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Critical role of the Mac1/NOX2 pathway in mediating reactive microgliosis-generated chronic neuroinflammation and progressive neurodegeneration

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Abstract

As average life expectancy rises throughout the world, neurodegenerative diseases have emerged as one of the greatest global public heath challenges in modern times. Substantial efforts have been made in researching neurodegenerative diseases over the last few decades, yet their predominantly sporadic nature has made uncovering their etiologies challenging. Mounting evidence has suggested that factors like damage-associated molecular patterns (DAMPs) released by stressed and dying neurons are likely involved in disease pathology and in stimulating chronic activation of microglia that contributes to neuronal oxidative stress and degeneration. This review focuses on how the microglial integrin receptor Mac1 and its downstream effector NADPH oxidase (NOX2) contribute to maintaining chronic neuroinflammation and are crucial in inflammation-driven neurotoxicity in neurodegenerative diseases. Our hope is to provide new insights on novel targets and therapies that could slow or even halt neurodegeneration.

Introduction

Neurodegenerative diseases are characterized by a progressive, yet selective regional neuronal loss, whereby chronic neuroinflammation has been verified to contribute to the degenerative process [1–4]. Microglia are widely considered the predominant resident effector cells of the immune system in the central nervous system (CNS) and become activated in response to stimuli such as chemicals and toxins detected in their microenvironment. Microglia typically are activated until they, and other infiltrating leukocytes that may have been recruited, have sufficiently cleared the insult or source of

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deleterious stimuli. Yet in condition where the deleterious stimuli persist, this results in unresolved neuroinflammation that can damage proximal neurons. Collateral damage to neurons releases endogenous proteins and cell membrane fragments that can re-stimulate the activation of microglia forming a chronic state of activation known as reactive microgliosis. Evidence suggests that this low-grade chronic neuroinflammation contributes to progressive neurodegeneration in diseases such as Parkinson's and Alzheimer's diseases. Though the receptors that detect microbial pathogens are shared among all innate immune cells including microglia, these cells are also attuned to detect endogenous cellular components released by cells during stress, injury and death through the same receptors [5]. Though the structure of these intrinsic and extrinsic stimuli are highly variable, they are thought to activate microglia through receptor-mediated signaling of pattern recognition receptors such as Toll-like receptors (TLRs), Nod-like receptors (NLRs), and RIG-1 like receptors (RLRs) or through scavenger, integrin, or RAGE receptors that have similarly promiscuous binding capabilities [1,6–11].

Traditionally, neuroinflammation was also described through the immunological activation of astrocytes, yet today the functional role of astrocytes during neuroinflammation is less clear. The expression of the above-mentioned receptors by astrocytes has been inconclusive and many of their immune functions are still controversial since they were historically confirmed *in vitro* using isolation methods that sill resulted in significant microglial contamination that could alter the outcome of the studies. A recent study from our group demonstrated that, contrary to much of the reported literature, astrocytes may not possess the ability to directly recognize innate immune stimuli such as the bacterial endotoxin LPS. In fact, they rather depend on crosstalk with activated microglia to elicit their activation and promote the release of neurotrophic factors as a counterbalance that supports neuronal survival from the collateral damage generated by activated microglia during neuroinflammation [12]. For this reason, we have chosen to focus on microglia with this review. Furthermore, even though circulating monocytes are known infiltrate the CNS and differentiate into microglial-like macrophages during neuroinflammation [13,14], we will also not be addressing the role of these infiltrating leukocytes since they are only thought to play a pivotal role in disease with high-grade neuroinflammation such as traumatic brain injury and multiple sclerosis and are not thought to be as important in conditions of low-grade neuroinflammation.

Neuroinflammation is a self-defense reaction to combat pathogen infection or clear and restore injuries in the CNS; this reaction is initially carried out by microglia and shaped by reactive astrocytes and other infiltrating leukocytes. Neuroinflammatory responses are typically transient and help restore CNS homeostasis, however in pathological conditions neuroinflammation may continue unresolved becoming persistent [15]. Yet the mechanism by which acute neuroinflammation turns to chronic in neurodegenerative diseases remains largely unknown. Here we have reviewed the literature on reactive microgliosis and hypothesize that persistent Mac1 signaling on microglia is required for continuous activation of NADPH oxidase (NOX2), the main catalytic enzyme responsible for generating extracellular superoxide required to maintain self-propelling reactive microgliosis found in chronic neuroinflammation.

