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REVIEW

THE COMPLEX RELATIONSHIPS BETWEEN MICROGLIA, ALPHA-SYNUCLEIN, AND LRRK2 IN PARKINSON'S DISEASE

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Abstract—The proteins alpha-synuclein (αSyn) and leucine rich repeat kinase 2 (LRRK2) are both key players in the pathogenesis of the neurodegenerative disorder Parkinson's disease (PD), but establishing a functional link between the two proteins has proven elusive. Research studies for these two proteins have traditionally and justifiably focused in neuronal cells, but recent studies indicate that each protein could play a greater pathological role elsewhere. αSyn is expressed at high levels within neurons, but they also secrete the protein into the extracellular milieu, where it can have broad ranging effects in the nervous system and relevance to disease etiology. Similarly, low neuronal LRRK2 expression and activity suggests that LRRK2-related functions could be more relevant in cells with higher expression, such as brain-resident microglia. Microglia are monocytic immune cells that protect neurons from noxious stimuli, including pathological αSyn species, and microglial activation is believed to contribute to neuroinflammation and neuronal death in PD. Interestingly, both αSyn and LRRK2 can be linked to microglial function. Secreted αSyn can directly activate microglia, and can be taken up by microglia for clearance, while LRRK2 has been implicated in the intrinsic regulation of microglial activation and of lysosomal degradation processes. Based on these observations, the present review will focus on how PD-associated mutations in LRRK2 could potentially alter microglial biology with respect to neuronally secreted αSyn, resulting in cell dysfunction and neurodegeneration.

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Key words: synuclein, LRRK2, neurodegeneration, microglia, neuroinflammation, autophagy.

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Abbreviations: αSyn, alpha-synuclein; ALP, autophagy/lysosomal degradation pathway; CSF, cerebral spinal fluid; ERK5, extracellular-signal-regulated kinase 5; IL, interleukin; KI, knock-in; KO, knockout; LPS, lipopolysaccharide; LRRK2, leucine rich repeat kinase 2; MHC, major histocompatibility complex; PD, Parkinson's disease; Ser935, serine residue 935; TLR4, toll-like receptor 4; TNF-α, tumor necrosis factor alpha; UPS, ubiquitin–proteasome system; WT, wild-type.

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Contents

INTRODUCTION

Leucine rich repeat kinase 2 (LRRK2) is the most commonly mutated gene in both idiopathic and familial Parkinson's disease (PD) (Healy et al., 2008; Paisán-Ruíz et al., 2008; Kumari and Tan, 2009; Lesage et al., 2010). The gene product is a large protein of 260 kDa with multiple enzymatic and protein-binding domains, which may imply a complex role in its cellular function. A consensus for a bona-fide substrate or canonical cellular function of LRRK2 has remained elusive. This is likely due to the complex nature of its architecture or perhaps its weak kinase activity or expression in neurons, which has represented the major areas of investigation in the field (Galter et al., 2006; Mandemakers et al., 2012; Schapansky et al., 2014; West et al., 2014). Recent observations confirm the relative high expression of LRRK2 in certain cells of the immune system, which might indicate that the contribution of LRRK2 signaling to human neurologic disease is more complicated than initially assumed.

Expression of LRRK2 has been detected in most immune cells, including T cells, B cells, and various subtypes of monocytes (Gardet et al., 2010; Hakimi et al., 2011; Thévenet et al., 2011; Schapansky et al., 2014). Consistent with a functional role within these cells, genomic studies have implicated LRRK2 in the development of autoimmune disorders including Crohn's disease, colitis, and leprosy (Cardoso et al., 2011; Umeno et al.,

2011). Among the immune cell types, however, the highest expression is observed in monocytes (Gardet et al., 2010), which implicates LRRK2 as a significant player in the innate immune system. One of many monocytic populations that express LRRK2 is microglia, the resident immune cells of the brain. Microglia are responsible for homeostatic and trophic support of the nervous system, phagocytosis of extracellular debris, and the initiation of inflammation. This review will explore the complex biologically and pathologically relevant interactions between microglia and α-synuclein (αSyn), a protein intimately linked to PD, and the multiple opportunities for disease-linked mutations in LRRK2 to perturb these associations.

