

# Microglial Activation and Chronic Neurodegeneration

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**Summary:** Microglia, the resident innate immune cells in the brain, have long been implicated in the pathology of neurodegenerative diseases. Accumulating evidence points to activated microglia as a chronic source of multiple neurotoxic factors, including tumor necrosis factor- $\alpha$ , nitric oxide, interleukin-1 $\beta$ , and reactive oxygen species (ROS), driving progressive neuron damage. Microglia can become chronically activated by either a single stimulus (e.g., lipopolysaccharide or neuron damage) or multiple stimuli exposures to result in cumulative neuronal loss with time. Although the mechanisms driving these phe-

nomena are just beginning to be understood, reactive microgliosis (the microglial response to neuron damage) and ROS have been implicated as key mechanisms of chronic and neurotoxic microglial activation, particularly in the case of Parkinson's disease. We review the mechanisms of neurotoxicity associated with chronic microglial activation and discuss the role of neuronal death and microglial ROS driving the chronic and toxic microglial phenotype. **Key Words:** Microglia, inflammation-mediated neurodegeneration, oxidative stress, chronic neurotoxicity, reactive microgliosis.

## INTRODUCTION

Neurodegenerative diseases (e.g., Alzheimer's disease, Parkinson's disease (PD), Huntington's disease, amyotrophic lateral sclerosis, and so forth) share many common characteristics, such as changes in microglial number and morphology, elevated cytokine levels, oxidative stress, and progressive neuronal loss. Increasing evidence reports that microglia can become a chronic source of cytokines and reactive oxygen species (ROS) to drive progressive neuron damage, and it is implicated in the chronic nature of neurodegenerative diseases.<sup>1</sup> Although we are predominantly focusing on PD in this article, we also discuss the mechanisms through which microglia can become neurotoxic, we explain current views on why the microglial response is chronic, and we discuss the meaning of these findings in respect to the progressive nature of neurodegenerative disease.

## MICROGLIAL ORIGIN AND MAINTENANCE IN THE CNS

Microglia reside in the CNS, comprise approximately 12% of the brain<sup>2</sup> (depending on brain region, health, or pathology), and serve as the brain's immune defense. Microglia are unique from neurons, oligodendrocytes, and astrocytes, in that they are not derived from the neuroectoderm. Instead, it is generally accepted that the original microglial population in the CNS differentiates from the cells of the myeloid lineage that originate in bone marrow,<sup>2</sup> which occurs early in embryonic development.<sup>3</sup> These myeloid cells can be found within the CNS by embryonic day 8 in rodents,<sup>4</sup> and by week 12 of gestation in humans.<sup>5</sup> At this stage, the cells are referred to as fetal macrophages, and these are the earliest form of microglial precursor cells present in the embryonic CNS.

Once fetal macrophages reside in the developing CNS, they begin the differentiation process that will result in the formation of fully-matured microglia. Although the course of this differentiation is not fully understood, one of the early steps is the formation of rounded "ameboid" microglia that cluster within distinct anatomical regions in the developing brain and may act as a source of microglial progenitors.<sup>6</sup> Later in embryonic and early fetal development, these progenitors will follow a path of migration and differentiation leading to the mature microglia, a process that extends into neonatal development. Differentiation first involves the formation of par-

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tially ramified microglia followed by the development of fully ramified, or branched, microglia that express cell surface molecules characteristic of resting microglia (discussed as follows).<sup>7</sup>

Although the origin of initial microglia populations within the CNS has been well supported, the replacement of microglial populations is matter of more debate. Due to the presence of the blood brain barrier, it was originally perceived that the circulating immune cells did not have immediate access to the CNS, keeping populations of microglia distinct from similar, circulating blood cells. However, there is increasing evidence that bone-marrow derived cells are capable of entering the CNS and differentiating into microglia in adults.<sup>8</sup> For example, studies using bone marrow-chimeras have shown that circulating monocytes infiltrate the CNS under different conditions of injury, inflammation, or disease.<sup>9–11</sup> This has been shown to be possible even when the blood brain barrier remains intact, suggesting a mechanism for entry into the CNS.<sup>9</sup> A possible mechanism for this could be replenishment from perivascular cells, which are bone marrow-derived and have been shown to enter the CNS and differentiate into microglia.<sup>12,13</sup> The mechanisms through which circulating cells are recruited to the CNS, and whether they enter the CNS under normal, resting conditions, however, are poorly understood. At present, there is also ongoing debate regarding the function of infiltrating cells *versus* resident microglia for which it has been suggested that infiltrating precursor cells may be predominantly beneficial cellular actors of wound healing<sup>14</sup> and an underutilized therapeutic resource.<sup>8</sup>

What also remains unclear is whether microglia are capable of a level of self-renewal that is sufficient to support the population of microglia in both resting and activated states. Microglia have a low mitotic rate when at rest, but are capable of high rates of proliferation when activated, suggesting that they have at least a partial ability to counteract cell turnover.<sup>15,16</sup> In addition, populations of microglial progenitors have been proposed to persist in the adult brain that are capable of proliferating to replace microglial populations.<sup>4,17</sup> At present, we are just beginning to understand these basic principles regarding the origin and replacement of microglia, and further research is needed to fully elucidate the mechanisms involved in the complicated life cycle of the immune system of the CNS.