Role of Toll-like receptors in the pathogenesis of acute and chronic neuroinflammation

Acute inflammation

Acute neuroinflammation is typically initiated by detecting the pathogen-associated molecular patterns (PAMPs) from microorganisms, or the damage-associated molecular patterns (DAMPs) molecules released from injured or dying neurons. At this stage, microglia are recruited to clear these signals by preventing infection or restoring injuries in the brain [16]. Toll-like receptors are the most extensively studied receptors stimulated by both PAMPs and DAMPs and are readily expressed on microglia [17]. TLRs signal through various adaptor molecules to stimulate the activation of nuclear factor-κB (NF-κB) and the mitogen-activated protein kinases (MAPKs) like extracellular signal-regulated kinase ½ (Erk1/2), p38, and JNK to induce the production of proinflammatory cytokines and chemokines [18]. DAMPs associated with the pathology of several neurodegenerative diseases act on TLRs [19] such as α-synuclein that acts on TLR2 [20] and both β-amyloid [21–23] and HMGB-1 that act on TLR2, TLR4 and TLR9 [24,25]. Furthermore, brains from patients with neurodegenerative diseases or that have undergone normal aging had far greater TLR gene expression and expression of pro-inflammatory genes associated with TLRs signal transduction [26,27] likely due to increased DAMPs during neurodegeneration. These results put forward the hypothesis that an aberrant TLR activation may contribute to the process of aging and neurodegenerative diseases.

Chronic inflammation

It is widely accepted that chronic low-grade neuroinflammation plays a key role in the pathogenesis of neurodegeneration. However, the molecular mechanism mediating chronic neuroinflammation is less clear. To avoid the excessive damage of host cells by harmful and inappropriate inflammatory responses during the acute phase of inflammation, TLR signals are often promptly dampened or terminated through multiple mechanisms, such as dissociation of adaptor complexes, degradation of signaling proteins, and transcriptional regulation (also known TLR tolerance) [28]. Although TLRs have been widely implicated in neuroinflammation, their role in maintaining reactive microgliosis and chronic neuroinflammaiton has not been well studied. For this reason, we suspect that although TLRs are crucial in the initiation of immune responses during the early phases of neurodegenerative diseases, the maintenance of chronic neuroinflammation is likely mediated by another receptor that detects neuronal DAMPs.

Microglial Mac1 is essential in maintaining chronic neuroinflammation

Our group and others have demonstrated in rodents that the integrin receptor Mac1 (also known as CD11b/CD18, complement receptor 3 [CR3], or $\alpha_M\beta_2$) is essential in maintaining chronic reactive microgliosis and in driving inflammation-mediated neurodegeneration [29–31]. Historically, Mac1 has been recognized as an adhesion molecule that participates in cell signaling during cell-to-cell contact. Yet, during inflammation, Mac1 is required for chemotaxis and phagocytosis in activated neutrophils and macrophages [32,33]. Additionally, the expression of Mac1 is elevated in brains from patients with Alzheimer's disease [34] and in MPTP animal models of Parkinson's disease [35]. Most importantly, microglial Mac1 is capable of binding neuronal DAMPs including α-synuclein [36], β-amyloid [8], HMGB-1 [9], and myelin [37] that are typically associated with neurodegeneration to induce signaling and their subsequent activation. Furthermore, *in vivo* and *in vitro* studies have shown that the genetic ablation of Mac1 results in decreased neurodegeneration when stimulated with the neurotoxicant MPTP [31] or the administration of the exogenous DAMP peptides α-synuclein [36] and β-amyloid [8]. Based on these findings we suspect Mac1 on microglia is the DAMP receptor responsible for maintaining the self-perpetuating reactive microgliosis required in chronic neuroinflammation (Figure 1; [9]).

