Perspective

Interplay among norepinephrine, NOX2, and neuroinflammation: key players in Parkinson’s disease and prime targets for therapies

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Abstract

The role of norepinephrine (NE) in the pathogenesis of Parkinson’s disease (PD) has not been well investigated until recently. The purpose of this perspective article is to review evidence supporting the idea that dysfunction of the locus coeruleus (LC)/NE system in the brain may be fundamentally linked to the pathogenesis of PD. Compelling evidence demonstrates that loss of NE neurons in the LC is sufficient to initiate chronic neuroinflammation, resulting in a progressive and sequential loss of neuronal populations in the brain. This article summarizes the critical role of both microglial and neuronal NADPH oxidase 2 (NOX2), the superoxide and reactive oxygen species generating enzyme, as an important regulator of chronic neuroinflammation. Moreover, NOX2 inhibitors show high efficacy in halting chronic neuroinflammation, oxidative damage, and neurodegeneration in several animal PD models. This line of research offers a promising disease-modifying therapeutic strategy for PD.
Keywords: Parkinson’s diseases, progressive neurodegeneration, chronic neuroinflammation, locus coeruleus, noradrenergic system, motor/nonmotor symptoms

INTRODUCTION

Parkinson’s disease (PD) is a neurological disorder characterized by progressive neurodegeneration in the nigrostriatal system, resulting in the development of progressive movement disorders\(^1\). Pathological examination revealed cytoplasmic inclusions known as Lewy bodies or Lewy neurites in the survival dopaminergic (DA) neurons\(^2,3\). About 15% of PD cases occur in familial clusters at early age\(^4\), which are attributed to mutations in genes, including parkin, leucine-rich repeat kinase 2, and \(\alpha\)-synuclein\(^5\). By contrast, the remaining PD cases are sporadic and may represent the final outcome of a complex set of interactions among the innate vulnerability of DA system, genetic predisposition, and environmental toxins exposure\(^6\). Exposures to infectious agents, pesticides, or heavy metals in humans increase the risk of acquiring PD\(^7-13\). We and others have proposed that exposures to these risk factors trigger neuroinflammation, which plays a key role in the pathogenesis of PD\(^14\). However, this concept has not been proved until recently\(^15,16\).

Microglia and astroglia are the two major types of glial cells involved in the initiation and maintenance of neuroinflammation. Microglia, the resident macrophages in the brain\(^17\), play critical roles in the programmed elimination of neural cells in the early stage of neuronal development\(^18,19\). As the brain’s main immune cells, microglia can rapidly be activated in response to brain injuries and immunological stimuli\(^20-23\). Activated microglia undergo morphological and functional changes\(^20\) and increase the expression of many surface molecules\(^24,25\). Activated microglia release a variety of immune factors to recruit more cells and phagocytize foreign substances. In normal physiological conditions, microglia exerts beneficial functions in immune surveillance and depletion of noxious stimuli. By contrast, in pathological conditions, such as chronic inflammation in the brain, microglia can cause neurotoxicity and significantly lead to neurodegeneration.

Different from microglia, astroglia are not derived from immune cell lineage, but they are essential to the integrity and function of the brain\(^26\). Besides serving as an component of the blood-brain barrier (BBB), astroglia provide physical support and nutrition to neurons, buffer excess neurotransmitters, and maintain ionic homeostasis\(^26\). Astroglia also become activated under immunologic challenges or brain injuries\(^27,28\). Activated astroglia secrete a host of neurotrophic factors, such as BDNF, GDNF, and NGF\(^29,30\), which are crucial for the survival of neurons. It has been reported that many anticonvulsant drugs exert potent neuroprotection through astroglia-derived neurotrophic factors\(^31\). These findings suggest that astroglia are promising targets for developing novel therapies for PD.

Interactions among microglia and astroglia are an important yet not fully studied area. It was found that, in response to immunologic challenges, activation of astroglia often depends on the presence of microglia. Secreted immune factors from prior activated microglia can act and turn astroglia into different phenotypes depending on the immune conditions. In physiological condition, increased release of neurotrophic factors, such as GDNF, BDNF, and NGF, benefits neuronal survival\(^32,33\). By contrast, neurotoxic reactive astrocytes (A1 astroglia) induced by activated microglia can exaggerate neurotoxicity in pathological condition\(^34\). Since the role of astroglia in inflammation-related neurodegeneration is less well-documented, this review mainly focuses on the role of microglia in neuroinflammation and neurodegeneration.
Scope of this article
Recent research revealed that low-grade, chronic neuroinflammation is a key to cause progressive neurodegeneration\[^{35,36}\]. However, the detailed mechanisms involved in the onset and maintenance of chronic neuroinflammation and related neurodegeneration still require additional studies. Emerging evidence suggests critical roles of central norepinephrine (NE) in the pathogenesis of disease progression and manifestations of a variety of nonmotor dysfunctions in PD patients. Thus, this perspective article focuses on the following three aspects.

Neuroinflammation-based rodent PD models
We review several toxin-elicited PD models, which show some of the cardinal characteristics of observed in PD patients, such as chronic neuroinflammation, sequential neurodegeneration, and progressive motor and nonmotor dysfunction.

Roles of central NE in neuroinflammation
Based on common features observed from inflammation-based animal models, we discuss immune factors involved in the initiation and maintenance of low-grade neuroinflammation. The possibility that the loss of locus coeruleus-norepinephrine (LC/NE) neurons may be the focal initiating point in producing a similar pattern of progressive caudal-rostral degeneration by various toxins is evaluated. Furthermore, cellular and molecular mechanisms underlying chronic neuroinflammation-induced progressive neurodegeneration are discussed.