THE BIOCHEMICAL PROPERTIES OF LRRK2 IN NON-NEURONAL TISSUES

The observation that LRRK2 is expressed in cells of the immune system was the first evidence that it could play a broad role in human biology with functions outside of the nervous system. Higher expression in both B cells and monocytes as compared to T cells (Gardet et al., 2010; Thévenet et al., 2011) suggests a specific role in the innate immune system, or the first line of defense against infection. Furthermore, monocytes have greater basal LRRK2 expression than neurons (Schapansky et al., 2014), raising the possibility that normal monocytic function may more heavily involve LRRK2-dependent processes and signaling than neurons. Monocyte activation also results in biochemical changes to LRRK2 that have not been formally investigated in neurons, including alterations in expression, phosphorylation state, and subcellular distribution. Treatment of monocytes with interferon gamma, a cytokine responsible for priming monocyte activation, significantly increases LRRK2 expression (Gardet et al., 2010; Kuss et al., 2014). In addition, lipopolysaccharide (LPS)-induced monocyte activation through stimulation of toll-like receptor 4 (TLR4) also elevates its expression (Moehle et al., 2012) and results in rapid and sustained (minutes to hours) LRRK2 phosphorylation at serine residue 935 (Ser935) (Dzamko et al., 2012; Schapansky et al., 2014). This particular biochemical event was once thought to be an early indicator of LRRK2 activation state (Nichols et al., 2010; Deng et al., 2011), but current data now question whether the status of this phosphorylation site is truly related to intrinsic kinase activity. Nonetheless, it is still believed that LRRK2 phosphorylation correlates well with conditions of increased LRRK2 function (Dzamko et al., 2012; Schapansky et al., 2014; Vancraenenbroeck et al., 2014). In our hands, stimulation-induced phosphorylation of LRRK2 at Ser935 preceded the dimerization and translocation of LRRK2 to membranous organelles (Schapansky et al., 2014). These findings may shed light on a previous study which showed that disruption of LRRK2 phosphorylation results in accumulation of the protein within the cytosol (Dzamko et al., 2010). LRRK2 phosphorylation has also been shown to regulate its association with 14-3-3 adapter proteins (Nichols et al., 2010; Li et al., 2011), and a disruption of 14-3-3/LRRK2 interactions can alter LRRK2 localization in the cell (Nichols et al., 2010; Mamais et al., 2014) and potentially impact membrane-related functions, such as exosome release (Fraser et al., 2013). Lastly, multiple PD-associated missense mutations in LRRK2 at position R1441 have been shown to reduce LRRK2 phosphorylation and prevent 14-3-3 binding (Muda et al., 2014), suggesting that mutations in LRRK2 could affect LRRK2 phosphorylation and activation upstream of its cellular function. Thus, the upregulation of LRRK2 expression and phosphorylation observed early in monocyte activation is likely an initial requirement for downstream changes in its kinase activity or interaction with cofactors, important events to its role within the innate immune system.

EMERGING CLUES AND CONSENSUS ON LRRK2 FUNCTION

A number of functions across multiple cellular systems have been implicated for LRRK2. These include rather diverse processes such as cell migration (Caesar et al., 2013), phagocytosis (Marker et al., 2012), and neuronal excitability (Cirnaru et al., 2014). Other proposed functions include an involvement in mitochondrial fission (Wang et al., 2012), synaptic vesicle endocytosis (Matta et al., 2012), and modulation of the expression of cell-surface markers in monocytes (Thévenet et al., 2011). Many of these observations were made by employing compounds that inhibit the kinase activity of LRRK2. Several inhibitors have now been generated for use as both tool compounds and potential therapeutic agents, but the significant off-target effects of earlier compounds likely complicate interpretability. Early research relied on broad range ROCK kinase inhibitors that also affected LRRK2, preventing its phosphorylation of a pseudo-substrate (Nichols et al., 2009). The first inhibitor developed specifically for LRRK2, named LRRK2-IN-1, was a highly potent agent with an IC_{50} in the low nanomolar range (Deng et al., 2011). However, the inhibitor also strongly inhibits the kinase extracellular-signal-regulated kinase 5 (ERK5) (Deng et al., 2011) (http://graylab.dfci.harvard.edu/index.php?id=157). This begs the question as to whether the effects of LRRK2-IN-1 on ERK5 better explain early results originally ascribed to LRRK2. The first off-target effects were suggested in a study that found an increase in neurite outgrowth by LRRK2-IN-1 in LRRK2 knockout (KO) neurons, while a structurally distinct LRRK2 inhibitor had no effect (Luerman et al., 2013). Data from our lab revealed that LRRK2-IN-1 could suppress LPS-induced phagocytosis, but two newer, structurally distinct inhibitors could not (Schapansky et al., 2014). Importantly, in our study the effects of LRRK2-IN-1 persisted in cells where LRRK2 expression had been silenced. We have since extended these findings and now show that LPS-induced reactive oxygen species generation can be prevented by selective ERK5 inhibitors and LRRK2-IN-1, but not other LRRK2 kinase inhibitors (Fig. 1). Further complicating the issue, a direct interaction between ERK5 and LRRK2 has been discovered, with the ERK5 inhibitor XMD-98 preventing LRRK2 phosphorylation at Ser935 (Kuss et al., 2014). Thus, the promiscuity of pharmacological kinase inhibitors requires that care be taken to not rely exclusively on a single compound. Ideally, a panel of reagents in conjunction with

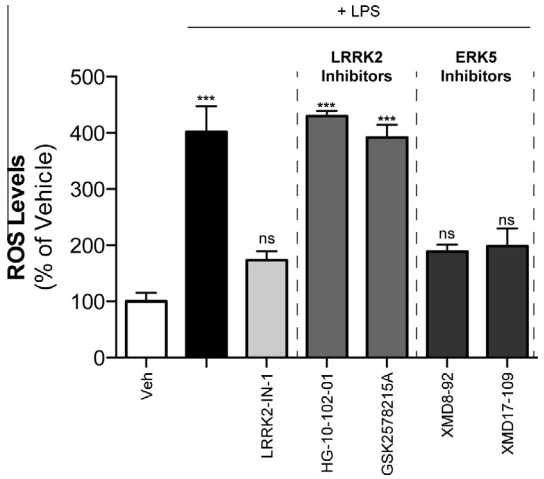

Fig. 1. LRRK2-IN-1 prevents ROS generation in a similar manner to ERK5 inhibitors, but not other LRRK2 inhibitors. RAW264.7 macrophage cells were treated with LPS (100 ng/ml) with and without LRRK2-IN-1 (1 μM), newer LRRK2 inhibitors (1 μM), or ERK5 inhibitors (2.5 μM) for 16 h. Media were removed, and cells were treated with the fluorescent dye DCFDA (10 μM, in PBS) for 30 min. Cells were washed once, lifted in 500 μl PBS containing 10 mM EDTA and transferred to round-bottomed tubes. Cytosolic ROS was measured using FACScalibur flow cytometer under the FL1 channel, counting 30,000 cells/treatment. Results are from 3 independent experiments. $^{***}p < 0.001$, $n = 3$, ANOVA followed by Dunnett's post hoc analysis.