Mac1–NOX2 signaling bridges chronic neuroinflammation and progressive neurodegeneration

Recent studies have indicated microglial NOX2 activation as an important downstream effector of Mac1 signaling [9,29,38]. NOX2 is composed of cytosolic subunits ($p47^{phox}$, $p67^{phox}$, $p40^{phox}$, and the small Rho GTPase, Rac1 or Rac2) and membrane-bound subunits $p22^{phox}$ and $gp91^{phox}$ [39]. Upon stimulation, cytosolic subunits of NOX2 translocate and bind to the membrane subunits to assemble the catalytically active form of NOX2 that produces extracellular superoxide [40]. When HMGB-1 binds to Mac1 it induces the expressions of several proinflammatory factors including TNF-α, IL-1β and NO through the activation of NF-κB signal pathway and the production of superoxide through the activation of NOX2 [9]. Further interrogation into the mechanism that links DAMPs binding to Mac1to NOX2 activation using β-amyloid shows that the conformational changes of Mac1 upon binding increases the level of PI3K, phosphorylating $p47^{phox}$ and PIP_3 to trigger their translocation from the cytosol to membrane-bound NOX2 to generate superoxide production [8]. Interestingly, when examining the chemotactic role of Mac1 towards aggregated α-synuclein, NOX2 activation was required to produce superoxide that rapidly transmutes into H_2O_2 that activates tyrosine protein kinase Lyn to phosphorylate the F-actin–associated protein cortactin—mediating actin rearrangement required for directional microglia migration [36].

During chronic neuroinflammation, microglia maintain chronic low-grade neuroinflammation through continuous transcription of mRNA of pro-inflammatory factors such as TNF-α, IL-1β, COX2 as well as NOX2 [41]. The importance of NOX2-generated superoxide in the progression of inflammation-driven degeneration in both *in vitro* and *in vivo* models has been established by using microglia derived from NOX2 knockout mice and using NOX2 inhibitors diphenyleneiodonium (DPI) or apocynin [42–44]. Interestingly, the genetic ablation of CD11b, the alpha subunit of the Mac1 receptor in mice, showed a similar ability to prevent superoxide release and attenuated neurodegeneration during neuroinflammation [31,38]. This attenuation occurs by preventing reactive microgliosis along the Mac1-NOX2 axis, whereby microglial Mac1 re-stimulated microglial activation in response to neuronal-derived DAMPs to induce receptor-mediated signal transduction that activates NOX2 to generate superoxide [31]. This pathway, beyond that of TLRs, is crucial for maintaining chronic neuroinflammation and driving inflammation-mediated oxidative stress that leads to neurodegeneration.

Neuronal NOX2 increases neuronal oxidative stress in aging and inflammatory conditions

How microglia-produced proinflammatory factors cause neuronal damage or death is a critical question in neurodegeneration that has not been clarified yet. Evidence suggests that ROS produced from the Mac1-NOX2 signaling pathway plays a crucial role in neuroinflammation-mediated oxidative stress in neurons that results in their degeneration [40]. Neurons are highly sensitive to oxidative stress. During inflammatory conditions, continuous bombardment of pro-inflammatory factors released from microglia gradually increase intracellular ROS (i.e. hydrogen peroxide and peroxynitrite produced by a reaction between superoxide and nitric oxide) within neighboring neurons. ROS-related oxidative stress impairs neuronal mitochondrial functions by the reduction of membrane potential, inhibition of ATP production, and greater production of mitochondria-derived ROS [41,45]. Moreover, upregulation of neuronal NOX expression results from the increased level of ROS and further enhances the production of ROS inside neurons. This feed forward chain reaction likely drives another vicious cycle within neurons generating even more ROS as mitochondria begin to fail until ROS over-production and oxidative stress begin to form protein aggregates, lysosomal malfunction and impaired clearance of dysfunctional mitochondria overall driving neuron death (Figure 2) [40].