Molecular mechanisms of anti-inflammatory and neuroprotective functions of NE
Anti-inflammatory therapy for neurodegenerative diseases has been emerging as a promising disease-modifying therapeutic strategy. We review the current status in the development of PD therapies by focusing on drugs that affect the NE system.

NEUROINFLAMMATION-BASED RODENT PD MODELS

Disease progression in PD patients
One of the cardinal characteristics of PD is the progressive nature. However, the mechanism of PD progression remains unclear. Currently, PD therapies are limited to symptoms relief, while disease-modifying therapies aimed at stopping PD progression are still lacking. The understanding of PD progression has been greatly facilitated by both basic and clinical research. Braak’s group was the first to document a caudal-rostral pattern of disease progression\[^{37}\]. In PD patients, neuronal loss starts from the lower brain (raphe nucleus, LC, and olfactory bulb) and gradually affects the higher centers of the brain\[^{38}\]. Further studies uncovered that peripheral inflammation occurs years before PD patients show movement dysfunction. The proposed route of disease progression originating from the gut and spreading to the brain fits well with the symptom progression of PD patients\[^{39}\]. Before symptoms of movement disorder are observed, gut dysfunction, such as constipation and other premotor disorders, including smell loss, sleep disorder, and other autonomic dysfunction, are often found in patients with PD. Recently, creating animal models mimicking the pattern of neurodegeneration observed in PD patients and investigating its underlying mechanisms has become a widely pursued research area.

Role of neuroinflammation in disease progression
Accumulating evidence strongly indicates that brain inflammation plays a critical role in progressive neurodegeneration. Both gene-mutated and toxin-induced animal PD models have been generated to investigate neurodegenerative pattern and associated motor and nonmotor behavioral changes. This perspective article focuses on only a few commonly accepted toxin-elicited animal PD models, which are inflammation based and show some of the cardinal progressive features observed in PD patients.

LPS model
Peripheral inflammation induces neuroinflammation and neurodegeneration
Most rodent PD models, including those generated by MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) or 6-hydroxydopamine, display acute toxicity within days but fail to recapitulate the delayed, progressive pattern of DA neurodegeneration. To investigate whether chronic neuroinflammation plays a role in the progressive neurodegeneration of PD, several environmental risk factors implicated in the pathogenesis of PD (e.g., pesticides, herbicides, and infectious agents) and whether they could recapitulate the delayed, progressive nature of PD have been determined in rodents by our lab and others[35,40,41]. Gram-negative bacterial endotoxin LPS is one of the commonly used toxins. Following a systemic injection of lipopolysaccharide (LPS, 5 mg/kg), mice show delayed, progressive neurodegeneration of DA neurons[35]. Further studies indicate that this model not only provides an excellent tool for studying the role of neuroinflammation-related neuronal damage but also serves as a useful platform for exploring drug therapies in PD. Clinically, the relevance of the LPS model for PD is supported by several case reports, in which a significant correlation between infections and the risk of developing PD was found[42].

To investigate the role of gene-environment interactions in the etiology of PD, we created an accelerated rodent PD model by LPS in transgenic mice over-expressing mutant human α-synuclein (A53T). After a single intraperitoneal injection of LPS (1 mg/kg) in seven-month-old male mutant A53T mice (Tg mice) and wild type controls (WT mice), the delayed, progressive degeneration of nigral DA neurons was observed in Tg mice, but not in WT mice[43]. After five months of LPS treatment, Tg mice lost more than half of their nigral DA neurons, while the striatal TH levels were reduced by a comparable degree. By contrast, LPS-induced neuronal damage was not observed in WT mice or saline-injected Tg mice. These results demonstrate synergistic neurotoxicity of LPS and A53T α-synuclein overexpression, thus strongly indicating the critical role of gene-environment interactions in PD. Selective DA degeneration was assessed by immunofluorescence double-labeling with antibodies against TH and Neu-N[43]. An about 52% decrease in nigral DA neurons was found in LPS-injected Tg mice, whereas only 9.2% of non-DA neurons were lost. Collectively, this two-hit PD model recapitulated the signature lesion of PD by its chronic, progressive, and selective neurodegeneration of nigral DA neurons.

LPS-elicited chronic neuroinflammation exerts progressive ascending neurodegeneration and behavioral changes
The involvement of neuroinflammation in the pathogenesis of PD was identified decades ago. Positron emission tomography (PET) imaging reveals prominent and heterogeneous neuroinflammation in the brains of patients with PD[44,45]. Strong evidence indicates that LPS-induced chronic neuroinflammation is sufficient to not only induce nigral DA neurodegeneration[15,46,47] but also drive progressive loss of other vulnerable neuronal populations outside the basal ganglia. Mechanistically, LPS-generated sub-lethal septicemia in the periphery is able to activate microglia, resulting in low-grade chronic neuroinflammation in the brain for the remaining lifetime of the mice[35]. The pattern of delayed neurodegeneration in this model is dissimilar to that of the intracranial LPS model that produces acute neurodegeneration. Chronic neuroinflammation elicited by systemic LPS injection enables steady production of cytotoxic factors to damage bystander neurons. In turn, damaged/dying neurons can re-active neighboring microglia through the release of danger-associated molecular pattern (DAMP), forming a self-propelling cycle that eventually leads to sustained neuronal damage[48].

