HHS Public Access

Author manuscript

Prog Neurobiol. Author manuscript; available in PMC 2018 August 01.

Published in final edited form as:

Prog Neurobiol. 2017 August ; 155: 57–75. doi:10.1016/j.pneurobio.2016.04.006.

Microglial phenotypes in Parkinson's disease and animal models of the disease

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Abstract

Over the last decade the important concept has emerged that microglia, similar to other tissue macrophages, assume different phenotypes and serve several effector functions, generating the theory that activated microglia can be organized by their pro-inflammatory or anti-inflammatory and repairing functions. Importantly, microglia exist in a heterogenous population and their phenotypes are not permanently polarized into two categories; they exist along a continuum where they acquire different profiles based on their local environment. In Parkinson's disease (PD), neuroinflammation and microglia activation are considered neuropathological hallmarks, however their precise role in relation to disease progression is not clear, yet represent a critical challenge in the search of disease-modifying strategies. This review will critically address current knowledge on the activation states of microglia as well as microglial phenotypes found in PD and in animal models of PD, focusing on the expression of surface molecules as well as pro-inflammatory and anti-inflammatory cytokine production during the disease process. While human studies have reported an elevation of both pro- or anti-inflammatory markers in the serum and CSF of PD patients, animal models have provided insights on dynamic changes of microglia phenotypes in relation to disease progression especially prior to the development of motor deficits. We also review recent evidence of malfunction at multiple steps of NFκB signaling that may have a causal interrelationship with pathological microglia activation in animal models of PD. Finally, we discuss the immune-modifying strategies that have been explored regarding mechanisms of chronic microglial activation.

Keywords

microglia; neurodegeneration; neuroinflammation; Parkinson's; immune; neuroprotection

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1. Introduction

The pathological hallmarks of Parkinson's disease (PD) are a loss of dopaminergic (DA) neurons in the substantia nigra pars compacta (SN) and the presence of proteinaceous inclusions termed Lewy bodies and neurites. In addition, numerous studies have highlighted a potential role for neuroinflammation in PD, the hallmark of which is microgliosis (Tansey and Goldberg, 2010). Microglia are the resident immune cells in the brain derived from progenitors originating in the yolk sac during embryogenesis (Alliot et al., 1999). They serve various functions that include eliminating and remodeling synapses during development, surveying the environment to maintain homeostasis, and responding to injury to limit tissue damage, initiate repair processes, and promote neuronal survival. On the other hand, in response to an insult or in various neurodegenerative disorders microglia can proliferate, display morphological changes, alter their gene expression and surface markers to those that are characteristic of chronic activation. Together this response is termed microgliosis, yet it is not clear if microglia play a beneficial or detrimental role in the neurodegenerative process. Similarly, it is not well understood if microglia solely contribute to PD as a pathogenic response that facilitates disease or as a disease-initiating component. Although microglia are the primary cell in the brain responsible for immune surveillance, under pathological conditions, peripheral macrophages and other immune cells can traffic across a disrupted blood brain barrier (BBB) and most likely contribute to the role of neuroinflammation in PD. A new and growing area of PD research is now focused on understanding whether microglia exert a protective function via production of neurotrophic factors, or contribute to neuronal death by producing inflammatory factors that are toxic to DA and other vulnerable neurons. In order to address the impact of microglia on neurodegeneration in PD, whether protective or deleterious, we must better understand the behavior of microglia during disease progression. We posit that the microglial phenotypes in the aging brain become further altered in PD giving rise to a prevalence of pro-inflammatory microglia, which likely contribute to progressive neuronal loss (Figure 1). To support our view we will discuss the complex microglial phenotypes found in PD and in animal models of PD and the immune-modifying strategies that have been explored regarding mechanisms of chronic microglial activation.

1.1 Microglia Activation States: complex and heterogeneous phenotypes

Over the last decade the important concept has emerged that microglia, similar to other tissue macrophages, assume different phenotypes and perform specific effector functions depending on the precise nature of the stimulus, its intensity and duration (Olah et al., 2011; Perry et al., 2007; Tansey and Goldberg, 2010). Activation of microglia through various pattern recognition receptors such as toll-like receptors (TLRs) leads to the synthesis of a range of different cytokines, inflammatory mediators, growth factors and cell surface molecules. These secondary effects of microglial activation have been used to classify them as either pro- or anti-inflammatory. However it is crucial to understand that activated microglia exist in a heterogenous population and that their phenotypes are not necessarily polarized into two categories, either pro- or anti-inflammatory, but instead exist along a continuum where they acquire different profiles based on their local environment. And as the brain ages, a progressive pro-inflammatory status also referred to as inflammaging manifests

increasing the risk of age-related disorders and leading to microglial priming that may increase the propensity to develop neurodegenerative diseases such as PD (Perry and Holmes, 2014).

Microglia undergo morphological changes when they shift from a quiescent to an activated state. Morphologically, resting, quiescent microglia have long ramified processes with small cell bodies *in vivo*, while activated microglia take on an amoeboid shape with shorter processes and larger cell bodies. Functionally, one way that microglia remain in a quiescent state is via the interaction between the glycoprotein CD200 located on surrounding neurons and the receptor CD200R located on microglia (Hoek et al., 2000). There are multiple other ligand-receptor pairs that make up an important system for cross-talk between neurons and microglia including CD45-CD22 and fractalkine-CX3CR1. Recent evidence suggests that morphological changes should not be interpreted as a sign of activation but instead that the microglia are impaired and can no longer efficiently perform their intrinsic functions (Heppner et al., 2015). Microglia Aβ-binding scavenger receptors that are largely involved in Aβ plaque clearance were downregulated in aged Alzheimer's disease mice (Hickman et al., 2008), suggesting alterations in microglial receptors may be a process that coincides with a shift in morphology. Furthermore, in a mouse model of Alzheimer's disease, the phagocytic capacity of microglia was reduced and inversely correlated to the Aβ plaque burden, suggesting that microglia function declined with severity of disease (Krabbe et al., 2013). Similar defective phagocytic activity was described in aged mice whereby primary microglia isolated from aged mice had less effectively taken up exosome associated α-synuclein oligomers compared to microglia from young mice (Bliederhaeuser et al., 2015). On the contrary, results from other studies in animal models of PD have suggested long-term phagocytic activation of microglia (Barcia et al., 2013; Rodriguez et al., 2007). These studies used immunohistochemical techniques to highlight presence of phagocytic markers, thus potentially suggesting that some microglia in the heterogeneous population maintain their phagocytic capacity in late stages of the disease but the presence of phagocytic markers does not necessarily correlate with *in vivo* phagocytic capacity. Therefore we must be cautioned to evaluate more than the morphological changes in order to understand the functions of the microglia and their relationship with neurodegeneration in PD.

The term "activated microglia" is an oversimplified term that lacks information about the heterogeneous nature of activated microglia and their various and complex effector functions. Activated macrophages also have diverse functions that historically have been categorized by their distinct patterns of secreted factors and cell surface markers following stimuli such as cytokines. The original concept of macrophage polarization described an M1 stage as cytotoxic and maintaining a pro-inflammatory phenotype, while a M2 stage was associated with repair and healing or an anti-inflammatory phenotype (Mackaness, 1962; Mills et al., 2000). Stereotypical M1 phenotypes occur when challenged with LPS and IFN-γ which are produced in large quantities during bacterial and viral infections, respectively, leading to the production of inflammatory cytokines such as tumor necrosis factor (TNF), interlukin-6 (IL-6), IL-12, and IL-1β and lead to the upregulation of cell surface markers such as major histocompatibility complex-II (MHC-II) and CD86 (Martinez et al., 2006; Nau et al., 2002). The M2 state is divided into 3 different sub-classes each having distinct activation mechanisms: M2a, M2b, and M2c (Biswas and Mantovani, 2010; Edwards et al.,

2006; Martinez and Gordon, 2014; Stout et al., 2005). Macrophages stimulated by IL-4 adopt an M2a-like phenotype that functions to suppress inflammation. Alternatively, M2b responses control selective phagocytosis and regulate inflammation. This response is stimulated by TLR ligands that become activated by the fusion of Fc gamma receptors (FcγRII) and immunoglobulin G (IgG) complexes. Lastly, the M2c phenotype has been implicated in tissue remodeling and immunoregulation and is stimulated by the binding of IL-10 to its receptor and can be enhanced by glucocorticoids (Gratchev et al., 2008). The proposed distinct macrophage activation states became controversial in 2011 when activation pathways were found to overlap, suggesting that states of activation may be intermittent and dynamic. A recent publication authored by 25 senior investigators proposes new nomenclature to describe the complex landscape of macrophage responses to various stimuli (Martinez and Gordon, 2014; Murray et al., 2014).

Based on the similarities between microglia and macrophages, microglia activation responses were also initially classified using M1/M2 polarization terminology. Based on the emergence of new cutting-edge technologies such as single-cell RNA sequencing and cytometry time-of-flight (CyTOF) mass spectrometer for high-speed acquisition of highly multi-parametric single cell data it has become clear that describing the states of microglia activation by the M1 or classical activation and M2 or alternative activation phenotypes is not accurate. The phenotypic responses of microglia do not necessarily mimic those of macrophages. For example, IL-4 stimulus promotes CD163 and CD206 expression in macrophages but not in microglia (Mittelbronn, 2014). Furthermore, the responsiveness of primary human microglia to LPS and IFN-γ is minimal to absent depending on culture conditions in contrast to the robust induction of TNF and CCR7 observed in monocyte-derived macrophages. Moreover, primary microglia produce increased CD163 and CCL18 after stimulation with dexamethasone, while the response of monocyte-derived macrophages is largely restricted to increases in CD163 (Melief et al., 2012).