Novel anti-inflammatory therapeutic strategies for neurodegenerative diseases by targeting NOX2

Oxidative stress and neuroinflammation are among the most common features shared in all neurodegenerative diseases. Antioxidant therapy has been considered as a strategy to treat neurodegenerative diseases, however the administration of antioxidants (e.g., vitamin C, vitamin E and co-enzyme Q10) showed marginal symptomatic improvements and was unable to halt disease progression in clinical trials for Alzheimer's and Parkinson's disease [46–49]. Since anti-oxidants are designed to neutralize unpaired electrons of free radicals, they are ineffective against hydrogen peroxide and peroxynitrite, the two species thought to be most effective at driving oxidative stress in neurons. Furthermore, targeting inflammation as a method of neutralizing inflammation-mediated oxidative stress in neurodegenerative diseases has also been investigated using nonsteroidal anti-inflammatory drugs (NSAIDs) in clinical trials with similar ineffective results. This is partly because anti-inflammatory therapies target the production of specific cytokines or prostaglandins rather than the mechanism underlying chronic neuroinflammation generation (i.e., the Mac1-NOX2 axis). For this reason, therapies that disrupt the Mac1-NOX2 axis could be more effective strategies in extinguishing chronic neuroinflammation, limiting the neuronal oxidative stress that contributes to neurodegeneration. There are two known anti-inflammatory compounds that reduce Mac1 expression, the natural flavonoid baicalin derived from the roots and leaves of the *Scutellaria baicalensis* plant [50] and the pharmaceutically synthesized leumedin NPC 15669 [51]. Unfortunately, NPC 15669 failed during Phase I clinical trials for undisclosed safety reasons. Interestingly, our group has shown that inhibition of NOX2 in the Mac1-NOX2 axis provides great efficacy at preventing inflammation-driven neurodegeneration in pre-clinical studies of Parkinson's disease. Our laboratory has identified several compounds including morphinans (e.g. dextromethorphan, sinomenine, naloxone and naltrexone) [52–55], peptides (e.g. dynorphine and Gly-Gly-Phe) [56,57], and adrenergic receptor agonist (e.g. salmeterol) [58] that inhibit NOX2-generated superoxide to limit inflammation-driven oxidative stress and neurodegeneration (Fig. 3). Among the most recently published compounds, the established NOX inhibitor diphenyleneiodonium (DPI) was precluded from use in humans due to its high toxicity at micromolar concentrations. Interestingly, when DPI was administered *in vivo* at subpicomolar concentrations it not only had high specificity with long-term NOX2 inhibition but also resulted in no detectable acute cytotoxicity [42]. DPI was so effective, as it was shown to protect mice in multiple models of Parkinson's disease even when administered post disease onset [42]. Though DPI had such great initial preclinical success, we are currently screening several novel blood-brain-barrier permeable NOX2 inhibitors with even greater efficacy and specificity that might become promising clinical therapeutics to extinguish chronic neuroinflammation in neurodegenerative diseases by targeting the Mac1-NOX2 axis.

Conclusions

The role of low-grade, chronic neuroinflammation in the pathogenesis of neurodegenerative diseases continues to be strengthened. Yet our lack of understanding of the cellular and molecular mechanisms that shift inflammation from a tightly controlled acute event into a self-propelling cycle that causes collateral damage has prevented us from developing better targeted interventions to prevent chronic neuroinflammation. The most recent hypothesis based on data collected from healthy and diseased human brain tissue and animal models suggests that gradual increased release of neuronal DAMPs likely drive and maintain reactive microgliosis in neurodegenerative diseases. Although studies have shown that TLRs could be the central receptors for DAMPs during the progression of neurodegenerative disease, we present data that supports the Mac1-NOX2 signaling pathway is not only stimulated by DAMPs but also necessary for neuroinflammation to become sustained and pathological. Unlike TLRs, Mac1 can undergo multiple activations without tolerance-like modulations which more closely resemble the pattern of chronic neuroinflammation seen in neurodegenerative diseases. Thus, we believe designing therapies that disrupt the Mac1-NOX2 axis will likely show great promise in breaking the vicious cycle of uncontrolled neuroinflammation that drives oxidative stress and neurodegeneration in neurodegenerative diseases.

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Highlights

1. Neuroinflammation is a key risk factor in neurodegenerative diseases.
2. Mac1 signaling bridges chronic neuroinflammation and progressive neuronal loss.
3. NADPH oxidase could be a novel therapeutic target for neurodegenerative diseases.
4. Neuroinflammation is a key risk factor in neurodegenerative diseases.
5. Mac1 signaling bridges chronic neuroinflammation and progressive neuronal loss.
6. NADPH oxidase could be a novel therapeutic target for neurodegenerative diseases.