LRRK2-null cells would provide an opportunity for a more precise determination of the contribution of LRRK2 protein and/or kinase activity to a cellular function.

Cytokine production, a key feature of activated monocytes (Shi and Pamer, 2011), was suggested to be regulated by LRRK2. However, a summative analysis from numerous studies on both sides of the debate indicates that a direct link is unlikely. Bone marrow-derived macrophages from LRRK2 KO animals have increased interleukin (IL)-12p40 and IL-6 secretion in one study (Liu et al., 2011), but two other studies found secreted cytokine levels in LRRK2 KO macrophages were the same as those seen from wild-type mice (Hakimi et al., 2011; Dzamko et al., 2012). Similar contradictions have been observed in transgenic mice expressing disease-linked mutations in LRRK2. Microglial cultures from R1441G knock-in (KI) mice demonstrate increased cytokine expression (Gillardon et al., 2012), but macrophages from R1441C KI mice have similar IL-6 secretion compared to wild-type cells (Hakimi et al., 2011). Data obtained from the use of LRRK2-IN-1 also supported a LRRK2 kinase-dependent regulation of cytokines with reduced cytokine production and secretion in primary cultured microglia (Marker et al., 2012; Moehle et al., 2012) that is fully in agreement with some KO data mentioned above. However, this is likely due to aforementioned off-target effects, as LRRK2-IN-1 and the specific ERK5 inhibitor XMD-98 had very similar dose-dependent effects on cytokine production (Luerman et al., 2013). The opposing nature of these results brings into question the true effect of LRRK2 on cytokine secretion, and demonstrates the need for orthogonal methods when addressing LRRK2 function.

Future efforts to address the cellular role of LRRK2 activity will require careful and appropriate use of multiple inhibitors as well as KO animals, resources that the field only now has available. The tools employed may not be the only considerations for future investigation. LRRK2 inhibition, resulting in reduced LRRK2 phosphorylation in an acute manner, occurs within minutes of treatment. Thus, it may seem reasonable to consider short incubation periods of an inhibitor, with the expectation that modulation of a subsequent LRRK2 function will occur within this acute time frame. However, we and others have shown that changes in LRRK2 biochemistry, including homodimerization and membrane translocation, can take several hours following cell activation (Mamais et al., 2014; Schapansky et al., 2014), and may suggest that the consequences of LRRK2 inhibition occur hours after stimulation. This indicates the potential requirement for extended inhibitor treatment to adequately interrogate LRRK2-dependent biology. For example, regulatory changes in the autophagy-lysosome pathway, to which LRRK2 has been functionally linked by many labs (see summary below), can take several hours to evolve. In addition, LRRK2 phosphorylation at key serine residues seems to be less dependent on an autophosphorylation event than originally thought (Doggett et al., 2011; Li et al., 2011; Dzamko et al., 2012), and thus the removal of these phosphate groups by phosphatase enzymes may contribute great complexity to the effects of LRRK2 and LRRK2 inhibitors in intact cells (Lobbestael et al., 2013; Vancraenenbroeck et al., 2014). In these regards, the future identification and validation of bona fide LRRK2 kinase substrates will dramatically clarify the experimental use of LRRK2 kinase inhibitors, and remains one of the greatest challenges in the field.

LRRK2 AND THE AUTOPHAGOSOME/LYSOSOME PATHWAY

The autophagy/lysosomal degradation pathway (ALP) is common to virtually all cells, and involves the cytoplasmic engulfment of cellular organelles, protein aggregates, or foreign bodies by a lipid bilayer encapsulated organelle prior to digestion by proteases provided by lysosomal fusion. In monocytes, autophagy is often relied upon to degrade internalized single-cell organisms or other toxins as part of their immune function. LRRK2 has been shown to be involved at both sides of the pathway, functionally and physically associating with autophagosomes and lysosomes. Autophagic regulation by LRRK2 was originally suggested when the protein was detected on autophagosome vesicles via immuno-electron microscopy (Alegre-Abarrategui et al., 2009; Gómez-Suaga et al., 2011), and later to be shown present on autophagosomal membranes biochemically isolated from activated macrophage cells (Schapansky et al., 2014). The interaction of LRRK2 with numerous organelles suggests that migration of cytoplasmic LRRK2 to membranes is likely part of its normal function across many cell types (Biskup et al., 2006; Berger et al., 2010; Schapansky et al., 2014). Some of these functions could include the formation or functioning of synaptic vesicles (Matta et al., 2012), secretory exosomes (Fraser et al., 2013), and centrosomes, (Mamais et al., 2014). Thus, the redistribution of

LRRK2 to autophagosomes is likely not part of its degradation, but rather a necessary component of their maturation. However, direct association of LRRK2 with LAMP2A during chaperone-mediated autophagy, and with the late-endosomal marker Rab7, a regulator of lysosome biogenesis, are indications that LRRK2 may alter lysosomal biology directly (Higashi et al., 2009; Dodson et al., 2012; MacLeod et al., 2013; Orenstein et al., 2013; Gómez-Suaga et al., 2014). It remains unclear with which organelles LRRK2 can play a definitive role, but its involvement at various points in the autophagy/lysosomal axis indicates a direct function in critical homeostatic degradation pathways.

