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Environmental neurotoxicant-induced dopaminergic neurodegeneration: a potential link to impaired neuroinflammatory mechanisms

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ABSTRACT

With the increased incidence of neurodegenerative diseases worldwide, Parkinson's disease (PD) represents the second-most common neurodegenerative disease. PD is a progressive multisystem neurodegenerative disorder characterized by a marked loss of nigrostriatal dopaminergic neurons and the formation of Lewy pathology in diverse brain regions. Although the mechanisms underlying dopaminergic neurodegeneration remain poorly characterized, data from animal models and postmortem studies have revealed that heightened inflammatory responses mediated via microglial and astroglial activation and the resultant release of proinflammatory factors may act as silent drivers of neurodegeneration. In recent years, numerous studies have demonstrated a positive association between the exposure to environmental neurotoxicants and the etiology of PD. Although it is unclear whether neuroinflammation drives pesticide-induced neurodegeneration, emerging evidence suggests that the failure to dampen neuroinflammatory mechanisms may account for the increased vulnerability to pesticide neurotoxicity. Furthermore, recent studies provide additional evidence that shifts the focus from a neuron-centric view to glial-associated neurodegeneration following pesticide exposure. In this review, we propose to summarize briefly the possible factors that regulate neuroinflammatory processes during environmental neurotoxicant exposure with a focus on the potential roles of mitochondria-driven redox mechanisms. In this context, a critical discussion of the data obtained from experimental research and possible epidemiological studies is included. Finally, we hope to provide insights on the pivotal role of exosome-mediated intercellular transmission of aggregated proteins in microglial activation response and the resultant dopaminergic neurodegeneration after exposure to pesticides. Collectively, an improved understanding of glia-mediated neuroinflammatory signaling might provide novel insights into the mechanisms that contribute to neurodegeneration induced by environmental neurotoxicant exposure.

Contents

Abbreviations: PD, Parkinson's disease; SNpc, substantia nigra pars compacta; DA, dopamine; α-syn, α-synuclein; LB, Lewy bodies; CSF, cerebrospinal fluid; ROS, reactive oxygen species; NO, nitric oxide; SOD, superoxide dismutase; 6-OHDA, 6-hydroxydopamine; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; ROT, rotenone; PQ, paraquat; MB, maneb; BBB, blood-brain barrier; GWAS, genome-wide association study; SNP, single nucleotide polymorphism; HLA, human leukocyte antigen; IgG, immunoglobulin G; PET, positron-emission tomography; SN, substantia nigra; IL, interleukins; TGF-β, transforming growth factor- β; ARG-1, arginase-1; ECM, extracellular matrix; GFAP, glial fibrillary acidic protein; GDNF, glial-derived neurotrophic factor; DAT, dopamine transporter; NOX2, NADPH oxidase 2; PAMPs, pathogen-associated molecular pattern molecules; DAMPs, damage-associated molecular pattern molecules; NLRP3, nucleotide-binding oligomerization domain-like receptor pyrin domain-containing-3; mtDNA, mitochondrial DNA; CB2, cannabinoid type-two; STAT3, signal transducer and activator of transcription 3; PACAP, pituitary adenylyl cyclase-activating polypeptide; MRI, magnetic resonance imaging; NMDPEF, N-[2-(2-methoxy-6H-dipyrido {2,3-a:3.2-e}pyrrolizin-11-yl)ethyl]-2-furamide; QR2, quinone oxidoreductase 2; OP, organophosphorus; AchE, acetylcholinesterase; NRG-1, neuregulin-1; DFP, diisopropylfluorophosphate; ARIAs, acetylcholine receptor-inducing activities; GGF, glial growth factor; NDF, neu differentiation factors; SE, status epilepticus; CPF, chlorpyrifos; DZ, diazinon; DZO, diazoxon; HRW, hydrogen-rich water; Mn, manganese; Glu, glutamate; YY1, Yin Yang 1; HDAC, histone deacetylase; VPA, valproate; GLT-1, glutamate transporter-1; COX-2, cyclooxygenase-2; AQP4, aquaporin-4; MVB, multivesicular body; ILV, intraluminal vesicle..

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1. Overview of PD-associated oxidative stress and neuroinflammation

PD is the second-most common progressive neurodegenerative disorder, affecting between seven million and 10 million people worldwide (Poewe et al., 2017; Schapira, Chaudhuri, & Jenner, 2017). The cardinal clinical manifestations of the disease include four motor symptoms: bradykinesia, rest tremor, rigidity, and postural instability (Jankovic, 2008). In recent years, non-motor symptoms, including mood disturbances, sleep disorders, cognitive decline, and autonomic impairment, have been recognized to precede the motor symptoms (Maass & Reichmann, 2013). The pathology of PD is associated with the pronounced loss of dopaminergic neurons in the substantia nigra pars compacta (SNpc) and the resultant depletion of dopamine (DA) in the caudate putamen (Braak, Ghebremedhin, Rub, Bratzke, & Del Tredici, 2004), which has been linked to the motor symptoms of the disease (Jiang & Dickson, 2018). Another hallmark of PD is the accumulation of misfolded pathological α-synuclein (α-syn) aggregates throughout the central and peripheral nervous system, eventually leading to cognitive decline, owing to its accumulation in the cerebral cortex. The pathological α-syn aggregates found within Lewy bodies (LB) and Lewy neurites have been associated with abnormally phosphorylated α-syn, namely, the phosphorylation of α-syn at serine 129, a marker of CNS synucleinopathy. The accumulation of oligomeric species of α-syn has been implicated in both sporadic and familial cases of PD, including the A53T α-syn mutation, which is linked to the early onset of the disease (Polymeropoulos et al., 1997). Although the exact mechanisms underlying dopaminergic neuronal demise are not fully understood, mounting evidence suggests that oxidative stress and inflammation are critical determinants in the cascade of events leading to dopaminergic neuronal degeneration (Gaki & Papavassiliou, 2014; Stojkovska, Wagner, & Morrison, 2015). Several genes that can cause or increase the risk of PD have been linked to inflammatory pathways (Lema Tome et al., 2013; Russo, Bubacco, & Greggio, 2014). In fact, inflammatory cytokines have been found to be elevated in the brain, cerebrospinal fluid (CSF), and serum of PD patients (Qin, Zhang, Cao, Loh, & Cheng, 2016; Sawada, Imamura, & Nagatsu, 2006). In addition, activated microglial cells and astrogliosis have been evidenced in brain regions susceptible to PD-related neuropathology. Nevertheless, little is known about the inflammatory pathways activated in response to agrochemicals.

Reactive oxygen species (ROS) have been recognized as critical cellular mediators of brain injury. It has been hypothesized that a persistent oxidative process may account for the gradual dysfunction of several cellular mechanisms throughout PD. Numerous sources have been identified to participate in ROS generation, including DA, mitochondrial dysfunction, iron, inflammatory responses, calcium, and aging. Importantly, PD-causing gene products, including DJ-1, PINK1, parkin, α-syn, and LRRK2, have been shown to harm mitochondrial functioning, thereby leading to the accentuation of ROS generation and increased cellular susceptibility to oxidative stress (Cookson, 2012; Hu & Wang, 2016; Puspita, Chung, & Shim, 2017). In fact, oxidative stress has been shown to exert deleterious effects on the ubiquitin-proteasome system and mitophagy. It has been posited that the interplay between these mechanisms may contribute to PD-associated neurodegeneration via a feedforward cycle whereby primary insults trigger oxidative stress, leading to the oxidation of key cellular proteins that, in turn, exacerbates ROS production (Dias, Junn, & Mouradian, 2013).

Oxidative stress occurs because of an imbalance between the production of free radicals and the body's ability to neutralize toxic effects through antioxidants. Free radicals that can damage cells include hydrogen peroxide, the hydroxyl radical, nitric oxide (NO), and the superoxide radical, while antioxidants include superoxide dismutase (SOD), catalase, glutathione, and uric acid. Both oxidative damage and mitochondrial dysfunction have been shown to contribute to the cascade of events leading to dopaminergic neurodegeneration (Hauser & Hastings, 2013; Subramaniam & Chesselet, 2013). The antioxidant system's failure can lead to oxidative stress that can have detrimental effects on DNA, lipids, and proteins (Blesa, Trigo-Damas, Quiroga-Varela, & Jackson-Lewis, 2015; Dias et al., 2013). The link between oxidative stress and dopaminergic neuronal degeneration has been further supported by the toxin models of PD, including 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), rotenone (ROT), 1,1-dimethyl-4,4'-bipyridinium dichloride (paraquat; PQ), and 6-hydroxydopamine (6-OHDA) (Blesa & Przedborski, 2014; Dias et al., 2013). Collectively, these studies suggest the failure of the antioxidant system to protect against the deleterious effects of free radical generation, leads to cellular damage and eventually cell death, which has implicated in the progression of PD.

In fact, there is mounting evidence that agrochemical exposure is connected to the etiology of PD (Brown, Rumsby, Capleton, Rushton, & Levy, 2006; Dardiotis et al., 2013; Dick, 2006; Freire & Koifman, 2012; Gorell, Johnson, Rybicki, Peterson, & Richardson, 1998; Pezzoli & Cereda, 2013; F. Sun, Kanthasamy, Anantharam, & Kanthasamy, 2007; Tanner et al., 2011). Occupational exposure to both herbicides and pesticides showed a significant association with PD (Allen & Levy, 2013). The results of a recent meta-analysis study suggest the existence of a statistically positive association between PD and pesticide exposure (Allen & Levy, 2013). The hypothesis that pesticide exposure increases the risk of PD was initially suggested by the demonstration of the neurotoxic effects of the MPTP metabolite, which is converted to a Parkinsonian neurotoxicant that bears a close structural resemblance to the herbicide PQ (Ascherio & Schwarzschild, 2016). Exposure to agrochemicals that induce oxidative stress such as PQ and maneb (MB) has been shown to increase the risk of PD by 200% (Berry, La Vecchia, & Nicotera, 2010; Pezzoli & Cereda, 2013; Tanner et al., 2011). Combined exposure to PQ and MB has been shown to increase the risk of developing PD (Costello, Cockburn, Bronstein, Zhang, & Ritz, 2009). Indeed, a definite causality has been identified between pesticide exposure and mitochondrial dysfunction. Tanner and colleagues have demonstrated that exposure to pesticides capable of inhibiting mitochondrial complex I and the increase of oxidative stress make one more susceptible to idiopathic PD (Tanner et al., 2011). Likewise, a recent report demonstrated a gene-environment interaction in PD whereby agrochemical exposure to ROT and PQ/MB was selectively found to impair mitochondrial transport via the nitration of microtubules in neurons harboring the A53T mutation (Stykel et al., 2018). Thus, it is likely that an interaction between environmental and genetic factors contributes to sporadic PD; however, the risk associated with the exposure to specific pesticide remains poorly understood. Nevertheless, several epidemiological studies have failed to establish a positive association between pesticide exposure and PD development owing to differences in study design, duration and level of exposure, variation in the number of agricultural workers, diagnostic criteria, and statistical tools (Bellou, Belbasis, Tzoulaki, Evangelou, & Ioannidis, 2016; Kamel et al., 2014; Sparling, Martin, & Posey, 2017). Thus, further epidemiological research should implement

prospective cohort studies that will incorporate accurate exposure assessments (Allen & Levy, 2013).

1.1. Role of microglia during PD-associated neuroinflammation

Neuroinflammation, the inflammation of the CNS, is considered a classic hallmark of numerous nervous system pathologies, including PD (Glass, Saijo, Winner, Marchetto, & Gage, 2010). Although the exact role of neuroinflammation in neurodegenerative diseases remains a conundrum, studies from animal and preclinical models suggest that sustained neuroinflammation resulting from the persistent activation of astrocytes and microglia is a key contributor to disease progression (Glass et al., 2010; Lucin & Wyss-Coray, 2009).

Microglia are macrophages residing in the brain that originate from early erythro-myeloid precursors in the embryonic yolk sac and migrate to the brain mesenchyme before the blood-brain barrier (BBB) is formed. They have been proposed to form a grid-like distribution pattern in the CNS that is maintained during the entire lifespan (Waisman, Ginhoux, Greter, & Bruttger, 2015). During the early half of the 19th century, Pio del Rio Hortega, a Spanish scientist, provided a visualization of microglia and their potential ability to transition from a ramified to an amoeboid morphology under pathological conditions (Eyo & Dailey, 2013; Waisman et al., 2015).

McGeer and colleagues (McGeer, Itagaki, Boyes, & McGeer, 1988) demonstrated for the first time the presence of reactive microglial cells in the SNpc of postmortem PD brains, which is suggestive of the involvement of neuroinflammation in PD pathogenesis. The involvement of the immune system in PD pathogenesis has been demonstrated in a large genome-wide association study (GWAS) that showed a single nucleotide polymorphism (SNP) in the human leukocyte antigen (HLA-DRA) gene, a genetic risk factor for late-onset PD (Hamza et al., 2010). Moreover, α-syn deposition in neurons was found to correlate with microglia expressing HLA-DR (a human homolog of MHCII) and immunoglobulin G (IgG) expression in neurons (Croisier, Moran, Dexter, Pearce, & Graeber, 2005; Orr, Rowe, Mizuno, Mori, & Halliday, 2005). These results suggest the significant contribution of the microglial activation response to the progressive nature of the disease's process.

Positron-emission tomography (PET) imaging studies have demonstrated the occurrence of ongoing microglial activation in PD patients. For example, [11C]-(R)-PK11195 PET imaging has revealed pronounced microglial activation in the pons, basal ganglia, and frontal and temporal cortical regions of PD patients (Alexander Gerhard et al., 2006). Another PET analysis confirmed microglial activation in the substantia nigra (SN) and putamen in PD patients (Iannaccone et al., 2013). Like PET analysis, in postmortem PD brains, microglial activation has been identified in diverse brain regions including the SN, putamen, hippocampus, and transentorhinal, cingulate, and temporal cortex, besides the limbic system (Imamura et al., 2003). Reactive microglial cells were found to be in close proximity to the α-syn lewy body (LB) in PD brains (A. Gerhard et al., 2003; Mackenzie, 2000) and around dying neurons (Imamura et al., 2003). Additional biochemical studies revealed higher levels of proinflammatory mediators such as TNF-α, IL-1β, and IFN-γ in the midbrain regions of postmortem PD brains (Qin, Zhang, et al., 2016; Sawada et al., 2006). These data suggest that microglial cells are inextricably linked to PD via the secretion of proinflammatory cytokines and ROS.

