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Neuroinflammation, Oxidative Stress and the Pathogenesis of Parkinson's Disease

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Abstract

Neuroinflammatory processes play a significant role in the pathogenesis of Parkinson's disease (PD). Epidemiologic, animal, human, and therapeutic studies all support the presence of an neuroinflammatory cascade in disease. This is highlighted by the neurotoxic potential of microglia . In steady state, microglia serve to protect the nervous system by acting as debris scavengers, killers of microbial pathogens, and regulators of innate and adaptive immune responses. In neurodegenerative diseases, activated microglia affect neuronal injury and death through production of glutamate, pro-inflammatory factors, reactive oxygen species, quinolinic acid amongst others and by mobilization of adaptive immune responses and cell chemotaxis leading to transendothelial migration of immunocytes across the blood-brain barrier and perpetuation of neural damage. As disease progresses, inflammatory secretions engage neighboring glial cells, including astrocytes and endothelial cells, resulting in a vicious cycle of autocrine and paracrine amplification of inflammation perpetuating tissue injury. Such pathogenic processes contribute to neurodegeneration in PD. Research from others and our own laboratories seek to harness such inflammatory processes with the singular goal of developing therapeutic interventions that positively affect the tempo and progression of human disease.

Keywords

Parkinson's disease; inflammation; oxidative stress; microglia dopaminergic neurons; diffusion tensor imaging (DTI); free radicals; dopaminergic neurodegeneration

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1. Introduction

Parkinson's disease (PD) is the most common movement disorder and second, only to Alzheimer's disease as a cause of age-linked neurodegeneration [1-3]. The primary pathological characteristics of PD are the progressive loss of dopaminergic neurons in the substantia nigra pars compacta (SNpc) and reductions in their terminals within the dorsal striatum [4]. These lead to profound and irreversible striatal dopamine loss. Indeed, extrapolated cell modeling data [5] demonstrate that 100–200 SNpc neurons degenerate per day during PD [6]. However, the SNpc is not the sole site for neuronal damage involving, in measure, the locus coeruleus, raphe nuclei, and the nucleus basalis of Meynert. Nonetheless, progressive degeneration of the nigrostriatal pathway is the predominant mediator for clinical manifestations of PD including rigidity, resting tremor, slowness of voluntary movement and postural instability, and in some cases, dementia [2].

In regards to disease epidemiology, the mean age of PD onset is 55. This increases dramatically with time [7]. While the cause of PD is not known, data obtained from familial disease and from animal models of PD support a pathogenic process that is closely linked to mitochondrial dysfunction and oxidative stress [7-9] and is strongly linked to dopaminergic neuronal loss. Moreover, increased risk of localized oxidative damage for dopaminergic neurons is linked to dopamine metabolism itself [8]. With the exception of rare familial forms, the majority of PD cases are sporadic and due, in part, to mitochondrial defects at complex I [7,10]. Indeed, complex I inhibitors (for example, rotenone) recapitulate many of the pathological features of disease [7]. Moreover, the presence of ubiquitinated and misfolded proteins suggests the dysregulation of protein assembly or defects in protein degradation pathway as a critical part of disease pathogenesis. Misfolding and abnormal degradation of brain proteins are linked to dopaminergic neuronal death [11].

A key player in the pathogenesis of PD is the microglial cell. Our own knowledge of the structure and function of macrophages and microglia has evolved considerably since Elie Metchnikoff, over a century ago, discovered an inflammatory cell type in starfish larvae capable of engulfing foreign objects and cellular debris. This led him to postulate that these cells were both beneficial and essential to the host. Metchnikoff's famous theory of phagocytosis wherein the macrophage serves as the first line of defense and protects an organism against pathogenic microbes is ingrained in the fundamentals of immunology. The past century has realized a great deal of progress in understanding the role macrophages play in immunity. Macrophages orchestrate a number of cellular processes, including but not limited to, intracellular killing of pathogenic microbes, antigen presentation, and secretion of biologically active factors, as well as mediation of pathological processes. Underlying such cellular functions is inflammation; the same response that often proves detrimental in localized and systemic diseases, including those operative in PD. Inflammation is the frontline defense of multi-cellular organisms against infection and its absence is incompatible with life. Inflammation enables the host to fend off various disease-causing microbes including bacteria, viruses, and parasites. At the time virulent microorganisms bypass initial skin and mucosal barriers of immune defense and enter the body, inflammatory processes are engaged in attempts to eliminate the invader along with clearance of damaged tissue. All together, this process is orchestrated primarily by mononuclear phagocytes (MP; monocyte, tissue macrophage, dendritic cell) serving as a vital sensor against microbial invasion and later as a control for wound healing following infection or injury.

However, inflammatory responses can also prove deadly to tissue and to the host. Inflammatory responses are closely linked to a number of human diseases including cancer, arthritis, cardiovascular disease, and autoimmune diseases. With regards to the nervous system recent data suggests that neuroinflammation, perpetrated through activation of brain MP (perivascular

and parenchymal macrophages and microglia) along with other glial elements, including astrocytes and endothelial cells, may act through paracrine pathways to accelerate neuronal injury in highly divergent diseases such as Alzheimer's disease (AD), Huntington's diseases (HD), HIV-1-associated dementia (HAD), and spongiform encephalopathies or prion-mediated neurodegeneration (Figure 1). Importantly, such pathologic processes also underlie the neurodegeneration in PD. In these disorders, central nervous system (CNS) inflammatory infiltrates are complex and multifaceted. The initial responders or the MP cell elements of innate immunity set up a cascade, which later involve the activation and recruitment of the adaptive immune system and ultimately neurodegeneration. For PD this can occur through release of aggregated proteins such as α -synuclein contained within Lewy bodies from damaged or dying neurons that later serve as an impetus for microglial neurotoxic activities. Nonetheless, on balance, microglia are the primary MPs in the CNS that respond to injury [12] and whose principal function is brain defense. Activated microglia participate in inflammatory processes linked to neurodegeneration by producing neurotoxic factors including quinolinic acid, superoxide anions, matrix metalloproteinases (MMP), nitric oxide, arachidonic acid and its metabolites, chemokines, pro-inflammatory cytokines and excitotoxins including glutamate. On the other hand, neuroprotective functions of microglia may be mediated through their abilities to produce neurotrophins and to scavenge and eliminate excitotoxins present in the extracellular spaces [13]. Indeed, neuronal survival after brain injury is known to be positively affected by microglial activities [14,15].

Thus, during PD-associated neurodegeneration, a spectrum of environmental cues and more notably, aggregated or misfolded proteins are known to evolve in many locations within and outside of the CNS (e.g. autonomic nervous and gastrointestinal systems) and form as a result of disease progression, which likely affect glial function, serving to accelerate the tempo of neurotoxic processes. These lead to neuronal excitotoxicity, synaptic dysfunction, and cell death (apoptosis and/or necrosis). Whether the environmental cues are dysregulated proteins or exogenous toxic/metabolic events, the inevitable amplification of primary disease processes results in the disruption of CNS homeostasis. In all, microglia can affect the evolving stages of neurodegeneration. This review articulates specific features of the inflammation that occur in response to or as part of the ongoing PD process.