How does a single intraperitoneal injection of LPS induce long-lasting brain inflammation and progressive neuronal loss?
An intriguing question arises: why can a single injection of LPS produce such robust and long-lasting effects in the brain, since the half-life of LPS in mouse blood is only approximately 12 h[49]? It is well-documented
that, under the physiological conditions, very minimal LPS can enter to the brain due to the poor passage through BBB\textsuperscript{[50]}. Therefore, LPS-induced neuroinflammation appears to be an indirect effect. Studies showed that a single intraperitoneal LPS injection initially produced large amounts of proinflammatory cytokines or chemokines from Kupffer cells, the resident macrophage-like cells in the liver\textsuperscript{[41]}. We found that the levels of cytokines in blood were greatly elevated at early times but declined to basal levels by 6–9 h. Remarkably, proinflammatory levels and microglial activation sustained in the brain for up to 10 months. Further mechanistic studies revealed that blood immune factors can pass through BBB\textsuperscript{[52]}. After entering to brain parenchyma, these proinflammatory factors can activate microglia to continually produce more cytokines, reactive oxygen, and nitrogen species and other cytotoxic factors\textsuperscript{[53]}. These microglia-generated toxic factors cause neuronal damage to release DAMPs, which further reactivates microglia. Through this process, a vicious cycle is formed to maintain neuroinflammation and cause additional neuronal loss [Figure 1].

To further investigate the mechanism of transition of chronic neuroinflammation from the periphery to the brain, mice were administered with TNF\textsubscript{α} peripherally. The results show that both TNFs and LPS injections elevated the production proinflammatory factors (TNF\textsubscript{α}, MCP-1, and IL-1\textsubscript{β}) in the brain. Further, mice deficient in TNFR1/R2/2 receptors failed to show brain neuroinflammation in response to LPS and TNF\textsubscript{α} challenges, supporting that TNF\textsubscript{α} was one of the critical factors in bridging inflammation from the periphery to the CNS. Long-lasting and enhanced microglial activation, indicated by the immunohistochemical analysis of brain sections with anti-Iba-1 or anti-CD11b antibodies, was observed in brain regions, such as the SN, hippocampus, and motor cortex. The LPS-elicited long-lasting inflammatory and neurotoxic effects in the brain were consistent with previous findings. Exposed to MPTP, a selective DA neurotoxicant, in humans\textsuperscript{[53]} and monkeys\textsuperscript{[54]}, led to sustained microglial activation up to years after exposure. In addition, in utero LPS exposure during a critical window of development (E11) rendered a 30% loss of nigral DA neurons in offspring at the age of seven months\textsuperscript{[55]}. Taken together, these findings suggest that early or brief exposure to toxins/toxicants can induce chronic, self-propelling neuroinflammation and lead to progressive neurodegeneration.

Rotenone model

Rotenone, a previously widely used pesticide, reproduces Parkinsonism associated with increased risk for PD. Since the first publication by Greenamyre et al.\textsuperscript{[6]} rotenone has been commonly used as a tool to create a rodent PD model\textsuperscript{[6]}. Chronic rotenone exposure in rodents induces key features of Parkinsonism\textsuperscript{[56]}. Mechanistically, rotenone is believed to impair mitochondrial complex I\textsuperscript{[57,58]} and microtubule-based transport of neurotransmitter vesicles\textsuperscript{[9,40]}. Although the role of mitochondrial complex I deficits has been demonstrated in rotenone-induced Parkinsonism\textsuperscript{[8,61]}, inhibition of mitochondrial complex I appears not to be the only mechanism for rotenone-induced DA degeneration\textsuperscript{[62]}. A mouse strain lacking functional Ndufs4, a gene encoding a subunit required for complete assembly and function of complex I, has been used to further address this issue. Genetic ablation of Ndufs4 gene suppressed complex I activity but did not affect DA neuron survival in midbrain cultures prepared from E14 mice\textsuperscript{[61]}.

The involvement of microglia in mediating rotenone-elicted neurotoxicity has also been reported. In midbrain neuron and glia cultures, rotenone showed much higher potency in reducing the survival of DA neurons than that in neuron-enriched cultures\textsuperscript{[63]}. Further studies revealed that microglial NADPH oxidase 2 (NOX2)-derived superoxide markedly exacerbated DA degeneration in rotenone-treated cultures\textsuperscript{[64]}, suggesting that microglial NOX2 is an alternative target of rotenone. This finding was further confirmed by a study showing that rotenone directly interacted with the catalytic gp91phox subunit of NOX2\textsuperscript{[64]}.
Figure 1. How does a single ip injection of LPS induce long-lasting brain inflammation and produce progressive neuronal loss? LPS reaches the liver via the portal vein circulation and causes it to secrete large amounts of various cytokines, such as TNFα. Some of these cytokines can pass through BBB by receptor-mediated mechanisms to activate microglia and produce additional proinflammatory factors to cause neuron damage. DAMP released from damaged neurons further reactivate microglia to form a self-propelling vicious cycle to maintain chronic neuroinflammation and lead to neurodegeneration. (This figure was modified from our previous paper with permission[136]).

The involvement of microglia in generating NOX2-dependent superoxide in rotenone-treated neuron-glial cultures further suggests a critical role of neuroinflammation in rotenone-induced neurotoxicity. Indeed, a recent report indicated that daily intraperitoneal injections of rotenone for three weeks produce microglia-dependent neuroinflammation in mouse brain[65,66]. Moreover, not only was neuronal loss observed in the SN area, but it also showed a greater loss of LC/NE neurons[65]. Mechanistic studies unraveled that the integrin Mac1/NOX2 complex is a major pathway coupling the production of superoxide and neuroinflammation in rotenone-treated mice[65]. These results provide a novel insight into the pathogenesis of rotenone-induced neurodegeneration. However, how the microglial NOX2 activation is related to the inhibition of mitochondria dysfunction in the rotenone PD model is an interesting but not yet studied question.