Microglia have complex plasticity. Under normal physiological conditions, microglia display small cell bodies with slender processes and extensive branching. These processes are motile and constantly survey the environment for pathogens, sick cells, and cell debris or participate in synaptic remodeling or maintenance (Sierra, Tremblay, & Wake, 2014; Tay, Savage, Hui, Bisht, & Tremblay, 2017; Wake & Fields, 2011). Upon detecting signals of injury, microglia polarize toward a classical proinflammatory phenotype also known as M1. The M1 state is characterized by hypertrophic cell bodies with fewer, stubby, and shorter processes compared with surveillance microglia. These cells secrete proinflammatory cytokines such as TNF-α, interleukins (IL-1,6,12-23), as well as chemokines and proteases (Rojo et al., 2014). All these events help with the tissue defense arising from external or internal tissue-damaging agents. Furthermore, these processes are reinforced by oxidative stress-related factors such as ROS and RNS released by both microglia and the surrounding cells. The proinflammatory stage is followed by a prolonged reparative state also known as the alternative or M2 phase in which microglia display hypertrophic cell bodies with a thick, ramified process and elevated phagocytic capacity. The M2 state is activated by anti-inflammatory cytokines such as IL-4, IL-13, and IL-10, transforming growth factor-β (TGF-β), and glucocorticoids. The expression of wound-healing genes such as arginase-1 (ARG-1), mannose receptors (MMC and Mrc2c), dectin-1, chitinase-3-like-1 (Ym in rodents), CD36, CD163, MARCO, and PPARγ is elevated in M2 microglia (Rojo et al., 2014). The M2 phase plays a pivotal role in switching off the proinflammatory response, scavenging damaged cells, and restructuring the damaged extracellular matrix (ECM) (Rojo et al., 2014). Notably, microglia exist in a heterogenous population, and activation states are not considered distinct categories but a dynamic process that involves an overlapping continuum across M1 and M2 states, whereby they acquire differential profiles depending on the local micro environment (Joers, Tansey, Mulas, & Carta, 2017). Despite a growing body of evidence suggesting a differential role for M1 and M2 activation states in experimental PD models, the corroboration of these states in human PD patients has yet to be determined.

1.2. Role of astrocytes during PD-associated neuroinflammation

Although glial cells constitute over 50% of brain cells, astrocytes are the most abundant glial cells (Herculano-Houzel, 2009; Jäkel & Dimou, 2017; Verkhratsky et al., 2012). For a long time, astrocytes have been believed to play a supportive role by maintaining neuronal homeostasis; however, emerging evidence supports an active regulatory role, including neuronal electrical excitability, neurogenesis, synaptogenesis, plasticity, synaptic transmission, ion homeostasis, production of neurotrophic factors, maintenance of the blood-brain barrier, and glial scar formation. In response to injury, infection, or disease, astrocytes undergo astrogliosis, a process involving the upregulation of the intermediate filament protein glial fibrillary acidic protein (GFAP), cell body swelling, and proliferation (Koob, 2017). Astrogliosis has been considered a graded response culminating in glial scar formation, which has been shown to exhibit both protective and deleterious effects. Mounting evidence supports a concept whereby astrocytes contribute to the progression of CNS disorders owing to the loss of normal protective function or the gain of toxic function, resulting in the release of neurotoxic mediators, ultimately leading to a heightened inflammatory response. Alternatively, astrocytes have been shown to participate in the generation of neurotrophic factors such as glial-derived neurotrophic factor (GDNF), which plays a central role in dopaminergic neuronal development and survival (Rocha, Cristovao, Campos, Fonseca, & Baltazar, 2012; Sandhu et al., 2009).

The role of astrocytes in PD remains a conundrum owing to the limited number of studies in PD brains and the discrepancies observed between human studies and experimental models of Parkinsonism. In patients with PD, minimal astrocyte activation has been evidenced in the regions undergoing neurodegeneration. Non-fibrillized α-syn was shown to accumulate in the cytoplasm of protoplasmic astrocytes early in the disease (Y. J. Song et al., 2009), which has been hypothesized to lead to the aggregation of α-syn and LB formation. Intriguingly, α-syn immunoreactive astrocytes were found to parallel LB distribution in the regions devoid of LBs, including the striatum and dorsal thalamus, especially in the vicinity of degenerating nerve terminals (Braak, Sastre, & Del Tredici, 2007), suggesting that astrocytes take up neuronally released altered α-syn. Indeed, subsequent studies revealed that neuronal α-syn was transferred to astrocytes (H.-J. Lee, Kim, & Lee, 2010). Thus, it is plausible that astrocytes cannot maintain a healthy environment near

damaged neurons, rendering them more vulnerable to disease and injury.

Most PD cases are idiopathic; however, monogenic mutations in 17 genes have been implicated in the development of familial PD (Klein & Westenberger, 2012; Lee et al., 2010). Proteins encoded by eight of these genes, namely DJ-1, α-syn, iPLA2, ATP13A2, LRRK2, GCase, PINK1, and parkin, have been implicated in astrocyte functioning. For example, astrocytes from parkin (an E3-ubiquitin ligase) and PINK-1 (involved in the phosphorylation of parkin) knockout (KO) mice have been shown to exhibit mitochondrial dysfunction, including the loss of mitochondrial structural integrity and membrane permeabilization, increased ROS generation, and decreased ATP generation. In a similar fashion, astrocytic mitochondrial defects were observed in transgenic mice expressing α-syn in glial cells including astrocytes (Schmidt et al., 2011). DJ-1, a PD-related gene with a mitochondria-stabilizing property, is highly expressed in the microglia and astrocytes and has been shown to be upregulated in reactive astrocytes in postmortem brain samples from PD patients (Bandopadhyay et al., 2004; Neumann et al., 2004). Co-cultures of astrocytes from DJ-1-null astrocytes and wild-type neurons were found to cause apoptotic cell death upon ROT treatment (Larsen, Ambrosi, Mullett, Berman, & Hinkle, 2011). In this study, the authors concluded that astrocytic DJ-1-mediated neuroprotection is specific to the mechanisms involving mitochondrial complex I inhibition rather than a generalized oxidative stress response. Alternatively, the astrocyte-targeted overexpression of Nrf-2 was found to delay the disease's onset and improve survival in mice that overexpressed human mutant α-syn in neurons (Gan, Vargas, Johnson, & Johnson, 2012). These studies collectively raise the possibility that the loss of function of PD-related proteins exacerbates neurotoxicity via two mechanisms involving oxidative stress and mitochondrial defects. The question of how the altered gene expression of PD-related genes contributes to PD pathogenesis remains a hotly debated issue. An improved understanding of whether differential transcriptional regulation of PD-related genes in astrocytes in a brain region-specific manner contributes to PD pathogenesis may lead to the development of novel therapeutic strategies for abrogating detrimental astrocytic responses.

The neurotoxicants (metals, pesticides, herbicides, and synthetic drugs) that cause PD-like neuropathology are functionally diverse; however, the question of whether neurotoxicant-induced astrocytic mitochondrial dysfunction leads to dopaminergic neuronal damage is currently an active area of investigation (Kubik & Philbert, 2015). MPTP is converted to its toxic metabolite MPP^+ in astrocytes (Ransom, Kunis, Irwin, & Langston, 1987), which are subsequently released into the synapse and eventually taken up into neurons via the dopamine transporter (DAT) (Pifl, Giros, & Caron, 1993). In addition, the direct inhibition of astrocytic mitochondrial enzymes has been demonstrated following MPTP treatment (Sundar Boyalla, Barbara Victor, Roemgens, Beyer, & Arnold, 2011). In this regard, the blockage of ATP-sensitive potassium channels on astrocytes has been shown to attenuate MPP^+-induced mitochondria-mediated apoptosis via the maintenance of astrocytic ATP levels (Zhang et al., 2009). Taken together, although many studies involving research on neuronal dysfunction in PD pathogenesis have been conducted, a growing body of evidence supports the pivotal role of non-cell autonomous processes, including astrocyte dysfunction in PD pathogenesis. A further understanding of the brain region-selective role of astrocytes in the disease physiology will not only improve our understanding of the role of this glial cell in the disease process but may also lead to the development of novel treatment strategies for PD.

In this review, we will summarize the experimental findings that link neuroinflammation to dopaminergic neurotoxicity in response to environmental neurotoxicants and discuss the mechanisms by which they induce neuroinflammation and potential neuropathological outcomes consequent to a persistent neuroinflammatory response. We also provide information on the mechanistic basis of anti-inflammatory agent-induced dopaminergic neuroprotection, which provides novel insights regarding novel therapeutic avenues that can be exploited to minimize dopaminergic neurodegeneration in response to environmental neurotoxicant exposure.

2. Selected environmental agents

2.1. Rotenone

Rotenone (ROT) is a botanical pesticide used as an insecticide to kill nuisance fish in lakes. This pesticide is highly lipophilic and can readily cross the BBB independent of transporters for cellular entry (M. E. Johnson & Bobrovskaya, 2015; Martinez & Greenamyre, 2012). As shown in Fig. 1, we demonstrated that exposure of the N27 rat mesencephalic dopaminergic neuronal cells to ROT leads to significant mitochondrial structural impairment and functional loss, thus supporting its function as a mitochondrial complex I inhibitor. Despite the short half-life of ROT and its failure to accumulate in the environment, several groups have shown that the continuous systemic administration of ROT to rats and mice recapitulates several of the etiologic mechanisms implicated in PD pathogenesis, including the loss of nigral dopaminergic neurons and elevated oxidative stress and neuroinflammation in the nigrostriatal dopaminergic pathway (Betarbet, Sherer, & Greenamyre, 2002; Greenamyre, Betarbet, & Sherer, 2003; Sherer, Betarbet, Kim, & Greenamyre, 2003; T. B. Sherer, R. Betarbet, C. M. Testa, et al., 2003; Sherer, Kim, Betarbet, & Greenamyre, 2003). The accumulation of α-syn cytoplasmic inclusions in DA neurons, LB pathology, DJ-1 decrements and translocation, and nigral iron accumulation (Betarbet et al., 2006) have also been evidenced in brain regions vulnerable to PD following ROT treatment. Rotenone faithfully replicates several of the clinical features of PD, including bradykinesia, rigidity, tremor, as well as non-motor symptoms evidenced in PD. Therefore, the rotenone rat model has been routinely used for the investigation of oxidative stress and neuroinflammation associated with PD. Interestingly, to date, the epidemiologic data supporting a role for ROT exposure in humans are sparse. In a case control study performed in pesticide applicators, Tanner and colleagues demonstrated a significantly (2.5-fold) increased risk of PD in those who were exposed to a combination of ROT and PQ or only ROT (Tanner et al., 2011). Another study conducted in agricultural workers from East Texas also revealed an increased risk (OR = 10.9) of developing PD upon the continued use of ROT (Dhillon et al., 2008). However, future studies are needed to investigate the detailed global epidemiology of PD following ROT exposure.

2.1.1. Microglia and rotenone

The ROT-mediated inhibition of mitochondrial electron transport chain (ETC) complex I has been shown to result in ATP depletion, mitochondria-derived ROS (mROS) generation, and the associated loss of mitochondrial membrane potential (Won, Park, Hong, Son, & Yu, 2015; Xiong et al., 2012). We recently demonstrated that ROT treatment leads to a dramatic loss of mitochondrial respiration in mouse microglial cells (Fig. 2).

2.1.1.1. Role of NADPH oxidase 2 (NOX2) in ROT-induced microglial activation and resultant dopaminergic neurodegeneration. Studies performed in ROT-exposed neuron-glia models have demonstrated that neurons are less sensitive to the toxic effects of ROT compared to neurons cultured in the vicinity of microglia (Gao, Hong, Zhang, & Liu, 2002). In this context, microglia-derived superoxide was found to be toxic to DA neurons via an NOX2-dependent mechanism, as $NOX2^{-/-}$ cultures were resistant to ROT-induced neurotoxicity (Gao, Liu, & Hong, 2003), further supporting the notion that NOX2-derived microglia might serve as a critical intermediary to enhance ROT-induced dopaminergic neurotoxicity. Consistently, in another study (Gao et al., 2011), HMGB1 (high-mobility group box 1) released from either inflamed microglia or damaged neurons was found to bind to microglial Mac1 (macrophage

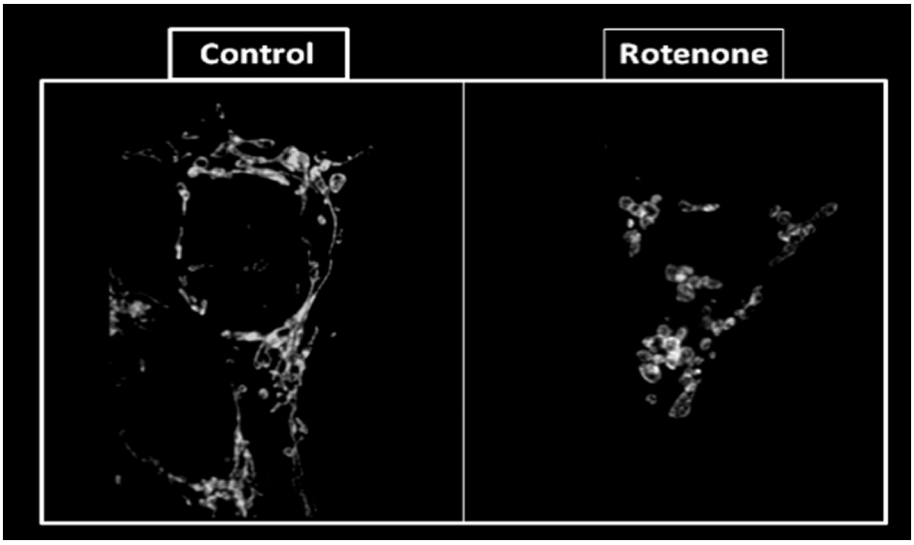

Fig. 1. Rotenone induced architectural structure damage to the mitochondria of N27 dopaminergic neuronal cells. N27 cells were exposed to rotenone (1 μM) for 3 h. Structural changes to mitochondria were then stained by the MitoTracker red dye. The z-stack images were captured on using a 63X oil immersion lens of the Leica Confocal Microscopy system and the processing of images were performed using IMARIS 10.0 software. Health mitochondrial looked hair or string like structures, whereas the damaged mitochondria exhibited a fragment or circular appearance.

antigen complex-1), subsequently leading to the activation of the NF-κB pathway and NADPH oxidase to stimulate the production of multiple inflammatory mediators. This study also showed that exposure of microglial cells to ROT resulted in the activation of NADPH oxidase and the consequent release of superoxide in a Mac1-dependent manner. These findings identified the importance of the HMGB1-Mac1-NADPH oxidase-signaling axis in mediating the ROT-induced microglial activation response. Thus, it is plausible that activated microglia and damaged neurons formed a vicious cycle leading to progressive neurodegeneration.