2. Microglial cells: structure and function in health and disease

Microglia are bone marrow-derived macrophage-lineage cells that enter the brain early during embryogenesis and develop in parallel with the maturation of the nervous system. They are the resident phagocytes of the CNS and can react promptly in response to brain insults of various natures, ranging from pathogens to aggregated proteins and to more subtle alterations in their micro-environment such as alterations in ion homeostasis that can affect pathological processes [12]. In the normal brain, microglial cells are in a resting state as shown in Figure 1A; their cell bodies barely visible and only few fine ramified processes are detectable. However, in pathological settings (Figure 1B), resting microglial cells quickly proliferate, become hypertrophic, and increase or express *de novo* a large number of marker molecules such as CD11b and major histocompatibility complex (MHC) antigens while transforming to macrophage-like cells [12,16,17]. Activated microglia, now readily visible, increase their numbers at the affected site and exhibit a "spider-like" or macrophage-like appearance. Ramified microglia change appearance by means of retracted processes and enlarged cell bodies. Within the damaged area, the maximal density of activated microglia is located at the epicenter of the lesion, close to injured cells (e.g., degenerating neurons). Following activation and during tissue regeneration, microglia gradually return to a ramified morphology exhibited prior to injury or insult. While such changes are clearly implicated in neurodegenerative processes of the CNS, the innate immune system has also been tasked with alternative functions. In addition to guarding the nervous system from invading pathogens, this system is

involved in many physiological functions such as tissue remodeling during development or after damage [18,19], transportation of blood lipids [19,20], and scavenging apoptotic cells [21]. Neuroprotective responses are elicited through elimination of the ongoing infectious agents by innate immune activities and subsequently through adaptive immune functions orchestrated in the CNS by microglia and other antigen presenting cells (see below). All together MP, including macrophages and microglia, are the Dr. Jekyll and Mr. Hyde of the nervous system. In health, they support critical regulatory immune and homeostatic functions, whereas in disease their roles progress from supportive, to reactive, and ultimately to destructive. The functional transformation of brain MP from neurotrophic to neurotoxic phenotypes is believed to underlie the pathogenesis in PD.

3. Microglia and neuroinflammatory responses in PD

As noted, the key cell element in neuroinflammatory responses is the brain MP. Supporting this idea, PD is characterized by activation of microglial cells found in and around degenerating neurons [22-26]. Evidence for a neuroinflammatory role in disease onset and progression is significant and profound from several independent lines of investigation [26-31]. *First*, reactive microglia are commonly seen within the SNpc of PD brains investigated at autopsy [22,25, 26]. A six-fold increase in numbers of reactive microglia has been shown phagocytosing dopaminergic neurons [32] and correlates with the deposition of α -synuclein [22]. Such microglia are reactive and over-express a variety of inflammatory markers including, HLADR of the human MHC II complex [22,26], complement receptor type 3 (CR3, Cd11b/CD18, Mac-1, Mo 1) [17,33], CD68 (EMB11) [17,22], CD23 (Fc receptor for IgE) [31], ferritin [33], CD11a (LFA-1) and CD54 (ICAM-1) [34]. These reactive microglia are functionally active and secrete a plethora of proinflammatory cytokines such as interferon- γ (IFN- γ) tumor necrosis factor- α (TNF- α) [30,31], interleukin 1- β (IL-1 β) [31], and upregulate enzymes such as inducible nitric oxide synthase (iNOS) [31,35], and cyclooxygenase (COX) 1 and 2 [23, 35]. Although the SN is relatively rich in microglia when compared to other brain regions [36], the total number of MHC class II positive microglia are also significantly increased in the putamen, hippocampus, transentorhinal cortex, cingulate cortex and temporal cortex of the PD brain [34]. *Second*, microglia activation is strongly associated with dopaminergic neuronal cell death during PD, suggesting that reactive microglia may be a sensitive biomarker for disease. Indeed, reactive microglia serve as *in vivo* indicators of neuroinflammatory responses and contribute significantly to progressive degenerative processes. This is supported by early-stage PD imaging, where PK11195 binding to peripheral benzodiazepine receptors present on reactive midbrain microglia inversely correlates with binding of 2-beta-carbomethoxy-3beta-(4-fluorophenyl) tropane (CFT) to the dopamine transporter (DAT) in the putamen as a measure of surviving dopaminergic termini. These observations also correlate with the severity of motor impairment [22]. *Third*, epidemiological data demonstrates that the use of nonsteroidal anti-inflammatory agents decreases the risk of PD [37]. *Fourth*, biochemical and histological evidence for oxidative stress in PD abounds and includes increased levels of carbonyl and nitrotyrosine protein modifications, lipid peroxidation, DNA damage, and reduction of glutathione and ferritin [38]. Indeed, nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, a primary producer of reactive oxygen species (ROS), is upregulated in PD and its expression coincides with activated microglia. Postmortem samples of SNpc from sporadic PD patients show elevated levels of the protein gp91^{phox} [39], the main transmembrane component of NADPH-oxidase [40], which co-localizes with microglia. Likewise, in 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-treated mice, large increases in gp91^{phox} immunoreactivity also co-localize in the SNpc with activated (Mac-1 immunopositive) microglia, but not with astrocytes or neurons [39]. Thus, microglia in the vicinity of dopaminergic neurons in disease appear to have an upregulated capacity for ROS production, consistent with an activated state leading to a continuous cycle of neuronal injury and neuroimmune activation. *Fifth*, a robust microglial response occurs in the midbrains of

MPTP-intoxicated animals [41], one of the foremost model systems for dopaminergic neurodegeneration in PD. Studies of post-mortem brains from three human subjects who injected MPTP and developed parkinsonian syndromes [42], demonstrated accumulations of activated microglial cells around dopaminergic neurons [43]. Thus, the initial acute insult to dopaminergic neurons likely leads to a secondary and perpetuated neuroinflammatory response. This neuroinflammatory reaction, serves to alter homeostatic neural mechanisms or to exacerbate disease processes by the production of proinflammatory factors.

4. Adaptive Immunity

While naïve T cells are precluded from CNS entry, neuroinflammation aggressively recruits activated components of the adaptive immune system to sites of active neurodegeneration by increasing expression of cellular adhesion molecules and inducing chemokine gradients [44]. Moreover, glial cells secrete toxic factors that disrupt blood brain barrier function. Nonetheless, much evidence indicates a far more complex relationship between the CNS and immunological systems than previously thought. For example, immune molecules such as Thy-1, interleukins, and chemokines are expressed at high levels by neurons and surrounding glia and may be involved in direct communication between the CNS and immune cells [45]. Signaling between neurons and glia during neuronal injury incite inflammatory responses and leukocyte migration [44]. Interestingly, molecules mediating specific antigen recognition and signaling by T lymphocytes, including MHC class I and CD3 ζ molecules, also have a role in axonal guidance, activity-dependent remodeling, and plasticity in the developing and mature mammalian CNS [46]. Within the neuronal synapse, MHC class I molecules may participate in the refinement or elimination of synaptic connections. The cognate receptor for MHC class I antigen peptide complexes is the T cell receptor (TCR) for antigen, expressed by T cells after a complex program of rearrangement. It was recently determined that neurons, particularly in the developing neonatal CNS, express mRNA transcripts for unrearranged β -chain subunit of the TCR [47,48]. Functional cooperation between these two molecules in neuronal populations has yet to be determined. Interestingly, during inflammatory states, MHC class I molecules are up-regulated on neuronal surfaces; however no direct evidence of cytotoxic T lymphocyte (CTL) mediated neuronal damage has been determined in common neurodegenerative disorders [49,50]. However, MHC class I molecules alone are unstable and must associate with proteasome-derived peptides and β 2-microglobulin to form stable complexes at the cell surface. Altered peptide profiles presented at the neuronal synapse during neuronal degeneration may, therefore also affect neuronal plasticity and remodeling during disease states.

Further challenging this view of the “immune privileged” status of the CNS are animal model systems wherein immune deficiencies translate into exacerbated neuronal loss following traumatic injuries [51-54]. Such injuries are corrected in animals that receive immune reconstitution prior to experimental injury. Rodents and humans that have sustained CNS injuries also have expanded T cell repertoires against myelin-associated antigens, yet do not appear to be at increased risk for the development of CNS autoimmunity. Any functional consequence of such T cell responses against CNS antigens following injury remains to be determined.