## MICROGLIAL ACTIVATION AND FUNCTION

### Resting microglia

Analogous to the role of macrophages and lymphocytes in the periphery, one role of microglia is to act as the brain's immune defense against disease and injury. In addition to these duties, however, microglia are involved in a number of processes in the normal, healthy CNS. In

a normal brain, microglia are said to be resting, and can be distinguished by both their morphology and pattern of gene expression. In this state, microglia take on a ramified appearance, in which long, thin processes extend from the cell body into the surrounding milieu. The immunological phenotype of this state is characterized by low expression of major histocompatibility complex (MHC) proteins and other antigen-presenting surface receptors.<sup>18</sup> This phenotype is in stark contrast to that of other macrophages, which exist in a more readily activated state. One of the reasons for this may be the absence of serum proteins in the brain that have been shown to cause macrophage activation.<sup>19</sup> In addition, the expression of certain receptors, such as CD200 and CX3CR1 on the microglia cell surface, may interact with ligands that keep microglia in a resting state.<sup>20,21</sup> Resting, ramified, microglia cell bodies are spaced throughout the CNS to avoid cell body overlap, but have been shown to be present with variable density in different brain regions.<sup>22</sup> Notably, this ramified morphology occurs only *in vivo* and is relatively absent from microglia in cell cultures.

Despite the fact that these ramified microglia are referred to as resting, they are constantly surveying the surrounding environment by extending and retracting their processes.<sup>23,24</sup> In doing this, microglia are able to sample the microenvironment, maintain homeostasis, and identify signals that require a response. When reacting to extracellular signals, such as the presence of pathogens, foreign material, and dead or dying cells, microglia may undergo a morphological change into an amoeboid shape with short or nonexistent processes.<sup>25</sup> This morphological change is also accompanied by changes in signaling and gene expression that can result in changes in surface receptor expression, the release of pro- or anti-inflammatory factors, recruitment molecules, and ROS, among others.<sup>26–29</sup> The cumulative effect of these changes in morphology and phenotype is a shift from resting to activated microglia.

### Microglial activation and function in the healthy CNS

Microglia are activated in response to brain injuries and immunological stimuli<sup>25,30–32</sup> to undergo dramatic alterations in morphology, changing from resting, ramified microglia into activated, amoeboid microglia,<sup>25</sup> which is believed to favor phagocytosis and mobility. Unfortunately, changes in morphology are unlikely to differentiate between benign and toxic microglial activation.<sup>33</sup>

In nonpathological states, microglia respond to extracellular stimuli in a number of ways. In the developing brain, and in areas of remodeling, microglia are responsible for the phagocytosis of cellular debris resulting from apoptosis and normal cell death.<sup>18</sup> Microglia are

also responsible for the phagocytosis of other debris present in the extracellular space, including damaged cells, plaques, and foreign matter. For microglia surrounding neurons, subtypes of microglia can provide trophic support to neurons through the release of nerve growth factors, neurotrophins, and other neurotrophic factors.<sup>34</sup> These microglia are also capable of assisting in synaptic plasticity, an observation that was first made in the mid-20th century.<sup>35</sup> Along these lines, microglia have been implicated as the “brain’s electricians,”<sup>36</sup> in which the release of neurotrophic factors and anti-inflammatory cytokines from microglia has been shown to promote synaptic plasticity.<sup>37–39</sup> Specifically in response to injury, activated microglia have been shown to surround damaged neurons and participate in synaptic stripping, a process of removing branches from damaged neurons to promote repair and regrowth,<sup>35,40,41</sup> although recent evidence shows that microglia may play an indirect, anti-inflammatory role in this process.<sup>42</sup> Notably, these beneficial microglial functions often involve changes resembling an activated morphology and protein expression, yet the function is distinct from a classic pro-inflammatory response.

In fact, the majority of microglial functions are beneficial and necessary for a healthy CNS, as activated microglia are critical for CNS wound healing.<sup>36</sup> For example, ablation of infiltrating bone marrow derived cells (that become microglia) in spinal cord injury at specific times has been shown to have disastrous neurotoxic consequences.<sup>14</sup> In addition, microglia have also been shown to release anti-inflammatory and trophic molecules to enhance the survival of surrounding neurons.<sup>43,44</sup> Thus, evidence supports that when microglia become neurotoxic, this is due to both the loss of the beneficial functions<sup>36</sup> and/or a shift to a pro-inflammatory phenotype.<sup>1,45,46</sup>

### Pro-inflammatory microglial activation

Microglia detect and respond to pro-inflammatory triggers by changing to an activated phenotype, resulting in a shift of cellular function to releasing cytotoxic factors (e.g., tumor necrosis factor- $\alpha$  [TNF- $\alpha$ ], NO, and ROS) aimed at destroying the invading pathogens. Surface molecules associated with the innate immune response, such as complement receptors and MHC molecules, are also upregulated upon microglial activation.<sup>47,48</sup> For example, upregulation of MHC proteins enable microglia to act as antigen-presenting cells to T-cells that will enter the brain during active infections.<sup>18</sup>