Although microglia can be manipulated *in vitro* to elicit polarized responses, the *in vivo* profiling of microglia activation is more complex and much less clear with distinctions by species and/or location. Anatomical regional differences have been reported for microglia frequency with greater density found in the telencephalon, while within the same region, more microglia are found in myelinated areas compared to non-myelinated (Lawson et al., 1990; Mittelbronn et al., 2001). In relation to PD, early reports describe, within the less dense mescencephalon, that microglia are more numerous in the SN, particularly in the pars reticulata, compared to adjacent structures (Lawson et al., 1990). Interestingly, microglial density is positively correlated to LPS-induced neurodegeneration suggesting regional differences in cell responsiveness (Kim et al., 2000). Potentially contributing to their functional response, variability in microglia basal gene expression exists among brain regions. For example, TNF mRNA is greater in microglia freshly isolated from the SN compared to the hippocampus of Wistar rats, suggesting that microglial profiles may contribute to regional vulnerability and susceptibility of neurons in a diseased state such as PD (Doorn et al., 2015). This idea is further supported by the non-uniform transcriptional identity of aged microglia across brain regions. Recent investigations have shown that microglia isolated from aged mice lose their younger, more distinct gene expression profile with greatest changes in found in the hippocampus which may result in a lack of

homeostatic support to the surrounding tissue and further influence the location dependent susceptibility to neurodegenerative disorders (Grabert et al., 2016). Future studies to better understand regional functionality of microglia should be conducted using the translating ribosome affinity purification (TRAP) methodology (Heiman et al., 2008). Furthermore, microglial surface markers also have demonstrated regional diversity. Using flow cytometry, isolates from gray matter (occipital cortex) had increased levels of CD45 compared to white matter (corpus callosum), suggesting that gray matter microglia may be in a more immunosuppressed state (Melief et al., 2012). Understanding region-specific differences may provide insight into the contribution of microglial responsiveness to disease and their vast capacity to produce a spectrum of responses versus merely polarized responses.

2. Microglia in Parkinson's disease (PD)

Research into disequilibrium and complexity of microglial activation responses became an area of intense focus in hope to identify a potential mechanism that could contribute to neuroinflammation and potentially underlie PD pathogenesis. The idea was that identifying neuroinflammatory profiles in the brains of PD patients may help define potential targets for anti-inflammatory neuroprotective therapies and could help determine the success of immunotherapies (Sanchez-Guajardo et al., 2013; Tansey and Goldberg, 2010). However, the challenges of such targeted therapies require sensitive methods to detect microglial activation states and phenotypic changes which remains a challenging undertaking given these factors are likely to display complex temporal associations with PD depending on Braak disease stage, rate of disease progression and duration of disease. In order to move forward and conquer these challenges we will discuss current understandings of the activation states of microglia in PD as determined by post-mortem histological evaluation and *in vivo* imaging and the microglia phenotypes found in PD tissues and biofluids.

2.1 Evidence for Microglia Activation in PD

Multiple studies have described reactive microglial in postmortem brain samples of PD patients. Specifically, major histocompatibility complex class II (MHC-II) immunoreactive microglia were identified in areas of the SN and striatum in PD patients and in patients with induced parkinsonism from a self-administered analog of synthetic meperidine analogue known as 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) that resulted from faulty chemistry and was responsible for "frozen addicts" with extreme parkinsonian bradykinesia (Imamura et al., 2003; Langston et al., 1999; McGeer et al., 1988). Located in the vicinity of the few remaining nigral DA neurons, these microglia displayed morphologies characteristic of activated and phagocytic cells, similar to those seen in aging (Jyothi et al., 2015), and displayed increased HLA immunoreactivity with some microglia containing visible evidence of phagocytized neuromelanin, the characteristic pigment of nigral DA neurons (Langston et al., 1999). Interestingly, Croisier et al. found an inverse correlation of CD68 immunoreactivity with disease duration, and suggested that elevated phagocytosis was associated with ongoing tissue destruction, decreasing in final disease stages when it was no longer functionally relevant (Croisier et al., 2005). These and other associations between the presence of activated microglia and advanced parkinsonian stages as well as the presence of α-synuclein aggregation suggest a significant contribution of microglial activation to the

progressive pathological process in PD. However, given that most of these observations were taken at the end stage of disease, it remains unclear whether the activation of inflammatory responses and microgliosis could play a detrimental or beneficial role at earlier stages of the neuropathology. In either case, the involvement of the immune system and potentially microglial activation in the pathogenesis of PD has been supported by a large genome-wide association study (GWAS) that identified a single nucleotide polymorphism (SNP) in the human leukocyte antigen (HLA-DRA) gene, which encodes HLA-DR antigens that are expressed by antigen-presenting cells such as microglia, as a genetic risk factor for late-onset PD (Hamza et al., 2010).

Evidence for the ongoing activation of microglia in PD has been confirmed using positron emission tomography (PET) imaging. Radioligands such as ^{11}C-PK11195, ^{11}C-PBR, or ^{18}F-FEPPA selectively bind to peripheral benzodiazepine receptors (PBR), more recently termed translocator proteins (TSPO), which generally have low expression in a healthy brain, but are greatly enhanced in the presence of neuroinflammation and therefore widely accepted as a measurement of activated microglia (Banati et al., 1997; Papadopoulos et al., 2006). Widespread microglial activation in PD as reflected by increased binding of ^{11}C-PK11195 was reported in the pons, basal ganglia and frontal and temporal cortex of PD patients compared to age-matched normal controls (Gerhard et al., 2006). Yet longitudinal evaluation of ^{11}C-PK11195 binding potential 18-28 months later did not show significant changes in radioligand binding regardless of the significant increases in UPDRS scores, suggesting that PBR binding does not correlate with disease progression. However, other studies have demonstrated a positive correlation in the levels of ^{11}C-PK11195 in the midbrain with the UPDRS in early stage PD patients (Ouchi et al., 2005), and although not significant, trends of higher levels of ^{11}C-PK11195 were found in advanced stages of PD compared to unmedicated patients within a year of their diagnosis (Bartels et al., 2010). Diffuse binding of ^{11}C-PK11195 has also been described in PD patients with a 25-30% increase in the temporal and occipital cortical regions (Edison et al., 2013); whereas, other studies demonstrated selective binding potential increases in the SN and putamen (Iannaccone et al., 2013) or the midbrain (Ouchi et al., 2005). While these disparate findings may partly reflect the large variability of neuroinflammation in PD patients, the largest inherent limitation of these studies is that quantitative interpretations of binding signal are confounded by large inter-individual variability in binding affinity due to genetic polymorphisms in TSPO (Owen et al., 2012; Yoder et al., 2013); therefore, caution should be used when comparing between individuals in cross-sectional studies. Even in a study that controlled for the *rs6971* polymorphism, ^{18}F-FEPPA binding was not significant in the striatum of PD patients compared to controls, yet interestingly within the PD group demonstrated trends of increased TSPO expression in high compared to mixed affinity binders (Koshimori et al., 2015). The most conservative interpretation of these findings is that the progressive degeneration in PD may not be associated with increasing microgliosis, suggesting there is not a parallel relationship and instead there may exist a threshold of microglial activation or dysfunction that continues to contribute to cell death. Yet, it also leaves open the possibility that microglia phenotypes fluctuate along with disease progression. However, PBR ligands are unlikely to distinguish across the spectrum of microglia activation states, therefore nowadays it is not possible to evaluate microglia phenotype in the brain and make a clear

correlation with different stages of PD progression in parkinsonian patients. Clearly it would be advantageous to develop PET radioligands that can detect microglial phenotypes or functional behavior. Furthermore, development of radioligands for α-synuclein aggregation would allow comparisons between Lewy pathology and not only DA neuron dysfunction but also microglial activation.

Future application of microglial imaging to evaluate patterns of temporal activation in PD are needed. Animal models of ischemia have demonstrated microglial temporal activation and distribution shifts. Mice induced with focal cerebral ischemia show CD68 expressing microglia bordering the ischemic core 24 hours after lesion, but then migrated into the core by 7 days post-ischemic lesion (Perego et al., 2011), suggesting that phagocytic behavior increased by 7 days because nerve loss was irreversible. However, little is known about the exact mechanisms by which microglia influence the outcome of disease, especially a progressive neurodegenerative disorder.

2.2 Microglia phenotypes in PD

Given the complex profile of microglia, the presence of reactive microglia alone does not define their helpful or harmful role in PD neuropathology. Evaluation of soluble factors such as cytokines in brain tissue, cerebral spinal fluid (CSF) or serum from PD patients may provide hints to the pathogenic impact of microglia activation (Table 1). Elevated levels of cytokines including TNF-α, IL-6 and IL-1β were reported in CSF and striatal samples of individuals with PD (Blum-Degen et al., 1995; Mogi et al., 1994a; Mogi et al., 1994b; Mogi et al., 2007; Muller et al., 1998). Additionally with the use of immunohistochemistry, nitric oxide synthase, cyclo-oxygenases 1 and 2 (COX1 and COX2) were found elevated in amoeboid microglia from PD samples of the SN (Knott et al., 2000), while microglia in the putamen (Imamura et al., 2003) or SN (Hunot et al., 1999) of PD patients were also immunoreactive for cytokines such as IL-6, TNF-α and IL-1β. More recently, evaluation of CSF from PD patients compared to a reference healthy control group revealed no significant differences in the inflammatory markers Flt3 ligand and fractalkine (Shi et al., 2011) or C-reactive protein (CRP), IL-6, TNF-α, eotaxin, interferon gamma-induced protein-10 (IP-10), monocyte chemotactic protein-1 (MCP-1), and macrophage inflammatory protein-1β (MIP-1β) (Lindqvist et al., 2013). Interestingly, and perhaps not surprisingly, increased CSF levels of CRP correlated with non-motor symptoms including depression and fatigue, while MCP-1 expression was significantly associated with depression (Lindqvist et al., 2013), suggesting a role for cytokine production in non-motor disease symptoms with significant inter-individual variability of inflammatory phenotypes in relation to disease pathology. The interaction of neuroinflammation and non-motor symptoms is further supported by a significant correlation between ^{11}C-PK11195 binding in the cortical regions and cognitive impairment in both PD patients with dementia and Alzheimer patients (Fan et al., 2015). Another cytokine transforming growth factor-beta (TGF-β), known to inhibit microglia activation, has been observed in the brains and CSF of PD patients (Mogi et al., 1995; Vawter et al., 1996). Therefore, it is possible that adaptive changes in the brain occur at some point along disease progression to induce a protective microglia phenotype, which would account for pro- and anti-inflammatory cytokines co-existence in CSF. To date, the

relationship between aggregated or soluble α-synuclein and CSF inflammatory markers has not been evaluated in-depth in PD patients.