Hydrogen sulfide (H_2S) has been shown to confer neuroprotection in experimental models of neurological diseases partly via the inhibition of microglial neuroinflammation (Hu et al., 2010; Yin et al., 2013). In a recent study, ROT-induced ROS formation was found to suppress cystathionine β-synthase (CBS), the predominant H2S-producing enzyme in the CNS-H_2S pathway, thereby promoting the polarization of microglia toward a proinflammatory M1 phenotype (Du et al., 2014). In this context, ROT was found to upregulate the levels of M1 phenotypic genes such as TNF-α, iNOS, and COX-2/PGE2 and reduce the levels of M2 markers, including Ym1/2 and IL-10 in mouse primary and immortalized microglia. This effect was accompanied by the downregulation of CBS expression as well as the levels of H_2S in ROT-treated primary microglia, implicating CBS as a negative regulator in the ROT-mediated inflammatory cascade. Elevating H2S via CBS overexpression in immortalized microglia not only attenuated the M1 phenotype but also enhanced M2 markers in response to ROT stimulation. Likewise, in another study, Hu et al. showed that the systemic administration of NaHS (an H2S donor) inhibited the accumulation of proinflammatory factors such as TNF-α and nitric oxide via the NF-κB pathway in the striatum and SN (Hu et al., 2010). Conditioned media from microglia

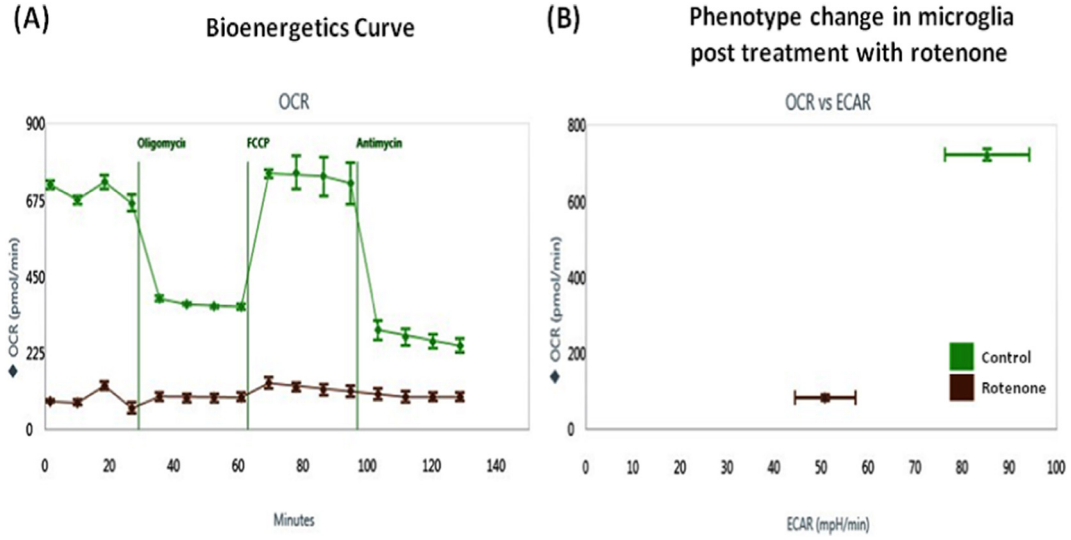

Fig. 2. Inhibition of mitochondrial respiration dynamics in the mouse microglial cells post exposure to the complex I inhibitor rotenone. (A) Bioenergetics study to measure and analyze the effects of rotenone on mitochondrial dynamics in microglia. The microglial cells were incubated with rotenone (20 nM) for 24 h and then the plate was de-gassed and made ready for the OCR measurement using the Seahorse XFe24 analyzer. Mitochondrial dynamics were measured using three-stage injections of mito-stressors with stage 1: 0.75 μM oligomycin, stage 2: 1 μM FCCP, and stage 3: 1 μM antimycin A/Rotenone. Throughout these stages, the analyzer continuously measures OCR. (B) Phenogram (OCR vs. ECAR) depicting the comparison of oxidative phosphorylation and glycolytic states of the microglial mitochondria post treatment with rotenone.

incubated with both NaHS and ROT were found to induce less neurotoxicity compared with ROT-only-treated microglia, suggesting that the protection of dopaminergic neurons might be partially attributed to the suppression of microglial activation. Another study using the parkin KO mice that bear a close resemblance to the abnormalities associated with PD demonstrated that ROT increased the number of glia more in parkin KO than in WT midbrain neuronal cultures (Casarejos et al., 2006). The addition of microglia from PK-KO to WT neuronal cultures increased the sensitivity of dopaminergic neurons to ROT, further confirming the role of microglia in ROT-mediated dopaminergic neurodegeneration (Casarejos et al., 2006).

2.1.1.2. NLRP3 inflammasome contribution to ROT-induced microglial activation response. Several studies have shown that the inhibition of mitochondrial complex I by ROT leads to NLRP3 inflammasome activation (Liang et al., 2015; Liang et al., 2017; Won et al., 2015). Inflammasomes are a multiprotein complex assembled in response to pathogen-associated molecular pattern molecules (PAMPs) or damage-associated molecular pattern molecules (DAMPs) via a sensor such as nucleotide-binding oligomerization domain-like receptor pyrin domain-containing-3 (NLRP3). Upon the assembly of the inflammasome complex, the inactive form of IL-1β (pro-IL-1β) is proteolytically cleaved via caspase-1, leading to the formation of active IL-1β. IL-1β is released at the site of injury, triggering an inflammatory response. Dysregulated inflammasome activation has been linked to neurodegenerative diseases, including Alzhimer's disease (AD). Recent evidence implicates the release of ROS and mitochondrial DNA (mtDNA) from damaged mitochondria as critical triggers for NLRP3 inflammasome activation. Numerous studies have indicated that the induction of autophagy, a cellular degradative pathway that plays an important role in the maintenance of cellular homeostasis via the degradation of damaged organelles and aggregate-prone proteins, may limit IL-1β production via the degradation of pro-IL-1β directly or indirectly by suppressing NLRP3 inflammasome activation (Harris et al., 2011; Liang et al., 2017; Shi et al., 2012). We recently demonstrated that the induction of mitochondrial dysfunction via ROT amplifies microglial NLRP3 inflammasome activation through mROS generation and autophagy dysfunction (Lawana et al., 2017). We also found that the blockade of mROS by Mito-TEMPO or the inhibition of c-ABL, a redox-sensitive kinase, via the non-receptor tyrosine kinase inhibitor dasatinib attenuated ROT-induced NLRP3 inflammasome activation in LPS-primed microglia through the restoration of autophagy (Lawana et al., 2017). Likewise, in another study, rifampicin was found to suppress NLRP3 inflammasome activation in microglia in part via the modulation of autophagy (Liang et al., 2015; Liang et al., 2017). Moreover, ROT was found to act as a selective priming signal for NLRP3 inflammasome activation via aberrant mROS production and mitochondrial hyperpolarization in BMDMs (Won et al., 2015). These studies provide convincing evidence that an ROT-induced microglial activation response is partly mediated via the activation of the NLRP3 inflammasome and that mROS may serve as a critical trigger of the microglial NLRP3 inflammasome activation response.

2.1.1.3. Role of endocannabinoid in rotenone-induced microglial activation. The endocannabinoid system has been an area of increased interest in PD owing to the role of the CB1 receptor in the basal ganglia. Cannabinoid type-two (CB2) receptors, a critical component of the endocannabinoid system, have emerged as novel therapeutic targets for neurodegenerative diseases, including PD. Microglia express high levels of CB2 receptors and upon activation have been shown to exhibit anti-inflammatory and immunosuppressant effects on neuroimmune cells (Concannon, Okine, Finn, & Dowd, 2016; Mecha, Carrillo-Salinas, Feliú, Mestre, & Guaza, 2016). CB2 receptor downregulation has been reported in PD patient brains (Price et al., 2009). CB2 has gained considerable attention owing to its ability to modulate neuroinflammation and to attenuate activated microglia and astrocytes in the SN and striatum (Bento et al., 2011; Concannon, Okine, Finn, & Dowd, 2015). In a recent study, the upregulation of the CB2 receptor was evidenced in an ROT model of PD (Concannon et al., 2016). Thus, the activation of CB2 receptors has been shown to protect dopaminergic neurons against neurodegenerative effects in toxin models of PD. β-caryophyllene (BCP), a naturally occurring CB2 receptor agonist, was found to attenuate proinflammatory cytokines such as IL-1β, IL-6, and TNF-α concomitant with the decreased generation of inflammatory mediators including NF-κB, COX-2, and iNOS in the striatum of ROT-challenged rats (Javed, Azimullah, Haque, & Ojha, 2016; Ojha, Javed, Azimullah, & Haque, 2016). These changes coincided with the rescue of dopamine neurons and fibers. On the other hand, the administration of AM360, a CB2 receptor antagonist, prior to BCP supplementation to ROT-treated rats demonstrated an increased number of activated microglia and astroglia, further supporting the CB2 receptor-mediated activity of BCP. Furthermore, previous studies supported the current findings whereby reduced microgliosis and astrogliosis were found to accompany reduced levels of COX-2 protein and proinflammatory cytokines (Cheng, Dong, & Liu, 2014). These studies suggested that CB2-activating compounds may afford dopaminergic neuroprotection through the attenuation of the microglial activation response. Therefore, the emerging preclinical evidence highlights the therapeutic advantage of targeting the CB2 receptor to break the vicious cycle of persistent neuroinflammation and resultant progressive neurodegeneration.

2.1.2. Astrocytes and rotenone

DJ-1 has been shown to participate in numerous functions, including responses to the cellular redox environment (Martinat et al., 2004; Yokota et al., 2003), protein-protein interactions (Meulener et al., 2005; Xu et al., 2005), and potential chaperone activity (Martinat et al., 2004). Despite this, the exact role of DJ-1 in cellular homeostasis remains elusive, as well as to what extent dysregulated DJ-1 contributes to the pathogenesis of PD.

Although early-onset PD is linked to DJ-1 mutation, DJ-1 KO mice fail to display hallmarks of PD, including LB formation and dopaminergic neuronal loss (Blesa & Przedborski, 2014; Goldberg et al., 2005). Thus, it is unlikely that mutations in DJ-1 per se are sufficient to induce Parkinsonism. On the other hand, studies performed in DJ-1 KO mice suggest that other genetic or environmental factors may be essential for the development of the PD phenotype in response to the DJ-1-associated loss-of-function mutations. Indeed, the DJ-1 knockdown in astrocytes was found to attenuate astrocyte-mediated neuroprotection upon the ROT challenge (Mullett & Hinkle, 2009), suggesting that neurotoxins may serve as adjunct factors for the development of DJ-1 phenotypes. Conversely, DJ-1 overexpression in astrocytes has been shown to protect against neuronal thiol oxidation compared to wild-type astrocyte co-cultures. These findings were replicated following the addition of astrocyte-conditioned media to neuron-enriched cultures. Together, these studies suggest that the DJ-1-associated neuroprotective capacity is linked to the release of soluble factors from astrocytes (Mullett & Hinkle, 2009, 2011). These studies highlighted for the first time the impact of manipulating the expression of the PD-causing gene in astrocytes on the preservation of neuronal integrity against the oxidative stress mechanism.

ROT at doses of 2–3 mg/kg/day, similar to that reported in platelets from PD patients, was found to induce nigrostriatal neurodegeneration via complex I inhibition with concomitant astroglial activation (R. Betarbet et al., 2000; Sherer et al., 2003). Similarly, the subcutaneous administration of ROT is associated with selective dopaminergic damage and the accompanying aggregation of α-syn akin to the LB of PD (Hoglinger et al., 2005; Sherer, Betarbet, Testa, et al., 2003). The mechanistic basis of ROT-induced α-syn aggregation remains poorly understood; however, it has been hypothesized that oxidative modifications of α-syn owing to the excessive generation of ROS following complex I inhibition might contribute to the aggregation process (Cabezas, El-Bachá, González, & Barreto, 2012; Greenamyre et al., 2003). In addition,

neurons and astrocytes treated with ROT (25–50 nM) showed the altered expression of γ-tubulin and disorganized centrosomes with concomitant α-syn aggregation (Diaz-Corrales, Asanuma, Miyazaki, Miyoshi, & Ogawa, 2005). These studies suggest that the inhibition of mitochondrial complex I and the promotion of α-syn aggregation may be critical determinants of ROT-resultant dopaminergic neurotoxicity and, presumably, glial activation (R. Betarbet et al., 2000; Sherer, Betarbet, Testa, et al., 2003).

Finally, using an intragastrically administered ROT (5 mg/kg) mouse model, α-syn aggregation in neurons of the dorsal motor nucleus of the vagus (DMV) and the intermediolateral nucleus in the spinal cord were evidenced with a concomitant selective loss of dopaminergic neurons and astrogliosis, suggesting that α-syn is taken to the CNS via retrograde axonal transport, leading to the replication of neuroanatomical and neurochemical correlates of PD staging (Drolet, Cannon, Montero, & Greenamyre, 2009; Pan-Montojo et al., 2010). To further elucidate the mechanistic basis of ROT-induced astrocytic dysfunction, in a recent study, ROT was shown to alter the expression of the signal transducer and activator of transcription 3 (STAT3). The deletion of STAT3 in astrocytes was found to impair astrocyte mitochondrial function and ATP generation. Many of the effects observed in STAT3 conditional KO astrocytes were altered following treatment with ROT, suggesting a role for the mitochondrial electron transport chain (Sarafian et al., 2010). ROT was also found to modulate connexin 43, a major gap junctional protein in astrocytes, suggesting that altered gap junctional cellular communication in astrocytes may play a key role in PD pathology (Zhang et al., 2011). These studies suggested that local effects of pesticide might be sufficient to reproduce the neuroanatomical and neurochemical features associated with PD progression. It appears that ROT-induced dopaminergic neurodegeneration results from the excessive activation of microglia and astrocytes, which in turn leads to the amplification of oxidative stress and neuroinflammatory mechanisms, both of which have been linked to PD-related dopaminergic neuropathology.

2.2. Paraquat (PQ)

Paraquat (PQ) belongs to the bipyridylium family of broad-spectrum herbicides and is commonly used to control pests in crops such as soybeans, sorghum, sugar cane, cotton, corn, and apple, among others. PQ has a long environmental half-life. It has been demonstrated that PQ leaches into groundwater and remains in the environment for prolonged periods of time, which represents 80% retention in river water for a period of two years (Fernandez, Ibanez, Pico, & Manes, 1998; Wang, Yen, Hsieh, & Chen, 1994). The improper handling and ingestion of PQ has been linked to pulmonary damage and death. PQ has been shown to cross the BBB via neutral amino acid transporters and preferentially cause dopaminergic neurodegeneration via the inhibition of complex I of the mitochondrial respiratory chain, leading to the generation of free radicals (Mandel, Adami, & Cole, 2012). PQ has been shown to induce the hyperacetylation of histones, leading to the epigenetic dysregulation of dopaminergic neuronal cells (Song, Kanthasamy, Jin, Anantharam, & Kanthasamy, 2011). PQ was found to be excluded from the rhesus macaque brain via the BBB, arguing against a role for PQ in the etiology of PD (Bartlett et al., 2009). Chronic exposure to PQ has been linked to PD, although it is controversial owing to inconsistencies in epidemiological and animal studies. In this regard, Berry et al. argued that the environmental link of PQ to PD is not convincing owing to the lack of information on exposure levels (Berry et al., 2010).