Microglia are readily activated by an extensive list of pro-inflammatory stimuli, such as lipopolysaccharide (LPS),<sup>49–51</sup> pesticides (e.g., paraquat,<sup>52</sup> dieldrin,<sup>53,54</sup> lindane,<sup>54</sup> and rotenone<sup>55</sup>), disease proteins (e.g., beta-amyloid peptide [A $\beta$ ],<sup>56</sup>  $\alpha$  synuclein,<sup>57</sup> and human immunodeficiency virus (HIV)-Tat<sup>58</sup>), air pollution,<sup>59</sup> and even

neuron damage.<sup>46</sup> In fact, it has been proposed that many disease proteins and environmental toxicants trigger a toxic microglial response because they are misinterpreted as a pathogen.<sup>1</sup> In response to these triggers, microglia can produce of a large array of cytotoxic factors, such as superoxide (O<sub>2</sub><sup>•-</sup>),<sup>27</sup> nitric oxide (NO),<sup>60,61</sup> tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ),<sup>62,63</sup> and inflammatory prostaglandins.<sup>64</sup> Although the detailed components of the microglial response can be stimulus specific, there are common factors of microglial activation.<sup>46</sup>

LPS is a cell wall component of gram-negative bacteria and is a potent stimulus of the microglial innate immune response. The microglial response to LPS has been well-characterized and provides excellent insight into the timing of the multiple factors produced in a pro-inflammatory response. Notably, extracellular O<sub>2</sub><sup>•-</sup> is an immediate toxic factor released by microglia in response to LPS. Interestingly, unique to microglia, LPS-induced production of O<sub>2</sub><sup>•-</sup> is not mediated through the traditional LPS receptor, the toll-like 4 receptor.<sup>50</sup> In fact, recent work has indicated that the MAC-1 receptor is responsible for the LPS-induced activation of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase and the consequent production of O<sub>2</sub><sup>•-</sup> in microglia.<sup>49</sup> Thus, the microglial (O<sub>2</sub><sup>•-</sup>) response is typical (although less robust) when compared with other professional phagocytes, but the mechanisms mediating this response may be cell-specific.

Although the microglial superoxide response is immediate, microglia also respond to LPS with a later pulse of pro-inflammatory factors, such as TNF- $\alpha$ , IL-1 $\beta$ , and NO, both *in vivo* and *in vitro*.<sup>65</sup> Specifically, there is a delay in the production of TNF- $\alpha$ , NO, prostaglandin E<sub>2</sub> (PGE<sub>2</sub>), and interleukin (IL)-1 $\beta$ , in which the TNF- $\alpha$  peaks at 6 hours, the NO peaks at 12 hours, and both the PGE<sub>2</sub> and the IL-1 $\beta$  peak at approximately 24 hours.<sup>65</sup> Thus, one mechanism through which microglia are believed to cause neuron damage is through the excessive and inappropriate release of toxic factors.

### Microglia in disease

Neuroinflammation is characteristic of many neurodegenerative diseases and microglia have been implicated to play both causative and exacerbating roles. In fact, microglia and inflammation-mediated neurodegeneration has been implicated in numerous other diseases and conditions, such as hypoxia,<sup>66</sup> stroke,<sup>67</sup> and neuropathic pain.<sup>68</sup> Neurodegenerative diseases are characterized by chronic and progressive neuronal loss, and pathological levels of cytotoxic substances, such as extracellular debris, elevated levels of pro-inflammatory factors, and production of reactive oxygen species, resulting in oxidative stress. These factors, in addition to the release of others that can activate and recruit microglia, support a role for microglia in diseases, such as Alzheimer’s dis-

ease,<sup>69</sup> PD,<sup>69</sup> multiple sclerosis,<sup>70</sup> amyotrophic lateral sclerosis,<sup>71,72</sup> and HIV-associated neurocognitive disorder.<sup>73</sup>

Although microglia undergo changes as a result of normal aging, including increases in activation or decreases in function and proliferation,<sup>74</sup> the activation of microglia in age-dependent neurodegenerative diseases, such as Alzheimer's disease and PD, seem to be a distinct process.<sup>75</sup> One of the hallmarks of Alzheimer's disease pathology is the existence of  $\beta$ -amyloid plaques, an extracellular protein aggregate, which is normally cleared by microglia. Activated microglia and their toxic effects have been associated with Alzheimer's disease for decades.<sup>76–78</sup> This has led to research showing that not only is  $\beta$ -amyloid directly toxic to neurons,<sup>79</sup> but it also causes microglia to cluster around plaques and become activated, which may perpetuate neuronal damage and death.<sup>80,81</sup> Similarly, activated microglia are associated with damaged neurons in patients with PD,<sup>69</sup> which is discussed in more detail as follows.