Phenotypes observed in the kidneys and lungs of LRRK2 KO animals also imply a significant role for LRRK2 in ALP biology. LRRK2 KO mice have deformed kidneys and protein accumulation indicative of defective autophagic clearance, with increased levels of autophagy substrates (Tong et al., 2010; Herzig et al., 2011; Hinkle et al., 2012). In addition, the lungs of LRRK2 KO mice also have increased size and number of lamellar bodies (secretory lysosomes). These animals display the classic foamy cytoplasm and poor surfactant production/secretion in Type II pneumocytes that are typical of lamellar body defects (Herzig et al., 2011; Miklavc et al., 2014). LRRK2 KO rats have a similar phenotype, with age-dependent increases in lysosomal size and lamellar body number in the kidney and lung, respectively (Baptista et al., 2013). These rats also have increased levels of lipofuscin, a lipid-based by-product indicative of poor lysosomal turnover, which further indicts LRRK2's involvement in ALP (Baptista et al., 2013). The kinase activity of LRRK2 has also been directly implicated in autophagosome and lysosome function, as LRRK2-kinase dead KI mice develop abnormal kidneys with elevated lysosomal numbers as well (Herzig et al., 2011). The convergent nature of these *in vivo* data demonstrate that either reduced expression or dysregulation of endogenous LRRK2 activity can result in severe tissue pathology related to the ALP.

DO PD-LINKED MUTATIONS IN LRRK2 AFFECT ALP FUNCTION?

The G2019S mutation within the LRRK2 kinase domain results in a multi-fold increase in LRRK2 kinase activity (West et al., 2005; Greggio et al., 2006; Jaleel et al., 2007), and is the most common causal mutation in LRRK2 PD patients (Healy et al., 2008). The mechanism by which LRRK2-G2019S induces PD pathology remains unclear, but multiple studies have implicated this mutation as a dysregulator of autophagic function. Given that loss of LRRK2 expression results in impaired autophagy, one might expect the gain-of-kinase function G2019S mutation to promote protein degradation. However, the dysregulation of LRRK2 kinase function through an autonomous increase in activity may result in complex and unexpected behaviors. Cells expressing G2019S LRRK2 consistently demonstrate increased autophagic vesicles or markers when compared to wild-type LRRK2 (Plowey et al., 2008; Bravo-San Pedro et al., 2012; Sánchez-Danés et al., 2012), which may be a result of increased autophagic flux (Plowey et al., 2008; Bravo-San Pedro et al., 2012), leading to neuritic loss in neurons (MacLeod et al., 2006; Plowey et al., 2008; Lin et al., 2010; Chan et al., 2011; Winner et al., 2011). Yet these increased vesicle numbers could also be an indication of an arrested autophagy/lysosomal network, following the inability of autophagosomes to fuse with lysosomes (Sánchez-Danés et al., 2012). Increases in lysosomal size and clustering in G2019S expressing cells, which can be rescued by the lysosomal marker Rab7, would further support the interpretation of a defect in the degradation pathway rather than an accelerated autophagic flux (MacLeod et al., 2006, 2013; Dodson et al., 2012). A model of insufficient lysosomal clearance would certainly explain Lewy body pathology in PD, which can be viewed as a selective proteinopathy involving disrupted proteostasis of the αSyn protein (Bosco et al., 2011; Lee et al., 2011). Yet G2019S KI mice do not demonstrate the same pathology in lung or kidney as observed in LRRK2 KO mice (Herzig et al., 2011), and thus the mutant LRRK2-directed defects in protein clearance are likely more subtle than the complete loss-of-function observed in the KO animals.

Arrested fusion at various time points during autophagolysosome formation may underlie the various reported G2019S-induced cellular phenotypes, such as increased autophagosomes versus enlarged lysosomes (MacLeod et al., 2006; Sánchez-Danés et al., 2012). Difficulties in the interpretation of autophagy data could partially explain previous confusing and contradictory results. The fastidious application of appropriate controls could provide additional clarity (for review, see (Mizushima et al., 2010; Klionsky et al., 2012)). Further work will be required to understand the precise mechanism by which PD-linked LRRK2 mutations, G2019S and otherwise, affect ALP biology and direct the development of the disease. We propose that the nature of LRRK2-dependent deficits along the ALP continuum may manifest itself in unique ways that are likely to be highly dependent on cell type. The foamy cytoplasm phenotype in the Type II pneumocytes of LRRK2 KO animals suggests a predominant LRRK2-lysosome dynamic in these cells, for example, while the emphasis in monocytes seems to indicate an important role in the autophagolysosome function. Differential expression of LRRK2 splice variants in cells of the brain would support a diversity or alteration of LRRK2 function (Giesert et al., 2013). The nature of LRRK2's association with the ALP may be questionable, but it is important to note that any disruption to normal LRRK2 signaling could result in profound changes to ALP/lysosomal function that have deleterious effects to the cell.