2.2.1. Paraquat and microglial activation

PQ administration to mice and rats was found to support an activated microglial phenotype characterized by enlarged cell bodies and the loss of processes. These changes were found to occur at earlier time points (two days) although they subsided at day 12, suggesting they might represent an early response, rendering neurons more vulnerable to PQ-induced dopaminergic neurotoxicity (Cristóvão, Choi, Baltazar, Beal, & Kim, 2009). In another study, Mangano et al. showed that the genetic deletion of IFN-γ was found to blunt the microglial activation response, the expression of NADPH oxidase subunits, iNOS, COX-2, TNF-α, IL-1β, and signaling factors such as JNK, p38 MAP kinase, STAT1, and NF-κB in the SN of PQ-treated mice (Mangano et al., 2012). The studies concluded that IFN-γ may be a critical cytokine linking toxin exposure, oxidative stress, and inflammatory response to the PD-associated pathological process. The neuropeptide PACAP (pituitary adenylyl cyclase-activating polypeptide) has been shown to protect dopaminergic neurons from 6-OHDA-induced neurotoxicity via the elevation of TH and VMAT2 expression. In a recent study, an interaction between PACAP and an environmental exposure to PQ was implicated in PD-associated dopaminergic neuropathology. Watson et al. hypothesized that decreased levels of PACAP may interact with environmental factors such as PQ to increase the risk of PD (Watson et al., 2013). For this purpose, PACAP KO mice were given a single (10 mg/kg) dose of PQ, a regimen that failed to elicit dopaminergic neurodegenerative effects in WT mice. This dose was found to cause a preferential loss of TH-positive cell bodies in the SNpc of PACAP KO mice. Strikingly, microglial activation was evident in the basal state of the PACAP mice. PQ administration to PACAP KO mice was found to enhance TNF-α expression and elevate the abundance of proinflammatory Th17 cells, while the induction of Tregs, which are anti-inflammatory regulatory T cells, was impaired following the administration of PQ. The authors concluded that PACAP's ability to modulate microglia and/or immune cells may underlie its ability to preserve the integrity of dopaminergic neurons during PQ administration, further pointing to the importance of examining inflammation-related factors in epidemiological and genetic studies of PD (Watson et al., 2013). Several other researchers have utilized *in vitro* studies such as the immortalized microglial cells BV2 to elucidate the mechanisms underlying the PQ-induced microglial activation response. For example, PQ was found to induce the transformation of microglia from a resting to an activated state, which was characterized by the increased expression of TNF-α and IL-1β, as well as the release of TNF-α, IL-1β, and IL-6, suggesting that it induces an inflammatory response. PQ was also found to activate NF-κB and AP-1, leading to the increased expression of proinflammatory cytokines in PQ-treated BV2 cells (Sun, Zheng, Xu, & Zhang, 2018). In another study performed in BV2 microglia, PQ was found to activate microglial NOX-mediated superoxide generation (Miller, Sun, & Sun, 2007b).

2.2.1.1. Paraquat and iron. Increased levels of iron have been demonstrated in both postmortem brains and magnetic resonance imaging (MRI) studies (Dexter et al., 1987; Du et al., 2016; Riederer et al., 1989; Wang et al., 2016). The exact relationship between iron accumulation and the pathogenic process of the disease remains poorly understood. The question still unanswered is whether iron is a trigger or a consequence of the disease. Free iron in the brain has been shown to activate microglia and promote neurodegeneration via ROS generation that, in turn, has been linked to PD pathogenesis (Kruszewski, 2003). Paraquat (PQ) has been shown to oxidize iron, which reacts with hydrogen peroxide, leading to the formation of hydroxyl radicals (Przedborski & Ischiropoulos, 2005). Thus, there is a consensus that PQ can act as a prooxidant. In another study, Peng et al. reported that PQ itself failed to elicit neurodegenerative effects on TH^+ neurons; however, in mixed neuron-glia cultures, PQ was found to cause a 40% reduction in TH immunoreactive neurons (Peng, Stevenson, Oo, & Andersen, 2009). The addition of iron was found to increase PQ neurotoxicity by 10%. This suggests that glia is an integral component of PQ-induced dopaminergic neurotoxicity and that iron exacerbates PQ neurotoxicity (Peng et al., 2009). In the same study, microglial activation was positively linked to iron-related oxidative stress. In this context, the inhibition of superoxide generation was found to inhibit the detrimental effects of PQ and PQ+Fe on dopaminergic neuronal survival in neuron-glia co-cultures (Peng et al., 2009). Furthermore, Wu et al. demonstrated increased iron concentration in the SN (pars reticulate), globus pallidus,

and red nucleus in humans at 6, 12, and 24 months following acute intoxication (B. Wu et al., 2012). Collectively, it appears that iron availability may impact dopaminergic neurotoxicity. In fact, iron has been shown to activate microglia, leading to the conversion of PQ^{2+} to PQ^{+}, which enters neurons via DAT and organic cation transporter-3 (Rappold et al., 2011). Therefore, these findings support the notion that environmental risk factors may act synergistically to induce the dopaminergic neurodegeneration associated with this debilitating disorder and that iron and PQ may share common oxidative stress mechanisms via the microglial activation response.

2.2.1.2. Role of NOX2 in paraquat-induced dopaminergic neurodegeneration. It is well established that PQ activates microglial oxidative stress via NADPH oxidase, leading to the generation of proinflammatory cytokines and oxidative nitrosative stress. For example, PQ has been shown to induce dose-dependent superoxide production in primary microglial cultures (Bonneh-Barkay, Reaney, Langston, & Di Monte, 2005; Wu et al., 2005), and the genetic depletion of NOX2 was found to block PQ-induced dopaminergic neurotoxicity in both *in vitro* (Wu et al., 2005) and *in vivo* models (Purisai et al., 2007). Lower doses (0.5 μM and 1.0 μM) of PQ have been shown to induce the microglial activation phenotype, resulting in a significant increase in NOX2 expression and O_2^- production (Bonneh-Barkay et al., 2005; Purisai et al., 2007; Wu et al., 2005). Neuron-glia cultures exposed to PQ also displayed a decrease in DA uptake and a decline in TH-positive cells (Wu et al., 2005). The authors concluded that, at lower doses, the indirect insult generated from microglial NADPH oxidase is critical for mediating DA neurotoxicity. However, it is unclear whether intracellularly generated O_2^- would lead to the damage of surrounding neurons.

Several hypotheses have been proposed to explain O_2^- generation. For example, PQ has been shown to enter microglia via active transport to directly interact with NADPH on NOX2 to promote redox cycling and the resultant O_2^- generation. Intriguingly, this contrasts with another hypothesis that PQ induces O_2^- outside the microglia (Kreutzberg, 1996). According to a second hypothesis, PQ has been proposed to remain extracellular relative to microglia and could utilize the electron transferred across the membrane from NOX2 prior to its interaction with molecular oxygen to produce extracellular O_2^- at more efficient rates compared with the intracellularly located NOX2 enzyme complex alone (Taetzsch & Block, 2013). In this case, it would lead to the amplification of the basal activation of NOX2 rather than initiating superoxide production. A third hypothesis states that NOX2 activation occurs via the activation of intracellular cell signaling events, leading to the assembly of the NOX2 complex in response to PQ. In fact, PKCδ has been implicated in the membrane translocation of p67PHOX, leading to the assembly of the NOX2 complex (Miller, Sun, & Sun, 2007a; Zhao et al., 2005). It has been proposed that this could serve as an initial signal for the amplification of extracellular redox signaling, as proposed in the previous hypothesis. Despite the pivotal role of microglial NOX2 in PQ-induced dopaminergic neurotoxicity, the mechanisms triggering NOX2 activation remain enigmatic, and more research is needed.

2.2.1.3. Dual hit pesticide-induced PD model. Given that PQ and MB are used in geographically overlapping areas, both MB and PQ are well documented as triggers for the PD phenotype in mice (Cicchetti, Drouin-Ouellet, & Gross, 2009). Epidemiological studies also demonstrate that combined exposure to MB and PQ increases the risk of developing PD in humans (Costello et al., 2009; Wang et al., 2011). Several studies have used PQ and MB dual exposure to develop a PD phenotype in mice (Thiruchelvam, Brockel, Richfield, Baggs, & Cory-Slechta, 2000; Thiruchelvam, Richfield, Baggs, Tank, & Cory-Slechta, 2000). In fact, co-exposure to PQ and MB in experimental animal models has been shown to synergistically enhance dopaminergic neurodegeneration in the SNpc and associated glial reactivity via an oxidative stress mechanism (Thiruchelvam, Brockel, et al., 2000; Thiruchelvam, Richfield, et al., 2000). The exposure of C57BL/6 mice to PQ (10 mg/kg) and MB (30 mg/kg) once per week for four weeks has been shown to decrease locomotor activity and to induce a loss of dopaminergic fibers and altered levels of DA and its metabolites, DOPAC and HVA (Thiruchelvam, Richfield, et al., 2000). Thus, pesticide-induced neurotoxic cellular and animal models may be directly relevant to humans, given that humans are exposed in their everyday life.

Pattern recognition receptors (PRRs) play critical roles in the innate immune response via PAMPs or DAMPs (Jang et al., 2015). Previous studies showed that complement receptor 3 (CR3 or CD11b/CD18), a microglia-specific pattern recognition receptor, is involved in the regulation of NOX2 activation (Canton, Neculai, & Grinstein, 2013; Ehlers, 2000). The suppression or genetic deficiency of CR3 was found to inhibit dopaminergic neurodegeneration via blocking the PQ/MB-induced membrane translocation of the NOX2 cytosolic subunit p47 phox, a critical step in NOX2 activation (L. Hou et al., 2018). In addition, the inhibition of Src and Erk (extracellular regulated protein kinases) impaired NOX2 activation in response to PQ and MB. The authors concluded that CR3-regulated NOX2 activation and dopaminergic neurodegeneration via an Src-Erk-dependent pathway contributed to PQ/MB-induced neurotoxicity supporting the pivotal role of pesticide-induced immunomodulatory mechanisms in PD pathogenesis. Likewise, in another study using a two pesticide-induced PD model (Hou et al., 2017), co-exposure to PQ and MB was found to drive noradrenergic locus coeruleus neurodegeneration through NADPH oxidase-mediated microglial activation. Importantly, the inhibition of NADPH oxidase by apocynin was found to confer resistance to PQ/MB-induced neurotoxicity (Hou et al., 2017). In a similar fashion, co-exposure to MB and PQ for six weeks was found to induce protein radical formation in the midbrain of mice with concomitant active microgliosis, NADP oxidase activation, and iNOS induction (Kumar, Leinisch, Kadiiska, Corbett, & Mason, 2016). Additionally, protein radical formation was attenuated in p47 $phox^{-/-}$ and $iNOS^{-/-}$ mice and in mice treated with apocynin and 1400W (an iNOS inhibitor) (Kumar et al., 2016). These studies demonstrated for the first time a link between α-syn radical formation and neuronal death via NADPH oxidase and iNOS activation in the midbrains of MB and PQ co-treated mice.

To further highlight the pivotal role of the dysregulation of protein processing in PQ/MB-induced dopaminergic neurotoxicity, Saint-Pierre et al., using a PQ-MB dual pesticide-exposed aged rat model (six-month-old rats), reported that PQ/MB-induced dopaminergic neurodegeneration can be seen by six weeks following PQ/MB treatment, which was preceded by microglial activation at four weeks, although it abated by six weeks (Saint-Pierre, Tremblay, Sik, Gross, & Cicchetti, 2006). Interestingly, EM studies revealed an abnormal morphology of the Golgi apparatus at four weeks after PQ/MB treatment as assessed via double immunolabeling for TH and Golgi (Li, He, Sun, & Li, 2016). These studies suggested that PQ/MB may cause dysfunction in the protein processing of nigral neurons that might be mediated either directly by PQ/MB or the result of microglial activation. These studies suggest that PQ/MB-induced dopaminergic neurotoxicity may be linked to microglia-mediated oxidative stress mechanisms.

To further emphasize the role of oxidative stress in PQ/MB-induced dopaminergic neurotoxicity, Singh and colleagues demonstrated a role for Cyp2d22, an ortholog of human cytochrome P450, in the PQ-induced degeneration of nigral dopaminergic neurons (Srivastava et al., 2012). The MB and PQ combination was found to induce oxidative stress, reduce the numbers of TH-positive neurons, and trigger neuroinflammation and apoptosis. A Cyp2d22 inhibitor, ketoconazole, was found to exacerbate neurodegenerative indices (Srivastava et al., 2012). On the contrary, resveratrol co-treatment was found to elicit anti-inflammatory effects via reduced microglial activation and an associated reduction in the NF-κB activation and generation of TNA-α and IL-1β, further confirming the anti-inflammatory effects of resveratrol (Srivastava et al., 2012). This study provided evidence that Cyp2d22 offers neuroprotection against MB- and PQ-induced nigrostriatal dopaminergic neurodegeneration via the attenuation of the proinflammatory

response. These findings support the notion that environmental exposure to diverse toxicants may act synergistically to produce dopaminergic neurodegeneration via microglial activation.

2.2.2. Astrocytes and paraquat

A recent topic of interest in the field of PQ-induced neurotoxicity is the positive association between senescent astrocytes and PQ-induced dopaminergic neurodegeneration. As described in the above sections, under normal physiological conditions, astrocytes exert a reparative role to maintain normal neuronal functioning (Booth, Hirst, & Wade-Martins, 2017). However, under pathological conditions, astrocytes, owing to their antioxidant machinery (which serves as the first line of defense against the toxic effects of the brain), are targeted by toxins (Episcopo et al., 2013; Shih et al., 2003; Yokoyama, Uchida, Kuroiwa, Kasahara, & Araki, 2011). Despite numerous studies implicating senescent cells outside the brain in age-related pathologies, to date, their contribution to age-related neurodegenerative changes remains poorly characterized (Bhat et al., 2012; Chinta et al., 2015). In a recent study, Chinta et al. demonstrated that PQ can induce senescence in cultured human astrocytes and *in vivo* PQ models (Chinta et al., 2018). Notably, the *in vivo* depletion of age-related senescent markers was associated with the absence of the development of neurodegenerative phenotypes upon systemic PQ exposure, suggesting that senescent astrocytic cells are critical contributors to neurodegenerative changes in an *in vivo* neurodegenerative disease model. The studies concluded the accumulation of senescent cells in the aging brain can be a critical contributor to dopaminergic neurodegeneration in which environmental factors such as PQ are implicated.

