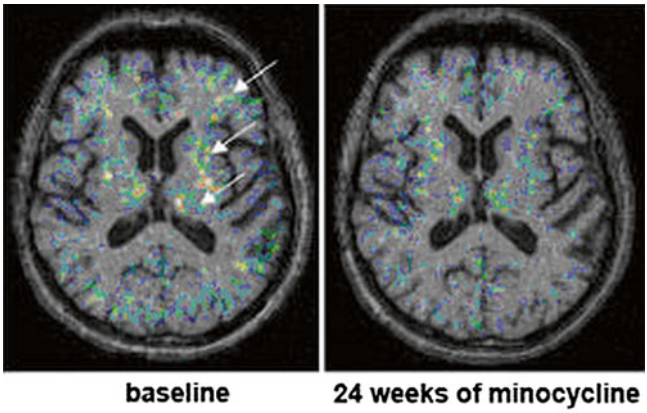

Fig. 2 ^{11}C-PK11195 PET scans in a multiple system atrophy patients before and after a 6-month course of minocycline. The microglial activation signal has fallen by 30 % in the posttreatment image [25]

Atypical Parkinsonian Disorders

Atypical Parkinsonian disorders include multiple system atrophy (MSA), progressive supranuclear palsy (PSP), and corticobasal degeneration (CBD). MSA-P is associated with asymmetric parkinsonism along with early autonomic dysfunction, postural instability, and ataxia. It is usually poorly responsive to levodopa. The pathology is characterized by argyrophilic glial cytoplasmic inclusions (GCIs) that contain alpha synuclein and target the substantia nigra, putamen, ponto-cerebellar connections, and the lateral columns of the spinal cord. At postmortem widespread microglial activation is associated with the MSA pathology [22].

An initial ^{11}C-PK11195 PET study involved five patients with multiple system atrophy (MSA-P). Activated microglia were detected in the head of caudate nucleus, putamen, substantia nigra and pons, and frontal regions in a more extensive distribution than that normally associated with PD [23].

A proteolipid protein-alpha synuclein (PLP-αSYN) overexpression transgenic mouse model of MSA has been developed [24]. This mouse shows a correlation between levels of nigral microglial activation and dopamine neurone loss. Minocycline is known to both block and reverse microglial activation in cell cultures. Its administration suppressed the inflammation present in the PLP-αSYN transgenic mouse and preserved dopamine neurones.

A human trial has been performed to determine the neuroprotective efficacy of minocycline in MSA. The drug failed to slow clinical disease progression at doses that are licensed for the treatment of acne. MSA cases receiving active medication, however, showed a 30 % reduction in brain ^{11}C-PK11195 uptake relative to placebo-treated cases (Fig. 2) [25]. This finding suggests that markers of microglial activation may be valid biomarkers when testing anti-inflammatory agents as potential neuroprotective agents.

PSP is associated with axial parkinsonism, impaired volitional eye movements, early bulbar dysfunction, and balance and gait difficulties. Dementia of a frontal type can be present, and the syndrome is poorly levodopa responsive. At postmortem cases show atrophy of the brainstem, internal globus pallidus, amygdala, frontal and parietal lobe, and nigral depigmentation. The characteristic pathology is intra-neuronal neurofibrillary tangles containing hyperphosphorylated 4-repeat tau protein and neuropil threads. Neuronal loss is seen in brainstem oculomotor nuclei, pallidum, substantia nigra, subthalamic nucleus, and frontal cortex. There is accompanying widespread brainstem, basal ganglia, and frontal microglial activation [26].

Four PSP patients who were studied with ^{11}C-PK11195 PET showed significantly increased signal in their caudate nucleus, putamen, pallidum, midbrain, substantia nigra, the frontal lobe, and the cerebellum [27]. Two of these patients were rescanned after 6–10 months, and the level of microglial activation was unchanged.