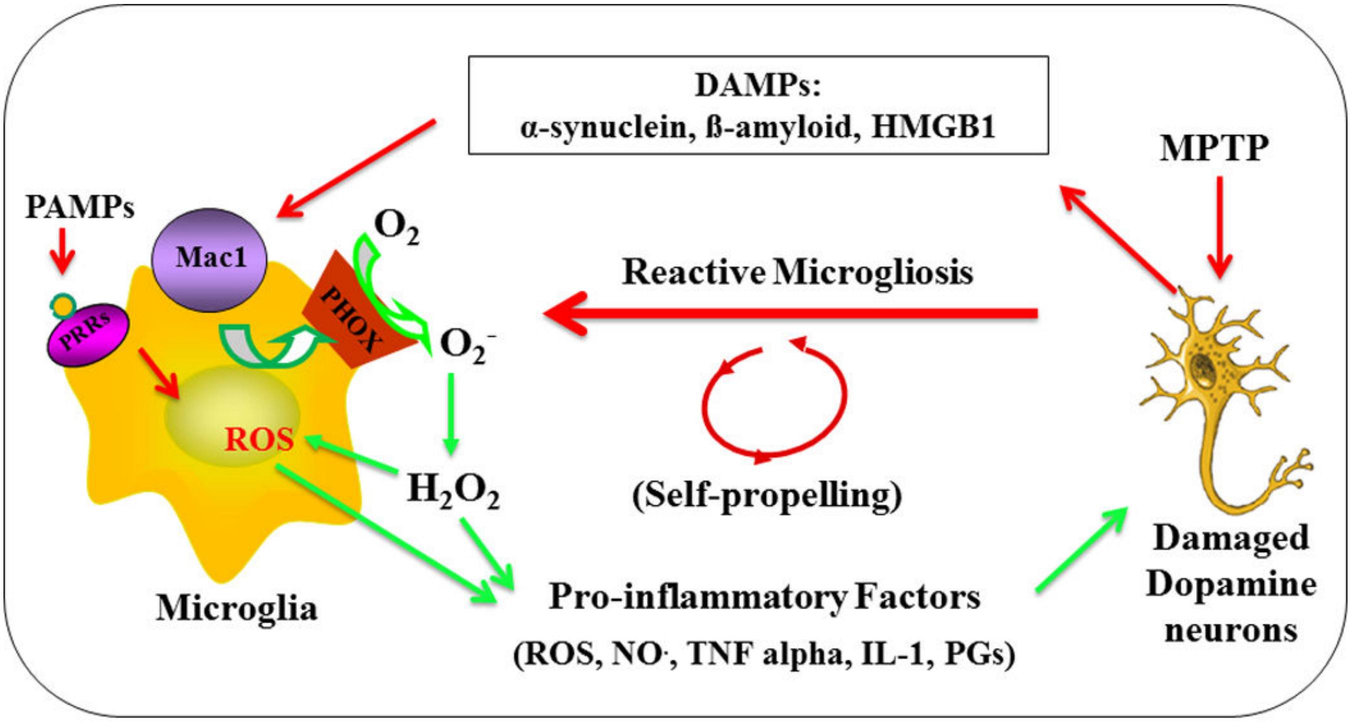

Figure 1.

Reactive microgliosis drives chronic and progressive neurotoxicity. Microglia can initiate neurotoxicity by recognizing pro-inflammatory stimuli, such as cytokines, pathogen associated molecular patterns (PAMPs) from microbial pathogens to become activated and producing cytotoxic factors to damage neurons. Damage-associated molecular patterns (DAMPs) released from damaged/dead neurons can sustain microglia activation (reactive microgliosis), which cause further neuronal damage/death. Microglia Mac1 could recognize DAMPs and activate downstream NADPH oxidase (NOX2) to produce superoxide anions and its associated reactive oxygen species (ROS), such as hydrogen peroxide, which play a critical role in reactive microgliosis and driving the chronic neurodegeneration.