CD8⁺ T cells have been reported in close proximity to activated microglia and degenerating neurons within the SN of PD patients; however, those numbers are consistently low in frequency [26]. Whether these T cells are activated, antigen-specific or migrating in response to microglial inflammation has yet to be determined; however the presence of a major T cell subset in ratios exceeding those typically found in the periphery suggests a more profound role in PD than merely performing a surveillance function [12,55-57]. Numerous aberrations in peripheral lymphocyte subsets have also been detected in PD patients [58-60]. In both drug-

naïve and treated PD patient cohorts compared to age-matched controls, numbers of total lymphocytes were shown to be diminished by 17%, while CD19⁺ B cells were diminished by 35% and CD3⁺ T cells were diminished by 22% [58]. Among CD3⁺ T cells, numbers of CD4⁺ T cells were diminished by 31%; whereas, numbers of CD8⁺ T cells were not significantly changed. The frequencies of cells within CD4⁺ T cell subsets are differentially diminished, with a greater loss of naïve helper T cells (CD45RA⁺) and either unchanged or increased effector/memory helper T cell subset (CD29⁺ or CD45RO⁺) [58,60]. Increased mutual co-expression of CD4 and CD8 by CD45RO⁺ T cells [59] as well as upregulation of CD25 (α -chain of the IL-2 receptor) [58], TNF- α receptors [61], and significant downregulation of IFN- γ receptors [62,63] indicate that at least some T cell subsets from PD patients are activated; however, evaluation of these parameters to assess whether activated T cell phenotypes are derived from any one T cell subset or many subsets have yet to be incorporated into one study. Interestingly, a significantly greater number of micronuclei and unrepaired single strand DNA breaks, which have been shown to result from exposure to higher levels of ROS and inflammation [64], are detected in lymphocytes and activated T cells from PD patients compared to age-matched controls [65,66].

5. Pathways and mechanisms for neuroinflammation

Cytokines

As microglia and peripheral macrophages share the same cell surface markers, it is difficult to distinguish the cell types in postmortem PD brain tissues. Lipopolysaccharide (LPS) stimulated peripheral macrophages from PD patients produce less TNF- α , IL-1 α/β , IFN- γ , IL-6 than healthy controls and their levels correlate inversely with disability, thus suggesting that impaired cytokine production may progress with disease [66]. In contrast levels of several cytokines, including TNF α , IL-1 β , IL-3 and IL-6 are increased in the postmortem striatum, SN and cerebral spinal fluid (CSF) of PD patients [29,67-70] and elevated levels of TNF- α receptor R1 (TNF-R1, p55), bcl-2, soluble Fas (sFas), caspase-1 and caspase-3 [29] support the existence of a proinflammatory/apoptotic microenvironment in PD patients. However, other regulatory cytokines, including IL-4, transforming growth factor (TGF)- α , TGF- β 1, and TGF- β 2 are also increased [29], which may indicate an attempt to regulate the predominantly proinflammatory environment. Additionally, hippocampal tissues from PD patients bind increased levels of IL-2 compared to controls indicating that IL-2 receptors (IL-2R) on cells contained within the hippocampus are also upregulated in PD patients [71]. Although likely expressed by both neuronal and glial cells, the localization of IL-2 and IL-2R primarily to the frontal cortex, septum, striatum, hippocampal formation, hypothalamus, locus coeruleus, cerebellum, and the pituitary and fiber tracts of the corpus callosum suggests possible regulatory interactions between peripheral tissues and the CNS [72]. Most likely, IL-2 acts in an auto- and paracrine fashion in the brain as in the peripheral immune system, but exhibits characteristics of a neuroendocrine modulator under different physiological conditions. For instance, IL-2 regulates neuronal and glial growth and differentiation during development, but has pleiotropic effects in the mature brain being involved in modulation of sleep/arousal, memory and cognition, locomotion, and neuroendocrine activities [72].

Reactive molecular species generation, oxidative stress, and nigrostriatal degeneration

As previously indicated, the innate arm of the immune system, comprised primarily of myeloid-derived MP (neutrophils, dendritic cells, macrophages, and microglia) represents the host's primary line of defense to foreign microorganisms. Once activated, they can produce noxious factors including pro-inflammatory cytokines, chemokines, quinolinic acid, arachidonic acid and its metabolites, and excitatory amino acids among others. A major defense mechanism provided by MP is the production of free radicals and reactive molecular species; all potentially toxic to invading organisms. The cellular machinery of myeloid lineage cells that has evolved

to produce these toxic products is NADPH oxidase (expressed by microglia, macrophages and neutrophils) and myeloperoxidase (expressed predominately by neutrophils). Among neurons, astrocytes and microglia of the CNS, the microglia are, in large measure, responsible for generating free radicals (Figures 1 and 2) [73]. Microglia possess the NADPH oxidase complex, which when assembled and activated, produces free radicals in abundance on the external cell surface; which can lead to tissue damage (Figure 2) [74]. Another producer of these destructive oxidants is accredited to mitochondrial metabolism [75]. Within the inner membrane of the mitochondria, protein complexes of the electron transport chains are responsible for the transfer of electrons in a process known as oxidative phosphorylation. Most cellular ROS is generated through this process during incomplete metabolic reduction of oxygen to water. The mitochondrial electron transport chain is a major source of ROS and is estimated that up to 1% of the mitochondrial electron flow leads to the formation of superoxide radicals at ubiquinone and NADH dehydrogenase (complex I) [76,77].

Free radicals, which are highly reactive molecules or chemical species that are formed by electron transfer, react to form a series of even more reactive species, which if not neutralized lead to oxidative stress, exacerbated inflammation, and tissue damage [78]. Free radicals include ROS such as singlet oxygen ($^1\text{O}_2$), superoxide ($\text{O}_2^{\cdot-}$), hydroxyl radical ($\text{OH}^{\cdot}$), peroxy radical ($\text{ROO}^{\cdot}$) and hydrogen peroxide (H_2O_2), as well as reactive nitrogen species (RNS) such as nitric oxide (NO), and peroxynitrite ($\text{ONOO}^{\cdot}$), and reactive chlorine species (RCS) such as hypochlorous acid (HOCl) [79-82]. These chemical species are abundant in nature and are the byproducts of normal cellular metabolism, but are also abundantly found in the environment and are easily formed by excessive exposure to sunlight, pollution, alcohol, insecticides, radiation, fried foods, strenuous exercise and chemicals. Protective antioxidant enzymes and mechanisms that neutralize free radicals abound and include superoxide dismutase (SOD), catalase, glutathione, glutathione peroxidases and reductase, vitamin E and vitamin C; all free radical scavengers, which neutralize or reduce the formation of free radicals. Free radical scavengers often work by donating an electron to the free radical, and once paired with another electron, the free radical is reduced and no longer toxic to the cells. Oxidative damage occurs within cellular organisms due to an imbalance between reactive species production and cell antioxidant defenses. Interestingly, a recent study indicated that catalase attenuated microglia proliferation induced by $\text{TNF}\alpha$, $\text{IL-1}\beta$, arachidonic acid, or phorbol myristate acid (PMA) suggesting that NADPH oxidase generated H_2O_2 [83]. Moreover, oxidase-generated H_2O_2 , whether from NADPH-, xanthine-, or glucose-oxidase was sufficient to stimulate vigorous microglia proliferation demonstrating the importance of ROS to maintaining an previously initiated inflammatory response.