INFLUENCE OF AUTOPHAGY IN THE IMMUNE SYSTEM

The uptake and digestion of pro-inflammatory molecules is an integral part of immune cell activation. A recent review describes how normal autophagic degradation can limit inflammation, and details the importance of lysosomal function to this process (Deretic et al., 2013). Firstly, cytokine production and secretion appears to be

affected through either activation or inhibition of the autophagolysosomal pathway. Stimulation of autophagy in macrophages with the mammalian target of rapamycin (mTOR) inhibitor rapamycin resulted in a degradation of IL-1β that prevented its release (Harris et al., 2011). Conversely, a blockade of autophagy increased secretion of the cytokine IL-23 from macrophage cells (Peral de Castro et al., 2012). Another group observed a reduction in tumor necrosis factor alpha (TNF-α) production and secretion from peripheral blood mononuclear cells when autophagy is prevented, but an increase in IL-1β secretion (Kleinnijenhuis et al., 2011). These actions are likely related to an inhibitory role on the activation of inflammasomes, autophagy-sensitive cytosolic protein complexes responsible for the proteolytic cleavage of pro-cytokines prior to secretion (Deretic et al., 2013).

Secondly, monocyte phagocytosis can also be driven by autophagy, to promote clearance of apoptotic cells, for example (Zang et al., 2012). However, macrophages deficient in autophagy demonstrate increased phagocytosis of bacteria (Bonilla et al., 2013). In addition, LC3-associated phagocytosis, a hybrid autophagy-phagocytosis process that is LC3-dependent, would also would be affected by the availability of autophagy machinery, including proteins such as beclin-1, ATG5, and ATG7 (Martinez et al., 2011; Vernon and Tang, 2013). Hence, the ALP system can influence the uptake of extracellular material in monocytes in addition to their subsequent degradation.

Finally, and perhaps most importantly, the processing of self-antigens for major histocompatibility complex (MHC) class II presentation is potentially regulated through lysosomal proteolysis (Münz, 2009). This is required to permit immune tolerance, and the inability for adaptive immune cells to recognize these self-antigens can result in an autoimmune response that promotes inflammation and cell death. Therefore LRRK2-dependent ALP deficits could result in autoimmune dysregulation, as suggested for inflammatory disorders such as Crohn's disease, colitis, and leprosy (Wellcome Trust Case Control Consortium, 2007; Henckaerts et al., 2011; Jostins et al., 2012; Yang et al., 2014). Indeed, co-morbidity between PD and Crohn's disease indicates that people diagnosed with PD could suffer from this dysregulation within the periphery, evidence for the potential involvement of the immune system as a player in vulnerability of PD, as well (Nalls et al., 2014). A recent review assesses the role of monocytes of the innate immune system in PD, including the promotion of neuroinflammation and the infiltration of peripheral macrophages, and supports a hypothesis of broad monocyte dysfunction in PD (Kannarkat et al., 2013). Thus, the etiology of PD could be partially rooted in an improper reactivity of monocytes such as microglia in response to native stimuli, including the protein αSyn, a central player in the pathogenesis and neuropathology of PD.

αSYN AND MICROGLIA ACTIVATION

Microglial activation is a normal biological process in which microglia respond to changes in their local environment, dynamically modifying their contributions to central nervous system function accordingly. These functional changes often are accompanied by conspicuous alterations in cellular morphology, where quiescent microglia exist in a ramified state and occupy minimal cytoplasmic volume. Upon activation, there is expansion of the cytoplasmic volume and contraction of processes and further ramification (hyper-ramified), or complete loss of processes and emergence of anameboid state (Perry et al., 1985; Giulian and Baker, 1986; Ransohoff and Cardona, 2010). These adaptations are further accompanied by changes in the expression of various cell-surface markers including but not limited to CD14, MHC molecules, and chemokine and fractalkine receptors (Cho et al., 2006; Sheridan and Murphy, 2013). Regulated glial activation (a.k.a reactive gliosis) can be homeostatic, as both astrocytes and microglia have been implicated in the regulation of blood–brain barrier integrity (Banerjee and Bhat, 2007; Ryu and McLarnon, 2009), synaptic plasticity (Fourgeaud and Boulanger, 2010; Schafer and Stevens, 2013), and the trophic support of neurons through the regulated release of growth factors (Nakajima and Kohsaka, 2004). In contrast, aberrant prolonged gliosis can lead to a harmful neuro-inflammatory state (Smith et al., 2012; Suzumura, 2013) that could contribute to neuropathology.

Lewy bodies are the pathological hallmark of PD (Spillantini et al., 1998) and αSyn is the major protein component of these insoluble inclusions. Aberrant accumulation of αSyn can promote both neuronal dysfunction and neuroinflammation. Missense mutations in αSyn associated with familial PD are argued to make the protein more aggregate-prone than wild-type (WT) αSyn (Conway et al., 1998; El-Agnaf et al., 1998), providing a mechanistic link between protein aggregation and disease. Moreover, duplication or triplication of the WT αSyn gene is sufficient to cause disease(Singleton et al., 2003; Chartier-Harlin et al., 2004), demonstrating that even modest increases in the WT protein are pathogenic. The accumulation of αSyn correlates with a near selective loss of dopaminergic neurons and an increase in activated glial cells in the substantia nigra in the PD brain (Croisier et al., 2005). To model αSyn-dependent processes in PD, a wide array of αSyn overexpression models in rodents have been developed. The sub-chronic adeno-associated virus-mediated overexpression of αSyn in the substantia nigra of mice leads to MHCII-dependent microglial activation, inflammatory cytokine release, lymphocyte infiltration and the subsequent degeneration of dopaminergic neurons (St Martin et al., 2007; Theodore et al., 2008; Cao et al., 2012; Harms et al., 2013; Rockenstein et al., 2014). Importantly, numerous transgenic αSyn animal models have corroborated these more acute results, further linking αSyn exposure to the promotion of neuroinflammation, *in vivo* (reviewed in (Sekiyama et al., 2012). Early studies examined αSyn immunization as a potential therapeutic treatment for PD-like synucleinopathy and found that human αSyn-expressing transgenic mice vaccinated against the human protein displayed reduced αSyn aggregates and decreased neurodegeneration (Masliah et al., 2005). This study was one of the first to suggest that immune cells can play a role in mitigating αSyn-dependent neuropathology.