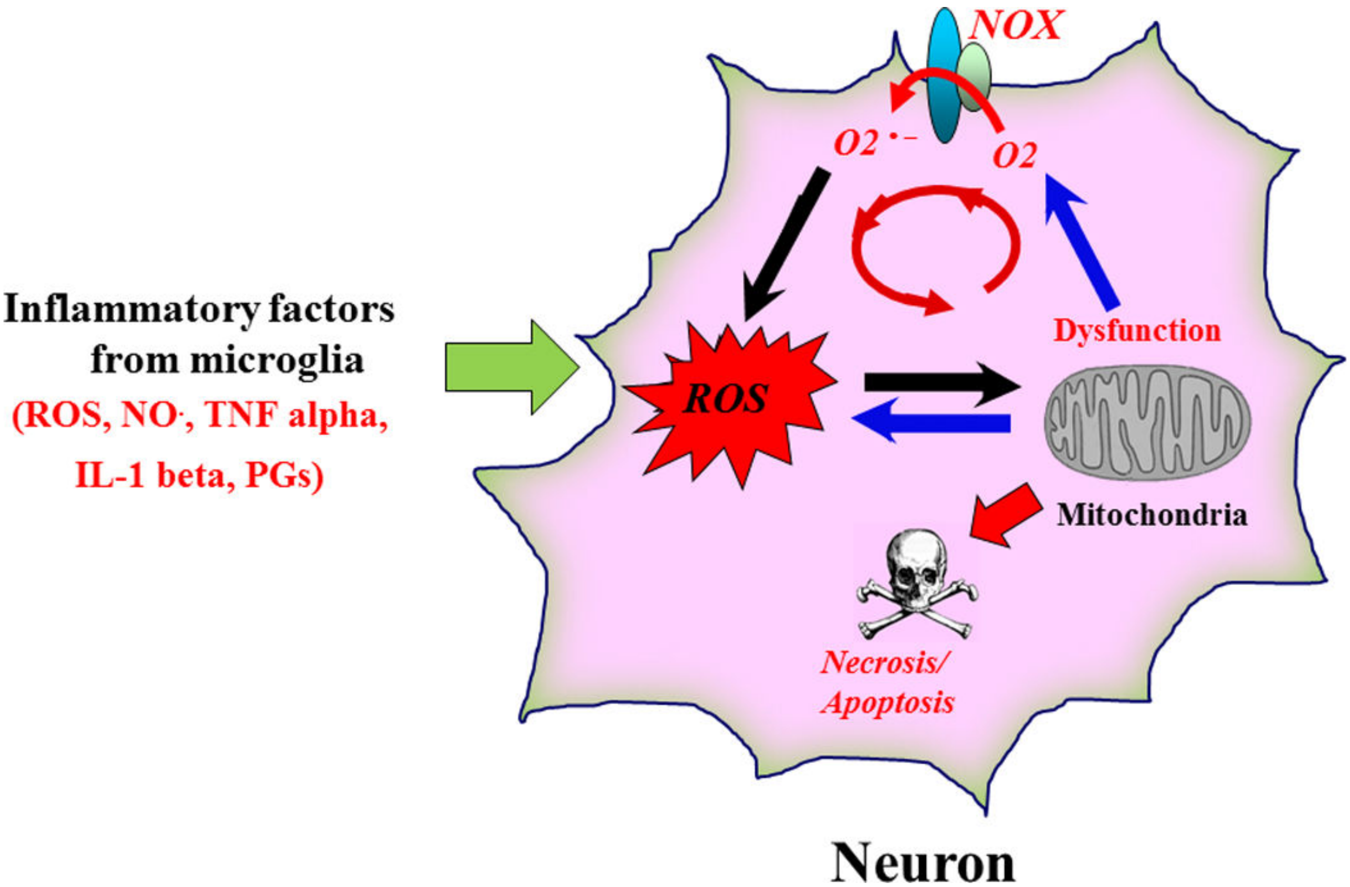

Figure 2.
Inflammation-derived oxidative stress leads to a vicious cycle inside the damaged neurons and causes neuronal death. Sustained release of neurotoxic factors from activated microglia continually bombards neurons and increases neuronal oxidative stress during chronic neuroinflammation. The oxidative stress causes mitochondria dysfunction, which could upregulate the expression of neuronal NOX, produce more ROS and lead to progressive neurodegeneration.