From the respiratory burst, as well as from dysregulated mitochondrial respiration, large amounts of ROS can be produced [84,85], which may have disastrous effects on delicate neuronal networks in the CNS. Indeed, oxidative stress is implicated as a major cause of neuronal injury in a wide range of neurological diseases including PD, however whether oxidative stress is causal or consequential is unclear. In either case, oxidative stress contributes to the cascade leading to dopamine cell degeneration in PD. Brain regions that are rich in catecholamines such as, adrenaline, noradrenaline and dopamine are exceptionally vulnerable to free radical generation [38,78]. Catecholamines particularly dopamine, can be metabolized by endogenous enzymes such as monoamine oxidases (MAO) or spontaneously break down by autooxidation to yield H_2O_2 and dopamine-quinones [86-88]. Metabolism of dopamine can therefore exacerbate inflammation and tissue damage by feeding H_2O_2 into the ROS cycle and/or by dopamine-quinone modification of protein sulfhydryl groups via nucleophilic additions [88-90]. Altered configuration of proteins including aggregation may trigger aberrant cellular processes such as oxidative phosphorylation resulting in the accumulation of reactive oxygen and nitrogen byproducts, which are typically produced by microglia and serve to destroy invading microorganisms. Reactive molecular species include superoxide, hydrogen peroxide

and hydroxyl free radicals as well as nitrogen intermediates (nitric oxide and peroxynitrite) and can cause damage to neurons if produced in excess as occurs during prolonged neuroinflammatory responses. Much of the microglial-derived ROS such as superoxide cannot efficiently traverse cellular membranes [81], making it unlikely that these extracellular ROS gain access to dopaminergic neurons and trigger intraneuronal toxic events [39]. However, superoxide can rapidly react with NO in the extracellular space to form a more stable oxidant, peroxynitrite [39], which can readily cross cell membranes and damage intracellular components in neighboring neurons. Nitrated species have been associated with the disruption of mitochondrial electron transport chain, lipid peroxidation, DNA damage, and the nitration of tyrosine residues in cellular proteins. This suggests that microglial-derived superoxide, by contributing to peroxynitrite formation, is a significant contributor to the pathogenesis of PD. NADPH-oxidase is a large multi-subunit complex and is the main enzyme known to produce ROS in activated macrophages and microglia. Moreover, genetic deletion of gp91, an essential subunit of NADPH oxidase, mitigates neuronal loss in numerous models of neurodegeneration including the MPTP model of PD [39].

NO is a biological messenger molecule that has numerous physiological roles in the CNS. In addition, NO plays an important role in innate immunity and is associated with tumoricidal and bactericidal activities of macrophages [91,92]. Three distinct forms of nitric oxide synthase (NOS) have been identified to date and are designated neuronal NOS (nNOS), inducible NOS (iNOS), and endothelial NOS (eNOS). In contrast to the physiological roles of normal NO levels, excessive NO produced under pathological settings can act as a potent neurotoxin in a number of neurodegenerative models [93-96]. For example, nNOS and iNOS are both upregulated in sporadic PD and some animal models; however, genetic ablation or pharmacological inhibition of excess NO production is neuroprotective in the MPTP model [96,97]. Although NO may generate much of its toxicity through the formation of peroxynitrite, it also reacts with sulfur containing cysteine residues in protein (S-nitrosylation), which may modify protein structural conformations or enzymatic activities [98].

Production of ROS and NO in neurons is buffered primarily by the glutathione system, which is compromised in the brain of PD patients [99-101], leading to an imbalance in redox homeostasis and consequent oxidative stress. The tripeptide glutathione (GSH; gamma-L-glutamyl-L-cysteinylglycine) is the major cellular thiol present in brain tissue, and the most important redox buffer in mammalian cells [102]. This antioxidant molecule cycles between reduced glutathione (GSH) and oxidized glutathione disulfide (GSSG) and serves as a vital sink for control of ROS levels in cells. GSH reacts directly with oxygen and nitrogen free radicals nonenzymatically and donates electrons in the enzyme-catalyzed reduction of peroxides [77,103]. Determination of the relative levels of glutathione and glutathione-related enzymes in neuronal and glial compartments is incompletely understood and remains an active area of research in our laboratories.

GSH content in the SNpc of PD patients is decreased by 40-50% but not in other regions of the brain, nor in age-matched controls or in patients with other diseases affecting dopaminergic neurons [99-101]. This diminution continues with progression and severity of disease, suggesting a correlation with concomitant increases in reactive species [99,100,104-106]. GSH depletion has been suggested as the first indicator of oxidative stress during PD progression, possibly occurring prior to other hallmarks of PD including the decreased activity of mitochondrial complex I [8,106-108]. Also, elevated GSSG/GSH ratios in PD patients [101, 109] argue strongly for a role of oxidative stress in this disease [102]. An increase in glutathione peroxidase immunoreactivity, exclusive to glial cells surrounding surviving dopaminergic neurons, has also been observed in PD brains [110]. Interestingly, the SN and striatum have lower levels of GSH relative to other regions of the brain, which include, in increasing order: SN, striatum, hippocampus, cerebellum, and cortex [111-113]. Although varying in different

regions of the brain, all GSH levels diminish by about 30% in the elderly [113], suggesting a possible link with the age associated risk factor for PD. GSH depletion cannot be explained by increased oxidation of GSH to GSSG as levels of both are diminished in the nigra of PD patients [101,113]. Diminished GSH levels do not appear to be caused from failure of GSH synthesis as γ -glutamylcysteine synthetase is unaltered, as are glutathione peroxidase and glutathione transferase activities [109]. Other possibilities of mechanisms for diminished levels include increased removal of GSH from cells by γ -glutamyltranspeptidase [109] and the formation of adducts of glutamyl and cysteinyl peptides of GSH with dopamine [114-116]. Nevertheless, depletion of GSH may render cells more sensitive to toxic effects of oxidative stress and potentiate the toxic effects of reactive microglia [117,118].

Inflammatory responses induced by reactive microglia, macrophages, and proinflammatory T cells, provide a primary source of free radicals with the capacity to modify proteins, lipids, and nucleic acids (Figure 2). This induces a condition of oxidative stress whereby the increased production of highly reactive species and decreased scavenging of free radicals results in increased modification and damage of biomolecules, and decreased clearance of those damaged macromolecules that are potentially toxic for neurons. The highly reactive nature and short half-lives of reactive species, combined with the restrictive nature of the neuroinflammatory foci to clinical sampling, preclude the direct measurement in disease processes of these reactive species. However, modifications of proteins, lipids and nucleic acids provide surrogate biomarkers, which can be directly measured as indirect assessments of the extent of oxidative stress. Postmortem analyses of PD patients have consistently demonstrated the increased presence of these biomarkers for oxidative stress. Protein modifications are among the many biomarkers detected in the brains of PD patients. Compared to brains from control donors, elevated levels of nitrated proteins are found in brains and CSF of PD patients [119,120]. Most notable are modifications of proteins that comprise Lewy bodies (LB), the neuronal inclusions that are considered the hallmarks of PD and consist primarily of α -synuclein, ubiquitin, and lipids. increased expression of 3-nitrotyrosine in LB as determined by immunoreactivity and primarily due to the presence of a nitrated form of α -synuclein, identifies peroxynitrite modifications of tyrosine moieties [121-123] suggesting an increased participation of inflammatory responses and reactive molecular species; however, whether those modifications occur before or after inclusion into LB remain unclear. Also S-nitrosylated forms of parkin, an E3 ubiquitin ligase involved in protein ubiquitination has been isolated from the temporal cortex from PD patients, but not from brains of Huntington's or AD patients [124]. *In vitro* and *in vivo*, S-nitrosylation of parkin induces an initial increase in ligase activity leading to autoubiquitination of parkin [124], eventual inhibition of ubiquitin ligase activity, and decreased activity in the E3 ligase-ubiquitin-proteasome degradative pathway [98,124]. Carbonyl modifications, which are reflective of protein oxidation, are increased by greater than 2-fold in the SN compared to the basal ganglia and prefrontal cortex of normal subjects [125]. Increases in protein carbonyls have been found in substantia nigra, basal ganglia, globus pallidus, substantia innominata, cerebellum and frontal pole, but not in patients with incidental LB disease (ILBD), a putatively presymptomatic PD disorder [126]. The involvement of the latter two brain regions are unexpected based on the restricted neuropathology of PD, but may reflect a consequence of L-DOPA treatment or a more global consequence of the inflammatory spread of oxidative stress in PD. Other evidence for oxidative damage to proteins in PD is the increased expression of neural heme oxygenase-1 [127] and increased immunostaining of glycosylated proteins by nigral neurons [128].