In the plasma, inflammatory cytokines have been found and correlated to PD severity as determined by Hoehn and Yahr staging (Brodacki et al., 2008; Hofmann et al., 2009; Koziorowski et al., 2012). Another study evaluated a total of 17 cytokines and chemokines in plasma of PD patients that were homozygous carriers of the *rs3129882* G SNP, but found no changes with the exception of increased CCL-3, also known as MIP-1α (Kannarkat et al., in press). The rs3129882 SNP is located in the first intron on the HLA-DRA gene and homozygous carriers of the high-risk G allele have elevated baseline and inducibility of MHC-II expression on antigen presenting cells, suggesting that cellular immune phenotypes may be an alternative marker to the soluble markers found in biofluids.

Based on the above reports, conclusions of microglial phenotypes throughout the different stages of PD are difficult not only because of the limited number of studies and the effect of method and timing of collection, but are also likely distorted by an exceeding number of patients who die in late stages of the disease. A recent study examined mRNA levels of cytokines and mediators of the immune response PD patients across stages and found increased expression of IL-6 signal transducer in the SN of patients in stage 1-2 while at later stages of the disease the same marker was downregulated (Garcia-Esparcia et al., 2014). This observation further reveals the complex responses dependent on the stage of PD-related pathology; yet again the low number of samples collected from patients in early stage PD and the inability to corroborate their findings at the protein level limited this study. Moreover, long-term L-DOPA therapy or antidepressant therapy often administered to patients in late disease stage, may affect immune responses in opposite directions, adding a factor of complication to human studies (Barnum et al., 2008; Baumeister et al., 2015; Lindqvist et al., 2013). Unfortunately, these limitations restrict our ability to fully understand the state of microglia during progressive neurodegeneration and α-synuclein deposition. Future evaluation of microglial gene expression in post-mortem brains of PD patients who died at different stages of disease may aid investigators to better define microglial phenotypes. It is becoming increasingly clear that in many diseases, microglia largely express complex gene expression signatures (Butovsky et al., 2014; Moore et al., 2015; Pisanu et al., 2014; Tang and Le, 2015), and mixed phenotypes most likely reflect a dynamic state or may be simply due to the presence of a heterogeneous population of microglia during disease. Until human studies can be conducted to evaluate the neuroinflammatory interaction with neurodegeneration and other histopathological hallmarks like Lewy Body formation, conclusions about these relationships will have to be drawn from animal models of the disease.

In summary, it remains to be established whether the activation of inflammatory responses and reactive microglia could play a beneficial role at some stage during the progression of PD in humans. As described above, human studies have reported an elevation of both pro- and anti-inflammatory cytokines in the serum and CSF of PD patients, suggesting that pro- and anti-inflammatory microglia may coexist in the PD brain and complex changes in microglia phenotypes are likely to maintain and exacerbate PD neuropathology (Brodacki et al., 2008; Mogi et al., 1995; Mogi et al., 1994a; Mogi et al., 1996; Rentzos et al., 2009).

Noteworthy, in Alzheimer's disease and in the elderly brain an imbalance of pro- and anti-inflammatory microglia phenotypes have been reported (Hoozemans et al., 2006; Jimenez et al., 2008). Therefore, it is reasonable to speculate that there is an array of activated microglia that coalesce in the PD brain and potentially drive disease progression via cytokine release (Tansey and Goldberg, 2010). Improvements in single-cell phenotyping and development of PET ligands for neuroimaging that can distinguish specific phenotypes among the vast array of heterogeneous microglia profiles will be needed to resolve these unanswered questions.

3. Microgliosis in models of PD-like degeneration

It is generally believed that stress signals produced by damaged neurons or aggregated misfolded proteins released by dead neurons activate microglia, which in turn lead to secondary degeneration, being responsible for the massive neurodegeneration in PD. Animal models have added pivotal insights to the understanding of microglia involvement in PD neuropathology. Neurotoxins such as MPTP and 6-hydroxydopamine (6-OHDA) induce different degrees of microgliosis in lesioned areas. Moreover, a number of pro-inflammatory stimuli have been used to activate microglia in order to mediate DA neuron toxicity including lipopolysaccharide (LPS), α-synuclein, and inflammatory cytokines. While all models induce some degree of activated microglia surrounding damaged DA neurons, they display differences in the time-course of microglia activation in response to the toxic stimulus. Moreover, different results have been reported on the sequence of toxic events in DA areas, where microglia activation may either precede neuronal damage or be a secondary event, depending on the animal model investigated (Frank-Cannon et al., 2009; Ramsey and Tansey, 2014). Differences mostly relate to the species used and to the primary trigger of degeneration, that is whether a neurotoxin or an inflammatory insult is delivered. Moreover, the protocol chosen to administer the neurotoxin, either an acute or chronic protocol, thereby determining the time-course of the degenerative process, will also influence microgliosis progression. Since microglia may display different phenotypes and perform specific effector functions depending both on the type and strength of the stimulus, a careful evaluation of the choice of animal model is pivotal in the experimental design so that it is suitable for the purpose of the study.

3.1 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) models

Monkey studies have consistently reported HLA-DR-stained reactive microglia in the SN of MPTP-injected monkeys years after neurotoxin injection, suggesting that an ongoing and long-lasting microgliosis was triggered and persisted in the absence of MPTP (Barcia et al., 2004; Hurley et al., 2003; McGeer et al., 2003; Vazquez-Claverie et al., 2009). Moreover, a similar extent of microgliosis was reported after subacute or chronic MPTP administration, which was independent from the extent of neuronal loss in the SN (Vazquez-Claverie et al., 2009). In rodents, the majority of studies aimed at investigate microgliosis in PD have been performed in acute models of MPTP toxicity. Pioneer studies performed in mice administered acute doses of MPTP reported early and transient presence of reactive microglia in the SN, which preceded neuronal loss and disappeared within a few weeks after toxin exposure (Czlonkowska et al., 1996; Kohutnicka et al., 1998; Kurkowska-Jastrzebska et al., 1999; Liberatore et al., 1999; Smeyne and Jackson-Lewis, 2005; Vazquez-Claverie et

al., 2009). More recent studies have investigated reactive microglia in chronic regimens of MPTP administration, which more closely reproduces the progressive nature of neurodegeneration and symptom development observed in PD neuropathology. These models show an early time course of microglia activation in the SN that is detectable in the absence of nigral cell loss in the premotor phase of the neuropathology when non-motor symptoms such as olfactory dysfunction have been observed (Rodriguez et al., 2007; Schintu et al., 2009). Along with the chronic administration regimen, reactive microglia proliferate as the DA degeneration progresses, and persist at least six months after discontinuation of MPTP. Moreover, in presence of advanced nigral degeneration, microglial cells are closely associated with apoptotic cells, suggesting an active role in the scavenging of dying neurons (Meredith, 2005).

3.2 6-Hydroxydopamine (6-OHDA) models

6-OHDA injections induce strong microglial activation in the SN or the striatum; yet microglia activation has been described as an early, simultaneous or delayed event with respect to DA degeneration depending on the site of 6-OHDA injection (Marinova-Mutafchieva et al., 2009; Sanchez-Pernaute et al., 2004; Walsh et al., 2011). After intrastriatal 6-OHDA infusion, reactive microglia were detectable histologically in the striatum days after terminal degeneration (Walsh et al., 2011), while microgliosis in the SN was absent or elicited subsequently to DA degeneration (Depino et al., 2003; Henry et al., 2009; Walsh et al., 2011). In contrast, microgliosis preceding DA degeneration was detected in both the striatum and SN of rats intrastriatally treated with 6-OHDA (Armentero et al., 2006). Interestingly, when the neurotoxin was infused directly into axons, microgliosis preceded striatal terminal degeneration and occurred together with nigral degeneration (Walsh et al., 2011). Evaluation of ^{3}H-PK11195 autoradiography from rats administered unilateral intrastriatal 6-OHDA showed early strong microgliosis in the striatum starting 3 days after toxin delivery, but delayed and progressive increases in radiotracer density in the SN, synchronous with the timeline of degeneration (Maia et al., 2012). Overall, studies performed in toxin-based models support early and enduring microglia activation in the course of DA neuronal loss.

3.3 Lipopolysaccharide (LPS) models

The bacterial endotoxin LPS administered either intranigrally or peripherally, induces a potent activation of microglia and secretion of pro-inflammatory factors which precede the degeneration of the nigrostriatal DA pathway, and is therefore used to investigate direct neuroinflammation-induced dopamine loss (Castano et al., 1998; Dutta et al., 2008). Depending upon the dose of LPS administration, the resulting DA neurodegeneration has been described as rapid or delayed and progressive. Acute LPS intranigral infusion in C57BL/6 mice or rats induced rapid activation of microglial cells and degeneration of dopamine neurons in the SN within 7 days, which remained stable up to one year (Arai et al., 2004; Herrera et al., 2000). Subacute or chronic infusion of low-dose LPS into rodent brain triggered a rapid and persistent activation of microglia, followed by a delayed and gradual loss of nigral DA neurons that began at 3-4 weeks, reaching 70% of neuronal loss by 10 weeks (Gao et al., 2002; McCoy et al., 2006; Tanaka et al., 2013). When LPS was administered as a single intraperitoneal injection, it caused rapid microglia activation and

increased brain TNF, that remained elevated for 10 months followed by delayed and progressive loss of nigral DA neurons starting at 7 months after LPS treatment that reached 47% loss at 10 months (Qin et al., 2007). Therefore, data from the LPS models indicate that neuroinflammation may be both an initiating stimulus and a self-propelling mechanism of progressive neuron degeneration.