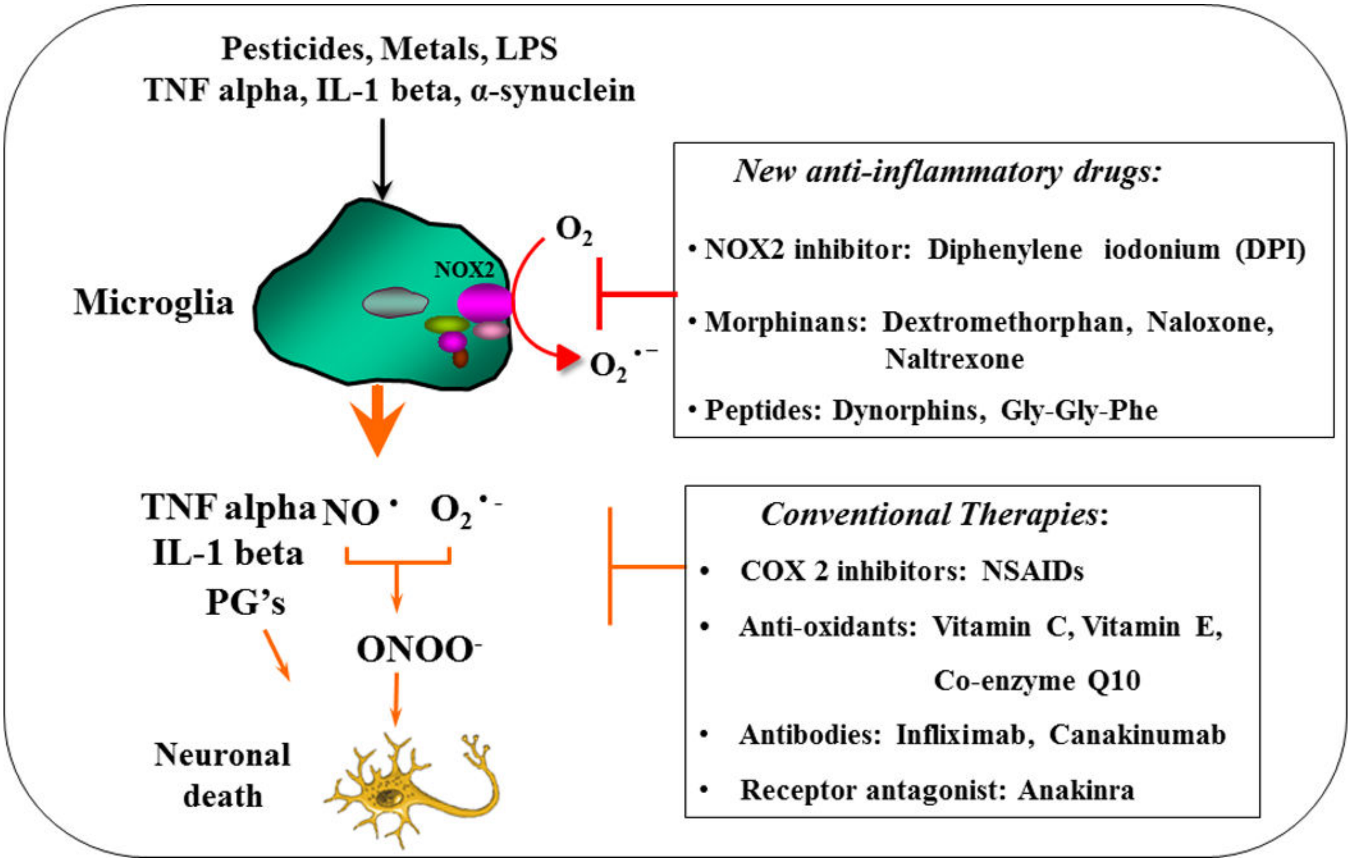

Figure 3.

The novel promising anti-inflammatory therapies. The conventional therapies target a limited number of pro-inflammatory factors and failed to block disease progression. The novel anti-inflammatory therapy targets upstream neuro-inflammatory signaling by inhibiting microglial NOX2, which in turn reduces superoxide production and over-activation of microglia and thereby reducing the release of most pro-inflammatory and detrimental factors.