Free radicals and nucleic acid modifications

Modification of nucleic acids by free radicals and reactive species can induce chromosomal aberrations with a high efficiency [64], suggesting that chromosomal damage exhibited in neurons of PD patients might be related to an abnormally high oxidative stress. Among the

most promising biomarkers of oxidative damage to nucleic acids is nucleoside 8-hydroxyguanosine (8-OHG) for RNA or 8-hydroxy-2'-deoxyguanosine (8-OHdG) for DNA. 8-OHG is an oxidized base produced by free radical attack on DNA by C-8 hydroxylation of guanine and is one of the most frequent nucleic acid modifications observed under conditions of oxidative stress [129]. In PD patients, levels of 8-OHG nucleic acid modifications are commonly increased in the caudate and SN compared to age-matched controls [130-133]. Immunohistochemical characterization of these modifications indicates that the highest levels of 8-OHG modifications are found in neurons of the SN and to a lesser extent in neurons of the nucleus raphe dorsalis and oculomotor nucleus, and occasionally in glial cells [132]. Given that (1) 8-OHG nucleic acid modifications are rarely detected in the nuclear area and mostly restricted to the cytoplasm, and (2) immunoreactivity is significantly diminished by RNase or DNase and ablated with both enzymes [132], suggest that targets of oxidative attack include both cytoplasmic RNA and mitochondrial DNA. Of particular interest are the findings that concentrations of 8-OHG in CSF of PD patients are higher than in age-matched controls; however, serum concentrations of 8-OHG appear highly variable [134,135].

Lipid peroxidation

4-Hydroxy-2-nonenal (HNE) is a reactive α,β unsaturated aldehyde that is one of the major products during the oxidation of membrane lipid polyunsaturated fatty acids, and forms stable adducts with nucleophilic groups on proteins such as thiols and amines [136,137]. HNE modification of membrane proteins forms stable adducts that can be used as biomarkers of cellular damage due to oxidative stress [137]. Immunochemical staining on surviving dopaminergic nigral neurons in the midbrains of PD patients show the presence of HNE-modified proteins on 58% of the neurons compared to only 9% of those in control subjects, weak or no staining on oculomotor neurons in the same midbrain sections from PD patients [138], and the presence of HNE modified proteins in LB from PD and diffuse LB disease patients, but not age-matched controls [139]. HNE species are typically more stable than oxygen species, thus they can easily spread from site of production to effect modifications at a distant site [140]. HNE modifications of DNA, RNA, and proteins have various adverse biological effects such as interference with enzymatic reactions and induction of heat shock proteins, and are considered to be largely responsible for cytotoxic effects under conditions of oxidative stress [136,141,142]. The cytotoxic effects of HNE modifications may be founded in part due to inhibition of complexes I and II of the mitochondrial respiratory chain [143]; induction of caspase-8, -9, and -3, cleavage of poly(ADP-ribose) polymerase (PARP) with subsequent DNA fragmentation [144]; inhibition of NF- κ B mediated signaling pathways [145]; and diminution of glutathione levels [144]. Consistent with an abundance of data showing the dysregulation of proteasomal function in PD, direct binding of HNE to the proteasome also inhibits the processing of ubiquitinated proteins [146]. Concentrations that induce no acute change in cell viability *in vitro* initially cause a decrease in the proteasomal catalytic activity to the extent that it induces accumulation of ubiquitinated and nitrated proteins, reductions in glutathione levels and mitochondrial activity, and increased levels of oxidative damage to DNA, RNA, proteins, and lipids [146-148].

Another reactive aldehyde species produced from the peroxidation of lipids is malonyldialdehyde (MDA), which is formed from the breakdown of endoperoxides during the last stages of the oxidation of polyunsaturated fatty acids; particularly susceptible are those containing three or more double bonds [136,149,150]. MDA can exist as free aldehydes or react with primary amine groups of macromolecules to form adducts with cellular structures [136,151,152]. Evidence of increased levels of MDA-modified proteins in the SN [153,154] and CSF [155] of PD patients, but not in controls is indicative of increased lipid peroxidation and supports the existence of chronic inflammatory responses in those patients.

F2-isoprostane (F2-IsoP) and isofuran (IsoF) are other products of lipid peroxidation and both are well-established as specific biomarkers of *in vivo* oxidative stress [156-158]. Under conditions of relatively low oxygen tension, the F2-IsoP species is favored; whereas, under higher oxygen tensions, IsoF is heavily favored [156]. Increased F2-IsoP concentrations in affected tissues from patients of most neurodegenerative disorders have provided general support for the role of inflammation and oxidative stress in those disorders [157], but the failure to detect similar levels in tissues from PD patients was particularly perplexing [159]. However comparison of tissues for IsoF species as well as F2-IsoPs has shown that levels of IsoF, but not F2-IsoP in the SN of patients with PD and dementia with LB are significantly higher than those of controls [156]. This preferential increase in IsoF species in PD patients indicates that the microenvironmental oxygen tension is typically greater in PD than other disorders, and suggests a unique mode of oxidant injury in PD, which may be indicative of an increased intracellular oxygen tension resulting from mitochondrial dysfunction or a greater intensity of inflammatory response in PD. These data certainly indicate that oxidative stress in the SNpc region is elevated in PD, however yet to be determined is whether innate immune cell activation of microglia and/or astrocytes during the progression of PD shifts the homeostatic balance towards increased protection from or exacerbation of ROS damage, and whether this dynamic changes with disease progression.

Iron and oxidative stress

Investigators using a variety of methods have provided a consensus that iron levels naturally increase with age and are significantly increased (reported from 25% - 100%) in the SN and CSF of postmortem PD patients compared to age-matched controls [104,160-172]. Iron in its ferrous (Fe^{2+}) form catalyzes the formation of strong oxidants and ferric iron (Fe^{3+}). With disease progression, levels of Fe^{3+} increase within the SN suggesting an increased state of oxidative stress [173]. Although most of the total iron in healthy brains is stored in ferritin, and levels are typically depleted under inflammatory conditions, ferric ions are readily released after damage to neuronal tissues by yet unknown mechanisms, making those ions available for oxidative catalysis [174]. In PD, proteins such as transferrin, ferritin, and iron regulatory proteins (IRP), which control iron homeostasis, could be modified by ROS and lose their regulatory capacity. Indeed, *in vitro* and *in vivo* nitrosylation of IRP2 leads to rapid ubiquitination and degradation in the proteasome [175]. Additionally, IRP knockout mice exhibit high levels of iron and ferritin with extensive axonopathy in the white matter tracts and reactive microglia and vacuoles SN [176]. These mice also manifest motor impairments when axonopathy is prominent, however dopaminergic cell loss is minimal.