There is growing evidence supporting a link between astrocyte dysfunction and dopaminergic neuronal loss. For example, the treatment of rat primary mesencephalic cultures with PQ resulted in the increased generation of H_2O_2 and Fe^{2+} preceding cell death. The adenoviral-mediated overexpression of mitochondrial aconitase (m-aconitase) leads to its increased expression in the astrocyte with an accompanying elevated generation of the aforementioned oxidative stress markers and dopaminergic neuronal cell death (Cantu, Schaack, & Patel, 2009). Given that catalase and an iron chelator attenuated cell death, the authors postulated that PQ-induced cell death was mediated in part via astrocyte-derived H_2O_2 (Cantu et al., 2009). In another study to elucidate the development-dependent effects of PQ, Sandstrom et al. used 3D rat brain cultures to demonstrate that the perturbation of GSH-mediated defenses against oxidative stress and impairments in dopaminergic neurons were accompanied by stronger astrogliosis in immature cultures compared with mature cultures (Sandström et al., 2017). The authors concluded that the increased vulnerability of immature cultures to the toxic effects of PQ might be attributed to insufficient protective mechanisms. To further highlight the pivotal role of oxidative stress mechanisms in PQ-induced neurotoxicity, another study examined how impairment in astrocytic oxidative stress mechanisms might lead to autophagic impairment, thereby promoting PQ-induced neurotoxicity. In this regard, N-[2-(2-methoxy-6H-dipyrido {2,3-a:3.2-e} pyrrolizin-11-yl)ethyl]-2-furamide (NMDPEF), a quinone oxidoreductase 2 (QR2) inhibitor, was found to attenuate PQ toxicity *in vitro* and *in vivo,* suggesting a key role for QR2 in the regulation of oxidative stress (Janda et al., 2013). The same authors in a later study (Janda et al., 2015) using astrocytic cultures demonstrated that prolonged PQ-induced oxidative stress in astrocytes impeded autophagosome formation, thereby leading to impairment in autophagic flux. These findings unveiled the novel neuroprotective role of astrocyte autophagy and highlighted the inhibitory role of quinone oxidoreductase 2 in mediating PQ-induced autophagic impairment and the resulting dopaminergic cell death. Consistent with this *in vitro* report, co-culturing astrocytes was found to block PQ-induced neuronal cell death via the induction of astrocytic glutathione homeostasis and the gamma-glutamyl cycle (Rathinam et al., 2012). Conversely, the characterization of the transcriptional profile following PQ exposure revealed that PQ upregulated the mRNA of antioxidative enzymes as well as the mRNAs of enzymes involved in the protection against oxidative stress, suggesting that the impairment of antioxidant mechanisms might be linked to PQ-induced neurotoxicity. These studies illustrate the importance of astrocyte-mediated antioxidant mechanisms in preserving the integrity of dopaminergic neurons against PQ-induced neurotoxicity.

Many discrepant findings have been reported regarding PQ-induced neurotoxicity. The controversy has been related to inconsistent dopaminergic neuronal loss and the associated motor impairment, the variable depletion of striatal DA, and/or the generation of LB impairments (Bastias-Candia, Zolezzi, & Inestrosa, 2018; Jackson-Lewis, Blesa, & Przedborski, 2012; Jones, Huang, Mailman, Lu, & Williams, 2014; Tieu, 2011; L. Yin et al., 2011). PQ has been shown to induce the moderate loss of dopaminergic neurons when administered alone. For example, a previous study demonstrated that PQ treatment induced a significant loss of dopaminergic neurons in the SN, although a reduction in striatal DA fibers was not found (Cicchetti et al., 2005). Smeyne et al. demonstrated that repeated exposure to PQ (either 20 mg/kg once or 10 mg/kg twice weekly for three weeks) failed to induce neuronal cell loss in the SNpc or striatum (Smeyne et al., 2016). Additionally, signs of apoptosis, microgliosis, or astrocytosis were absent. Likewise, Breckenridge et al. reported that repeated exposure to PQ failed to alter the levels of DA and its metabolites HVA or DOPAC (Breckenridge, Berry, Chang, Sielken, & Mandel, 2016). Evidence of degenerating dopaminergic neurons as well as astrogliosis and microgliosis was absent in PQ-treated mice in this study. Thus, it is possible that individual genetic variations in susceptibility led to differences in neurotoxic risk and functional outcomes (Jones et al., 2014). The question regarding whether PQ exposure increases the risk of developing PD remains a hotly debated issue.

2.3. Organophosphorus (OP) and neuroinflammation

Organophosphorus (OP) compounds are used worldwide as insecticides to protect crops, animals, and humans (Jr & Nidiry, 2002; Voorhees, Rohlman, Lein, & Pieper, 2016). The main sources of OP contamination include dietary ingestion and occupational exposure. OP exposure has been suggested as a risk factor for the development of neurodegenerative disorders (Sánchez-Santed, Colomina, & Herrero Hernández, 2016), and the major concerns related to OP exposure include delayed effects following high-level exposure as well as low-level exposure during the entire lifespan. Acute OP intoxication is characterized by the irreversible inhibition of acetylcholinesterase (AChE). AChE inhibition increases the levels of acetylcholine in cholinergic synapses, leading to the hyperstimulation of cholinergic receptors followed by cholinergic syndrome-related symptoms. On the contrary, it is posited that the downregulation of cholinergic receptors may be linked to chronic OP neurotoxicity (Costa, 2006; Yang & Deng, 2007). However, a growing body of evidence supports non-cholinergic mechanisms, including oxidative stress and neuroinflammation independent of AChE inhibition in OP-induced neurotoxicity. Both acute OP intoxication and chronic repeated low-level exposure to OP have been shown to elicit a heightened neuroinflammatory response; however, the detrimental impact of an inflammatory response on neuronal integrity and neurobehavioral deficits remains a highly debated issue. Given that anti-inflammatory agents are neuroprotective following acute OP intoxication (Amitai et al., 2006) and the positive correlation between the induction of proinflammatory markers and the resultant neurodegeneration and neurobehavioral deficits, neuroinflammation has proven to be an attractive candidate for study, compared to other molecular events that mediate OP-induced neurotoxicity (Banks & Lein, 2012; Dziedzic, 2006; Mrak & Griffin, 2005). Therefore, in this section, we will provide a brief overview on the *in vivo* models that have simulated occupational OP exposure to better understand its impact on the nervous system. In addition, we will discuss the knowledge

gathered from *in vitro* models that has shed light on inflammatory cell signaling events related to OP exposure.

2.3.1. Organophosphorus and microglia

There is convincing evidence that inflammatory responses are involved in OP-mediated neuronal injuries, leading to a poor prognosis of neurological outcomes (Banks & Lein, 2012; Oostingh, Wichmann, Schmittner, Lehmann, & Duschl, 2009; Proskocil, Lein, Jacoby, & Fryer, 2017; Viviani, Boraso, Marchetti, & Marinovich, 2014). Much of this evidence has been derived from experimental models of OP intoxication (Moser, 2007; Pereira et al., 2014; Voorhees et al., 2016). It is well established that OP intoxication leads to the induction of proinflammatory responses, eventually leading to delayed neuronal injury and neurological deficits (Banks & Lein, 2012; Viviani et al., 2014). One among the early events associated with OP intoxication involves the activation of microglia (Collombet et al., 2005; Raveh et al., 2003; Zimmer, Ennis, & Shipley, 1997). Activated microglia have been shown to induce inflammatory cytokines such as IL-1α, IL-1β, IL-6, and TNF-α in rodent brains following OP intoxication (Dhote et al., 2007; Dillman III et al., 2009; Johnson & Kan, 2010; Svensson, Waara, Johansson, Bucht, & Cassel, 2001; Williams et al., 2003). Notably, activated microglia were found to be a key source of IL-1β following OP treatment (Banks & Lein, 2012). Experimental evidence suggests that the inflammatory response in the brain can reduce cell survival via enhanced neuronal excitability (Shimada, Takemiya, Sugiura, & Yamagata, 2014; Vezzani & Granata, 2005). Conversely, anti-inflammatory agents have been shown to serve as effective therapeutic agents for the treatment of humans poisoned with OP neurotoxins and nerve agents. In a recent study, the impact of neuregulin-1 (NRG-1) in diisopropylfluorophosphate (DFP)-induced microglial activation was investigated (Li et al., 2015). NRG-1 belongs to a family of multipotent neuroprotective and anti-inflammatory growth factors that include acetylcholine receptor-inducing activities (ARIAs), glial growth factors (GGFs), heregulins, and neu differentiation factors (NDFs) (Falls, Rosen, Corfas, Lane, & Fischbach, 1993; Holmes et al., 1992; Marchionni et al., 1993; Wen et al., 1992). In this context, DFP was found to induce microglial activation and neuronal injury in multiple brain regions; this response was inhibited by NRG-1 (Y. Li et al., 2015). To elucidate the molecular mechanisms underlying the NRG-1-mediated inhibition of microglial activation, the authors analyzed the gene expression profile of the hippocampal region in animals exposed to NRG-1 and DFP (Y. Li et al., 2015). The bioinformatics analysis of NRG-1 genes revealed the modulation of immune cell trafficking and IL-6 signaling, among others. In addition, NRG-1 was found to attenuate the levels of chemokines, namely CXCL2, CXCL11, and LECT1. The authors concluded that NRG-1 protects neurons against DFP-induced delayed cell death via the inhibition of neurotoxic proinflammatory factor generation (Y. Li et al., 2015).

OP nerve agent-induced status epilepticus (SE) is accompanied by a rapid and sustained neuroinflammatory response involving the activation of microglia and astrocytes (Angoa-Pérez et al., 2010; Collombet et al., 2005; Zimmer et al., 1997) and the resulting elevation of proinflammatory mediators such as cytokines (Chapman, Kadar, & Gilat, 2006; Dhote et al., 2007; Dillman III et al., 2009; Johnson & Kan, 2010; Svensson et al., 2001; Williams et al., 2003) and arachidonic acid metabolites (Angoa-Pérez et al., 2010; Zaja-Milatovic, Gupta, Aschner, & Milatovic, 2009) in multiple brain regions. Neuroinflammatory responses were found to precede neurodegeneration, seizures, and significant behavioral deficits (Banks & Lein, 2012; Y. Chen, 2012; de Araujo Furtado, Rossetti, Chanda, & Yourick, 2012; Pereira et al., 2014). However, it remains poorly understood whether neuroinflammation is causally linked to OP-induced neurological deficits. Recently, Flannery et al., using an acute DFP rat model, demonstrated that acute DFP exposure (9 mg/kg) triggered neuroinflammation in the hippocampus and cortex during the first three days that peaked at seven days and persisted up to 21 days after exposure to DFP (Flannery et al., 2016). Neurodegeneration was evidenced in multiple brain regions from one to 14 days after exposure. The authors concluded the neuroinflammatory response is influenced by the severity of seizures and that neurobehavioral deficits can also occur independently of seizures or neuroinflammation. Collectively, these studies, together with previous reports, suggest that microglial activation is linked to OP-induced memory impairment and that delayed astrogliosis enhances neurogenesis and promotes the functional recovery of affective but not cognitive behavior. Furthermore, neuroinflammation may play a differential role as the inflammatory cell activation profile that evolves post-drug treatment. These studies suggest that neuroinflammation may elicit a differential response across the diverse neurological outcomes associated with acute OP intoxication.

Acute OP (nerve agent) intoxication such as soman exposure has been shown to be associated with the upregulation of protein and mRNA levels of proinflammatory cytokines including TNF-α, IL-1β, and IL-6 in the hippocampus, piriform cortex, and thalamus of rodents. Sarin has been shown to elevate these inflammatory cytokines in the rat cortex and hippocampus. In fact, inflammation has been shown to remain persistent even 30 days after the cessation of sarin treatment (Chapman et al., 2006), further supporting the persistent nature of the proinflammatory response elicited by acute OP intoxication. Regarding chemokines, soman has been shown to increase the protein levels of CXCL1, MIP-1α in the hippocampus, the piriform cortex, and the thalamus (Johnson et al., 2011). Sarin has been shown to increase the gene expression of IL-10, an anti-inflammatory cytokine (Damodaran et al., 2006). Increased prostaglandin (PGE2) production has been seen in the hippocampus and cortex of sarin-intoxicated rats (Chapman et al., 2006; Grauer et al., 2008). Likewise, soman has been shown to increase the expression of COX-2, the initial enzyme in the biosynthetic pathway of prostaglandins, in the neurons of the rat hippocampus, the piriform cortex, and the amygdala (Angoa-Pérez et al., 2010), which is further indicative of increased prostaglandin synthesis following OP intoxication. It would be interesting to investigate whether OP-induced neurodegeneration is associated with the perturbation of microglial polarization, such as the balance between M1 and M2 microglial phenotypes in a time-dependent manner and importantly whether OP-stimulated, microglia-derived toxic inflammatory mediators serve as the primary drivers of astrocyte activation, eventually leading to neurodegeneration. More importantly, the dose of OP that can alter the neuroinflammatory response should be compared with the dose that can cause AChE inhibition.