CBD is associated with asymmetrical parkinsonism, and the affected limbs are characteristically apraxic and exhibit stimulus-sensitive myoclonus and on occasion the alien limb phenomenon. Dysphasia, a supranuclear gaze palsy, and frontal dementia may all accompany the limb signs. Established cases show asymmetrical cortical atrophy targeting the superior frontal gyrus and inferior parietal areas. Microscopically ballooned achromatic neurones and astrocytic plaques containing tau are seen in affected cortical areas, the dentate nuclei, and the substantia nigra. Microglial activation is extensive in CBD, and its distribution correlates with the anatomical regions most affected [26]. The syndrome of CBD, however, can be associated with other pathologies including PSP and Pick's disease. Four patients with CBD have had ^{11}C-PK11195 PET, and they showed increased signal in caudate, putamen, substantia nigra, and frontoparietal cortex [27].

Imaging Inflammation in Dementias

Alzheimer's Disease

Dementia affects 10 % of the over sixties, the prevalence rising to one-third of the over eighties. It is characterized clinically by progressive impairment of memory along with word finding and recognition and perceptual difficulties in the absence of an altered conscious level. Personality changes occur and patients can regress a state of total dependency. At postmortem 60 % of dementia cases have Alzheimer pathology with fibrillar extracellular amyloid plaques and intra-neuronal neurofibrillary tangles of hyperphosphorylated tau. Activated microglia are seen surrounding the amyloid plaques, which target association cortex, cingulate, and striatum [28, 29]. They are also found in brain areas with dense tau pathology but sparse amyloid plaques, such as the entorhinal cortex and hippocampus.

^{11}C-PK11195 PET detects raised levels of microglial activation in patients with probable Alzheimer's disease (AD), binding potentials being raised by around 30 %

in frontal, temporal, and parietal association cortical areas [30, 31]. The newer TSPO ligand ^{11}C-DAA1106 has also been reported to show a 33 % increase in cortical uptake in AD [32]. The cortical distribution of inflammation in AD, like that of amyloid deposition, targets association areas when imaged with PET [33]. Interestingly, while levels of cortical ^{11}C-PK11195 and ^{11}C-DAA1106 uptake in AD correlate with the degree of cognitive impairment rated with the MMSE, this is not true of amyloid load [31, 34]. This suggests that it may be the cortical microglial activation rather than amyloid plaques in AD that is detrimental to cognitive function, possibly due to cytokine release by these cells. In AD activated microglia can also be detected in the thalamus, cerebellum, and brainstem as well as the association cortex; areas are not targeted by plaque or tangle pathology. The TSPO expression detected by PET is likely to reflect microglial activation in response to cortical disconnection, the cells probably acting to strip synapses and remodel connections.

Subjects with amnestic mild cognitive impairment (MCI) have isolated recall problems that are not severe enough to interfere with activities of daily living. 60 % of these cases progress to develop Alzheimer's disease over 5 years, and amnestic MCI can be regarded as a transitional state between normality and dementia [35]. ^{11}C-PK11195 and ^{11}C-DAA1106 PET have both been reported to detect microglial activation in MCI when present. In one series ^{11}C-PK11195 PET revealed increased cortical microglial activation in 40 % of amnestic MCI cases while 60 % showed evidence of amyloid plaque deposition with ^{11}C-PIB PET [36]. In another group of seven MCI cases studied with ^{11}C-DAA1106 PET there was a significant mean 27 % increase in tracer uptake across brain regions, two cases individually showing significantly elevated levels of microglial activation [37]. Five of the seven MCI subjects with ^{11}C-DAA1106 uptake raised more than 0.5 standard deviations above the normal mean progressed to dementia over a 2-year follow-up period.

Frontotemporal Dementia/Amyotrophic Lateral Sclerosis

Frontotemporal dementias are now though to be largely genetic in origin and account for around 10 % of dementia cases. Clinically they present as progressive personality change, dysphasia, and apraxia, with memory difficulties developing later. Semantic dementia where subjects recognize objects but cannot recall their function, progressive aphasias, and apraxias are all variants of FTD. There are three main pathological causes of FTD, all associated with abnormal protein aggregation: Pick's disease is associated with Pick bodies containing aggregated three-repeat hyperphosphorylated tau. A second variant is characterized by the presence of TDP-43 protein inclusions and spongiform degeneration targeting the frontal and inferior temporal lobes. These inclusions are associated with mutations of the progranulin gene. Abnormal aggregation of FUS protein represents a third type of FTD. A PET series of five clinically diagnosed FTD cases reported raised frontotemporal uptake of ^{11}C-PK11195 as would be predicted [38].