6. Genetics and Immunity

Recent evidence has shown that genetics may contribute to the onset of neurodegenerative disorders [177]. Linkages to the age at onset (AAO) for PD have been identified on chromosomes 1 and 10. The latter is significantly associated with glutathione transferase omega-1 (GSTO1) [178]; a provocative finding since GSTO1 is thought to be involved in the post-translation modification of IL-1, a major component in the regulation of inflammatory responses [179-181]. One factor associated with the chromosome 1p peak is the ELAVL4 gene [182], a human homologue of the *Drosophila* ELAV (embryonic lethal abnormal vision) [183] and essential for temporal and spatial gene expression during CNS development. Additionally, ELAVL gene products are known to bind to AU-rich response elements (ARE) in the 3'-untranslated region (3'UTR) of inflammation-associated factors [183]. Interestingly, PD patients homozygotic for allele 1 at position -511 of the IL-1 β gene have an earlier onset of the disease than those homozygotic for allele 2, which produces higher amounts of IL-1. Thus, higher production of IL-1 β might provide some neuroprotective effect for dopaminergic neurons [184,185].

The generalized toxicity of these inflammatory responses provides very little insight into the selective neurodegeneration patterns observed in various disease states. However, it is tempting to speculate that the shared phenotype of multiple genetic mutations identified in familial forms of PD suggests the dysregulation of a common pathway may be involved. Consistent with aberrant protein accumulation in PD, malfunction of the ubiquitin-proteasome system appears to be a common link in these familial forms of PD. Indeed, many of the genes identified are linked to protein misfolding and/or degradation pathways [11,186]. While these genetic mutations offer insight into common pathways involved in familial forms of PD, the information they offer for sporadic forms of the disease in individuals who lack these genetic lesions are not completely understood. Interestingly, recent data suggests that some of these PD associated genes are active targets of reactive nitrogen and oxygen species both of which are generated during chronic inflammation.

In keeping with this notion, three missense mutations (A53T, A30P, and E46K) have been identified in the gene encoding α -synuclein leading to an autosomal dominant inheritance of PD. Moreover, genomic triplication of the α -synuclein gene is associated with familial PD [187]. Transgenic overexpression of wild-type or mutant forms of α -synuclein in mice produces intraneuronal aggregates, but little if any nigral neurodegeneration [188], while overexpression of mutant forms in *Drosophila* results in both aggregate formation as well as dopaminergic neuronal cell death [189]. In sporadic PD, recent studies support a role for oxidative and/or nitrative stress in α -synuclein modification and aggregation [122]. Nitrating agents such as peroxynitrite can readily nitrate α -synuclein at tyrosine residues, and generate highly stable *o,o'*-dityrosine oligomers (Figure 2). These biochemical lesions enhance fibril formation *in vitro*, similar to the biophysical properties of α -synuclein isolated from PD brains [190]. Aberrant protein conformations of modified α -synuclein can potentially overload cellular proteasome and by doing so, may increase cellular stress associated with the accumulation of misfolded proteins in affected neurons [191].

Parkin is another gene associated with familial PD whose protein product may be a target of nitrosative stress-associated protein modifications. Parkin is an ubiquitin E3 ligase responsible for the addition of ubiquitin to protein substrates, including α -synuclein and its interacting protein, synphilin-1, that are marked for degradation by cellular proteasomes [192]. Over expression of parkin in α -synuclein transgenic flies rescues neurons from degeneration [193]. Mutations in parkin result in the loss of ubiquitin E3 ligase activity and are found in juvenile PD in an autosomal recessive fashion [194]. Posttranslational modifications such as S-nitrosylation of parkin also abolishes its E3 ligase activity and inhibits the ability to rescue cells from α -synuclein/synphilin co-expression in the presence of proteasome inhibition (Figure 2) [98,124]. Nitrosylation modifications on parkin were found in sporadic cases of PD in affected brain regions, as well as in both MPTP and rotenone animal models. Animal studies reveal that nitrosylation of parkin is dependent on both nNOS, as well as microglial-derived iNOS [98]. Thus, it is conceivable that inflammation contributes to oxidative modifications in parkin, which in turn predispose affected neurons to cytotoxic stress caused by altered protein catabolism.

7. Experimental Models of PD: Neuroinflammation and Disease

The prevalence of reactive microglia and biomarkers of inflammatory responses in PD necessitates the inclusion of an inflammatory component in most models of PD. Although reactive microglia in PD may have an initial function to scavenge dead or dying neurons after the primary etiological event, evidence of a more adverse role in neuroinflammation and neurodegeneration emerges from animal models of PD. Several models of PD exist that induce significant inflammatory responses as evidenced by reactive microglia and degeneration of dopaminergic neurons along the nigrostriatal axis, and include 1-methyl-4-phenyl-1,2,5,6-

tetrahydropyridine (MPTP), 6-hydroxydopamine (6-OHDA), rotenone, paraquat, LPS, and trisialoganglioside GT1b [96,195-202].

Arguably, of great importance among the compounds used to model PD is MPTP, the only agent reported to have dopaminergic effects in humans. MPTP is a neurotoxin that was discovered after induction of irreversible parkinsonian syndrome in addicts following injection of MPTP as a contaminant of illicitly and poorly synthesized meperidine [42,43]. Postmortem examination of several patients ranging from 3 to 16 years post-exposure and onset of parkinsonism, revealed not only evidence of progressive neurodegeneration, but also reactive microglial clusters surrounding nerve cells. This ongoing inflammatory reaction years after the original toxic exposure supports the notion of a self-perpetuating process of neurodegeneration mediated by localized inflammatory processes within the nigrostriatal axis. However, that many of these patients self-administered drugs both before and after MPTP exposure, and that only a few were affected from an estimated 300 individuals exposed to MPTP, warrants caution about extrapolating these data to extremes.

Among several animal models of PD, MPTP can reproduce many of the characteristics of the disease when administered to mice [203] and primates [41]. MPTP is converted to 1-methyl-4-phenylpyridinium (MPP⁺) in astrocytes, which is taken up by dopaminergic neurons where it inhibits mitochondrial electron transport complex I, resulting in decreased ATP production and cell death. This toxin has proven to be valuable for the study of PD pathology, both in murine and primate animal models and *in vitro* culture systems. MPTP induces peak microglia activation within 2 days after acute MPTP intoxication and produces a proinflammatory environment in the substantia nigra and striatum with predominant production of TNF α , IL-1 β , and IL-6, and upregulation of iNOS, COX-2, and MMP-9 [23,31,204-208]. In addition to increased reactive microglia in the MPTP model, a minor, but consistent T cell infiltrate occurs soon after MPTP treatment, but before peak neuronal loss, and is comprised mostly of CD8⁺ T cells with fewer CD4⁺ T cells [195,196]. These T cells express LFA-1 and CD44 suggesting they are an effector/memory phenotype and may be activated.

However, MPTP is not the only valid model of human PD. Intrastriatal injection of 6-OHDA induces increased numbers of reactive microglia in striatum and SN, as evidenced by increased expression of MHC II, Mac-1 and peripheral benzodiazepine receptors by day 1 after exposure, which peak after 6 to 10 days post injection, and gradually resolve 20-30 days thereafter [197,209-211]. Proinflammatory cytokines are also implicated in 6-OHDA-induced neurodegeneration as levels of TNF- α are elevated in striatum and CSF of treated rats [67]. Signs of inflammation remain after one month post-intoxication as shown by significantly increased levels of mRNA for IL-1 α and IL-1 β in lesioned tissues, however significant amounts of those cytokine proteins have not been demonstrated [211] suggesting a role for post-transcriptional regulation in regulation of the inflammatory response.

Rotenone is a lipophilic herbicide that causes a chronic, systemic defect of mitochondrial complex 1 and release of superoxide free radicals, inducing selective degeneration of nigrostriatal dopaminergic neurons along the nigrostriatal axis and leading to hypokinesia and rigidity [198,199,212]. However, behavioral abnormalities occur even in the absence of detectable dopaminergic neurodegeneration, suggesting that other systems may be affected by rotenone [213,214]. Additionally, neurons from treated rats accumulate fibrillary inclusions comprised of ubiquitin and α -synuclein. Rotenone induces a prominent inflammatory response of Mac-1⁺ reactive microglia in the striatum and substantia nigra, even in the absence of detectable dopaminergic neurodegeneration [199,213].