3.4 α-synuclein models

A variety of α-synuclein-based models have been developed in an attempt to replicate the α-synucleinopathy observed in PD patients. These models include overexpression of wild type human α-synuclein or mutant A53T human α-synuclein, and transgenic mice overexpressing truncated human α-synuclein in DA neurons, as extensively reviewed (Dawson et al., 2010; Giasson et al., 2002; Magen and Chesselet, 2010; Masliah et al., 2000; Wakamatsu et al., 2008). Although none of these models replicate the main hallmarks of PD, such as progressive and severe loss of DA neurons and motor deficits, they have greatly contributed to the understanding of α-synuclein-induced toxicity. Alternative models include mice overexpressing human wild type α-synuclein under the Thy-1 promoter (Chesselet et al., 2012) or the tyrosine hydroxylase promoter (Su et al., 2008), and viral vector driven α-synuclein overexpression in rodents (Decressac et al., 2012; Lo Bianco et al., 2002; Sanchez-Guajardo et al., 2010; Van der Perren et al., 2015; Yamada et al., 2004) and primates (Eslamboli et al., 2007). Viral vector-based models show a progressive degeneration of nigrostriatal neurons that is associated with gradual motor impairment (Decressac et al., 2012; Van der Perren et al., 2015). Independent of the extent of nigrostriatal degeneration, α-synuclein models offer the opportunity to study pathologic mechanisms specific to early disease stage, and to elucidate mechanisms of cellular dysfunction which precede cell death (Chesselet et al., 2012), including inflammatory responses and the relationship between α-synuclein accumulation and subsequent inflammation (Allen Reish and Standaert, 2015; Gao et al., 2008; Gao et al., 2011a). In Thy-1 α-synuclein mice an early and progressive increase of activated microglia and inflammatory cytokine TNF was specifically observed in striatum and SN that preceded dopamine fiber damage (Watson et al., 2012). Similarly, one-month old TH α-synuclein mice displayed an increase of Iba1-positive ameboid shaped microglia in the SN and increased expression of TNF in both the SN and striatum (Su et al., 2008). As mentioned above, different forms of wild type or mutated α-synuclein have been overexpressed in the rodent brain, which were shown to affect microglia activation in different ways. LPS induced a persistent microgliosis, production of inflammatory molecules, and a progressive degeneration of nigrostriatal neurons when administered to transgenic mice overexpressing human A53T mutant α-synuclein, but not in wild type α-synuclein mice, suggesting that microglia bridge neuroinflammation and α-synuclein-induced cellular damage in mediating chronic degeneration in PD (Gao et al., 2011a). Moreover, studies in α-synuclein overexpressing mice and primates have been pivotal to demonstrate that levels of α-synuclein-induced neuronal pathology, either in presence or absence of DA degeneration, strictly modulate the phenotype of activated microglia (Barkholt et al., 2012; Sanchez-Guajardo et al., 2010).

3.5 LRRK2 models

Cells or mice bearing a genetic mutation of the leucine-rich repeat kinase 2 (LRRK2) gene have provided direct evidence of the causal relationship between neuroinflammation and DA neuron degeneration in late-onset PD. LRRK2 expression is upregulated in activated microglia under pathological conditions, whereas LRRK2 ablation or inhibition prevents a full inflammatory response, resulting in attenuated inflammatory cytokine production following LPS, reduced nuclear factor (NF)-κB transcriptional activity, and reduced microglial morphological activation, chemotactic and phagocytic activity (Kim et al., 2012; Marker et al., 2012; Moehle et al., 2012). Missense mutations in LRRK2 are the most common cause of both genetic and idiopathic PD where disease penetrance is variable (Healy et al., 2008). The G2019S LRRK2 mutation is the most frequent mutation responsible for dominantly inherited PD, and is associated with increased kinase activity. PD patients with the G2019S LRRK2 mutation present clinical and pathological phenotypes nearly indistinguishable from idiopathic disease (Paisan-Ruiz et al., 2004; West et al., 2005; Zimprich et al., 2004). Remarkably, LRRK2 KO rats were resistant to DA degeneration when challenged by either intracranial LPS or α-synuclein overexpression, while LRRK2 overexpression accelerated the progression of neuropathology in A53T α-synuclein transgenic mice (Daher et al., 2014; Lin et al., 2009). Of note, one study reported an age-dependent partial loss of nigral neurons and damage to the nigrostriatal system in mice carrying the G2019S LRRK2 mutation (Ramonet et al., 2011). Overall, results from transgenic LRRK2 mutant mice suggest that there is another contributing factor in the brain environment leading to the progressive neurodegeneration in PD. Therefore, inhibition of LRRK2 protects from DA cell loss by preventing the recruitment of activated microglia and potentially infiltrating myeloid cells, while LRRK2 overexpression causes neuronal damage, placing the neuroinflammatory response center stage among causal factors of neurodegeneration in this PD model.

Recently, mice carrying null mutation for the c-Rel gene, a regulatory subunit of the transcriptional regulator of inflammation NFκB, have been characterized and suggested to be a useful model of progressive inflammation-induced nigral degeneration (Baiguera et al., 2012). c-Rel KO mice develop an age-dependent PD-like progressive neurodegeneration and accumulation of aggregated α-synuclein with activation of CD11b-positive microglial cells in the SN, highlighting NFκB as a potential downstream target for the regulation of microglial activation.

4. Microglia activation states in animal models of PD-like degeneration

As described above, PET imaging studies in human subjects have revealed that the extent of microglia activation does not increase as the clinical features of the disease progress (Gerhard et al., 2006). In line with this evidence, the extent of reactive microglia in monkeys was similar after a subacute or a chronic MPTP regimen and appeared independent from the lesion extent in the SN (Hurley et al., 2003; Vazquez-Claverie et al., 2009). These findings suggest that progressive degeneration of DA neurons and terminals may not be due to progressively increasing microglial proliferation, but leave open the possibility that changes in the microglia phenotype occur along with disease progression that confer to them a

permanent and increasing neurotoxicity for DA neurons that cannot be appreciated using the current PET imaging methodologies.

4.1 Pro-inflammatory microglia

In an attempt to explore changes in microglia phenotype in PD and their time-course, a number of studies have investigated the expression of surface immune molecules in microglial cells in animal models. These pioneer studies have revealed that the time-course of expression displayed by these molecules does not necessarily reflect the time-course of microglia activation during disease progression, suggesting that dynamic changes in the phenotype of activated microglia are occurring in experimental models of PD. Following an acute MPTP protocol in mice, an early upregulation of MHC-I and ICAM-I were found in reactive microglia on days 1-7 post treatment, while MHC-II was transiently upregulated on days 7-14, then became undetectable on day 21 and up to 6 months after the lesion; these findings provided the first suggestion of dynamic changes in microglia effector function in an animal model of PD (Kurkowska-Jastrzebska et al., 1999; Yasuda et al., 2007). Primary murine ventral midbrain cultures exposed to media with elevated IFN-γ from microglia stimulated with LPS, α-synuclein, or A53T mutated α-synuclein had significantly heightened proportions of MHC-I expression compared to the same cultures either plated with control media from microglia that were not stimulated or exposed directly to these stimuli, or compared to primary cortical and striatal cultures exposed to the same stimulated media. Furthermore, induction of MHC-I on murine catecholamine neurons by factors (IFN-γ) released from stimulated microglia triggered extensive neuronal death, suggesting that stimulated microglia can selectively promote MHC-I presentation in SN catecholamine neurons (Cebrian et al., 2014). In mice intravenously injected with peripheral bone-marrow-derived cells, chronic MPTP induced an early microglia proliferation greatest in areas of rich DA innervation such as the anterior, medial and posterior striatum, which began to express the marker of phagocytosis CD68 only at later stages of degeneration (Rodriguez et al., 2007). Following 6-OHDA injection into the medial forebrain bundle (MFB), an increase in OX-42 and MHC-II immunoreactivity was detected as early as day 1 post-lesion, while levels of CD68 started to increase after 3 days, reaching maximal levels 9-13 days post-lesion (Marinova-Mutafchieva et al., 2009). Interestingly, upregulation of CD68 was coincident with dopaminergic degeneration in a microglia subpopulation located near dopaminergic neurons (Kurkowska-Jastrzebska et al., 1999). Therefore, phagocytic mechanisms may be responsible for microglia-mediated secondary neuronal degeneration, being mostly upregulated in presence of intense tissue destruction, which is in line with human findings (Cebrian et al., 2014; Croisier et al., 2005). In support of this concept, similar results were reported after medial forebrain bundle (MFB) transection, where early microglia activation was followed by a delayed upregulation of CD68 that occurred together with DA degeneration (Cho et al., 2006). Although interpretation of data obtained from toxin-based models is undermined by the diverse induction of microglia activation depending on the model and protocol used (as discussed above), overall these studies strongly suggest that microglia phenotypes are modulated in a manner that depends on the degree of DA damage/degeneration in their microenvironment, perhaps first characterized by an antigen-presentation effector function, then followed by the induction of a heightened

pro-inflammatory phenotype, followed by phagocytic phenotype in the more advanced stages of degeneration.

Studies in rats and primates overexpressing viral vector driven human α-synuclein have provided convincing evidence that microglia acquire different phenotypes and display different effector functions at early versus late stages of neurodegeneration (Barkholt et al., 2012; Sanchez-Guajardo et al., 2010). In rats overexpressing viral driven human α-synuclein, microglia assumed different morphology and phenotype depending on the level of α-synuclein expression and the related degree of α-synuclein-induced DA degeneration (Sanchez-Guajardo et al., 2010). Upon exposure to levels of human α-synuclein that cause neuronal damage but not cell death perhaps modeling early disease stages, microglia displayed an antigen-presenting phenotype with increased MHC-II expression, in contrast to the more macrophage-like morphology with increased CD68 expression when exposed to human α-synuclein levels that induced DA degeneration (Sanchez-Guajardo et al., 2010). Similar results were reported in primates, where human α-synuclein overexpression induced a long-term microglia polarization (Barkholt et al., 2012). Importantly, in this study human A53T mutant α-synuclein overexpression induced neurodegeneration that resulted in nigral dopaminergic cell death, while wild type human α-synuclein overexpression induced by recombinant adeno-associated virus injections resulted in degeneration that was limited to DA fibers. In addition, although the extent of activated microglia was similar across primates that received α-synuclein compared to controls, microglia polarization into a macrophage-like morphology was more pronounced in the A53T over-expressing animals (Barkholt et al., 2012). The significance of this is unclear but highlights the complexity of the molecular interactions between different forms of α-synuclein and brain microglia that shapes microglial responses.