In contrast to diseases specifically associated with aging, microglial activation can also play a role in diseases not related to age, and may involve unique properties of microglial cells. A strong example of this is in multiple sclerosis, a disease associated with severe inflammation and demyelination of axons. Usually considered an autoimmune disease, multiple sclerosis is associated with lesions within the white and gray matter of the CNS that have increased levels of activated microglia.<sup>82,83</sup> In addition to increases in microglia-released ROS and pro-inflammatory cytokines,<sup>84–86</sup> microglia may play a large part in the initiation of disease by acting as antigen-presenting cells targeting myelin.<sup>87</sup> In HIV-associated neurocognitive disorder, microglia play a strong part in harboring the HIV and acting as a site of viral replication.<sup>88,89</sup> During this process, microglia become activated and release pro-inflammatory cytokines.<sup>90</sup> In addition, microglia are activated by HIV proteins, such as Tat,<sup>91</sup> to produce ROS. Thus, the interaction between microglia, viral replication/proteins, and the production of cytotoxic factors in HIV-associated neurocognitive disorder has strong implications for disease progression.

These neurodegenerative diseases, as well as others, such as amyotrophic lateral sclerosis, Huntington's disease, and prion diseases, highlight the role that activated microglia can play in cell damage and death. However, in cases of these pathologies, the exact role of microglia remains controversial. Ongoing research seeks to answer questions pertaining to microglial activation in both the development and progression of neurodegeneration.

### Microglia activation and PD

In contrast to the beneficial housekeeping duties of resting and moderately activated microglia, over-activa-

tion of microglia resulting in excess production of inflammatory mediators is in fact neurotoxic,<sup>92–94</sup> and microglial activation has been strongly linked to pathology in PD.<sup>1,95</sup> The term over-activation describes the state in which microglia continually produce inflammatory mediators, which accumulate to levels that are harmful to neurons,<sup>96–98</sup> and often in combination leads to neurodegeneration.<sup>94,98,99</sup>

Pioneering work by McGeer et al.<sup>69</sup> discovered increased staining of the MHC class II cell surface receptor HLA-DR in the substantia nigra (SN) of postmortem PD patient brains, indicating the presence of activated microglia, and first implicating that these cells may have an active pathological role in disease. Since then, many inflammatory mediators such as TNF- $\alpha$ , IL-1 $\beta$ , and IL-6 have been identified in the striatum in human PD postmortem brains,<sup>100–104</sup> in addition to an upregulation of inducible nitric oxide synthase (iNOS) and cyclo-oxygenase 2 in amoeboid microglia located in the SN of PD patients,<sup>105</sup> further suggesting a link between activated microglial cells and neuronal damage in disease. Research has identified a critical role for neuroinflammation in dopamine (DA) neuron damage, as cytokines such as TNF- $\alpha$ <sup>106–108</sup> have been shown to exert DA neuron damage. In fact, continuous expression of low levels of TNF- $\alpha$  in the SN induced by an adenoviral vector will cause chronic microglia/macrophage activation, progressive neurodegeneration, and delayed motor symptoms.<sup>109</sup> Thus, it is not surprising that anti-inflammatory approaches,<sup>95,110–112</sup> including those focusing on TNF- $\alpha$ ,<sup>99,113,114</sup> have been targeted as therapeutic strategies.

Subsequent studies provide a wealth of evidence confirming the presence of activated microglia in PD and PD-like brains. For instance, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) is an illicit drug contaminant linked to human Parkinsonism cases,<sup>115</sup> which is commonly used as an animal model of PD.<sup>116</sup> After MPTP administration, microglial activation in the SN of mouse brain has been confirmed by an increase in cell number, changed cell morphology, increased lectin staining, larger cell bodies, and thicker processes.<sup>117,118</sup> Activated microglia are also present in the brains of MPTP intoxicated monkeys.<sup>119,120</sup> Using positron emission tomographic imaging, a 6-hydroxydopamine model of DA neuron damage showed decreased binding of C-PK11195 in the striatum, indicating increased activation of microglia.<sup>121</sup> Importantly, these findings parallel what is seen in the human PD postmortem brain.<sup>100,122,123</sup> More recently, positron emission tomographic imaging has shown that over-activated microglia are also present in the SN of living PD patients and are associated with DA neuron damage.<sup>124,125</sup> Thus, evidence strongly supports that microglia may play a role in the active pathology driving PD.

## MICROGLIA-MEDIATED DOPAMINERGIC NEURON DAMAGE

Several studies using animal models have demonstrated that the presence of microglia and the production of neurotoxic factors can initiate and amplify neuron damage. Interestingly, multiple factors and toxins are shown to selectively damage DA neurons through microglial activation, such as rotenone,<sup>126</sup> diesel exhaust particles,<sup>127</sup> paraquat,<sup>52</sup>  $\alpha$ -synuclein,<sup>57</sup> and  $A\beta$ .<sup>128</sup> However, early studies using the immunogen LPS, demonstrate that a pro-inflammatory trigger, such as LPS, is toxic to DA neurons only in the presence of microglia,<sup>127,129,130</sup> whereas LPS was one of the first microglia-mediated selective DA toxins identified. At present, it is unclear why DA neurons are more vulnerable to microglial activation when compared with other cell types, but inherent vulnerability to oxidative stress has been implicated.<sup>1</sup>

## MICROGLIAL NADPH OXIDASE, ROS, AND NEUROTOXICITY

### Redox signaling

Unregulated, excessive ROS can indiscriminately damage biomolecules (e.g., protein carbonyls) to impair cellular function, a mechanism through which ROS is believed to contribute to neurodegenerative disease.<sup>131</sup> However, in addition to permanent damage, ROS is capable of a more elegant and selective modification of proteins, whereas ROS is known to target thiol functional groups on cysteine amino acid residues.<sup>131</sup> These reversible modifications can be likened to phosphorylation, and they can regulate protein function, acting as an important process of signal transduction in multiple cell types, including microglia.