Paraquat (PQ, 1,1'-dimethyl-4,4'-bipyridinium) is a herbicide that induces selective degeneration of dopaminergic neurons along the nigrostriatal axis [215-219]; thus PQ exposure

is implicated as a putative risk factor for PD [217]. PQ induces nigral astrogliosis and microgliosis; the latter showing a reactive phenotype with increased numbers of Mac-1 immunoreactive cells [217,220]. Co-culture with microglia is necessary to induce PQ-mediated degeneration of dopaminergic neurons *in vitro* [221]. Additionally, PQ mediates the accumulation of α -synuclein inclusions and 4-HNE modifications by nigral dopaminergic neurons [218,219], suggesting increased oxidative stress may contribute to proteasomal dysregulation.

To assess the role of microglial inflammation on dopaminergic neurodegeneration, known inducers of inflammation have been introduced intrastrially. Of those, LPS is a most potent activator of microglia. Injection of LPS into the nigrostriatal area induces a strong reactive microglial response that precedes a delayed and progressive dopaminergic neuronal loss along that axis [198,200,201,222], whereas injection into other brain regions, such as the hippocampus or cortex, has no detectable deleterious effect on neurons in those areas [201]. Injection of LPS between the substantia nigra and ventral tegmental area (VTA) affects only those neuronal bodies within the SN as similarly observed with dopaminergic-specific neurotoxins. Progression of neurodegeneration without an overt neurotoxin, but in the presence of LPS-induced reactive microglia suggests that reactive microglia are a primary neurodegenerative agent for dopaminergic neurons. LPS-induced neuronal death is subsequent to upregulation by nigral microglia of iNOS, TNF α , and IL-1 β , and increased production of NO and superoxide [200,201,222-225]. While LPS does not induce any detectable adverse effects to purified dopaminergic neurons *in vitro*, the presence of reactive microglia, but not astrocytes, is essential for LPS-induced neurodegeneration [200,226,227]. Inhibition of LPS binding to its cognate receptor inhibits activation of microglia, subsequent production and release of all proinflammatory factors and protection of dopaminergic neurons in culture [223]. Interestingly, inhibition of proinflammatory cytokines by neutralizing antibodies is also neuroprotective [225]. Thus, the salient features of these models are that prominent inflammatory responses precede a progressive dopaminergic neuronal degeneration, and a critical role for microglia and the products of inflammation in dopaminergic neurodegeneration exists.

8. Inhibition of Inflammation in PD and Experimental Models

Various sources of evidence suggest that inflammatory changes in the parkinsonian brain, rather than being mere secondary scavenging effects, may participate more actively in the neurodegenerative processes. Of greatest interest is the finding in a large cohort of health care professionals, that daily nonsteroidal anti-inflammatory drugs (NSAIDs) reduces the risk for PD by 45% compared to those that did not routinely take NSAIDs [37]. Additionally, evidence for the role of neuroinflammation is provided in several intoxicant models of neuroinflammation; whereby, attenuation of the inflammatory component protects subsequent dopaminergic neurodegeneration along the nigrostriatal axis.

As an inducible proinflammatory enzyme, iNOS is thought to play a major role in dopaminergic neurodegeneration. Ablation by genetic manipulation or inhibition with specific pharmaceutical agents protects nigral neurodegeneration induced by MPTP [96,228,229], LPS [222] or 6-OHDA [197], but is less active at protecting striatal termini [96,228]. Interesting, not all microglia express iNOS and inhibition of iNOS does not attenuate all reactive microglia suggesting that only a subpopulation of reactive microglia may participate in neurodegeneration [96,228].

Minocycline is a long-acting second generation tetracycline shown to have a high capability to penetrate the brain parenchyma and CSF. Minocycline acts on activated microglia to prevent upregulation of iNOS, inhibit phosphorylation of p38 mitogen-activated protein (MAP) kinase,

and reduce IL-1 β converting enzyme (ICE) and IL-1 β production [230-236]. In the MPTP model, the effects of minocycline have a combined effect to reduce reactive microglia and inhibit neurodegeneration of the dopaminergic neuronal bodies of the nigra as well as the termini in the striatum in a dose-dependent fashion, but does not effect the conversion of MPTP by astrocytes [197,233,234]. Similarly, in 6-OHDA treated animals, minocycline reduces the number of reactive microglia and protects dopaminergic neurons in the SN [197].

Similarly ablation or inhibition of COX-2, the rate-limiting enzyme in prostaglandin E2 synthesis markedly diminishes dopaminergic neurodegeneration along the nigrostriatal axis after treatment with MPTP [23,237-241], or 6-OHDA [242]. *In vitro* data shows that inhibition of COX-2 is more efficacious in 6-OHDA-induced toxicity compared to that induced with MPP⁺ suggesting that MPTP-induced dopaminergic neurodegeneration may be COX independent [243]. Indeed, in MPTP/MPP⁺ induced toxicity, COX-2 inhibition does not entirely attenuate microglia activation, but rather prevents the formation of reactive oxygen/nitrogen species [23,244].

On a more general level, MMPs are a class of extracellular soluble or membrane bound cysteine proteases involved in remodeling of the extracellular matrix and are regulated by tissue inhibitors of metalloproteinases (TIMPs). Both classes of proteins have been implicated in a range of neurodegenerative disease including HAD, AD, PD and stroke. Indeed, consistent with the possibility that alterations in MMPs/TIMPs may contribute to disease pathogenesis, samples from PD patients show levels of MMP-2, expressed primarily by microglia and astrocytes that are significantly reduced in the SN compared to age-matched controls, but remain unchanged in cortex and hippocampus [245]. Gu and colleagues reported that S-nitrosylation of N-terminal cysteine residues within proMMP-9 leads to the subsequent activation of MMP-9 protease activity, which identifies an extracellular proteolysis mechanism putatively involved in neuronal cell death in which S-nitrosylation activates MMPs [246]. Additionally, an increase in MMP-9 expression has been determined in the MPTP model and pharmacological inhibition of MMP-9 was neuroprotective [207].

9. Therapeutic Immunoregulation

To establish a disease diagnosis at earlier stages, as well as designing rational therapeutic modalities for this disease, efforts have been made in recent years to identify the neuropathological, biochemical, and genetic biomarkers of PD. α -Synuclein-containing LB and altered DAT imaging for PD are the most eminent biomarkers. Several potential markers of oxidative stress such as malondialdehyde, superoxide radicals, the coenzyme Q10 redox ratio, and 8-OHG from RNA oxidation have been measured in blood and the levels of these markers tend to be higher in PD compared with control groups [247]. Thus, therapeutic approaches to PD may target a number of factors that play a role in disease onset, inflammation and neurodegenerative progression

Studies involving pro-apoptotic proteins in PD animal models indicate that their suppression may lead to decreased rates of neuronal loss. Fas, a member of the TNF receptor family, shows pro-apoptotic and inflammatory functions, and is upregulated in the SNpc of both PD patients and MPTP mouse models [248]. However, Fas blockade with dominant-negative c-Jun adenovirus indicates that Fas deficiency does not significantly prevent the reduction of dopaminergic terminal fibers within the striatum or attenuate the activation of striatal microglia [248]. Numerous studies have demonstrated that Bax is a pro-apoptotic factor required for the programmed death of several types of neurons in the peripheral and central nervous systems [249]. Bax is upregulated in the SNpc of MPTP mice, and its ablation alleviates SNpc neuronal apoptosis, indicating that targeting Bax may provide a protective benefit in PD [250].