In addition to cell surface molecules, a number of studies have investigated soluble factors such as cytokines and chemokines, following administration of different neurodegeneration-inducing neurotoxins and inflammagens, highlighting their role in DA degeneration and revealing dynamic changes in the production of these molecules coincident with the neurodegenerative process. Genes involved in the inflammatory response including cytokines, chemokines and pro-inflammatory enzymes, contain in their promoter region the DNA binding site for the transcription factor NFκB, a master regulator of inflammatory responses (Hayden and Ghosh, 2004). In the SN of PD patients and of MPTP-intoxicated monkeys and mice, a robust expression of the p65 subunit of NFκB has been described in the nucleus and cytoplasm of glial cells (Ghosh et al., 2007; Hunot et al., 1997; Mondal et al., 2012). Accordingly, the concentration of a number of inflammatory cytokines is elevated in either CSF or affected brain regions in PD patients, and pro-inflammatory cytokines such as TNF and IFN-γ were found elevated years after neurotoxin injection in MPTP-treated monkeys (Barcia et al., 2011; Nagatsu et al., 2000). Emphasizing the cardinal role of inflammatory cytokines in DA degeneration, blocking of soluble TNF by intranigral administration of TNF dominant negative variants rescued DA neurons from intrastriatal 6-OHDA toxicity (Harms et al., 2011; McCoy et al., 2006; McCoy et al., 2008), while transgenic mice carrying homozygous mutant alleles for the TNF receptors or lacking TNF were completely protected against the DA neurotoxicity of MPTP, indicating that the pro-inflammatory cytokine TNF is an obligatory component of DA neurodegeneration (Ferger et

al., 2004; Sriram et al., 2002). Moreover, overexpression of TNF in the SN through adenoviral vector was sufficient to cause nigral degeneration, while IL-1β overexpression exacerbated 6-OHDA induced nigral degeneration and microglia activation (De Lella Ezcurra et al., 2010; Pott Godoy et al., 2008). Interestingly, in TNF and IFN-γ KO mice, MPTP caused microglia activation, but cells displayed limited phenotypical changes, suggesting that these cytokines were necessary for full microglia activation (Barcia et al., 2011; Harms et al., 2012). An increase of mRNA and protein for inflammatory cytokines, such as IL-1β, TNF and IL-6, has been consistently reported after acute MPTP exposure together with an increase of inducible nitric oxide synthase (iNOS) levels, suggesting that reactive microglia acquired an early pro-inflammatory phenotype in this model (Bian et al., 2009; Liberatore et al., 1999; Lofrumento et al., 2011; Pattarini et al., 2007; Yasuda et al., 2008). Interestingly, such increase of inflammatory cytokines was followed by a delayed upregulation of the microglial receptor CX3CR1 for the chemokine fractalkine CX3CL, presumably as a compensatory mechanism to limit neuronal damage and decrease levels of CX3CL (Cardona et al., 2006; Pattarini et al., 2007). In the chronic MPTP model, progressive dopaminergic degeneration was associated with an early and persistent increase of TNF, which preceded neuronal degeneration, followed by a later rise of IL-1β (Luchtman et al., 2009; Pisanu et al., 2014). In the rat intrastriatal 6-OHDA model, reactive microglia was observed early after toxin infusion, while an increase of IL-1β mRNA, but not protein, was reported 30 days after infusion, suggesting a different temporal profile of microglia activation relative to cytokine production in this model (Depino et al., 2003; Luchtman et al., 2009; Pisanu et al., 2014). Interestingly, in non-human primates administered chronic low doses of MPTP, serum levels of IL-6, TNF-β, IL-16 or IL-8 did not fluctuate significantly in either asymptomatic or parkinsonian monkeys, but increases in TNF and IFN-γ were observed in monkeys displaying parkinsonism (Barcia et al., 2011).

In summary, studies in animal models of PD suggest that overproduction of inflammatory cytokines is an early and persistent event that is associated with microglia activation from the early stages of injury, preceding and likely driving microglia polarization towards a phagocytic, CD68-expressing phenotype present in later stages of neurodegeneration. Increased phagocytic function clearly is not a detrimental feature per se, however the upregulation of phagocytosis in microglial cells which have mostly assumed a pro-inflammatory phenotype in earlier stages of the disease is likely to contribute to progressive neuronal loss.

4.2 Anti-inflammatory microglia

Despite evidence in humans, the expression of anti-inflammatory molecules has been poorly described in experimental models of PD. As a consequence, a pivotal yet unresolved question is whether microglia neurotoxicity is driven by the switch from a beneficial neuroprotective phenotype to one in which microglia are chronically activated to secrete pro-inflammatory cytokines that enhance apoptotic cell death cascades in neurons. Chronic MPTP treatment in mice has been reported to increase pro- and decrease anti-inflammatory markers in microglia, creating a ratio of inflammatory mediators that may perpetuate and accelerate the course of disease (Pisanu et al., 2014; Rojo et al., 2010). Specifically, expression of pro-inflammatory cytokines as well as COX-2 and iNOS was increased in

parkinsonian mice, while expression of anti-inflammatory cytokines such as IL-4, and surface markers including CD206, YM-1, Arg-1 and FIZZ-1 were decreased in the SN, suggesting that the toxin-induced degeneration of DA neurons was associated with a skewed microglia polarization and prevalence of pro-inflammatory microglia (Pisanu et al., 2014; Rojo et al., 2010). Moreover, while upregulation of pro-inflammatory cytokines was early and persistent during the chronic MPTP treatment in mice, downregulation of anti-inflammatory cytokines and CD206 was delayed and associated with appearance of motor symptoms and extensive degeneration of the SN (Pisanu et al., 2014). Therefore, while both pro- and anti-inflammatory phenotypes of microglia co-existed in the early phase of the disease, this balance slowly gave way to a prevalence of pro-inflammatory microglia in the late stages of degeneration.

Additional insights come from LRRK2 transgenic mice. Primary microglia from mice overexpressing the PD-linked LRRK2 R1441G mutation exhibited increased expression and secretion of inflammatory cytokines (TNF, IL-1β, IL-12) upon LPS stimulation compared to wild type microglia, while production of IL-10 was decreased. In addition, reduced expression of the pro-inflammatory marker iNOS was detected in CD68-positive microglia in LRRK2 KO mice compared to wild type mice. Moreover, conditioned medium from LPS-stimulated microglia from LRRK2 R1441G transgenic mice induced significant cell death when added to neuronal cultures, suggesting that enhanced neuroinflammation and a prevalent pro-inflammatory phenotype may be a key mechanism underlying neuroinflammation in LRRK2-associated PD (Gillardon et al., 2012). Although molecular mechanisms regulating the skewed microglia polarization in PD are currently under investigation, they remain largely unknown.

4.3 Role and regulation of NFκB in microglia activation states

Recent studies have highlighted a role for aberrant NFκB activation in PD pathogenesis, suggesting that enhanced NFκB activation may sustain the activated state of pro-inflammatory microglia. Persistent NFκB activation in both neurons and glia has been associated with progressive neurodegeneration in PD (Ghosh et al., 2007; Glass et al., 2010; Hunot et al., 1997; Mogi et al., 2007), while inhibition of the NFκB pathway has been shown to confer neuroprotection in animal models of PD by dampening inflammatory responses (Flood et al., 2011; Ghosh et al., 2007; Tran et al., 2008). Conversely, NFκB activity in neurons may promote neuronal survival (Mattson et al., 1997), suggesting that the source of NFκB may dictate its overall function, yet this distinction is still not clear.

NFκB is expressed in neurons and glia but its role has been better characterized in glia where it regulates the expression of inflammatory cytokines but also growth factors and effector enzymes in response to activation of many receptors involved in immunity (Grinberg-Bleyer et al., 2015; O'Neill and Kaltschmidt, 1997; Panet et al., 2001; Tansey and Goldberg, 2010). Therefore, NFκB plays a crucial role in maintaining cellular homeostasis, and the activation of NFκB signaling is tightly controlled (Grinberg-Bleyer et al., 2015; Ruland, 2011). NFκB is normally inactive in the cytoplasm in a complex with IκB family proteins. External stimuli lead to the activation of IκB kinases (IKK) complex, which then phosphorylates IκB allowing its subsequent degradation in the proteasome, and releasing

NFκB, which translocates to the nucleus where it binds to specific sequences in the promoter or enhancer regions of target genes (Baldwin, 2001; Hayden and Ghosh, 2004). Relevant to PD pathogenesis, NFκB signaling may take place through either the classical or alternative pathway (Bonizzi and Karin, 2004). In the classical pathway, binding of activating molecules including TNF or LPS to their respective receptors causes the activation of the IKKβ subunit (also called NEMO), leading to translocation of the NFκB dimer p65/p50 or p65/c-Rel to the nucleus, where they transcriptionally activate pro-inflammatory genes (Grinberg-Bleyer et al., 2015). In the non-canonical pathway, binding of NFB inducers such as LTβ, BAFF and CD40 to their receptors causes the activation of the IKKα subunit, which lead to phosphorylation of the precursor IkB protein p100 and its proteasomal processing into p52, which translocates to the nucleus upon dimerization with RelB, where it activates growth and survival genes that may be of particular importance in neurons during inflammatory stress.

In recent years, the NFκB activation pathway has been investigated in detail in relation to PD pathogenesis. One hypothesis that has emerged is that in PD a skewed activation of the canonical NFκB pathway may dominate over a more balanced stimulation of both the canonical and non-canonical pathways, leading to microglia polarization and DA degeneration. Of note, NFκB has been recognized as a key pathway in α-synuclein dependent activation of phagocytic microglia and increased production of inflammatory cytokines (Fellner et al., 2013). We will further discuss evidence for the protective effect of NFκB key regulators in animal models of PD including regulator of G-protein signaling 10 (RGS10), RING finger protein 11 (RNF11), and NEMO (Figure 2).

4.3.1 Regulator of G-protein Signaling-10 (RGS10)—Recently RGS10, a microglia-enriched GTPase activating protein (GAP), has been recognized as a key negative regulator of NFκB activity and microglia/macrophage polarization in inflammatory conditions (Fellner et al., 2013; Lee et al., 2013; Lee et al., 2011; Lee et al., 2008). RGS10-null mice display increased microglial burden, while RGS10-null microglia/macrophages treated with LPS display a pro-inflammatory phenotype, with increased production of inflammatory cytokines over anti-inflammatory phenotype markers as compared to wild type cells, and enhanced cytotoxicity on DA neurons (Lee et al., 2013; Lee et al., 2011). Interestingly, RGS10-null microglia showed higher levels of p50/p65 NFkB subunits both in resting and TNF-stimulated conditions, suggesting a dysregulated NFκB activation, with canonical pathway prevailing over the alternative pathway (Lee et al., 2011). Moreover, *in vivo* gene transfer of RGS10 into the rat SN reduced microgliosis and protected against 6-OHDA neurotoxin-induced death of DA neurons, while RGS10-null macrophages exerted higher cytotoxicity on DA MN9D neuroblastoma cells, suggesting a link between NFκB dysregulation, prevalence of pro-inflammatory microglia and nigral degeneration (Lee et al., 2013; Lee et al., 2011).