There is little debate that changes in the metabolism or aggregation properties of aSyn can cause PD, yet the precise species and/or conformation of the offending protein is not clear. Both a soluble-oligomeric form (El-Agnaf et al., 2006; Paleologou et al., 2009; Winner et al., 2011; Rockenstein et al., 2014) and the fibrillar specie (Lashuel et al., 2002; Karpinar et al., 2009; Volpicelli-Daley et al., 2011) have been implicated in pathology. Likewise, the true physiological form of the protein remains unknown (Croke et al., 2008; Bartels et al., 2011; Wang et al., 2011; Fauvet et al., 2012; Lashuel et al., 2013). Regardless, the accumulation of toxic levels of αSyn can lead to the activation of both microglia (Ahn et al., 2012; Béraud et al., 2013; Harms et al., 2013) and astroglia (Fellner and Stefanova, 2013), and foster neuroinflammation that likely contributes to neurodegeneration. Thus, understanding the interplay between the αSyn protein and microglia, as well as the role of microglia in promoting or attenuating the progression of PD pathology, is critical. Such future work will likely identify under-appreciated aspects of disease pathogenesis, and may ultimately afford novel opportunities to design effective therapeutic strategies.

IS THE NEURONAL SECRETION OF αSYN LINKED TO PATHOGENESIS?

The discovery that neurons naturally secrete αSyn, a protein once considered to be strictly intracellular, was the first indication that extracellular αSyn might play a role in disease. αSyn was first identified as a neuronal protein enriched within presynaptic terminals and involved in learning and memory (originally named synelfin) in songbirds (George et al., 1995). Over the past few years, however, several studies have now demonstrated that αSyn is not only secreted from neurons (Luk et al., 2012a; Ejlerskov et al., 2013; Paillusson et al., 2013) but also subsequently taken up by surrounding neurons (Kordower et al., 2008; Desplats et al., 2009; Ahn et al., 2012) and glia (Lee et al., 2008b, 2010b; Su et al., 2009; Fellner et al., 2013; Kim et al., 2013). The observation that cerebral spinal fluid (CSF) (Mollenhauer and Schlossmacher, 2010; Mollenhauer et al., 2012, 2013) and interstitial fluid of PD patients contain detectable levels of αSyn (Emmanouilidou et al., 2011; Shi et al., 2014) provide further support that the neuronal release of αSyn could contribute to its pathogenicity. Braak et al. have proposed a model correlating the progression of PD pathology with the subsequent clinical phenotypes. It postulates that Lewy body pathology originates in the dorsal motor nucleus of the vagus nerve (as well as in olfactory structures) and proceeds in a neuron-to-neuron manner rostrally through the medulla, midbrain, forebrain, before eventually reaching the cerebral cortex (Braak et al., 2004). In this particular model, the later stages of the disease are associated with subsequent (or concomitant) neuronal loss and the resultant clinical symptoms. While the Braak model fits well with more recent demonstrations of the spreading of αSyn pathology in animal models (described below), it is has also been the subject of some controversy (Burke et al., 2008). Similarly, three independent investigations of the postmortem evaluation of patients who had received fetal ventral midbrain cell transplantation revealed mixed results with respect to emergent Lewy body pathology and signs of neurodegeneration in the grafted cells (Kordower et al., 2008; Mendez et al., 2008; Desplats et al., 2009). Nevertheless, their findings were largely consistent with the hypothesis that αSyn spread from the host brain into implanted tissues, and suggested a pathological mechanism for secreted αSyn.

Currently, numerous studies have now described the transfer of neuronally secreted αSyn species to other neurons in culture and *in vivo* (Lee et al., 2005, 2008b, 2013; Hansen et al., 2011; Kordower et al., 2011; Braidy et al., 2014). While the mechanism of αSyn release is still not known, some have proposed that this process involves exosomes (Emmanouilidou et al., 2010; Alvarez-Erviti et al., 2011; Danzer et al., 2012), but this has not been broadly observed (Paillusson et al., 2013; Chutna et al., 2014). Recent *in vivo* studies have shown that that αSyn injected into the rodent olfactory bulb (Rey et al., 2013), vagus nerve (AAV-αSyn) (Ulusoy et al., 2013), or striatum (Luk et al., 2012b) displays a rostral spreading of αSyn into regions of the brain characteristic of the pathology described in the Braak model. The informative study by Rey and colleagues injected different preparations of human αSyn into the olfactory bulb of mice, and detected human αSyn within interconnected neural structures that was consistent with both retrograde and anterograde transport of the injected αSyn (Rey et al., 2013). Independent studies have modeled αSyn-induced disease progression through stereotaxic injection of protofibrillar αSyn material into the brains of mice, showing age-dependent seeding of endogenous αSyn into Lewy Body-like aggregates (Luk et al., 2012a) followed by substantial neuronal loss and glial activation weeks later (Luk et al., 2012b). Recently, one group purified aggregated αSyn from Lewy body deposits of PD patients, injected the material into the brains of mice and non-human primates, and likewise observed αSyn pathology and nigrostriatal neurodegeneration (Recasens et al., 2014). However, one caveat regarding the current generation of studies may be that the concentrations of αSyn used are orders of magnitude higher than the human CSF αSyn levels recently reported: the direct quantification of CSF αSyn has been variable (reviewed in (Mollenhauer, 2014)), but levels have been reported to be as low as 1.31 ng/mL (90.5 pM) (Mondello et al., 2014) and as high as 39.67 ng/mL (2.7 nM) (Parnetti et al., 2014). Nevertheless, these *in vivo* data support the hypothesis that αSyn is secreted from healthy neurons, that this pool of the protein can play a role in PD pathogenesis, and these models provide excellent opportunities to explore the regulatory mechanisms involved in the inter-neuronal transfer of αSyn in PD.