Paraquat/maneb model
Paraquat and maneb, two pesticides used in agriculture, are commonly used in many of the same crops. Epidemiological studies revealed an increased risk of PD in human when exposed to combined paraquat and maneb compared with either alone. Paraquat and maneb cotreatment has been widely employed to model PD in rodents. Systemic administration of combined paraquat and maneb led to synergistic damage to nigrostriatal DA neurons and reduction of motor activities in mice[41]. In addition to the DA system, paraquat and maneb co-exposure also damaged neurons in other brain regions and followed a time-dependent ascending neurodegenerative pattern. We recently reported that paraquat and maneb co-treated
mice displayed loss of LC/NE and nigral DA neurons at four weeks after exposure, which was two weeks earlier than that of hippocampal and cortical neurodegeneration\[40,67,68\]. Consistent with sequential neurodegeneration, paraquat and maneb co-exposure induced gait abnormality and cognitive decline in mice at four and six weeks after treatment, respectively\[40,67\]. Interestingly, inhibition of microglial activation and production of inflammatory factors by targeting CD11b, the α-chain of Mac-1, or NOX2 significantly mitigated combined paraquat- and maneb-induced neurodegeneration and behavioral abnormalities in mice\[40,68,69\]. Furthermore, attenuated neuronal damage in paraquat- and maneb-treated mice was also observed once these mice were co-administered with taurine, a major intracellular free β-amino acid with potent anti-inflammatory capacity\[67,70,71\]. Altogether, these findings suggest that microglia-mediated neuroinflammation contributes to progressive neurodegeneration in this two-pesticide-induced mouse PD model.

**NE DYSFUNCTION PLAYS A KEY ROLE IN THE PATHOGENESIS OF PD**

Chronic neuroinflammation and progressive neurodegeneration can be generated by various toxins with different modes of action

Animal PD models described in previous section are generated by a variety of toxins with different chemical structures and modes of action. In general, neuroinflammation can be generated by two ways: (1) agents that are infectious, such as microorganism or endotoxins; and (2) chemicals that are not infectious, such as rotenone, paraquat/maneb, or DSP-4, a selective NE neurotoxicant (see below). Despite their differences in initiating neuroinflammation, these toxins somehow produced a similar pattern of neurodegeneration. The pattern of ascending caudal-rostral neurodegeneration generated by a single systemic injection of LPS or DSP-4, (or repeated injections of rotenone) is of the utmost importance for two reasons: (1) it resembles the pattern of neurodegeneration observed in PD patients; and (2) it indicates that a common mechanism is operative to drive a similar pattern of neurodegeneration produced by various toxins, even if they are different in chemical structures and modes of action. Elucidation of this common pathway would greatly advance our understanding of the etiology and pathogenesis of PD. Therefore, rodent PD models generated by LPS (infectious) or DSP-4 (non-infectious) could be useful to investigate possible mechanisms underlying the similar ascending sequential pattern of neurodegeneration induced under different pathological conditions.

**Loss of LC/NE neurons is the focal point in producing similar patterns of progressive caudal-rostral degeneration by various toxins**

As mentioned above, despite high chemical disparity and toxicological actions, exposure to various toxins/toxicants produces similar patterns of neurodegeneration in mouse brain. Immunochemical analysis reveals a sequential caudal-rostral fashion: neuronal degeneration is first found in the brain stem region, such as LC, followed by neurons in the SN and thalamus, and lastly observed in the hippocampus and cortical regions\[36,65,67,72\]. Based on these observations, as well as our previous reports indicating anti-inflammatory and neuroprotective functions of NE\[73\], a logical hypothesis was proposed that loss of LC/NE neurons may be the critical focal point for producing similar patterns of progressive caudal-rostral degeneration by various toxins. Recent progress in this area of research has greatly advanced our understanding of the roles of NE in neurodegenerative diseases, particularly in PD. We review evidence supporting this hypothesis and discuss potential clinical implications of NE dysfunction in PD below.

**NE deletion by DSP-4 elicits progressive neurodegeneration**

The early loss of LC/NE neurons induced by LPS suggests a possibility that depletion of central NE is a key for progressive neurodegeneration in this neuroinflammatory PD mouse model and even possibly in PD patients. To test this hypothesis, the NE-depleting toxin DSP-4 was used. A single injection of DSP-4 (50 mg/kg; ip) reduced tissue levels of NE (ranging from 55% to 80%) one day after injection in NE-innervated
regions, such as the midbrain, motor cortex, and hippocampus. Brain NE levels remained significantly reduced for up to four months, but they slowly returned to normal by 10 months post injection. Depletion of brain NE levels was accompanied by a time-dependent sequential loss of neurons: as expected, a more than 60% decrease in LC neurons was found one day after DSP-4 treatment. Time-dependent decreases in nigral DA neurons were observed at 4, 7, and 10 months after DSP-4 injection, in comparison to age-matched vehicle controls. DSP-4 also led to reduction of Neu-N-positive neurons in the motor cortex and hippocampus, but not in caudate/putamen and ventral tegmentum area 10 months later. DSP-4-induced neurodegeneration was accompanied by decreased metabolism of glucose detected by PET imaging with [18F]-FDG. The reduced glucose levels were observed in the olfactory bulb, thalamus, hindbrain, midbrain, hippocampus, and across all cerebral cortices at 10 months in DSP-4 injected mice, implicating putative neurodegeneration in these brain regions. Again, it is interesting to note that no change of glucose utilization was observed in the cerebellum or the caudate/putamen.