4.3.2 Ring Finger Protein 11 (RNF11)—As mentioned above, NFκB is expressed in glia as well as neurons, where it can either mediate inflammation-induced toxicity or promote the expression of survival genes. RNF11, in association with A20 ubiquitin-editing complex, is a key negative regulators of NFκB signaling pathway, whose role has been

demonstrated in neurons and more recently in microglia (Dalal et al., 2012; Pranski et al., 2013). In microglia RNF11 expression levels were inversely correlated to NFκB activation, in that RNF11 silencing increased NFκB activation, inflammatory molecules production, and inflammation-mediated cytotoxicity, whereas RNF11 overexpression conferred protection against LPS-induced cell cytotoxicity through an inhibition of the inflammatory response, suggesting that RNF11 may be a potential target for modulating inflammatory responses in neurodegenerative diseases (Dalal et al., 2012). Similar results were observed in TNF-stimulated neurons in culture, where RNF11 silencing resulted in enhanced p65RelA nuclear translocation and heightened inflammatory responses (Pranski et al., 2013). Interestingly, targeted knockdown of RNF11 in nigral DA neurons of 6-OHDA-induced hemiparkinsonian rats, was associated with increased NFκB activity and promoted DA cell survival, while RNF11 overexpression inhibited NFκB activity and increased dopaminergic cell loss (Pranski et al., 2013). Of note, RNF11 suppression was associated with enhanced expression of antioxidants GSS and SOD1, neurotrophic factor BDNF and the anti-apoptotic factor Bcl2, whose transcription is believed to rely on non-canonical NFκB pathway activation. Overall these studies highlight the dual role of NFκB in neuroprotection, and emphasize the importance of tight regulation of NFκB pathway activation within the context of neuroinflammation during neurodegenerative conditions.

4.3.3 NFκB essential modulator (NEMO)—The selective NEMO inhibitor compound A is a small molecule that specifically blocks the catalytic activity of IKKβ, and therefore the activation of the p50/p65 NFκB heterodimer (May et al., 2000). Compound A inhibited the production of pro-inflammatory factors by LPS-activated microglia and p65 nuclear translocation, and protected DA neurons from LPS-induced degeneration *in vivo* (Zhang et al., 2010). Accordingly, another IKK-targeted molecule, the peptide corresponding to the IKK NEMO-binding domain (NBD), reduced nigral activation of NFκB, suppressed microglial activation in the SN, protected nigrostriatal neurons, and improved motor performance in MPTP-treated mice and monkeys, while suppressing inflammatory molecule production (Ghosh et al., 2007; Mondal et al., 2012).

Taken together, these pre-clinical studies strongly suggest that selective targeting of the canonical NFκB activation pathway may afford protection against neurodegeneration in PD (Ghosh et al., 2007; Zhang et al., 2010). More specifically, the identification of selective inhibitors of the canonical pathway, which may target specific components like IKKβ and IKKγ but not molecules belonging to the non-canonical pathway like IKKα or the P100/p52, may represent therapeutic agents in the treatment of chronic inflammation-induced neurotoxicity in PD.

5. Clinical studies to target microglia activation with immune-modulating drugs

It is reasonable to speculate that different phenotypes of activated microglia co-exist in the PD brain, given that functionally distinct microglia have been reported in Alzheimer's disease and in the aging brain (Hoozemans et al., 2006; Jimenez et al., 2008). Therefore, it is possible that the dynamic interactions between microglia and other pathogenic components

drive PD progression via excessive cytokine release (Tansey and Goldberg, 2010). For these reasons, microglia polarization has been proposed to be a reasonable target for neuroprotective therapies by several researchers (Sanchez-Guajardo et al., 2013; Tansey and Goldberg, 2010). The recognition that pro-inflammatory microglia are likely to play a critical role in responses that hasten neuronal death has prompted great interest in testing anti-inflammatory drugs as disease-modifying agents in PD. Non-steroidal anti-inflammatory drugs (NSAIDs) have provided positive results in preclinical models; however epidemiological studies have yielded conflicting results on the extent to which chronic intake of these drugs reduce the risk of PD (Bower et al., 2006; Chen et al., 2005; Esposito et al., 2007; Gao et al., 2011b; Noyce et al., 2012). More recent understanding of the complex immune-regulatory function of microglia in PD, and the recognition of phenotypes with diverse effector functions in the course of this pathology, have suggested that strategies aimed at modulating rather than inhibiting microglia activation may represent a promising therapeutic approach in the treatment of PD (Tansey and Goldberg, 2010).

5.1. Peroxisome proliferator receptor (PPAR) gamma (PPAR-γ)

PPAR-γ is a member of the nuclear hormone receptor superfamily. This receptor was first characterized in adipose tissue as a key regulator of lipid and glucose metabolism through modulation of gene expression. Endogenous ligands include oxidized fatty acids, unsaturated fatty acids, 15-deoxy-Δ-prostaglandin J2 (15d-PGJ2). Synthetic ligands are some non-steroidal anti-inflammatory drugs such as ibuprofen, fenoprofen, indomethacin, and thiazolidinediones (TZDs) including pioglitazone and rosiglitazone, that are FDA approved drugs for the treatment of type II diabetes mellitus (T2DM) (Carta, 2013). More recently, the main role of PPAR-γ in the regulation of immune responses and neuroinflammation, has gained increasing attention and led to the investigation of synthetic agonists as a therapeutic against pathologies with a neuroinflammatory component, such as neurodegenerative diseases. As a result, preclinical studies have consistently reported the neuroprotective efficacy of PPAR-γ agonists in different PD models in rodents as well as in primates (Carta et al., 2011; Dehmer et al., 2004; Schintu et al., 2009; Swanson et al., 2011). A phase II clinical trial with the agonist pioglitazone, tested as a disease modifying agent in early PD, has failed to confirm preclinical results (NCT01280123). However, several issues mostly related to the PK profile of this drug, including the scarce BBB permeability, leave uncertain whether the drug sufficiently reached the target, suggesting the need for further clinical evaluation of PPAR-γ agonists in PD (Carta and Simuni, 2015; NETPD-Investigators, 2014).

PPAR-γ is highly expressed in peripheral immune cells, including macrophages, lymphocytes, and dendritic cells, as well as in immune cells in the brain. Besides microglia, astrocytes, oligodendrocytes and neurons also express PPAR-γ (Bernardo et al., 2000; Varga et al., 2011). Gene expression is regulated by PPAR-γ through mechanisms involving transactivation, where the PPAR-γ/retinoid × receptor heterodimer binds to the PPAR-responsive elements (PPREs) in the promoter region of target genes (Ricote and Glass, 2007). Alternatively, inhibition of gene expression may occur via transrepression mechanisms, that are independent from DNA-binding, involving physical sequestration of activated transcriptional factors, including NFkB through a direct interaction with the p65

subunit (Ricote and Glass, 2007). Relevant to the present discussion, modulation of target genes involved in the inflammatory response includes: suppression of pro-inflammatory cytokines and induction of anti-inflammatory molecules, upregulation of antioxidant enzymes, upregulation of scavenger receptors such as CD36 (Ballesteros et al., 2014; Carta, 2013; Martin et al., 2012; Reddy et al., 2008). Targeting PPAR-γ is known to modulate the phenotype of peripheral macrophages via the above cited mechanisms. Hence, a number of studies have shown that PPAR-γ modulates the production of both pro- and anti-inflammatory cytokines by these cells, boosting the anti-inflammatory benefit while suppressing the harmful macrophage phenotype (Odegaard et al., 2007; Pascual et al., 2007; Satoh et al., 2010; Straus and Glass, 2007; Szanto et al., 2010). In contrast, in infected peripheral macrophages the natural PPAR-γ ligand 15dPGJ2 increased both anti-inflammatory polarization and phagocytic activity of these cells (Penas et al, 2013). We have recently shown in a chronic MPTP mouse model of PD, that PPAR-γ agonists administered in a neuroprotective regimen, hampered pro-inflammatory microglia while boosting an anti-inflammatory phenotype, suggesting that the immunomodulatory properties may contribute to the disease modifying potential of PPAR-γ agonists in PD (Pisanu et al., 2014).

5.2 Glucagon-like peptide-1 (GLP-1)

GLP-1 is a target for neuroprotection and has elicited much interest in recent years (Aviles-Olmos et al., 2013; Holscher, 2014). GLP-1 is an incretin hormone which facilitates insulin release in hyperglycemic episodes and overcome insulin desensitization, and GLP-1 mimetics are a successful strategy to treat T2DM (Holscher, 2014). Specifically, the GLP-1 synthetic agonist exenatide has shown neuroprotective properties in multiple animal models of PD (Bertilsson et al., 2008; Harkavyi et al., 2008; Kim et al., 2009; Li et al., 2009; Rampersaud et al., 2012). Exenatide has been recently evaluated in a proof of concept study using a single-blind trial design, where 45 patients with moderate PD were monitored for disease progression, and randomly assigned to receive subcutaneous exenatide for 12 months or to act as controls. In this pilot study, exenatide group suggested clinically relevant improvements across motor and cognitive measures as compared with the control group (NCT01174810) (Aviles-Olmos et al., 2013; Foltynie and Aviles-Olmos, 2014). The mechanism underlying GLP-1-mediated neuroprotection is still a matter of controversy. As a growth factor, GLP-1 neuroprotective effects may be partly due to classic growth factor effects such as increased expression of genes involved in cell growth and repair, increase of cell metabolism, and inhibition of apoptosis (Holscher, 2014). In addition, accumulating evidence indicates that GLP-1 mimetics display anti-inflammatory properties, which may largely support the neuroprotective action of these compounds, by suppressing the expression of inflammatory molecules as exemplified by the inhibition of IL-1β production in LPS-stimulated astrocytes treated with GLP-1 (Iwai et al., 2006). In mouse macrophages, exenatide treatment suppressed LPS-induced mRNA expression of TNF-α, monocyte chemoattractant protein-1, and nuclear translocation of p65, while in human monocytes it reduced the expression of CD11b (Arakawa et al., 2010). In MPTP-intoxicated mice, exenatide prevented the MPTP induced microglia activation and expression of TNF-α, IL-1β and MMP-3 mRNA in the SN (Kim et al., 2009). A recent study showed that increase of GLP-1 levels obtained by the delivery of dipeptidyl peptidase (DPP)-4 inhibitor saxagliptin, resulted in neuroprotection in the rotenone rat model of dopaminergic degeneration, and

decreased the rotenone-induced inflammatory response, by reducing NF-κB activation, iNOS, TNF-α, ICAM-1 and myeloperoxidase (Nassar et al., 2015).