### Microglial ROS production and NADPH oxidase

The production of ROS in phagocytes is derived from multiple sources, such as peroxidases inside the cell, NADPH oxidase on the membrane surface, or the oxidative processes of mitochondria.<sup>132</sup> NADPH oxidase is a multi-subunit enzyme that catalyzes the production of  $O_2^{\bullet-}$  from molecular oxygen, and it is the predominant source of extracellular ROS in phagocytes, such as microglia.<sup>129,130</sup> The enzyme complex is dormant in resting phagocytes and can be activated on exposure to specific stimuli, such as bacteria and inflammatory peptides.<sup>133</sup> In resting cells, the cytosolic subunits of NADPH oxidase (p47, p67, p40, and Rac2) are distributed between the cytosol and the membranes of intracellular vesicles and organelles.<sup>133</sup> However, activation induces the cytosolic subunits to translocate to the membranes, where they bind to the membrane-associated subunits (p22 and gp91), assembling the active oxidase that produces  $O_2^{\bullet-}$ .<sup>133</sup>

NADPH oxidase and intracellular ROS have been implicated in several cellular functions, such as migration<sup>134</sup> and phagocytosis.<sup>135</sup> Specific to microglia, ROS produced from NADPH oxidase has been shown to mediate changes in microglia morphology,<sup>130</sup> pro-inflammatory gene expression,<sup>130</sup> and upregulation of markers in response to immunological stimuli.<sup>136</sup>

Interestingly, NADPH oxidase is upregulated in neurodegenerative diseases, such as PD<sup>137</sup> and Alzheimer's disease,<sup>138</sup> indicating a potential role for microglial NADPH oxidase activation in disease and neuron death. In fact, the critical role of NADPH oxidase in mediating inflammation-related neurotoxicity has been well documented,<sup>130</sup> whereas the LPS-induced loss of nigral DA neurons *in vivo* and *in vitro* was significantly less pronounced in NADPH oxidase-deficient mice, when compared with control mice. NADPH oxidase has also been linked to microglia-derived oxidative stress from a variety of neurotoxic insults, such as rotenone,<sup>126</sup> diesel exhaust particles,<sup>127</sup>  $\alpha$ -synuclein,<sup>57</sup>  $A\beta$ ,<sup>128</sup> paraquat,<sup>139</sup> dieldrin,<sup>54</sup> DA neuronal injury,<sup>137,140</sup> prothrombin kringle-2,<sup>141</sup>  $\beta$ 2 adrenergic-receptor activation,<sup>142</sup> angiotensin,<sup>143</sup> and cerebral ischemia-reperfusion injury,<sup>144,145</sup> indicating that microglial NADPH oxidase activation may also be a common denominator of microglial activation associated with neurotoxicity. Currently, the precise species of ROS responsible for NADPH oxidase-induced neurotoxicity are unknown. However, SOD and catalase mimetics, which remove  $O_2^{\bullet-}$  and hydrogen peroxide ( $H_2O_2$ ), respectively, reduce LPS-induced DA toxicity,<sup>146</sup> indicating the critical importance of  $H_2O_2$  and  $O_2^{\bullet-}$  in microglia-mediated neurotoxicity. Thus, microglial-derived ROS may be an essential and common factor of toxic microglial activation.

### NADPH oxidase and redox signaling

The phagocytic oxidase-ROS signaling pathway is the signaling mechanism induced by the increase in intracellular ROS in phagocytes as a response to NADPH oxidase activation (pro-inflammatory redox signaling in phagocytes). The increase in intracellular ROS in phagocytes, such as microglia, includes a number of oxygen species, such as  $O_2^{\bullet-}$ , hydroxyl radical ( $OH^{\bullet-}$ ), lipid hydroperoxides, and their byproducts (e.g.,  $H_2O_2$ ).<sup>147</sup> Although multiple cellular organelles and enzymes contribute to intracellular ROS, it is not surprising that the amount of intracellular ROS produced by NADPH oxidase is dependent on the stimuli. For example, NADPH oxidase contributes to approximately 50% of the LPS-induced intracellular ROS increase<sup>130</sup> in microglia, but substance P-induced intracellular ROS is nearly completely dependent on NADPH oxidase.<sup>148</sup> In the traditional phagocyte, the phagocytic oxidase-ROS pathway has been shown to amplify pro-inflammatory gene expression through their function as second mes-

sengers to regulate several downstream signaling molecules, including protein kinase C, mitogen activated protein kinase, and nuclear factor- $\kappa$ B,<sup>135,149–151</sup> through redox signaling.