One salient finding of these studies is that the pattern of neurodegeneration in both LPS and DSP-4 models approximate the spatiotemporal progression of neuronal loss in PD. Following the degeneration of LC/NE neurons, both models show significant loss of DA neurons in the SN, yet without affecting DA-neurons in the VTA region. Cortical and hippocampal atrophy, which are often observed in the late-stages of PD, were also found months after LPS or DSP-4 injection. In agreement with neurocircuit degeneration, both LPS- and DSP-4-injected mice displayed behavioral dysfunction, including motor deficits and a variety of nonmotor phenotypes [Figure 2].

These findings approximate the neurodegeneration found in PD patients. The selective neurodegeneration pattern revealed a strong correlation between the concentration of NE and the vulnerability of the intrinsic neurons in LC/NE neuron-innervated regions in response to different toxins/toxicants, such as LPS, DSP-4, rotenone, paraquat, etc. Together, these findings strongly suggest that loss of LC/NE neurons play a pivotal role in producing a similar pattern of progressive caudal-rostral degeneration.

Comparison of LPS, DSP-4, rotenone, and paraquat/maneb models
Different toxins produce neurodegeneration with distinct modes of action. However, based on the initial cell types targeted, most toxins used for modeling PD can be generally classified into three groups.

Cell non-autonomous mechanism
Pathogen associated molecular pattern agents such as microorganisms, endotoxins, or proinflammatory cytokines belong to this class. The primary target cells are microglia in the CNS. Upon the activation of microglia, large amounts of cytokines are released and produce a high degree of acute neuroinflammation to combat the infectious agents. However, over-production of immune factors also causes collateral bystander neuronal damage. Subsequent release of DAMP substances from injured neurons in turn triggers reactive microgliosis through the activation of the MAC-1 receptor, which further activates microglial NOX2, increases the production of superoxide/ROS, and causes additional inflammation and neuronal death. Thus, a vicious cycle becomes operative to cause delayed and progressive neurodegeneration [Figure 3].

Cell autonomous mechanism
Common toxins used in animal PD models such as MPTP, 6-hydroxydopamine, and the aforementioned DSP-4 belong to this class. Initially, these toxins are selectively taken up by neurons and directly cause neuronal damage. Different from the LPS model, these direct-acting toxins usually cause neuronal loss within days without causing acute inflammation during the initial stage. If the damaged neurons are able to secrete enough DAMP to trigger reactive microgliosis, then the vicious cycle will start and drive
Figure 2. DSP-4 injection causes progressive neuronal loss along the gut-brain axis. DSP-4-induced chronic inflammatory models display progressive ascending neuronal loss along a caudal-rostral axis, which recapitulates the spatiotemporal order of neurodegeneration in PD. Furthermore, the colon is an early site affected after injection with DSP-4[137]. α-synuclein pathology and enteric neuronal loss were initially found in the large intestine at one month, while neurodegeneration in the brain was observed a few months later, indicating progressive neurodegeneration occurs along the gut-brain axis.

Figure 3. Comparison of LPS, DSP-4, rotenone, and paraquat/maneb. This figure illustrates that toxins produce neurotoxicity through different mechanisms: (1) LPS by activating microglia; (2) DSP-4 by directly damaging LC/NE neurons; and (3) rotenone and paraquat/maneb by exert directing neurotoxicity in high concentrations while in lower concentrations causing activation of microglia. However, between neuronal damage and reactivation of microglia, eventually, these toxins all generate a self-propelling vicious cycle to keep chronic neuroinflammation continued and drive progressive. (This figure was modified from our previous paper with permission[138]).
inflammation-based progressive neurodegeneration⁷⁸ [Figure 3].

**Mixed-mode mechanism**

Many environmental risk factors, such as pesticides, herbicides, fungicides, and heavy metals, display mixed modes of action in causing neurodegeneration. *In vitro* studies revealed that mixed-mode agents may target different cell types depending on toxin concentrations. Rotenone serves as a prototype agent for illustrating this class of toxins. In high concentrations, rotenone can directly damage neurons in neuron-enriched cultures by inhibiting mitochondrial complex I. By contrast, rotenone at low concentrations is not sufficient to directly damage neurons, but it exerts neurotoxicity through microglial activation in neuron-glial cultures⁶⁵. Microglia-dependent neurotoxicity of rotenone has also been reported in an animal study [Figure 3].

**Prolonged microglial activation plays a key role in DSP-4-elicited neurotoxicity**

A consistent pattern of progressive, ascending, and sequential loss of brain neurons was found in different models of NE-deficient mice, which is similar to that of LPS-treated mice. These findings align with the idea that loss of LC/NE neurons could play a key role in the subsequent neuron loss in other brain regions. To address this question, the time course study of microglial activation after DSP-4 injection was performed. Immunocytochemical analysis using CD11b, a marker for microglial activation, revealed that DSP-4 induced time-delayed microglial activation. Enhanced CD11b-immunoreactivity was not observed until seven days after injection, peaked at two weeks, and remained elevated for up to ten months in NE heavily innervated regions, such as SN, hippocampus, and cortex, but not in the caudate/putamen⁶⁵. PET analysis using [18F]-PBR translocator protein as a ligand for neuroinflammation in DSP-4-injected mice showed similar patterns of increased microglial activation at 10 months after injection⁶⁶. Genetic studies using DBH conditional KO mice showed a long-lasting increase in microglial activation compared with WT mice (Song et al., unpublished observations). Putting all the evidence together, a clear pattern emerges, indicating a high degree of correlation of prolonged microglial activation and neuronal loss in LC/NE-innervated regions in both DBH-genetic knock-out and NE-depleted mice. These findings clearly demonstrate a crucial role of LC/NE in the pathogenesis of PD.