2.3.2. Association between organophosphorus exposure and the risk of PD

A growing body of evidence suggests that the likelihood of developing PD is greater in patients with acute OP poisoning (Bhatt, Elias, & Mankodi, 1999; Chuang, Su, Lin, & Kao, 2017; Hashim et al., 2011), suggesting that OP can impact the nigrostriatal dopaminergic system. While oxidative stress has been implicated in OP-induced toxicity, recent evidence supports a role for impaired mitochondrial function in the mechanisms of OP-induced neuronal dysfunction (Terry, 2012). In the human brain, numerous cholinergic neurons are in the pontine reticular formation, striatum, and basal forebrain (Chuang et al., 2017; Dayall, 1993). In the striatum, efferent GABA projections to the globus pallidus are stimulated by large aspiny neurons that participate in maintaining balance between DA and GABA. The increased expression of AChE is found within the extrapyramidal system, including the nucleus caudate and globus pallidus. Therefore, it is likely that reduced AChE activity within the cholinergic neurons may lead to the development of Parkinsonian features (MÜLler-Vahl, Kolbe, Dengler, & MÜLler-Vahl, 1999). A previous study using a geographic information system (GIS)-based exposure assessment tool in 357 incidents of PD cases and 752 population controls living in the central valley of California demonstrated that exposure to an increasing number of OPs is associated with an elevated risk of PD (A. Wang, Cockburn, Ly, Bronstein, & Ritz, 2014). These studies further confirm that agents that inhibit mitochondrial function can elicit signs of Parkinsonism. To further investigate the

relationship between glial activation and the loss of nigral dopaminergic neurons in response to chlorpyrifos (CPF), Zhang et al. investigated whether neonatal exposure to CPF contributes to the initiation and progression of dopaminergic neurotoxicity and explored the possible underlying mechanisms (J. Zhang et al., 2015). In this study, the exposure of newborn rats to 5 mg/kg (a subtoxic dose) of CPF from post-natal day (PND) 11 to 14 daily induced a significant loss of dopaminergic neurons and an accompanying increase in microglial and astroglial activation in the SN at 16d and 46d post-exposure. The authors concluded that CPF may cause long-term neuronal damage in the SN via a heightened inflammatory response involving NF-κB, p38 MAPK activation (Zhang et al., 2015). Several studies have demonstrated that OP can induce microglial activation at levels that cause neuronal apoptosis. For example, chronic low-level exposure to the OP dichlorvos in adult rats was found to trigger neuronal apoptosis and impairment in mitochondrial complexes I, III, and IV activities, as well as an elevated inflammatory response (Kaur, Radotra, Minz, & Gill, 2007). Follow-up studies by the same authors demonstrated that dichlorvos induced the loss of nigrostriatal dopaminergic neurons, reductions in striatal DA and TH levels, microglial activation, as well as α-syn and ubiquitin-positive inclusions reminiscent of PD neuropathology (Binukumar, Gupta, Bal, & Gill, 2011; Bk, Bal, Kandimalla, & Gill, 2010). Thus, microglia-derived NADPH oxidase coupled with the generation of proinflammatory cytokines such as TNF-α, IL-1β, and IL-6 in the ventral midbrain may increase the vulnerability of dopaminergic neurons to oxidative stress-induced neuronal injury following OP exposure.

2.3.3. Astrocytes and OP

Astrocytes have been shown to modulate neuronal functioning via the secretion of numerous factors, including extracellular matrix proteins and growth factors as well as via the regulation of glucose and neurotransmitter levels. In a previous study, diazinon (DZ) and diazoxon (DZO) were found to inhibit neurite outgrowth via an oxidative stress mechanism (Binukumar et al., 2011). Owing to the neuroprotective effects of astrocytes, the authors hypothesized that astrocytes may increase the neuronal defense against oxidative stress. As anticipated, the inhibition of neurite outgrowth induced by DZ and DZO in primary hippocampal neurons was prevented when cultured in the presence of astrocytes (Binukumar et al., 2011). In fact, this astrocyte-mediated neuroprotective response was attributed to an enhancement of GSH levels in the neurons (Binukumar et al., 2011). The results of present studies have direct relevance for human exposure to OPs, especially neurodevelopmental effects. It is plausible that astrocytic functioning varies as a function of the developmental stage at the time of exposure (Banks & Lein, 2012). Likewise, in another study aimed at investigating the cross-talk between astrocytes and neurons in CPF-induced developmental neurotoxicity, human neural progenitor-derived neuron cultures exposed to CPF exhibited pronounced reductions in neuritic length, branching, and numbers, while in astrocyte-neuron co-cultures, a marked reversal of CPF's detrimental effects on neuritic integrity was evidenced (X. Wu et al., 2017). The cytochrome P450 inhibitor SKF525A partially negated the astrocyte-associated protective effects, permitting the reinstatement of CPF-induced reduction in neuritic branch points. This study highlighted the pivotal role of astrocytic P450 in affording protection against CPF-induced neurotoxicity. Likewise, using mixed cell aggregate cultures, the leaving group of CPF, namely trichloropyridinol, was found to exert toxic effects on glia via a reduction in glutamine synthetase activity, a marker of astrocytes (Zurich, Honegger, Schilter, Costa, & Monnet-Tschudi, 2004). Together, these studies suggest that compromised astrocytic functioning might be a critical contributor to OP-induced neurotoxicity.

A main concern regarding OP-induced neurotoxicity is related to neurobehavioral deficits following long-term exposure to low doses of OP. A recent study demonstrated that long-term exposure to the OP pesticide malathion at a dose (30 mg/kg) that does not influence hippocampal AChE activity caused deficits in spatial memory and discrimination and was found to be associated with mitochondrial dysfunction, apoptosis, and astrogliosis (dos Santos et al., 2016). Thus, upon extrapolation to humans, non-cholinergic mechanisms might account for the neurobehavioral and cognitive deficits observed following chronic OP exposure. On the contrary, in another study, a chronic low-level exposure to CPF (6.75 mg/kg-1/20 LD_{50}, oral gavage for two to eight weeks showed that, at the end of two weeks of CPF exposure, GFAP expression was increased compared with controls (Wang et al., 2014). Like this study, GFAP expression was significantly increased at the end of one week following CPF administration (sub toxic doses: 1/10 and 1/5 LD_{50} in mice) (Lim, Tay, Nadarajah, & Mitra, 2011; Mitra, Nadarajah, & Siong, 2009). However, no prominent increase in GFAP expression was found at the end of three weeks (Mitra et al., 2009). CPF-induced astrocyte activation was accompanied by a significant decrease in AChE activity and oxidative stress-induced neuronal injury in the hippocampus (Wang, Zhao, et al., 2014), suggesting that the early activation of astrocytes might elicit a neuroprotective response. To further emphasize the therapeutic significance of targeting neuroinflammatory mechanisms to assuage CPF-induced neurotoxicity, the intake of hydrogen-rich water (HRW) was found to attenuate CPF-induced astroglial activation and neurotoxicity (Wang, Zhao, et al., 2014), further highlighting the involvement of non-cholinergic mechanisms, in particular, oxidative stress-associated mechanisms, in CPF-induced neurotoxicity.

It appears that neuroinflammatory responses might elicit both beneficial and detrimental effects following OP intoxication. Based on the experimental models of OP intoxication, a concept emerges in which inflammation might be beneficial in initial stages; however, at later stages, due to an overwhelming oxidative stress response, the reparative capacity of glial cells might be compromised, leading to neurotoxicity, at least in dopamine-enriched brain regions, including SN and the striatum. Regarding chronic exposure to OPs, mounting evidence suggests that repeated low-level exposure to OP triggers a heightened inflammatory response encompassing the activation of glial cells and the elevation of neurotoxic mediators, including cytokines and chemokines, both in the CNS and the periphery. In addition, emerging evidence suggests that OP exposure can increase the risk of developing PD. However, mechanistic studies are warranted to investigate the association between neuroinflammation and neurodegenerative effects following OP intoxication.

2.4. Manganese (Mn)

Although Mn is considered to be an essential trace element, excessive exposure to Mn originating from welding fumes and mining has been linked to a neurodegenerative disorder referred to as *manganism* that bears a close resemblance to PD (Rodier, 1955; J. D. Wang et al., 1989). Manganism is characterized by neuronal injury to the cortex, globus pallidus, and basal ganglia (Michael Aschner, Guilarte, Schneider, & Zheng, 2007; Perl & Olanow, 2007). The clinical symptoms of manganism include facial masking, postural or kinetic tremor without the presence of dyskinesia, and resting tremor. This may partly explain why Mn-exposed patients are less responsive to levodopa therapy, which is commonly used for PD (Calne, Chu, Huang, Lu, & Olanow, 1994; Cersosimo & Koller, 2006; Pal, Samii, & Calne, 1999). In rodent and non-human primates, exposure to excessive levels of Mn results in the accumulation of Mn in basal ganglia, the dysregulation of GABAergic and dopaminergic neurotransmission, glial activation, and ultimately neuronal loss (Burton & Guilarte, 2009). Studies performed in experimental primate models have confirmed the occurrence of motor disturbance, gliosis in the globus pallidus, neuronal loss, and a lack of sensitivity to levodopa treatment (Olanow et al., 1996). In contrast, the exposure of the general population to low levels of Mn via the consumption of food products tainted with the pesticide MB and well water containing high levels of Mn have been identified as

alternative routes of non-occupational exposure (Tjalkens, Popichak, & Kirkley, 2017).

Despite the identification of Mn neurotoxicity syndrome 200 years ago, only a few postmortem neuropathological studies in humans with documented Mn exposure have been reported in the literature. For example, a histopathological report from a symptomatic Mn ore crushing factory worker revealed gross atrophy and discoloration of the globus pallidus as well as indices of neuronal loss and moderate activation of astrocytes in the corpus striatum and normal SN (Yamada et al., 1986). Several of these reports are qualitative in nature without quantitative data. To address this gap, Gonzalez-Cuyar et al. performed a cross-sectional exploratory neuropathological study of the corpus striatum in Mn and non-Mn mine workers in the Republic of South Africa (Gonzalez-Cuyar et al., 2014). During the preclinical state of chronic low-level Mn exposure, the authors reported a trend toward lower astrocyte and neuronal loss in the striatum and an activated microglial phenotype in the globus pallidus. The number of years worked in Mn mines was found to significantly correlate with increased microglial density in the GPi. The astrocyte microglial density was found to be lower in the caudate, putamen, GPe, and Gpi in Mn mine workers compared to non-Mn mine workers. Owing to the small sample size of Mn mine workers used in that study, more research will be needed to confirm the etiological role of Mn exposure in these pathological findings. Moreover, a detailed clinical examination during the preclinical stages will permit a clinical-pathological correlation. *Ex vivo* MRI imaging techniques that correlate tissue Mn concentrations with pathology will enable the identification of novel biomarkers for manganism. Therefore, unbiased stereological studies are needed to further confirm the trends evidenced in Mn and non-Mn miner reference subjects. Interestingly, epidemiologic studies fail to provide supportive evidence regarding the role of Mn exposure in PD development (Mortimer, Borenstein, & Nelson, 2012).

2.4.1. Role of oxidative stress in manganese-induced microglial activation response

Over the last two decades, a complex cross-talk between glia and neurons has been identified as a pivotal mechanism in Mn-induced neuronal damage. Despite the appearance of activated microglia and astrocytes in the brains of Mn-exposed patients (Perl & Olanow, 2007), only a handful of studies have investigated the mechanistic basis of the elevated glial response following Mn exposure, whereas the majority of the studies performed with neurons have revealed the direct toxic effects of Mn on neurons via the induction of the oxidative stress response in an ETC-dependent manner (Surong Zhang, Zhou, & Fu, 2003).

For example, Zhang et al. demonstrated significant dopaminergic toxicity in primary neuron-glia cultures following treatment with low micromolar concentrations of Mn (Zhang et al., 2009). ROS generation in Mn-stimulated microglia was identified as a major contributor to Mn-induced neurotoxicity. Despite studies using rat brain homogenates, studies performed in neuronal cells have reported that Mn^{2+} interrupts oxidative phosphorylation by inhibiting ETC complex or diminishing ATP production (Galvani, Fumagalli, & Santagostino, 1995; Gavin, Gunter, & Gunter, 1999; Surong Zhang, Fu, & Zhou, 2004; Zwingmann, Leibfritz, & Hazell, 2003); however, the involvement of mitochondrial ETC complex in Mn-induced microglial ROS generation was not determined. Interestingly, Zhang et al. demonstrated that mitochondria are the sites of Mn^{2+}-induced H_2O_2 generation in microglia and that mitochondrial complexes differentially contribute to this process. In this context, the succinate mitochondrial complex II substrate was required for Mn-induced mitochondrial H_2O_2 production. Furthermore, the pharmacological inhibition of complex II but not complexes I and III abolished Mn^{2+}-induced mitochondrial H_2O_2 production, suggesting that mitochondrial complex II is a key mediator of Mn^{2+}-induced H_2O_2 production. Likewise, in a recent study, Liu et al. identified the source of Mn-dependent ROS generation that serves as a major trigger of Mn-induced neurotoxicity (Liu, Barber, Zhang, & Liu, 2013). They demonstrated that Mn^{2+} ions induce the generation of H_2O_2 in isolated microglial mitochondria with succinate, but not with malate/glutamate as substrates, suggesting that mitochondrial complex II is the major source. The IIf-site was proposed to be the generator site, given that malonate (5 mM), an IIf-site inhibitor, suppressed Mn^{2+}-induced H_2O_2 production, while TTFA (10 μM), an IIq-site inhibitor, failed to exhibit any significant effect. Another study demonstrated that Mn promotes ROS generation in the presence of succinate at the IIq site of complex II (succinate-ubiquinone oxidoreductase) (Bonke, Zwicker, & Dröse, 2015). In heart sub-mitochondrial particles (SMP), Mn^{2+}-induced H_2O_2 production was blocked by the specific complex II ubiquinone binding site (IIQ) inhibitor atpenin A5. Mn (2+) ions also increased the rate of superoxide dismutation, which would partially explain the increased generation of H_2O_2 and levels of superoxide (Bonke et al., 2015). These findings advance our understanding of the mechanisms by which microglial ROS contributes to Mn neurotoxicity. The relevance of these *in vitro* studies to Mn^{2+}-induced ROS generation *in vivo* remains speculative. To further validate that mitochondrial dysfunction exacerbates Mn-induced dopaminergic neurotoxicity, we studied the vulnerability of MitoPark mice to Mn-induced dopaminergic neurotoxicity. The MitoPark mouse model is a transgenic model that harbors defects in mitochondrial functioning such as the knockout of TFAM (a gene that plays a key role in mitochondrial DNA maintenance and mitochondrial biogenesis). Our studies revealed that Mn-exposed MitoPark mice exhibited increased microglial activity that coincided with enhanced oxidative damage in the SN and striatum (Langley et al., 2018), suggesting that glia-mediated oxidative stress mechanisms may, at least partially, contribute to Mn-induced dopaminergic neurotoxicity. This model especially demonstrated the utility of using a progressive model of Parkinsonism to study the relationship between mitochondrial dysfunction and Mn-induced dopaminergic neuronal loss.

The ability of Mn to modulate the release of microglial proinflammatory factors in response to diverse inflammagens has been extensively characterized (J. Y. Chang & Liu, 1999; Filipov, Seegal, & Lawrence, 2005; Zhang, Hatter, & Liu, 2007; Zhang, Lokuta, Turner, & Liu, 2010). Mn was found to potentiate LPS-induced microglial iNOS expression and NO release in a dose- and time-dependent manner, whereas only a minimal inflammatory response was evidenced in cells stimulated with Mn alone (Filipov et al., 2005; Zhang et al., 2010). Likewise, Mn was found to potentiate LPS-induced proinflammatory cytokine release, including TNF-α, IL-6, and IL-1β (Filipov et al., 2005; Zhang et al., 2010), while Mn itself induced a minimal increase in the aforementioned cytokines. Additional studies further confirmed the transcriptional upregulation of proinflammatory cytokines (TNF-α and IL-6) that appeared as early as 4h posttreatment and remained elevated for up to 24h (Dodd & Filipov, 2011). In rodent models, intrastriatal Mn infusion caused increased microglial activation characterized by greater TNF-α, IL-1β, and iNOS expression in the SN (F. Zhao et al., 2009). Importantly, the inhibition of microglial activation was found to confer resistance against Mn-induced neurotoxicity, highlighting the central role of microglia in the mechanism of dopaminergic neurodegeneration. Furthermore, the exposure of rats to Mn via the inhalation route was found to increase the striatal expression of chemokines, such as CCL2 and CXCL2, and proinflammatory cytokines, such as TNF-α and IL-1β (Antonini et al., 2009). These studies suggest the heightened microglial activation response is an essential factor in Mn-induced dopaminergic neurotoxicity.