DOES α-SYNUCLEIN REPRESENT A PATHOLOGICALLY RELEVANT STIMULATOR OF MICROGLIAL ACTIVATION?

The literature supports multiple ways in which αSyn can influence microglia function and contribute to the

etiology of PD. One model that has been widely investigated is the ability of pathologic conformations or toxic levels of αSyn to activate microgliosis and stimulate neuroinflammation. The loss of dopaminergic neurons in the substantia nigra and Lewy-body pathology characteristic of PD brains is often accompanied by signs of neuroinflammation, as an accumulation of CD68+ ameboid microglia have been observed at the sites of neuronal loss (McGeer et al., 1988; Imamura et al., 2003; Hirsch and Hunot, 2009; Marinova-Mutafchieva et al., 2009). It is debatable whether microglial recruitment and activation occurs before or after the appearance of cytoplasmic αSyn inclusions and/or the first signs of neuronal dysfunction or loss. One could predict that the aberrant accumulation of αSyn in neurons would result in the increased release of a pathogenic αSyn specie, and the subsequent recruitment of microglia to affected brain regions prior to their activation by extracellular αSyn. Some groups have suggested specific cell-surface receptors such as TLR4 (Stefanova et al., 2011; Fellner et al., 2013), TLR2 (Kim et al., 2013), Fc-gamma receptors (FCγR) (Cao et al., 2012) or proteinase-activated receptor 1 (PAR-1) (Lee et al., 2010a) are required for αSyn-induced microglial activation. However, each of these studies has identified a different αSyn conformation as the primary ligand for microglial activation, as well as a distinct receptor with which it binds. This lack of consensus may indicate a non-specific nature of αSyn-induced microgliosis, or perhaps the capacity for multiple receptors to sense changes in the levels of extracellular αSyn, regardless of conformation. Investigations into the oligomeric secreted forms of αSyn are gaining momentum (Angot et al., 2012; Chai et al., 2013; Prots et al., 2013), but questions remain as to which αSyn specie is the most pathologically relevant. The recent use of neuronally secreted αSyn in this context may be particularly informative (Kim et al., 2013).

While it seems likely that extracellular αSyn can affect microglial cells, the efficacy of different secreted species in activating microgliosis is still undetermined (for an in-depth review see (Sanchez-Guajardo et al., 2013)). The monomeric and fibrillar αSyn species have both been shown to elicit an inflammatory response in microglia, either evidenced through analysis of cytokine secretion (Austin et al., 2006; Klegeris et al., 2008; Su et al., 2008; Lee et al., 2009; Couch et al., 2011; Béraud et al., 2013) or reactive oxygen species (ROS) production (Thomas et al., 2007; Fellner et al., 2013). However, it is difficult to accurately identify the pathogenic αSyn specie when both the function and the physiologically relevant form of αSyn are under debate (Cookson, 2009; Dettmer et al., 2013; Gurry et al., 2013). αSyn aggregates (soluble oligomers and fibrils) have displayed more consistent results with respect to activating microglia than monomeric αSyn (Zhang et al., 2005; Sanchez-Guajardo et al., 2010; Béraud et al., 2013; Kim et al., 2013). A recent study showed that soluble monomeric αSyn did not elicit an immune response in the microglia-like BV2 cell line, but relatively low doses of aggregated material dramatically altered TNF-α secretion from these cells (Béraud et al., 2013). These data suggest that aggregated αSyn is more likely to induce microgliosis than the monomeric conformation. One important point is that αSyn preparations utilized in the aforementioned microglial activation studies have been either recombinant, bacterially expressed unfolded monomer or fibrillar aggregate at concentrations 50–1000 fold higher than the highest recently reported CSF αSyn values (Parnetti et al., 2014) from healthy human control subjects, which must be taken into account. Given the robust literature recently accumulated on neuronal secretion and models for *in vitro* (and *in vivo*) seeding of synucleinopathy, it will be valuable to examine more physiologically relevant preparations and concentrations of neuronally secreted αSyn with respect to microglial activation in the near future.

THE CLEARANCE OF EXTRACELLULAR αSYN BY MICROGLIA

The removal and degradation of neuronally secreted αSyn could be a key measure taken by microglia to prevent the spread of αSyn and its deposition into Lewy body inclusions. αSyn released from neuronal cells is not only taken up by other neurons, but also the non-neuronal cells of the brain. Astrocytes express relatively low levels of αSyn (Solano et al., 2000; Mori et al., 2002; Miller et al., 2005), but display large αSyn deposits in the brains of PD (Braak et al., 2007; Lee et al., 2010b) and Dementia with Lewy-Body (DLB) (Wakabayashi et al., 2000; Terada et al., 2003) patients. Similarly, in Multiple Systems Atrophy, oligodendrocytes primarily accumulate αSyn protein into glial cytoplasmic inclusions despite not expressing aSyn, further implicating the glial accumulation of αSyn in this disease (Spillantini et al., 1998; Tu et al., 1998; Dickson et al., 1999). In contrast, microglia appear to be the most efficient scavengers of extracellular αSyn (Lee et al., 2008a), and it is plausible that they actively buffer neurons from the aberrant accumulation and/or spread of the αSyn protein in PD. In the aforementioned study by Rey et al., injected αSyn was rapidly transferred to interconnected brain regions, but after 72 h the exogenously added αSyn was absent from those neurons, and appeared in microglia of those same nuclei. These data provide experimental evidence that microglia rapidly internalize αSyn and may play a role in clearing secreted αSyn, not just *in vitro* but also *in vivo* (Rey et al., 2013).