Using neuron-glia cultures from NADPH oxidase-deficient mice, studies have shown that NADPH oxidase initiates an intracellular ROS signaling pathway<sup>152</sup> that can activate microglia and amplify the production of pro-inflammatory cytokines, such as TNF- $\alpha$ <sup>130</sup> or PGE<sub>2</sub>.<sup>153</sup> In addition, Min et al.<sup>154</sup> demonstrated that ganglioside induces the activation of microglia, whereas the production of IL-1 $\beta$ , TNF- $\alpha$ , and iNOS are attenuated by the addition of the NADPH oxidase inhibitor, diphenylene iodonium. Furthermore, NADPH oxidase inhibitors and catalase are shown to suppress LPS-induced expression of cytokines (IL-1 $\beta$ , IL-6, and TNF- $\alpha$ ), iNOS, mitogen activated protein kinase, and nuclear factor- $\kappa$ B phosphorylation.<sup>155</sup> Thus, both microglial cellular function and signaling pathways are modified by NADPH oxidase ROS production.

Accumulating evidence supports that NADPH oxidase contributes to microglia-mediated neurotoxicity through two mechanisms. First, activation of NADPH oxidase results in the production of extracellular ROS that is toxic to neurons. Second, activation of NADPH oxidase causes an increase in the microglial intracellular ROS, which enhances the production of pro-inflammatory factors that are toxic to neurons. Given the dual impact of NADPH oxidase activation on neurotoxicity, the role of NADPH oxidase in generating toxic ROS, and the prevalence of NADPH oxidase activation on microglial activation, suggests that microglial NADPH oxidase is a critical mechanism of neuronal death across multiple neurodegenerative diseases. However, the specific mechanisms defining how ROS causes this toxic microglial phenotype through redox-signaling is unknown.

### CHRONIC MICROGLIAL ACTIVATION—PROPAGATION OF DISEASE

Microglia are unique when compared with differentiated myeloid immune cells in the periphery for multiple reasons. In addition to the obvious morphological differences and unique resting profiles characterized in microglia, these cells are also more likely to establish chronic pro-inflammatory responses, rather than demonstrate resolution of the innate immune response, as is common in the peripheral immune system.<sup>156</sup> Although the mechanisms are unclear, we believe that this microglial tendency for a chronic pro-inflammatory response is a key factor driving progressive neuron damage, contributing to the chronic nature of neurodegenerative diseases.

### Reactive microgliosis-chronic response to a single stimulus

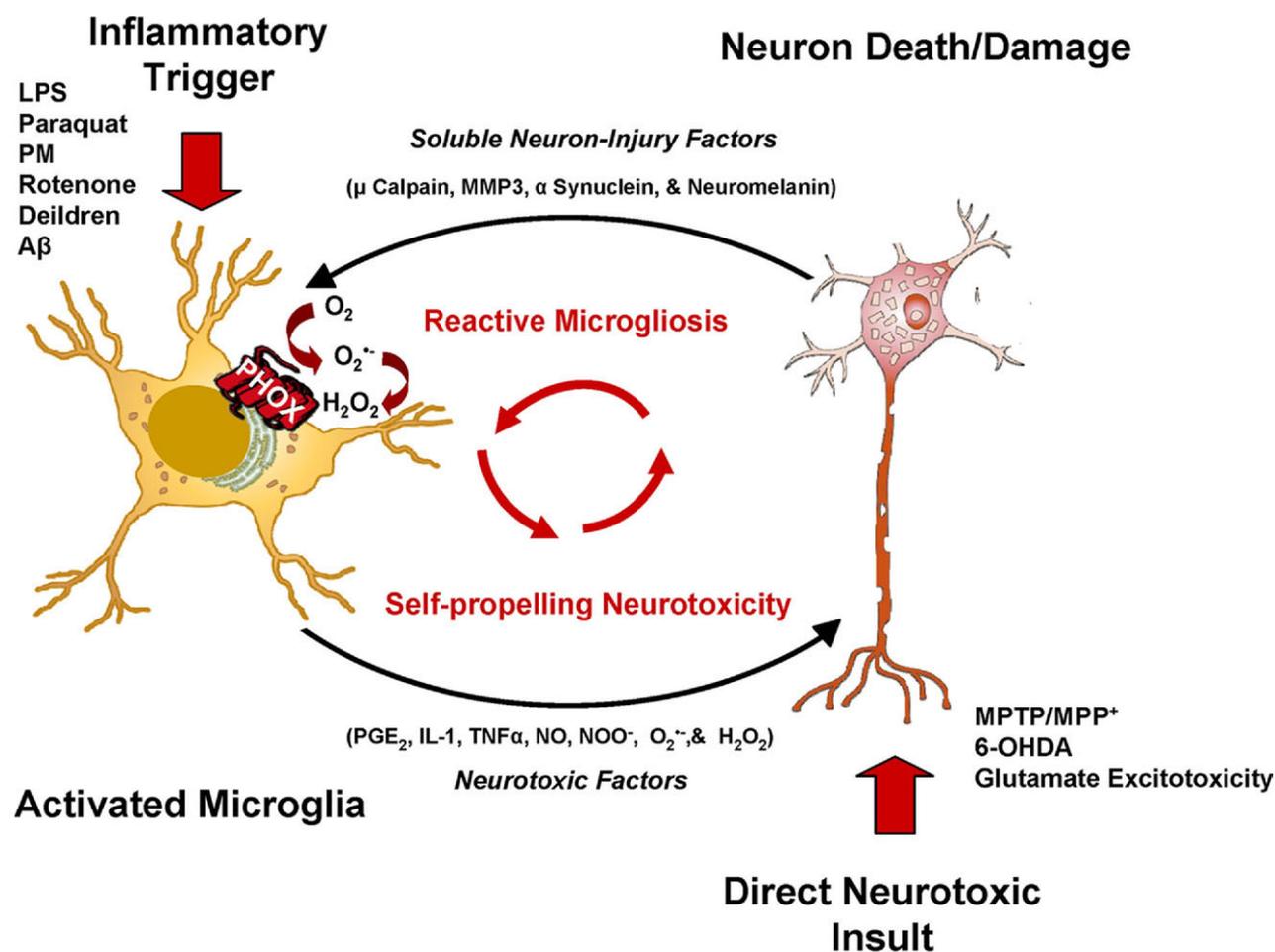
Although microglial activation was initially perceived as a transient event,<sup>31</sup> it is now believed to be chronic and culpable in the propagation of disease.<sup>1,94,157</sup> Reports have shown that microglia can remain chronically activated<sup>140,158,159</sup> in a process that has been termed reactive microgliosis. Reactive microgliosis can be defined as microglial activation, which occurs in response to neuronal damage, which is then perpetuated by further microglial activation and neurotoxicity (FIG. 1). Thus, a self-propelling and progressive cycle of microglial activation and neuron damage ensues.<sup>1</sup>