**Why is LC/NE particularly vulnerable to the insults of a variety of toxins?**

LC/NE neurons are more susceptible to oxidative damage following injections of a variety of toxins/toxicants: LPS, rotenone, paraquat, maneb, etc.⁷⁹,⁸⁰. In PD, the reduced level of NE following LC/NE degeneration is closely correlated with the development of a series of prodromal and nonmotor symptoms⁸¹–⁸⁴. It has been reported that depletion of brain NE significantly enhanced neuronal loss in many rodent PD models, including LPS, MPTP, 6-OHDA, and combined paraquat and maneb models⁷³,⁷⁵–⁹⁰. These findings were further confirmed by our recent studies on both NE-depleted and DBH-deficient conditional knock-out mice⁶⁶,⁷².

To further address the question, we explored the differential vulnerability among various groups of neurons in response to toxic insults. It is generally believed that distinct nuclei respond differently to microenvironments under chronic exposure to oxidative stress and may lead to PD with age⁸¹. The most vulnerable neuronal populations likely share three intrinsic features: (1) coexist with a large quantity of active microglia⁸²,⁸³; (2) impaired antioxidant buffering capabilities⁸⁴,⁸⁵; and (3) greater energetic demands in neurons with long-axon projections, multi-synaptic neurotransmission, and pacemaker firing⁹⁶,⁹⁷. In a DSP-4-treated chronic neuroinflammatory mouse PD model, the superoxide/ROS productions were significantly increased in LC and SN in comparison to age-matched vehicle control. However, the appearance of oxidative injuries in the cortex and hippocampus was not observed until a few months later. When antioxidant systems in those nuclei are overwhelmed by too much oxidative stress, it results in the
irreversible dysfunction of mitochondria and cell death\textsuperscript{[80,96–101]}. Thus, the vulnerability to oxidative injuries in different brain regions seems to be the driving force for a discrete, sequential spatiotemporal pattern of neurodegeneration\textsuperscript{[102]}. Indeed, the PET with \textsuperscript{[18F]}-Fluorodeoxyglucose (\textsuperscript{[18F]}-FDG) study clearly showed the high basal levels of glucose consumption in olfactory bulb, thalamus, midbrain, and hindbrain regions in control mice\textsuperscript{[56,72]}. Moreover, the drastic increase in microglial activation, as measured by \textsuperscript{[18F]}-PBR111 uptake, was found in the same brain areas after different toxins challenge\textsuperscript{[56,72]}. Taken together, these results further support the idea that the energy demand and neuronal susceptibility are the key factors that lead to the subsequent oxidative injury-related neurodegeneration in the caudo-rostral order.

**Dysfunction of noradrenergic system exacerbates inflammation-based ascending sequential neurodegeneration and behavioral deficits**

Besides producing the sequential caudal-rostral pattern of neurodegeneration, noradrenergic dysfunction is associated with both motor and nonmotor behavioral changes in mice. Since the level of NE content reduces with aging, so it is thought to be associated with the appearance of a wide range of nonmotor symptoms as well as contributing to the neurodegenerative process. We hypothesized that selective pre-depletion of NE in an LPS-induced chronic neuroinflammatory mouse PD model may not only accelerate the disease progression but also expedite PD-like nonmotor and motor symptoms. Indeed, we found that mice pre-treated with DSP-4 significantly potentiated LPS-induced neurodegeneration in different brain regions in a sequential, ascending, and time-dependent pattern, such as SN, hippocampus, and motor cortex, but spared in VTA and striatum\textsuperscript{[72]}. Most importantly, aligned with the enhanced neurodegeneration, this “two-hit” model also displayed greater deficits of both nonmotor (e.g., hyposmia, constipation, anxiety, sociability, exaggerated startle response, and impaired learning) and motor (e.g., decreased rotarod activity, grip strength, and gait disturbance) symptoms in a progressive fashion\textsuperscript{[72]}. It is interesting to comment on the clinical relevance of loss of LC/NE neurons in nonmotor dysfunctions of PD patients. The prodromal nonmotor PD symptoms, such as GI disturbance, constipation, orthostatic hypotension, anxiety, and loss of sociability, are likely related to the early loss of LC/NE neurons since adrenergic neurons directly control the autonomic nervous system regulating these functions. Furthermore, the loss of cognition ability in the late stage of PD patients may be related to dysfunction of higher centers, such as the hippocampus and cortex, which are heavily innervated by LC/NE neurons\textsuperscript{[103,104]}. Our DSP-4/LPS mouse PD model recapitulates many nonmotor dysfunctions in a similar temporal fashion\textsuperscript{[72]}. Our mechanistic study demonstrating the relationship among the loss of LC/NE function, chronic neuroinflammation, and neurodegeneration lends strong support for a pivotal role of the LC/NE system in the pathogenesis of PD. Taken together, this novel “two-hit” dosing regimen not only revealed a critical role of early LC lesion in the pathogenesis of PD but also provided an accelerated PD model that recapitulates both PD-like sequential neurodegeneration and progressive appearance of motor/nonmotor symptoms\textsuperscript{[72]}.