5.3 Minocycline

Minocycline is a tetracycline derivative used for treating a variety of infections. Besides antimicrobial effects, minocycline was shown to exert neuroprotective and anti-inflammatory activity in preclinical studies in the MPTP model of PD (Du et al., 2001; Thomas and Le, 2004; Wu et al., 2002). In vitro, minocycline inhibited MPP(+)-induced neurotoxicity and iNOS expression via suppression of p38 MAPK phosphorylation, but neuroprotection was afforded only in the presence of glial cells (Du et al., 2001). In MPTP-treated mice, minocycline inhibited MPTP-induced activation of microglia and production of IL-1β and iNOS in the SN (Wu et al., 2002). A Phase II futility trial in early untreated PD patients and an 18-month follow-up study indicated that minocycline could not be rejected as futile and did present safety concerns; it therefore received consideration for definitive Phase III trials in order to determine whether it had disease-modifying activity on the long term progression of PD (Investigators, 2006, 2008).

5.4 Cannabinoid receptor 2 (CB2)

Cannabinoids act as a major signaling regulator of various neurotransmitters and have anti-inflammatory properties (Giuffrida et al., 1999; Gomez-Galvez et al., 2015; Romero et al., 1996). The role of the cannabinoid system is thought to be dependent on which receptor is activated, CB1 or CB2. Earlier therapeutic studies focused on altering CB1 activity because of its high expression in the central nervous system, specifically in the basal ganglia, and its hypokinesia effect on levodopa induced motor complications (Fox et al., 2002; Morgese et al., 2007; Sieradzan et al., 2001). However, the therapeutic potential of CB1 antagonists is controversial due to inconsistent results including a clinical trial testing rimonabant, a CB1 antagonist, which did not demonstrate any antiparkinsonian effects on either the severity of motor deficits or levodopa-induced dyskinesias (Mesnage et al., 2004). Whereas, CB2 receptors are highly expressed on activated microglia and are upregulated in the SN of PD patients (Gomez-Galvez et al., 2015). Various studies have selectively blocked CB2 receptors to modulate the microglial activation state leading to neuroprotection in a mouse model of PD (Gomez-Galvez et al., 2015) and mice with mild traumatic brain injury (Reiner et al., 2015). Both studies demonstrate a reduction in pro-inflammatory mediators such as TNFα, IL-1 β, IL-6, IL-10 following CB2 antagonism in primary human microglial cells (Reiner et al., 2015) or intrastriatal LPS-treated mice (Gomez-Galvez et al., 2015). Although there are currently no clinical trials modulating CB2 activity, pre-clinical work demonstrates its great promise as a therapeutic target.

5.5 Granulocyte-macrophage colony-stimulating factor (GM-CSF)

GM-CSF is an immune modulator that has been safely used to reduce infections in immunosuppressed patients following chemotherapy by increasing myeloid progenitor proliferation, and more recently has shown to induce regulatory T cells (Tregs), which are dysfunctional in PD (Buchsel et al., 2002; Rowin et al., 2012; Sheng et al., 2011). GM-CSF treatment demonstrated neuroprotective properties in MPTP- (Kosloski et al., 2013) and paraquat-treated mice (Mangano et al., 2011), with increases in Tregs and downregulation of

CD11b+ microglia, suggesting a role for GM-CSF to effect the pro-inflammatory nature of microglia. After the success of GM-CSF in animal models of PD, a clinical trial is currently recruiting patients to evaluate the safety of an engineered GM-CSF called Leukine (sargamostrim) in idiopathic PD (NCT01882010).

6. Concluding remarks

The idea that microglia may assume a spectrum of phenotypes and serve multiple effector functions has revolutionized the classical view of “activated microglia” as an oversimplified concept pointing to the need for a deeper characterization of their heterogeneous nature in relation with disease progression. In light of this novel view a growing area of research is now focusing on understanding whether activated microglia may exert a protective function at some stage of PD via production of neurotrophic factors, or contribute to neuronal death by producing inflammatory factors that are toxic to DA and other vulnerable neurons, and whether they influence typical pathological mechanisms such as α-synuclein aggregation and propagation. Yet is likely that the age-associated changes in inflammatory responses, along with genetic and environmental factors, give rise to a prevalence of pro-inflammatory microglia in PD, which likely contribute to progressive neuronal loss.

Studies in biofluids from PD patients have shown an overproduction and coexistence of both pro- and anti-inflammatory cytokines, yet understanding the time course and degree to which microglia produce these mediators may provide hints to therapeutic immunomodulatory targets and identify appropriate candidates for successful intervention. Moreover, human studies are limited by an exceeding number of patients who die in late stages of the disease, and by a therapeutic history which may have affected disease-related immune responses, undermining the possibility to understand the complex dynamic of microglia states in the different stages of PD. Post mortem studies have demonstrated the presence of activated and phagocytic cells in the vicinity of remaining nigral DA neurons, suggesting the contribution of microglia to the progression of neurodegeneration, yet leaving undetermined whether they could play a beneficial role at earlier stages of the neuropathology. While imaging studies have provided evidence of ongoing, persistent activation of microglia in PD by detection of TSPO ligand binding, techniques that would distinguish different phenotypes of activated microglia are needed in the future for a better understanding of microglia phenotype in the brain particularly during the early stages of PD.

Animal studies have permitted a closer and more specific look at the activation state of microglia in relation to the extent of neurodegeneration, and have given the possibility to follow dynamic changes in phenotype and function along with disease progression. According to the concept that microglia may display different phenotypes depending both on the type and strength of the stimulus, a comparative look at animal studies reveals that progression of microgliosis and microglia polarization is to some extent dependent upon the animal model and species, whether a toxin or inflammatory stimulus has been administered, and the regimen of administration. This peculiarity has offered the great possibility to dissect and manipulate the inflammatory process at different stages of degeneration. Overall, studies conducted in parkinsonian monkeys and toxin-based models support an early microglia activation in the course of DA neuronal loss, while inflammation-based models as well as

LRRK2 transgenic mice have added the pivotal concept that neuroinflammation may be both an initiating stimulus and a self-propelling mechanism of progressive neuron degeneration. Studies based on α-synuclein overexpression have disclosed the relationship between the accumulation of different forms of α-synuclein and subsequent inflammation, and have been pivotal to understand that levels of α-synuclein-induced neuronal pathology strictly modulate the phenotype of activated microglia, inducing different phenotypes and effector functions at early versus late stages of neurodegeneration. A careful evaluation of cytokines content in the different PD models has attributed a specific pathological role to pro-inflammatory cytokines in disease progression, suggesting that an early and persistent overproduction of detrimental cytokines may precede microglia polarization towards the phagocytic phenotype expressing high levels of CD68, which characterize later stages of intense neurodegeneration, likely contributing to spreading of neuronal loss. On the other hand, a still scarce number of studies have described the expression and production of anti-inflammatory molecules from activated microglia. These studies have shown that markers typically associated with a beneficial phenotype were gradually decreased in the SN, and that the increased production of pro-inflammatory mediators was associated with motor impairment and massive cell death. All together animal studies lead to the suggestion that inflammation-driven DA neuron degeneration may be underpinned by a gradual process of skewed microglia polarization, with prevalence of pro-inflammatory microglia beginning early in the course of the disease, and further polarization to phagocytic phenotype which sustains massive cell destruction and its progression toward late end-stages. Further studies in this direction will be needed to fully address the still unresolved question whether microglia neurotoxicity is driven by the switch from a beneficial neuroprotective phenotype to one in which microglia are chronically activated to secrete harmful pro-inflammatory cytokines that enhance apoptotic cell death cascades in neurons.

From a mechanistic perspective, enhanced NFκB activation may hold a role in pathologically activated state of pro-inflammatory microglia, given the evidence that inhibition of the NFκB pathway confer neuroprotection in animal models of PD by dampening inflammatory responses. Based on this assumption, the NFκB activation pathway has been profoundly dissected and investigated in relation to PD pathogenesis. The emerging hypothesis is that in PD a skewed activation of the canonical NFκB pathway may dominate over a more balanced stimulation of both the canonical and non-canonical pathways, leading to microglia polarization and DA degeneration. Several studies support this concept by showing that manipulation of specific molecules belonging to the canonical NFκB signaling including regulator of G-protein signaling 10 (RGS10), RING finger protein 11 (RNF11), and NEMO can drive protection or degeneration effects in animal models of PD, leading to the hope that selective regulators of the canonical NFκB pathway may represent therapeutic agents in the treatment of chronic inflammation-induced neurotoxicity in PD.

Based on advancing knowledge on the spectrum microglia phenotypes, chronic microglia activation is proposed as a reasonable target for neuroprotective therapies in PD. Testing of drugs with anti-inflammatory and/or immunomodulatory properties such PPAR-γ agonists, CB2 modulators, the GLP-1 synthetic agonist exenatide, subclinical dosage of antibiotic minocycline and the immunomodulator GM-CSF goes in this direction. All of these drugs

have shown beneficial results in animal models of PD when administered in the early stages of degeneration and some are now being tested in clinical trials. Given that all evidence points to neuroinflammation being present in the early stages of the degenerative process, it will be critical to test the efficacy of these drugs in patient populations prior to significantly advanced disease in order to maximize chances for success in modifying the course of disease progression.

Acknowledgments

We thank members of the Carta and Tansey labs for useful discussions. Funding for the authors of this review came from NIH/NINDS R01NS072467-05 (MGT), the Michael J. Fox Foundation for Parkinson's Research (MGT), NIH/NIEHS 2T32ES012870-11 (VJ), The Perry & Ruby Stevens Foundation (ARC) and Fondazione Banco di Sardegna U629.2013/AI.553 MGB (ARC).