The JAK/STAT signaling mechanism is a critical mediator of adaptive immune mechanisms. STAT proteins, importantly STAT3, are latent cytoplasmic transcription factors that become activated following tyrosine phosphorylation by members of the JAK family, eventually leading to transcriptional activation (Heinrich et al., 2003; Levy & Darnell Jr, 2002). Increased STAT3 activation has been identified in neurodegenerative diseases such as PD (Qin et al., 2016). However, the role of JAK/STAT signaling events following chronic Mn exposure remains poorly

understood. Using an *in vitro* HAPI microglial culture model, Mn exposure was found to induce a dose- and time-dependent increase in the proinflammatory cytokines TNF-α and IL-1β with a concomitant upregulation of JAK2/STAT3 signaling. A JAK2 inhibitor was found to inhibit the generation of the aforementioned proinflammatory cytokines. Most importantly, the incubation of PC12 cells with microglia-conditioned media harvested from Mn-stimulated microglial cells was found to facilitate neuronal apoptosis. Following Mn administration for 30 days, a marked increase in the expression of p-STAT3 in microglial cells was observed. The authors concluded that the Mn-induced induction of the JAK2/STAT3 signaling pathway in microglia may elicit a heightened proinflammatory response, thereby leading to neuronal loss.

2.4.2. Role of autophagy in manganese-induced neurotoxicity

A growing body of evidence has implicated autophagy dysfunction during Mn-induced microglial activation. LRRK2, a key molecule linked to numerous neurodegenerative diseases, including PD, has been shown to be abundantly expressed in the microglia and play a key role in autophagy, a cellular degradative process that participates in the clearance of obsolete organelles and long-lived macromolecules. As Mn has been shown to induce autophagy dysregulation, a recent study examined the relationship between microglial activation, autophagy, and LRRK2 in Mn-stimulated cells (J. Chen, Su, Luo, & Chen, 2018). Using both *in vivo* and *in vitro* Mn models, the authors demonstrated that Mn activated microglia and promoted the release of proinflammatory factors in an LRRK2-dependent manner. The siRNA-mediated downregulation of LRRK2 not only mitigated Mn-induced autophagy dysfunction but also microglial activation, implying that LRRK2 may be involved in Mn-induced neuroinflammation and autophagy impairment. The NLRP3-CASP1 inflammasome pathway in the microglia has been implicated in numerous neurodegenerative diseases, including PD and AD. However, the mechanisms driving this phenomenon in response to Mn-intoxication remains poorly understood. In a recent study, Mn was found to induce the microglial release of proinflammatory cytokines via the NLRP3-CASP1 inflammasome pathway by triggering autophagy-lysosomal dysfunction (D. Wang et al., 2017). In this regard, the excessive accumulation of Mn in the lysosome was found to induce lysosomal damage, which caused the release of cathepsin B and the ensuing activation of NLRP3 inflammasome activation. The accumulation of proinflammatory cytokines, such as IL-1β and IL-18, with a concomitant impairment in autophagy coincided with neuronal dysfunction and impairment in hippocampal-dependent learning and memory, suggesting that the microglial activation of the NLRP3 inflammasome might be a critical determinant of Mn-induced impairment in hippocampal functioning. In contrast, autophagy activation in astrocytes was found to counteract Mn-induced neurotoxicity. In this regard, both *in vitro* and *in vivo* studies demonstrated that Mn induces neuronal injury via the alteration of LC3 expression levels in rat striatal astrocytes, implicating the induction of autophagy as a cytoprotective mechanism to mitigate ROS generation and the resultant cytotoxicity (Gorojod et al., 2015).

2.4.3. Role of astrocytes in manganese-induced neurotoxicity

Astrocytes serve as major storage sites and homeostatic regulators of Mn in the brain (M. Aschner, Vrana, & Zheng, 1999). Mn has been shown to be preferentially taken up by astrocytic cells via high-capacity transporters. The concentration within astrocytes has been shown to be 50 to 60 times greater than in neurons (Chen et al., 2015; Sidoryk-Wegrzynowicz & Aschner, 2013b). Astrocytes maintain brain homeostasis by promoting neurotransmitter recycling functioning (Sidoryk-Wegrzynowicz & Aschner, 2013b). The disruption of the astrocyte-neuron cross-talk via the impairment of the glutamine (Gln)/glutamate (Glu)-γ-aminobutyric acid (GABA) cycle (GGC) has been shown to contribute to alterations in GABAergic and glutamatergic neurotransmission, thereby leading to numerous neurological conditions, including Mn toxicity. At subcellular levels, mitochondria harbor the highest concentration of Mn. The exposure of primary cultures of astrocytes to Mn has been shown to cause mitochondrial dysfunction and ATP depletion via oxidative stress-dependent mechanisms (Aschner, Gannon, & Kimelberg, 1992; Filipov & Dodd, 2012; Gavin, Gunter, & Gunter, 1990). Neurotoxicity associated with Mn exposure may be related to impaired astrocytic glycine uptake and the resultant dysregulation of neurotransmitters (Milatovic et al., 2007; Sidoryk-Węgrzynowicz, Lee, Albrecht, & Aschner, 2009). Recently, we demonstrated that Mn altered astroglial mitochondrial bioenergetics, as evidenced by an impaired basal mitochondrial oxygen consumption rate as well as reduced ATP production (Sarkar et al., 2018). Mn was found to decrease mitochondrial mass, as indicated by a reduction in mRNA levels of mitofusin-2, a protein required for the fusion of the mitochondrial outer membrane, while mitochondrial fission was enhanced. Intriguingly, Mn was found not only to enhance inflammatory mediator generation but also to exacerbate the aggregated α-syn-induced inflammatory response. Conversely, the mitochondrial antioxidant mitoapocynin attenuated Mn-induced inflammatory gene expression, further supporting the role of mitochondria-dependent, oxidative stress-mediated proinflammatory events in Mn-induced astrogliosis. Interestingly, the intranasal delivery of Mn was found to increase GFAP expression and decrease TH expression levels in the olfactory bulb, providing further support to Mn-induced dopaminergic neurotoxicity. Likewise, the disruption of the antioxidant status of astrocytes, such as the deregulation of GSH synthesis, has been implicated in the Mn-induced impairment of astrocytes (Dukhande, Malthankar-Phatak, Hugus, Daniels, & Lai, 2006; K. V. Rama Rao, Reddy, Hazell, & Norenberg, 2007). These studies suggest that exacerbated oxidative stress in astrocytes is a major driver of Mn-induced neurotoxicity.

Glutamate (glu) is a major excitatory neurotransmitter in the mammalian CNS (Mayer & Westbrook, 1987). The regulation of synaptic glutamate levels is essential for the normal functioning of the CNS. The glutamate transporters present on astrocytes play a key role in the reuptake of synaptically released Glu, albeit small amounts taken up by adjacent presynaptic neurons (Filipov & Dodd, 2012; Sidoryk-Wegrzynowicz & Aschner, 2013a). It has been well accepted that Mn reduces the expression and functionality of glutamate transporters, including EAAT1/GLAST and EAAT2/GLT-1, in astrocytes (Mutkus, Aschner, Fitsanakis, & Aschner, 2005; Sidoryk-Węgrzynowicz et al., 2009; Sidoryk-Wegrzynowicz & Aschner, 2013a), thereby leading to glutamate dysregulation (Erikson, Dorman, Lash, & Aschner, 2008). Mn-induced TNF-α has been implicated in the impairment of glutamate transporter expression (GLT-1) and functioning (Sitcheran, Gupta, Fisher, & Baldwin, 2005). Furthermore, PKC-mediated signaling events have been implicated in the Mn-induced dysregulation of astrocytic glutamate transporters (Sidoryk-Wegrzynowicz, Lee, Mingwei, & Aschner, 2011). The activation of PKC was found to rapidly downregulate GLT-1 cell surface expression with an accompanying increased nuclear translocation of PKCδ. Additionally, the inhibition of PKC was found to attenuate the Mn-induced decrease in glutamate uptake, as well as GLT-1 and GLAST protein levels (Lee et al., 2012), further highlighting the ubiquitous role of PKC-signaling mechanisms in the regulation of GGC by Mn. Recently, Karki et al. revealed that the transcription factor Yin Yang 1 (YY1) is a critical repressor of GLT-1 in astrocytes (Karki et al., 2014). In astrocytes, YY1 is involved in the regulation of glutamate homeostasis by modulating the expression of GLAST and GLT-1. Mn was found to enhance YY1 promoter activity and protein levels via an NFκB-dependent mechanism. It was speculated that the Mn-induced release of TNF-α from astrocytes may mediate the repressive effects of Mn on GLT-1 expression via YY1, as TNF-α increased YY1 promoter activity, mRNA, and protein levels, whereas it represses GLT-1 mRNA levels. Furthermore, it was shown that YY1 using HDACs as co-repressors serves as a critical negative transcriptional regulator of GLT-1, thereby resulting in GLT-1 repression upon Mn stimulation. Emerging evidence indicates that the inhibition of histone deacetylases

(HDACs) via valproate (VPA) exerts neuroprotection in numerous brain disorders (Dokmanovic, Clarke, & Marks, 2007; Karki et al., 2014). Accordingly, in a recent study, VPA was found to attenuate an Mn-induced reduction in histone acetylation in astrocytes as well as brain tissues (Johnson Jr. et al., 2018). VPA was found to reverse the Mn-induced repression of astrocytic glutamate transporters concomitant with the attenuation of murine dopaminergic neurotoxicity, further highlighting the therapeutic potential of VPA in ameliorating Mn-induced neurotoxicity. These studies raise the possibility that the impairment of astrocyte glutamate transporters might be a critical determinant of Mn-induced neurotoxicity.

Despite numerous studies underscoring the pivotal role of glia-glia interactions in neurodegenerative diseases such as AD and PD, the importance of this interaction between microglia-astroglia in Mn-induced neurotoxicity remains poorly defined. In support of this interaction, in a recent study, microglia were found to amplify astrocytic inflammatory activation during Mn-induced neurotoxicity (Kirkley, Popichak, Afzali, Legare, & Tjalkens, 2017). The authors studied glial cross-talk in response to Mn treatment using purified cultures of primary microglia and astrocytes. Their studies revealed that microglia-conditioned media had increased levels of proinflammatory mediators, including IL-6, TNF-α, CCL2, and CCL5, and that, upon addition to astrocytes, it was found to enhance the gene expression of TNF-α, IL-1β, IL-6, CCL2, and CCL5 in astrocytes like exposure to Mn in the presence of co-cultured microglia. The inhibition of microglial NFκB was found to completely block microglia-induced astrocyte activation while TNF-α downregulation in primary microglia only partially inhibited the astroglia-associated neuroinflammatory response, suggesting that NFκb in microglia plays a critical role in the Mn-induced inflammatory response by regulating chemokines and cytokines that exacerbate astrocyte activation. These studies highlighted for the first time the importance of astroglia-microglia interactions in the Mn-induced neuroinflammatory response.

2.4.4. Influence of manganese on NOS activity and glutamate transporter functioning

The mechanisms underlying the cognitive and neurobehavioral abnormalities associated with Mn exposure during childhood are not well understood but may be influenced by nitrosative stress originating from glial activation via the expression of inducible nitric oxide synthase (iNOS/NOS2). Streifel et al using juvenile NOS2 KO exposed to 50 mg/kg of $MnCl_2$ by intragastric lavage from days 21 to 34 postnatal demonstrated that NOS2 (-/-) mice exposed to Mn were protected against neurobehavioral alteration, glial activation, and the formation of 3-nitrotyrosine adducts within neurons in the basal ganglia (Streifel, Moreno, Hanneman, Legare, & Tjalkens, 2012). The authors concluded that NO plays a pivotal role in Mn-induced neurological dysfunction in juvenile mice and that NOS2 activation in activated microglia plays a critical role in neuroinflammatory injury after Mn exposure. Mn has been shown to increase the astrocytic expression of iNOS and cyclooxygenase-2 (COX-2), which mediate the production of NO and prostaglandins upon the co-treatment of astrocytes with subtoxic concentrations of Mn and the inflammagen LPS or interferon-γ (Liao, Ou, Chen, Chiang, & Chen, 2007). The increased generation of proinflammatory cytokines, especially TNF-α and IL-1β, was found to directly correlate with impairments in glutamate transporter-1 (GLT-1) functioning (Prow & Irani, 2008; Sitcheran et al., 2005). Moreover, oxidative nitrosative stress has been implicated in Mn-induces astrocyte swelling given that L-NAME, a nitric oxide synthase inhibitor, and cyclosporine A, a mitochondrial permeability transition pore inhibitor, attenuated the swelling of astrocytes (Rama Rao et al., 2007). Additionally, Mn-induced astrocytic cell swelling was found to be secondary to NOS activation and increased expression of the water channel protein aquaporin-4 (AQP4) has been implicated in Mn-induced swelling (Kakulavarapu V. Rama Rao, Jayakumar, Tong, Alvarez, & Norenberg, 2010). Furthermore, genetic deletion of NOS2 was found to attenuate Mn-induced neuronal injury. Importantly, neuronal injury was found to coincide with increased expression of NOS2 in astrocytes in Mn-exposed mice (Abbott, Rönnbäck, & Hansson, 2006; Moreno, Sullivan, Carbone, Hanneman, & Tjalkens, 2008). Therefore, it appears that multiple mechanisms including enhanced inflammatory response, glutamate transporter dysfunction, and oxidative/nitrosative stress in astrocytes may lead to neuronal damage.