**Molecular mechanism of anti-inflammatory and neuroprotective functions of NE**

Besides functioning as a neurotransmitter, NE has also been well-studied in the periphery for its anti-inflammatory capacities\textsuperscript{[105–106]}. We hypothesized a lesion of NE neurons may disrupt brain immune homeostasis results in chronic neuroinflammation and subsequent neurodegeneration. Previous *in vitro* studies demonstrated that NE in micromolar concentrations or higher exert neuroprotective effects\textsuperscript{[109–111]}. Interestingly, a recent report showed that sub-micromolar concentrations of NE (10\textsuperscript{-9}–10\textsuperscript{-6} M) also exert anti-inflammatory and neuroprotective effects in LPS-treated midbrain neuron-glial cultures\textsuperscript{[73]}. The reason for using lower concentrations of NE was that, while micromolar NE can be reached in synaptic junctions\textsuperscript{[112]}, sub-micromolar concentrations of NE are probably more relevant for studying its extra-synaptic effects. In the brain, most of NE will be either re-taken up by nerve terminals or undergo enzymatic breakdown. Therefore, it was reasoned that the remaining NE, which escapes from both processes, is capable of acting on the surrounding microglia even at less than micromolar concentrations\textsuperscript{[113]} [Figure 4].
Figure 4. Initial loss of NE/LC neurons resulted from either LPS or DSP-4 injection renders neurons in the NE neuron-innervated regions more susceptible to inflammation-related damage. In normal condition (Left), NE released from the presynaptic terminals of LC/NE neurons performs multiple functions through different ways of transmission. During synaptic transmission, released NE functions as a neuromodulator by directly acting on postsynaptic β2-receptors to modulate the function of postsynaptic neurons. In volume transmission, extra-synaptic NE, diffused out of the synapse or released from dendrites, can act on other neighboring cells, such as microglia. NE exerts anti-inflammatory and neuroprotective functions through the inhibition of microglial NOX2. In pathological condition (Right), reduced NE release from LC/NE neurons not only disrupts the synaptic transmission, but also renders surrounding microglia prone to activation to release proinflammatory immune factors, leading to neuronal damage. Thus, we hypothesize that dysfunction of LC/NE neurons after LPS or DSP-4 injection renders neurons more sensitive to inflammation/oxidative insults and initiates neurodegeneration.

Further studies demonstrated that sub-micromolar NE exerts neuroprotective effects by way of reducing the release of a series of pro-inflammatory cytokines (e.g., IL-1β, IL-6, and TNFα) and free radicals (e.g., superoxide/ROS, nitric oxide, etc.) from LPS-treated microglia cultures.[73]

A novel β2-AR-independent pathway mediating sub-micromolar NE-induced anti-inflammatory effect: inhibition of microglial NOX2-produced superoxide

On immune cells, β2-Adrenergic receptor (β2-AR) plays a critical role in mediating the NE-elicited anti-inflammatory effect by suppressing the release of pro-inflammatory factors via activation of the cAMP/protein kinase A pathway. It is generally accepted that β2-AR mediates the anti-inflammatory effect of micromolar concentrations of NE. It is interesting to find that a novel β2-AR-independent pathway may mediate the sub-micromolar NE-elicited anti-inflammatory effect. We demonstrated that LPS-induced superoxide production was significantly inhibited by NE in a dose-dependent manner in primary midbrain neuron-glial cultures.[73] Two NE optical isomers were used to investigate the important role of ARs in inhibiting NE-derived superoxide production. Surprisingly, the active isomer (-)-NE showed over 100-fold AR-binding affinity than that of inactive isomer (+)-NE.[114,115]. However, both (+)-NE and (-)-NE were found equipotent in inhibiting superoxide production in LPS-treated mixed-glia cultures. The AR-independent inhibitory function of both NE isomers on superoxide production was further confirmed in a low AR-expressing cell line, COS7 cells, treated with phorbol myristate acetate (PMA).[116-118]. As expected, after transfected with NOX2, both isomers exerted a comparable inhibitory capacity on PMA-induced
superoxide in COS7 cells.\textsuperscript{119} Moreover, the AR-independent inhibitory capacity of both NE isomers has also been confirmed in mouse mixed-glia cultures with genetically depleting ARs.\textsuperscript{73}

Besides β2-AR, the possibility of the involvement of other types of ARs in regulating NE-elicited reduction of superoxide production has also been studied. However, blocking α1 and/or β1 ARs by pretreating with their non-selective antagonists (phenotolamine and propranolol, respectively) failed to show any changes in NE-induced superoxide reduction in LPS-treated mixed-glia cultures.\textsuperscript{120} Moreover, inhibition of PKA, a common enzyme in ARs signal transduction pathways, again failed to affect NE-induced superoxide production.\textsuperscript{73} Altogether, these findings reveal that NOX2 plays a critical role in regulating sub-micromolar NE-elicited microglial deactivation. It should be emphasized here that β2-AR is still activated by micromolar NE. In fact, our previous report showed that salmeterol, a long-acting β2 adrenergic receptor agonist, exerts a neuroprotective effect against LPS-elicited DA neuron damage mediated through the β2-AR/β-arrestin pathway.\textsuperscript{120}

**NOX2 IS A KEY PLAYER IN DISEASE PROGRESSION AND PRIME TARGET FOR DEVELOPING DISEASE-MODIFYING THERAPY**

Recent studies revealed a critical role of microglial NOX2-derived ROS in initiating neuroinflammation-mediated oxidative damage and progressive neurodegeneration.\textsuperscript{121} Neuroinflammation has been widely accepted as a crucial contributor to progressive neurodegeneration in a broad spectrum of neurodegenerative diseases.\textsuperscript{78,122,123} Microglia can be activated by a wide range of stimuli that are able to disrupt brain homeostasis, such as infection, ischemia, trauma, toxic insults, or autoimmune injury.