The role of neurotrophins in reducing neurodegeneration and promotion of neuroregenerative processes presents an exciting possibility for therapeutic benefit to PD. A study of lentiviral delivery of glial cell line-derived neurotrophic factor (GDNF) showed trophic effects on degenerating nigrostriatal neurons in a primate model of PD [251]. Results indicated augmented dopaminergic function in aged monkeys and reversal of functional deficits with complete prevention of nigrostriatal degeneration in MPTP-treated monkeys. These data indicate that GDNF delivery using a lentiviral vector system can prevent nigrostriatal degeneration and potentially induce regeneration in primate models of PD, showing the potential for a viable therapeutic strategy for PD patients. However, recent clinical trials of intraputamenally infused GDNF in PD patients are controversial with one 2-year phase I trial showing improved activity scores and no untoward effects in a limited cohort [252], while phase II trials were halted after six months due to lack of efficacy and adverse effects in patients and nonhuman primates.

Immune suppression through receptor modulation has been another approach attempting to alleviate or reverse PD progression. For example, agonists of peroxisome proliferator-activated receptor- γ (PPAR- γ), a nuclear receptor involved in carbohydrate and lipid metabolism, have been shown to inhibit inflammatory responses in a variety of cell lines, including monocyte/macrophages and microglial cells [253]. *In vivo* administration of PPAR- γ agonists modulate inflammatory responses in the brain. Pioglitazone, a PPAR- γ agonist used currently as an anti-diabetic agent, has been shown to have anti-inflammatory effects in animal models of autoimmune disease, attenuate glial activation, and inhibit dopaminergic cell loss in the SN of MPTP treated mice [253]. However, pioglitazone treatment had little effect on MPTP-induced changes in the striatum. This result seems to indicate that in the MPTP mouse model of PD, mechanisms regulating glial activation in the dopaminergic terminals compared with the dopaminergic cell bodies are PPAR- γ independent [253].

Another potential therapeutic avenue for PD may involve T cell mediated immune responses. Activation of T cells directed against antigens expressed at the injured areas of the CNS has been shown to be neuroprotective under acute and chronic neurodegenerative conditions [254-256]. However, immunization with such antigens might lead to development of an autoimmune disease. Immunization with Copolymer-1 (Cop-1, glatiramer acetate) or passive transfer of Cop-1 specific T cells has been shown to be beneficial for protecting neurons from secondary degeneration after injurious conditions [257]. Cop-1 reactive T cells have partial cross-reactivity with myelin basic protein (MBP) and other self-antigens expressed in the brain [258]. Therefore, immunization with Cop-1 leads to increased accumulation of T lymphocytes in areas of injury within the brain and spinal cord and is neuroprotective without causing any adverse effects; however, the molecular mechanism of this response is not fully understood. T cells reactive to Cop-1 could be a source of brain-derived neurotrophic factor (BDNF) and other neurotrophic factors [257] or can induce production of neurotrophins by microglial or astroglial cells.

Recently, the neuroprotective effect of immunization with Cop-1 was tested in the MPTP model of PD and demonstrated that adoptive transfer of Cop-1-specific T cells, but not ovalbumin-specific T cells, into MPTP-intoxicated mice attenuates reactive microglia neuroinflammation and inhibits dopaminergic neurodegeneration in both the SNpc and the striatum [259]. Additionally, by determination with quantitative proton magnetic resonance spectroscopic imaging (^1H MRSI), adoptive transfer of those T cells protect the loss of nigral N-acetylaspartate (NAA) levels associated with MPTP-induced neurodegeneration [260]. More recent advances in diffusion tensor imaging (DTI) afford greater sensitivities for detecting neuronal loss demonstrating the utility of DTI as a useful translational tool for detecting the loss of dopaminergic neurons and non-invasive monitoring of experimental therapies (Figure 3). Suppression of microglial-associated inflammation was associated with T cell accumulation

within the SNpc, induction of a TH₂ phenotypic T cell response with production of anti-inflammatory cytokines (IL-4, IL-10), and increased expression of GDNF by astrocytes, but not by infiltrating T cells or microglia [259]. These data suggest a putative mechanism for which regulatory T cells, induced by vaccination with cross-reactive epitopes, extravasate in response to neuroinflammation from neurodegenerative processes; secrete anti-inflammatory cytokines in response to cross-reactive self-epitopes (e.g. myelin basic protein) to attenuate reactive microglia; suppress the inflammatory response; induce a neurotrophic response by T cells and/or other glia, which can interdict ensuing neurodegenerative processes (Figure 4). This therapeutic vaccine approach using Cop-1 represents a potential interdictory modality for slowing or halting the progression of neuroinflammation and secondary neurodegeneration, and could be considered in strategies with other anti-inflammatory or anti-oxidant therapies for a combinatorial modality to protect against neuroinflammation and consequent neurodegeneration in PD.

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Figure 1.

Brain mononuclear phagocytes (MP; perivascular macrophages and microglia) in nervous system health and disease. (A, top panel) Under steady state conditions, microglia secrete neurotrophic factors and engage other glial elements to promote tissue homeostasis. (B, bottom panel) During disease states (for example, Parkinson's and Alzheimer's disease), MP inflammatory responses damage the BBB, increase oxidative stress and release pro-inflammatory and pro-apoptotic cytokines and other neurotoxic factors that affect neuronal damage or dropout. The damage and stress signals enhance microglial activation, resulting in positive feedback in the release of chemokines and cytotoxic cytokines that cause further ingress of immune cells into the brain and expand inflammatory responses.

Figure 2.

Neuroinflammatory and oxidative stress pathways in PD pathogenesis. Free radicals can arise several diverse ways, such as glial cell activation, mitochondrial dysfunction and protein aggregation. Microglial derived NO and superoxide species react in extracellular spaces to form peroxynitrite. Peroxynitrite readily crosses cell membranes where it contributes to lipid peroxidation, DNA damage and nitrotyrosine formation in α -synuclein and other cellular proteins. Damaged proteins are targeted to cellular proteasomes for degradation via the ubiquitination pathway. Excess NO produced by activated microglia can lead to S-nitrosylation of cellular proteins, including parkin. Such modifications may diminish E3 ubiquitin ligase activity necessary for efficient protein turnover by proteasomes. Excessive protein damage caused by oxidants and disruptions in the ubiquitin pathways may overload or inhibit protein degradation quality control measures leading to the accumulation of damaged proteins in cells. When reactive species exceed anti-oxidant defenses, oxidative stress is generated; destroying molecular structures, such as proteins, lipids and DNA, causing irreversible and detrimental damage, neuronal cell injury and death. Adapted from Gao et al. [261].

Figure 3.

Diffusion tensor imaging of brain in a mouse model of PD. (A) Color encoding of the direction of the primary eigenvalue of the diffusion tensor is used to identify anatomical regions for analysis. (B) Results of quantitation of apparent diffusion coefficient of water (ADC_w) and fractional anisotropy (FA) in striatum, corpus callosum, and substantia nigra in mouse brain before and after treatment with MPTP. No significant differences were found in the striatum and corpus callosum. Substantia nigra from mice treated with MPTP exhibited a significant increase in ADC_w ($P = 0.036$) and decrease in FA ($P = 0.002$).

Figure 4. Cop-1 induced T cell-mediated neuroprotection in a PD model. In MPTP-intoxicated mice, regulatory T cells infiltrate the inflamed nigrostriatal pathway where they encounter cross-reactive self-antigens (myelin basic protein) presented in the context of MHC by resident microglial cells. Activated T cells secrete anti-inflammatory cytokines such as IL-4, IL-10, and TGF- β that suppress toxic microglial activities. Neurotrophin expression may occur directly from T cells or T cell derived IL-4 and IL-10 may induce neurotrophin production in neighboring glia. These activities lead to neuroprotection indirectly by suppression of microglial responses and directly through the local delivery of neurotrophins.