For example, to damage DA neurons, MPTP is metabolized to 1-methyl-4-phenylpyridinium (MPP<sup>+</sup>), which is then selectively taken up by the dopamine transporter, resulting in inhibition of the mitochondrial electron transport chain complex I.<sup>160</sup> In addition to this mechanism of direct neurotoxicity, MPTP-induced neurotoxicity is also clearly linked with microglial activation *in vivo* and *in vitro*.<sup>137,140,159</sup>

It is particularly interesting that chronic microglial activation can continue years after MPTP exposure in humans<sup>122</sup> and primates,<sup>159</sup> despite the fact that the exposure to MPTP was brief, indicating an incessant, active pathologic process. Importantly, several studies have shown that microglia have an active role in the process of neuronal death in MPTP/MPP<sup>+</sup>-induced neurotoxicity.<sup>137,140,159</sup> Specifically, *in vitro* studies show that while MPTP directly damages DA neurons, both MPTP and MPP<sup>+</sup> fail to directly activate microglia.<sup>140</sup> Rather, microglial activation in response to MPTP or MPP<sup>+</sup> occurs only when neurons are present and this response takes time (days) to accumulate.<sup>140</sup> Furthermore, the addition of microglia to enriched neuron cultures greatly enhances MPTP-induced DA toxicity,<sup>140</sup> demonstrating that microglia cause DA neuron damage in addition to the direct toxic effects of MPTP/MPP<sup>+</sup> on the neuron.

*In vivo* studies also emphasize the important role of inflammation as a toxic component of MPTP/MPP<sup>+</sup> neurotoxicity,<sup>161</sup> in which DA neuron damage in response to MPTP is significantly reduced in mutant mice with deficient production of pro-inflammatory factors, such as nitric oxide,<sup>162</sup> superoxide,<sup>137,163</sup> prostaglandins,<sup>164,165</sup> and TNF- $\alpha$ .<sup>108</sup> Thus, several lines of evidence suggest that microglial activation initiated by neuronal damage may be toxic and persistent, continuing long after the initiating damaging/toxic stimulus is gone.

Recently, we used an *in vitro* approach to separate neuron injury factors from the cellular actors of reactive microgliosis in an attempt to begin to discover molecular signals (soluble neuron injury factors) responsible for chronic and toxic microglial activation.<sup>166</sup> We found that when injury with the DA neurotoxin MPP<sup>+</sup> occurred, DA neurons released soluble neuron injury factors that



**FIG. 1.** Reactive microglia drives chronic neuron damage. Both stimulation of microglia with pro-inflammatory triggers (e.g., lipopolysaccharide [LPS]) and direct neuron damage (e.g., glutamate excitability) result in microglial activation causing the release of neurotoxic factors, such as interleukin-1beta (IL-1β), nitric oxide (NO), tumor necrosis factor-alpha (TNF-α), peroxynitrite (NOO<sup>-</sup>), superoxide (O<sub>2</sub><sup>•-</sup>), and hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>). Subsequently, after damage with either a pro-inflammatory trigger or a direct neurotoxin, the neuron releases microglial activators (soluble neuron-injury signals), such as μ calpain, MMP3, α-synuclein, and neuromelanin, which activate microglial cells and propagate the cycle. This self-perpetuating cycle of neurotoxicity is known as reactive microgliosis. LPS = lipopolysaccharide; MMP3 = matrix metalloproteinase 3; MPTP = 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; MPP<sup>+</sup> = 1-methyl-4-phenylpyridinium ion; 6-OHDA = 6-hydroxydopamine; PGE<sub>2</sub> = prostaglandin E<sub>2</sub>; PM = particulate matter. This figure was slightly modified from Block and Hong (2007)<sup>1</sup>.

activated microglia and were selectively toxic to DA neurons in mixed mesencephalic neuron-glia cultures through NADPH oxidase.<sup>166</sup> This is consistent with other studies that have identified other soluble neuron injury factors that signal toxic microglial activation, such as matrix metalloproteinase 3,<sup>167</sup> α synuclein,<sup>57</sup> and neuromelanin.<sup>168</sup> In addition, we identified μ calpain as a key soluble neuron-injury signal released from damaged neurons, causing selective DA neuron death through activation of microglial NADPH oxidase and superoxide production, converging on the common mechanism of toxic microglial activation through ROS production.<sup>166</sup> Together, these findings support that DA neurons may be inherently susceptible to reactive microgliosis, providing much needed insight into the chronic nature of PD. Notably, although DA neurons may be more vulnerable to reactive microgliosis, it is likely that reactive microgliosis

is a contributing factor to most neurodegenerative diseases.