Once activated, microglia release innumerable cytotoxic factors, including cytokines, chemokines, proteases, excitatory amino acids, eicosanoids, and ROS. NOX2-derived superoxide has been recognized as one of the most crucial players in chronic progressive neurodegeneration.\textsuperscript{78,122,123} Those microglial NOX2-derived ROS (H$_2$O$_2$ and peroxynitrite) can directly enter neurons, resulting in impaired mitochondrial integrity, reduced ATP production, and increased mitochondria-derived ROS. They also cause a series of damages to enzymes and other proteins through oxidation, nitration, aggregation, or accumulation (e.g., α-synuclein). By dysfunction of the ubiquitin-proteasome system, ROS will not only reduce protein degradation but also exaggerate abnormal protein accumulation. Moreover, the impaired redox-sensitive signal transduction, products of oxidated DNA, RNA, and lipids, and/or ROS-induced autophagy also play a role in oxidative neuronal damages during neuroinflammation.\textsuperscript{10,124,125}

**Role of dysregulated NOX2 in PD**

It has been reported that the increase in microglial NOX2 was found in the SN of both PD patients and mouse PD models.\textsuperscript{124} In line with those pathological examinations, a crucial role of microglial NOX2 activation in driving DA neurodegeneration has also been extensively studied.\textsuperscript{78,127} For example, in a microglia and DA neuron co-culture system, the mis-folded α-synuclein is able to kill DA neurons by activating microglial NOX2 to release ROS.\textsuperscript{124} Moreover, the presence of microglia exacerbates DA neurodegeneration following diverse challenges, including fMLP and LPS, angiotensin II and nanometer-sized diesel exhaust particles, PD-producing neurotoxins (6-OHDA, MPTP, and MPP$^+$), and PD-associated pesticides (paraquat and rotenone); such neurodegeneration could be alleviated by NOX2 deletion, diphenyleneiodonium (DPI), or apocynin.\textsuperscript{129} In addition, the release of DAMPs and other cellular components to the extracellular space, such as high-mobility group 1, the active form of matrix metalloproteinase-3, or aggregated α-synuclein, could trigger reactive microgliosis and release NOX2-dependent ROS production, which further facilitates DA neurodegeneration.\textsuperscript{46} In a MPTP-induced mouse PD model, minocycline-induced neuroprotective effects were achieved by inhibition of microglial activation.
and membrane translocation of \textit{p67}^{\text{phox}}[130]. Furthermore, the neurotoxic effects induced by either systemic administration of MPTP or intra-nigral injection of LPS were significantly suppressed in NOX2-deficient mice in comparison to \textit{WT} mice[131].

\textbf{NOX2 is a prime target for anti-inflammatory therapy}

Chronic aberrant neuroinflammation, a ubiquitous feature among a variety of neurodegenerative diseases, has been targeted as a disease-modifying strategy for halting the diseases progression[120,122-134]. However, little progress has been made on the ground due to the lack of knowledge pinpointing the immune factors released during chronic neuroinflammation. Recent studies suggest that blocking the superoxide/ROS-generating enzyme NOX2 ameliorates neuroinflammation and reduces neurodegeneration[132].

A NOX2 inhibitor DPI has served as a useful tool to demonstrate the advantages of targeting NOX2 as a prime target for therapy. DPI is a widely used NOX2 inhibitor. However, commonly used concentrations (1-10 µM) of DPI are highly toxic in cell cultures and animals, thus preventing its use in humans. We discovered that an ultra-low dose of DPI (10 ng/kg/day) displayed potent anti-inflammatory and neuroprotective effects in LPS-treated mice[132]. Furthermore, post-treatment of DPI to LPS-treated mice that already shown marked loss of nigral DA neurons and motor symptoms could effectively stop the remaining neuronal population from degeneration and largely restore motor functions[132]. The dopaminergic neuroprotective effects of low-dose DPI, even in a post-treatment regimen, were also detected in an MPTP-induced mouse PD model[132]. Recent studies using similar post-treatment regimens demonstrated the same efficacy of DPI in DSP-4 injected mice[36]. DPI greatly reduced microglial activation, decreased oxidative stress, and most importantly protected DA neurons. Collectively, these findings suggest that DPI is effective at protecting neurons in either infectious agent (LPS)- or non-infectious agent (DSP-4)-induced mouse PD models, suggesting that targeting NOX2 can be a novel and promising therapeutic strategy for PD.

In addition to targeting microglial NOX2, the use of \textit{β}-AR agonists has also been tried as a potential therapy for PD. It is worth noting that, in LPS-injected mice, post-treatment with the \textit{β}2 adrenergic receptor (\textit{β}2-AR) agonist salmeterol significantly rescued DA neurons and improved motor function deficits[120]. Results from these animal studies corroborate a recently published human study. A meta-analysis showed that asthmatic patients prescribed with salbutamol, a \textit{β}2-AR agonist, had significantly reduced lifetime risk of developing PD[135].

\textbf{CONCLUSIONS}

This review provides clear and convincing evidence to demonstrate that low-grade chronic neuroinflammation is a key factor leading to the progressive neurodegeneration in PD. The reduction of brain NE resulted from the lesion of LC/NE neurons is sufficient to initiate and maintain chronic neuroinflammation, which is associated with progressive, massive, and sequential loss of vulnerable neurons that are sensitive to oxidative damage. Dysregulated microglial NOX2 plays a critical role in generating and maintaining chronic neuroinflammation, oxidative stress, and subsequent neurodegeneration among vulnerable brain regions. NOX2 may serve as a prime target for developing promising disease-modifying therapeutic strategies for PD.

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\textbf{Authors’ contributions}
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