Abbreviation List

BBB	blood-brain barrier
CSF	cerebral spinal fluid
COX1	cyclo-oxygenase 1
COX2	cyclo-oxygenase 2
CRP	C reactive protein
DA	dopamine
EGF	Epidermal growth factor
FACIT	functional assessment of chronic illness therapy
FcγRII	Fc gamma receptor II
HADS	hospital anxiety and depression scale
IFN-γ	interferon-gamma
IL-1β	interleukin-1 beta
IL-6	interleukin-6
IL-12	interleukin-12
IP-10	interferon gamma-induced protein-10
LRRK2	leucine-rich repeat kinase 2
LPS	lipopolysaccharide
MHC-II	major histocompatibility complex-II
MCP-1	monocyte chemotactic protein-1
MIP-1β	macrophage inflammatory protein-1β

MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
NFκB	nuclear factor kappa beta
NT-proCNP	N-terminal Pro-C-type natriuretic peptide
PET	positron emission tomography
RNF11	Ring Finger Protein 11
SN	substantia nigra
TGF-β	transforming growth factor β
TNF	tumor necrosis factor
TLR	toll-like receptor
6-OHDA	6-hydroxydopamine

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Highlights

- microglia exist in a heterogenous population and their phenotypes are not permanently polarized into two categories
- neuroinflammation and microglia activation are considered neuropathological hallmarks, however their precise role in relation to disease progression is not clear
- animal models have provided insights on dynamic changes of microglia phenotypes in relation to disease progression especially prior to the development of motor deficits microglial phenotypes in the aging brain become further altered in PD giving rise to a prevalence of pro-inflammatory microglia, which likely contribute to progressive neuronal loss
- malfunction at multiple steps of NFκB signaling that may have a causal interrelationship with pathological microglia activation in animal models of PD

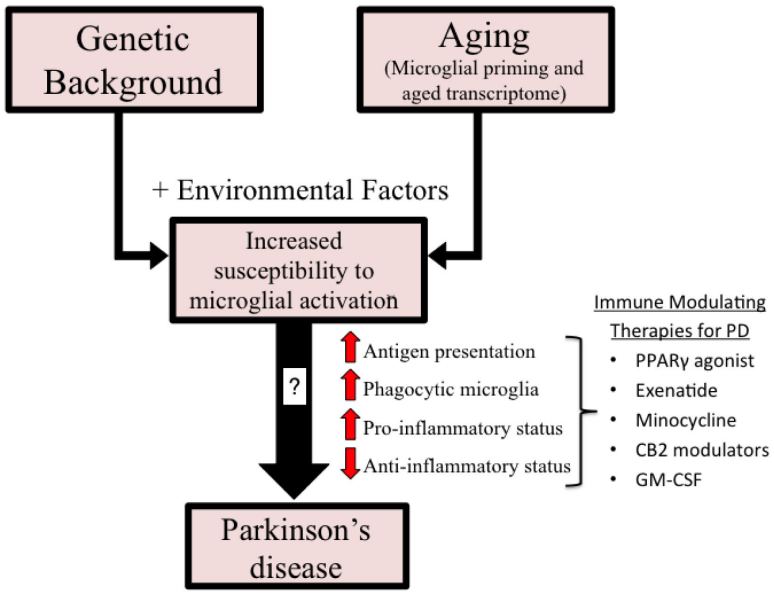

Figure 1. Potential role of inflammation in Parkinson's disease pathogenesis

Results of microglial phenotypes have varied across studies and PD patients. Genetic background and aging, along with other environmental factors (ie: illness) impact the susceptibility of microglia activation leading to a lack of homeostatic support (increased antigen presentation, phagocytic microglia, pro-inflammatory status and decreased anti-inflammatory status) to the surrounding tissue that may further influence the pathogenesis of Parkinson's disease. Multiple immune modulating therapies are currently being investigated for their effect on the phenotypes and effector functions of activated microglia and may represent promising approaches in the treatment of PD.

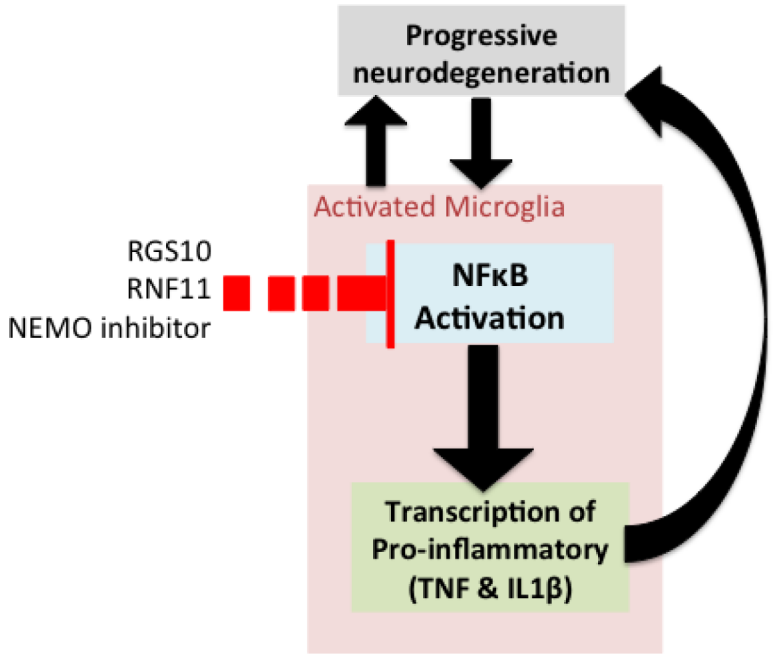

Figure 2. Microglial NFκB regulation of immune responses in animal models of PD

Malfunctions at multiple steps of NFκB signaling have been argued to have a causal interrelationship with pathological microglia leading to increased pro-inflammatory gene expression and further contributing to neurodegeneration. Regulator of G-protein signaling 10 (RGS10), RING finger protein 11 (RNF11), and NEMO inhibitor exert negative regulation on NFκB signaling producing a dampened immune response and reduced neurodegeneration.

Table 1

Cytokines detected in brain tissue, cerebral spinal fluid (CSF) or serum of Parkinson's disease patients. Results are in comparison with control patients. CRP, C reactive protein; EGF, Epidermal growth factor; FACIT, functional assessment of chronic illness therapy; HADS, hospital anxiety and depression scale; IFN-γ, interferon-gamma; IP-10, interferon gamma-induced protein-10; MCP-1, monocyte chemotactic protein-1; MIP-1β, macrophage inflammatory protein-1β; NT-proCNP, N-terminal Pro-C-type natriuretic peptide; SN, substantia nigra; TGF- β, transforming growth factor β; TNF, tumor necrosis factor

Tissue or Biofluid	Number of PD patients (M;F)	Mean age of PD (years)	Hoehn & Yahr	duration of disease (years)	Number of controls (M;F)	Mean age of controls (years)	Results	Comments	Reference
Caudate and putamen	5M;4F	72	-	-	6M;5F	71	Caudate/ Putamen: ↑ IL-1β, IL-6, TNF-α Caudate only: ↑ EGF	No significant changes found in the cerebral cortex of PD compared to controls.	Mogi et al., 1994a
Caudate and putamen	9	No mean defined (75 - 91)	-	-	8	No mean defined (75 - 91)	↑ TNF-α		Mogi et al., 1994b
Caudate and putamen	5M;4F	72	-	-	6M;5F	71	↑ TGF-β1		Mogi et al., 1995
Caudate, Putamen and SN	12M;3F	69.7	-	16.9	6M; 8F	60.8	Caudate/ Putamen/SN: ↑ IFNγ and NF-κBp65 Caudate: ↑ p53 (apoptosis related protein)	Similar levels of INFγ,NF-κBp65, and p53 in cerebellum and frontal cortex of PD and controls.	Mogi et al., 2007
CSF	6M;9F	58	-	-	6M;10F	46	↑ TNF-α		Mogi et al., 1994b
	12M;10F	61 ± 1.54	1-3	0.5 - 3	6M;6F	61 ± 4.15	↑ IL-1β, IL-6	No changes in CSF IL-2. No significant differences found in plasma. Measurements taken off medication.	Blum-degen et al., 1995
	7M;7F	68	-	-	10M;3F	46	↑ TGF-β1	Evaluations completed on ventricular CSF.	Mogi et al., 1995
	22M;8F	73.8 ± 1.4	-	-	11M;5F	72.7 ± 3.1	↑ TGF-β1 and TGF-β2	Evaluations completed on ventricular CSF	Vawter et al., 1996
	8M;4F	57.5 ± 2.6	-	-			↑ IL-6	Evaluated de novo patients. IL-6 negatively correlated with UPDRS scores.	Muller et al., 1998
	44M;27F (non-demented)	64.1 ± 10.5	1.9 ± 0.8	6.4 ± 10.5	14M;19F	65.8 ± 8.8	No significant differences in CRP, IL-	CRP and MCP-1 correlated with	Lindqvist et al., 2013

Tissue or Biofluid	Number of PD patients (M;F)	Mean age of PD (years)	Hoehn & Yahr	duration of disease (years)	Number of controls (M;F)	Mean age of controls (years)	Results	Comments	Reference
	12M;4F (demented)	72 ± 5.8	3.1 ± 0.9	15.8 ± 6.5			6, TNF-α, eotaxin, IP-10, MCP-1, and MIP-1β ↑ CRP (compared to non-demented PD and controls)	HADS depression score. CRP correlated with FACIT score (fatigue)	
	93M;33F	63.8 ± 10.4	-	-	76M;61F	58.9 ± 18.4	No significant difference in fractalkine	Frackalkine/ Aβ1-42 positively correlated to UPDRS score and H&Y.	Shi et al., 2013
Serum	17M;14F	61.5 ± 2.8	2-4	6.9 ± 1	10M;10F	67 ± 3.2	↑ TNF-α, IL-6, IFNγ, IL-10, IL-4, IL-2	Significant increases (1.5x) in TBARS (lipid peroxidation) compared to controls.	Brodacki et al., 2008
	7M;10F	65.3 ± 13.1 (without levodopa)	70.6% <3	2.8 ± 3.9	8M;15F	60 ± 7.8	No significant difference in IL-6	Negative correlation found between serum IL-6 and activities of daily living scale in levodopa group.	Hofmann et al., 2009
	15M;8F	66.5 ± 8.8 (with levodopa)	47.8% <3	8.1 ± 6.1					
	30M;30F	59.17 ± 15.48	2.35 ± 0.83	11.27 ± 10.69	12M;12F	64 ± 5.8	↑ TNF-α, NT-proCNP	Positive correlation between IL-6 and IL-1β; IL-6 and H&Y stage in PD.	Koziorowski et al., 2012