#### LPS-chronic response to a single stimulus

LPS is reported to activate microglia both *in vivo* and *in vitro* causing the progressive and cumulative loss of DA neurons with time.<sup>129,169,170</sup> Although traditionally believed to be a model of infectious insult, a recently developed PD animal model uses a systemic LPS administration, and shows that a single pro-inflammatory stimulus can persistently activate microglia to cause neuron death.<sup>156</sup> Specifically, we have recently shown that systemic LPS administration activates cells in the liver to produce TNF-α, which is distributed in the blood and transferred to the brain through TNF-α receptors to induce the synthesis of additional TNF-α and other pro-inflammatory factors, creating a persistent and self-pro-

elling neuro-inflammation that induces delayed and progressive loss of DA neurons in the SN of adult animals.<sup>156</sup> This work is the first to support that a single pro-inflammatory stimulus (whether pathogen or environmental in origin) in the adult animal can cause progressive neuron damage later in life, suggesting a wide therapeutic window for the effective use of anti-inflammatory therapy in neurodegenerative disease.

However, earlier reports have already shown that exposure to LPS early in life can induce and enhance DA neuron damage later in life. Studies show that during critical periods of embryonic development (embryonic day 10.5), maternal exposure to low concentrations of LPS in mice impacts microglial activation and DA neuron survival in offspring that persists into adulthood.<sup>170,171</sup> Interestingly, LPS has been implicated in the potential etiology of sporadic PD through sepsis and early life exposure during pregnancy.<sup>169,172,173</sup> Furthermore, diagnosed symptoms of PD, brain inflammation, and damage to the SN have been described in a patient who had received accidental systemic administration of LPS.<sup>174</sup> However, the most important implication of these findings is that not only can microglia induce neuron damage, but microglia can become persistently activated to produce continuous and uncontrolled neurotoxicity that fails to resolve long after the instigating stimulus has dissipated.

### **Priming: Lowering the threshold to initiate neurotoxicity**

The phenomenon of microglial priming offers valuable insight into why microglia continue to respond to additional stimuli in the chronic cycle of neuro-inflammation (FIG. 1). In the case of priming, microglia are not just exhibiting an enhanced toxic microglial response. Rather, in the case of priming, the microglial phenotype shifts, in which a much lower stimulus is needed to exact a toxic microglial response, which enhances the probability that the chronic cycle of toxic reactive microgliosis will continue.

Systemic LPS has been widely explored for the ability to amplify ongoing neuropathology in adults. Systemic LPS administration has been shown to enhance prion-induced cognitive deficits, neuroinflammation, and neuropathology.<sup>175</sup> Furthermore, systemic neonatal exposure to LPS is shown to significantly amplify neuronal death associated with ischemic insult.<sup>176</sup> Finally, immunological perturbation during critical periods of development<sup>170</sup> or aging<sup>177</sup> and senescence,<sup>178</sup> can prime microglia, in which additional stimuli results in an exaggerated and prolonged pro-inflammatory response that enhances neuron damage.

By altering concentrations of intracellular ROS and consequent redox-signaling, NADPH oxidase is reported to prime the microglial response to further insult. Trig-

gers of microglia activation, such as rotenone<sup>55</sup> and neuronal death,<sup>179</sup> are shown to prime microglia through NADPH oxidase and result in synergistic microglial activation, which is associated with neurotoxicity on additional insult with LPS. This has particular importance, given that a multiple hit hypothesis<sup>180</sup> has been proposed as a potential mechanism through which environmental toxicants induce neurodegenerative disease during an individual's lifetime and may provide significant insight into the progressive nature of neuroinflammation.

## **CONCLUSIONS AND IMPLICATIONS**

Microglia can be continuously activated to produce toxic factors (cytokines and reactive oxygen species) by either single or chronic exposure to disease proteins, environmental toxins, cytokines, and neuronal damage (reactive microgliosis), resulting in the progressive loss of neurons with time, a fundamental component of neurodegenerative disease. Recent work suggests that DA neurons may be inherently vulnerable to reactive microgliosis, providing much needed insight into the progressive nature of PD. Current research also suggests that redox signaling in microglia may be a critical mechanism of chronic neuro-inflammation that propagates the microglial pro-inflammatory response through amplification of cytokine production and lowering of the stimulus threshold to cause a neurotoxic toxic response. Future research will need to focus on why the microglial response fails to resolve when compared with the peripheral immune system, the mechanisms through which ROS is signaling toxic microglial activation, and to harness this information for the identification of novel therapeutic approaches.

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