There is a clinical and pathological overlap between frontotemporal dementia and motor neurone disease (amyotrophic lateral sclerosis). The neuropathology of both can be associated with cortical TDP-43 inclusions and spongiform degeneration, and neuropsychological and imaging studies have indicated that cortical dysfunction is present in many cases of ALS. In one series ten clinically probable ALS patients on El Escorial criteria and 14 healthy controls were studied with ^{11}C-PK11195 PET [39]. Significantly increased microglial activation was seen in the motor cortex, brainstem, thalamus, dorsolateral prefrontal cortex, and occipital lobes of the ALS patients. There was a significant correlation between ^{11}C-PK11195 uptake in motor cortex and severity of upper motor neuron signs clinically.

Detection of Preclinical Microglial Activation

Huntington's disease is an autosomal dominant inherited progressive neurodegenerative disorder associated with motor, cognitive, and psychiatric symptoms. It is associated with an abnormal CAG triplet repeat expansion of the huntingtin gene on chromosome 4, which leads to an elongated polyglutamine chain at the terminus of the huntingtin protein. The effect of this is to cause intranuclear aggregations of huntingtin to form resulting in progressive loss of medium spiny striatal GABAergic neurones [40]. These neurones express either dopamine D1 or D2 receptors, and subclinical striatal dysfunction can be detected as a loss of availability of these sites for ligand binding [41].

Loss of striatal neurones in HD is associated with microglial activation [42], which can be detected with ^{11}C-PK11195 PET. Levels of striatal microglial activation correlate with loss of D2 binding measured with ^{11}C-raclopride PET and also with locomotor disability rated with the Unified Huntington's Disease Rating Scale (UHDRS) [43]. This suggests, again, that microglial activation may play a role in driving the disease process. In support of this, ^{11}C-PK11195 PET studies have reported raised microglial activation in a majority of asymptomatic adult HD gene carriers [44]. Those carriers with active disease also showed reduced dopamine D2 binding with ^{11}C-raclopride PET. Cortical microglial activation can also be detected in pre-manifesting HD carriers confirming that this is not a purely basal ganglia disorder [45]. Levels of striatal ^{11}C-PK11195 uptake have been shown to correlate with the predicted time of clinical disease onset [44, 45].

Conclusion

Microglial activation is a non-specific reaction to all forms of neurodegeneration and can be detected with PET. In chronic neurodegenerative diseases, the role of activated microglia remains uncertain. Initially, these cells could act to ingest misfolded proteins and release growth factors, but this process appears to fail in later

disease. Local release of cytokines by microglia with the M1 phenotype may lead to neuronal death and disease progression. Conversely, in downstream disconnected areas, microglia could have a beneficial action leading to synaptic stripping and remodelling of connections as an adaptive response.

Currently, PET imaging of activated microglia is based on the use of TSPO substrate radioligands. While these provide a valuable in vivo tool for detecting disease activity and tracking the progression of neuroinflammation, binding of the newest ligands with lower non-specific background signals is influenced by the TSPO polymorphism expressed, and 40 % of the Caucasian population are either non- or mixed affinity binders. As TSPO polymorphism has only a small effect on ^{11}C-PK11195 binding, despite its lower affinity and higher non-specific signal, ^{11}C-PK11195 PET still provides a pragmatic approach for measuring brain inflammation. The early detection of microglia using PET provides a potential biomarker for measuring the efficacy of neuroprotective strategies designed to suppress the inflammatory response to neurodegenerative processes. In the future hopefully there will be development of both TSPO tracers uninfluenced by the genotype along with other markers of microglial activation, such as cannabinoid CB2 expression, allowing us to improve our understanding of the role of activated microglia in CNS disease.

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M. Thomas (ed.), *Inflammation in Parkinson's Disease: Scientific and Clinical Aspects*,
DOI 10.1007/978-3-319-08046-8, © Springer International Publishing Switzerland 2014

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