3. Exosomes and environmental neurotoxicants

3.1. Exosomes

Exosomes are membrane-bound spherical nanovesicles of 50-200 nm in size with endosomal origin. They are known to be released by almost all cell types and have been identified in all biological fluids, including urine, saliva, blood, amniotic fluid, malignant ascites, bronchoalveolar lavage fluid, synovial fluid, breast milk, and CSF (Simpson, Jensen, & Lim, 2008). The biogenesis of exosomes begins within the endocytic pathway with the formation of multivesicular bodies (MVBs) containing several intraluminal vesicles (ILVs). While most of the MVBs fuse to lysosomes and transport their cargo for degradation, some of them fuse with the plasma membrane and release their vesicular content to the extracellular space as exosomes (Bebelman, Smit, Pegtel, & Baglio, 2018; Isola & Chen, 2017). The mechanisms by which MVBs are directed toward degradation pathway or secretory exosome pathway are not well understood, but several lines of evidence suggest that switching of specific Rab proteins on the membrane of MVBs plays an essential role in determining the fate of ILVs in the MVBs (Ostrowski et al., 2010; Pfeffer, 2010). Initially thought to simply act as cellular waste disposal bags, a growing body of evidence reveals that exosomes play a pivotal role in cell-to-cell communication and influence both physiological and pathological processes. Although exosomal cargo composition varies depending on the cell and tissue of origin, as well as the health status, it was recently discovered that exosomes contain various biomolecules, including proteins, mRNA transcripts, miRNA, noncoding RNA, DNA and lipids (Howitt & Hill, 2016; Rashed M et al., 2017). Once released, exosomes can stimulate recipient cells via receptor-ligand interactions or get endocytosed by phagocytosis, micropinocytosis or endocytosis or even directly taken up by membrane fusion to release their content into the cytoplasm of the recipient cells, thereby modifying the physiological state of the recipient cell (Maia, Caja, Strano Moraes, Couto, & Costa-Silva, 2018). Importantly, recent studies have reported that exosomes carry aggregated or misfolded proteins that could induce deleterious effects on targets cells in neurodegenerative disorders, and this cell-to-cell transfer transmission of pathogenic aggregated proteins is thought to contribute to the progression of various neurodegenerative diseases (Arellano-Anaya et al., 2015; Grey et al., 2015; Kong et al., 2014). For example, oligomeric α-syn has been shown to be released from neurons through an exosome-dependent mechanism (Alvarez-Erviti et al., 2011; C. Chang et al., 2013; Danzer et al., 2012; Emmanouilidou et al., 2010; Grey et al., 2015; Tsunemi, Hamada, & Krainc, 2014), which enables misfolded α-syn to propagate to neighboring cells with detrimental consequences. Recent interest in exosome research also has been sparked by its potential role in disease biomarker discovery (Alderton, 2015; Anastasiadou & Slack, 2014; Couzin, 2005; Minton, 2015; Thery, Zitvogel, & Amigorena, 2002). However, quantifications of PD-related proteins in exosomes isolated from PD patients have led to contradictory results (Soria et al., 2017), which may be linked technical differences as well as distinctions in the patient population. To date, the mechanism of cell-to-cell exosomal transmission of α-syn pathology in PD is poorly understood (Harischandra, Ghaisas, Rokad, & Kanthasamy, 2017). Therefore, it is expected that future studies will characterize this exact mechanism, greatly increasing our understanding of α-syn propagation in PD pathogenesis with the potential for the discovery of reliable biomarkers for the diagnosis and prognosis of the disease.

3.2. Environmental neurotoxicants and exosomes

Given the association between the exposure to environmental factors and PD, one possible mechanism underlying neurotoxicant-induced dopaminergic neurodegeneration is through inducing the exosome formation and upregulating exosome-mediated α-syn propagation among neurons. The toxicological relevance of this hypothesis is supported by a previous study in which treatment with the neurotoxic pesticide ROT promotes the release of α-syn by enteric neurons in mice, and the released enteric α-syn can be taken up by presynaptic sympathetic neurites and retrogradely transported to the soma (Pan-Montojo et al., 2012). This study suggests that exposure to neurotoxicant pesticides may contribute to the progression of PD pathology via an exosome-mediated mechanism. Very recently, we reported that *in vitro* exposure to the environmental neurotoxicant manganese upregulates the exosome-mediated release of extracellular miRNAs (Harischandra et al., 2018). In this work, we used an miRNA profiling study to identify that certain exosomal miRNAs responsible for the regulation of protein aggregation, autophagy, inflammation, and hypoxia can be specifically enhanced to release the following manganese treatment (Fig. 3). Further characterization of the neurotoxicant-induced exosome release of pathogenic proteins will have major impacts on both biomarker discovery and translational strategies for environmentally linked neurodegenerative diseases.

4. Conclusion and future directions

Mounting evidence suggests that both microglial activation and environmental neurotoxicants may be linked to PD etiopathology via elevated mitochondria-driven oxidative stress mechanisms. Numerous pesticides associated with the risk of developing PD have been shown to cause dopaminergic neurotoxicity via the activation of microglial NOX2 at low concentrations (Taetzsch & Block, 2013). ROT and PQ have been shown to converge upon mitochondria, leading to its dysfunction via the induction of oxidative stress markers such as NOX2. Despite a consensus that NOX2 is a critical determinant of diverse pesticide-induced neuronal injury, the mechanisms triggering NOX2 activation appear to be diverse depending on the identity of the pesticide. Additional research focused on delineating the cellular mechanism underlying the mitochondrial dysfunction-mediated neuroinflammatory response will be necessary to develop disease-modifying therapies to halt the progression of environmental neurotoxicant-induced dopaminergic neuronal injuries and the associated neurobehavioral deficits. Given that glial activation can participate in both neuroprotective and neurotoxic effects, studies aimed at investigating the dynamics underlying glia-to-glia communication and, in particular, microglial polarization may lead to an improved understanding of the role of neuroinflammation in CNS neuropathology and the identification of novel therapeutics for neuroinflammation-associated disorders.

Pesticides may cause diverse acute and long-term detrimental effects on human health. Despite a lack of congruency, a large body of epidemiological studies suggests that long-term exposure to low doses of pesticide may increase the risk of developing neurodegenerative diseases such as AD and PD (Baltazar et al., 2014). Several different animal models have demonstrated a positive association between PD and pesticide exposure (Cicchetti et al., 2009; Drechsel & Patel, 2008; Moretto & Colosio, 2011). However, a crucial limitation in linking experimental animal studies to real-world pesticide exposure is attributed to the lack of availability of information on the tissue concentration reached, duration of exposure, route, and dose (Angelo Moretto & Colosio, 2013). Moreover, studies using animal models implement exposure to high doses or a single compound for several days or weeks, while the adverse health effects of pesticides on humans is related to prolonged low-level exposures (months to years) in addition to a combination of diverse environmental neurotoxicants (Baltazar et al., 2014). There is an urgent need for well-designed, standardized epidemiological and experimental studies mimicking real-world exposure to determine the impact of pesticides on the inflammatory response to develop useful biomarkers of exposure.

Studies using *in vitro* and *in vivo* models should explore the effects of exposure to mixtures of pesticides given that occupational exposure to a

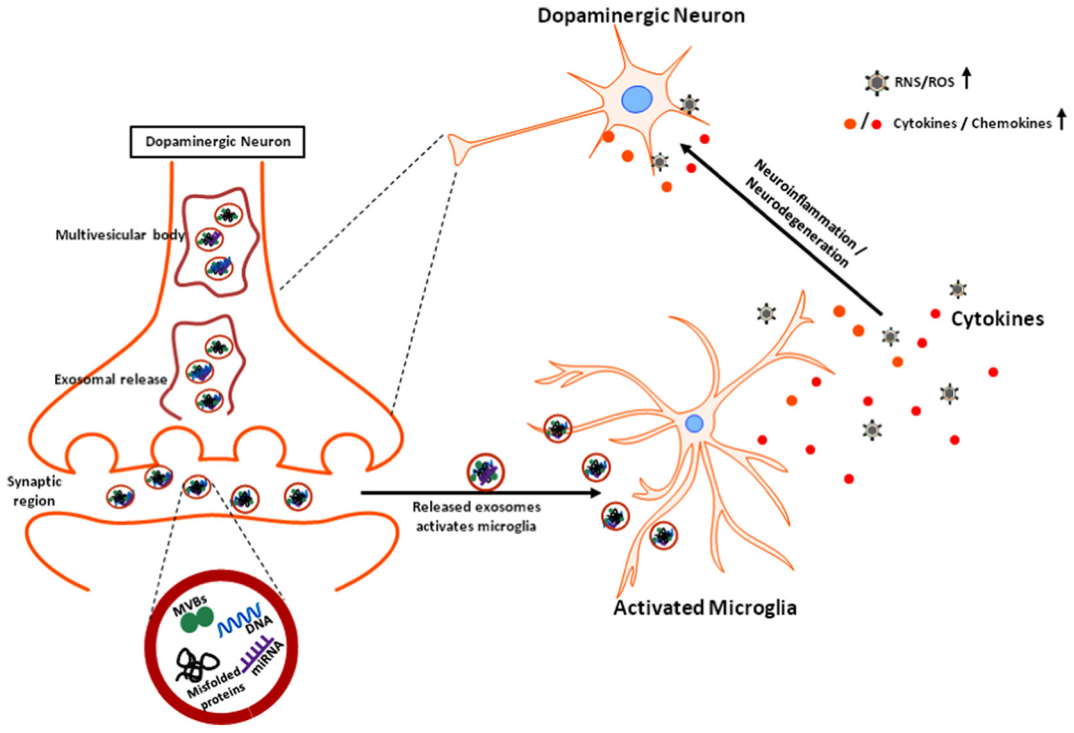

Fig. 3. Exposure of Dopaminergic neurons to neurotoxicants triggers exosome release-mediated activation of microglia, highlighting the interplay between neurodegeneration and neuroinflammation mechanisms involved in PD.

single pesticide is less likely. Exposure to multiple pesticides may lead to additive or synergistic effects. For example, mounting evidence suggests that exposure to a combination of MB and PQ increases the risk of PD (Baltazar et al., 2014; Freire & Koifman, 2012). ROT, PQ, and OP have been shown to recapitulate few cardinal features of PD in animal models; however, until now, no single neurotoxicant has been shown to reproduce all of the clinical manifestations of PD (Baltazar et al., 2014; Blesa, Phani, Jackson-Lewis, & Przedborski, 2012; Cicchetti et al., 2009). Therefore, it is imperative to develop an experimental animal model that bears a close resemblance to human PD, including an elevated inflammatory response, progressive loss of dopaminergic neurons in the SN, extranigral pathology, progressive spread of α-syn pathology leading to the formation of Lewy bodies, and detectable motor and non-motor symptoms (Betarbet et al., 2000; Moretto & Colosio, 2013). Despite numerous studies showing pesticide-induced dopaminergic neurotoxicity, there is a paucity of data on the relationship between pesticide exposure, modulation of peripheral markers of inflammation, expression of non-motor PD symptoms, and associated impairments in neurotransmitters such as serotonin, norepinephrine, and acetylcholine. Furthermore, investigation is warranted into whether the pesticide-induced modulation of proinflammatory markers alters the expression of prodromal PD symptoms.

There is emerging evidence that epigenetic mechanisms, especially DNA methylation, can have a fundamental role in gene-environment interactions implicated in PD (Miranda-Morales et al., 2017). Growing evidence supports a similar methylation pattern between blood and brain tissue DNA. The DNA hypermethylation of PD-related genes, namely PINK1-PARK2, was reported following the exposure of human neuroblastoma SH-SY5Y cells to Mn (Tarale et al., 2017), suggesting that it may influence mitochondrial functioning and promote Parkinsonism. Several studies investigating epigenetic alterations in PD have used experimental cell culture models of PD; however, there have been very few human studies that have investigated this link. Moreover, the impact of altered inflammatory gene promoter DNA methylation on disease development in agricultural workers exposed to high levels of pesticides (Rusiecki et al., 2017) will provide an opportunity to evaluate the influence of pesticide exposure on the alteration of epigenetic patterns associated with PD-related inflammatory genes. In particular, determining the association between high pesticide exposure events and the DNA methylation of inflammatory genes in the blood of male pesticide applicators may provide insights into the relationship between gene inactivation and the development of neurobehavioral deficits. It is also plausible that long-term adverse health outcomes may occur at levels associated with these events. Thus, an experimental animal model that incorporates the determination of serial epigenetic markers over time may improve our understanding of the timing and persistence of epigenetic modifications and how they correlate with the initiation and exacerbation of neurobehavioral deficits upon exposure to pesticides that increase the risk of developing PD. Future avenues that warrant further investigation:

- Is the glial accumulation of α-syn a key determinant in the pesticide-induced formation of α-syn-positive inclusions in neurons?
- Does microglia-mediated astrocyte dysfunction contribute to the progression of pesticide-induced neuronal injury?
- Does concurrent mitochondrial and lysosomal dysfunction in glial cells contribute to the exacerbation of neurotoxicant–induced dopaminergic neuronal injury?

Most importantly, there is an urgent need to explore the impact of gene-environment interactions on the neuroinflammatory response in relation to occupational OP exposure, as they are linked to the increased risk of PD progression (Wang, Cockburn, et al., 2014). Human genetic studies that evaluated gene-environment interactions have provided partial evidence that gene polymorphism in metabolic and oxidative stress genes may increase the risk of developing PD in individuals exposed to OP (Benmoyal-Segal et al., 2005; Dardiotis et al., 2013; Fitzmaurice, Rhodes, Cockburn, Ritz, & Bronstein, 2014). At the present time, this evidence is contradictory and limited; therefore, it is important that these interactions are addressed in future studies to better understand PD pathophysiology. More studies aimed at defining PD causation and pathophysiology should include pesticide exposure in combination with genetic predisposition to better address the role of gene–environment interactions in PD pathogenesis.

RNAseq studies aimed at specifically monitoring global changes in the gene expression of inflammatory markers may facilitate bioinformatics studies to pinpoint how changes in specific inflammatory transcript levels fit the overall scheme of gene networks implicated in pesticide-induced neurodegeneration and associated neurobehavioral deficits. Alternatively, single-cell RNAseq analysis can be implemented to study the expression pattern of inflammatory markers in discrete populations of microglia in a brain region-specific manner. Likewise, gut microbiome studies can be performed to elucidate the relationship between the alteration of inflammatory markers and gut microbiome composition upon exposure to pesticides linked to PD development in experimental animal models. The long-term effects of occupational pesticide exposure on central and peripheral inflammatory responses have not been routinely studied, although persistent neurological decrements have been reported. The potential of impaired innate and adaptive immunity to initiate or exacerbate long-term neurological deficits warrants further exploration.

Creating gene mutants using the CRISPR/Cas9 system may be beneficial in studying how the disruption of mitochondria-related genes in glial cells impacts the pesticide-induced inflammatory response and neurotoxicity, given that mitochondrial dysfunction in glial cells is increasingly linked to dopaminergic neurodegeneration. Moreover, unbiased proteomics technology may provide novel insights into cell signaling events underlying pesticide-associated neuroinflammation and the resultant neurodegeneration.

Conflict of Interest Statement

The authors declare no conflict of interest.

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