The data describing microglial internalization of αSyn varies greatly, and can largely depend on the cellular system employed (immortalized vs. primary cell cultures) and the αSyn species being analyzed. Some *in vitro* studies have suggested that monomeric αSyn enters microglia in a clathrin-, caveolae-, dynamin-independent manner, as chemical inhibitors of each pathway and over-expression of dynamin K44A, a dominant negative form of dynamin, had no effect on the microglial uptake of monomeric αSyn (Park et al., 2009). Some groups have even proposed that αSyn diffuses freely in and out of cells, avoiding lysosomal degradation (Park et al., 2008; Lee et al., 2008a), while the fibrillar form of αSyn has been reported to be readily taken up by microglia and degraded by the lysosome (Liu et al., 2007; Lee et al., 2008a). Most investigations

into the degradation of αSyn have been performed in neurons or neuronal-like cultures, and there is no consensus on the main cellular pathway of αSyn degradation. Both the ubiquitin–proteasome system (UPS) and the ALP have been implicated (Webb et al., 2003; Cuervo et al., 2004), and again outcomes appear to depend largely on the αSyn conformation being analyzed (for an in depth review see Xilouri et al., 2013). Briefly, soluble short-lived proteins are typically ubiquitinated and delivered for processing at the proteasome, while organelles, long-lived proteins, and aggregates of mis-folded proteins are degraded through microautophagy, macroautophagy, or chaperone-mediated autophagy via ALP. Consequently, in neurons, where αSyn is readily produced, an equilibrium likely exists between UPS degradation of soluble αSyn and ALP degradation of insoluble αSyn aggregates. Conversely, microglia do not express αSyn, and the internalization of aggregated αSyn likely results in the lumenal accumulation of the protein and clearance via ALP. As loss-of-function mutations in lysosomal proteins, such as the Gaucher's disease-linked protein glucocerebrosidase, result in synuclein accumulation, deficits in microglial ALP are a probable contributor in the progression of PD (for an in depth review see (Bourdenx et al., 2014)).

DO PATHOGENIC MUTATIONS IN LRRK2 ALTER THE INTERACTIONS BETWEEN MICROGLIA AND αSYN?

The robust responsiveness of microglia to extracellular αSyn and the relatively high LRRK2 expression in these cells compared to neurons suggest that LRRK2 plays a role in αSyn-induced microglial activation. The diversity of the changes in microglia due to αSyn are clear, and largely dependent on the αSyn species being analyzed, but taken together with the proposed role of LRRK2 in regulating microglia responsiveness, there is an enticing opportunity to investigate the relationship between these two PD-linked proteins across neurons and non-neuronal cells. We propose a model that may delineate how mutations in LRRK2 could exacerbate αSyn-induced microglial pathology and neuroinflammation, either by modulating the activation of microglia or regulating the clearance of internalized synuclein (Fig. 2).

LRRK2 could mediate αSyn-induced microglial activation, based on either a commonality of receptor pathways responding to αSyn, or a potential involvement in multiple microglial signaling cascades. Initially, different α-synuclein conformations in the neuron may exist in equilibrium, forming or dissociating as necessary. This synuclein could be degraded by some cellular mechanism, potentially by LRRK2-directed autophagic degradation (Point 1a, Fig. 2), or could exit the cell by an unknown process. One possible method could be through exosome secretion (Emmanouilidou et al., 2010; Alvarez-Erviti et al., 2011; Danzer et al., 2012), a process potentially mediated by LRRK2 (Fraser et al., 2013) (Point 1b, Fig. 2). This extracellular synuclein will also likely exist in some dynamic equilibrium of species and may then either be taken up by surrounding neurons, or interact in some capacity with microglia (Point 2, Fig. 2). Studies in microglia and other monocytes reported that LRRK2 expression and phosphorylation were increased upon TLR2 or TLR4 stimulation (Gillardon et al., 2012; Moehle et al., 2012; Schapansky et al., 2014), and both of these cell surface receptors have been implicated in the αSyn-induced activation of microglia (*in vitro*, *in vivo* or both) (Fellner et al., 2013; Kim et al., 2013) (Points 3–5, Fig. 2). In addition, a recent report demonstrates that a LRRK2 KO rat showed reduced microglial activation in response to both LPS (a TLR4 agonist) and αSyn (Daher et al., 2014), suggesting a requirement for intact LRRK2 signaling to initiate microglial activation. However, this finding awaits confirmation, as deletion of LRRK2 in mice was associated not with reduced monocyte activation but rather exaggerated inflammation in a model of colitis (Liu et al., 2011). However, the Daher study did show an increase in LRRK2 protein levels in the midbrain after treatment with LPS or αSyn that suggests that microglial inflammation is linked either directly or indirectly to LRRK2 function. These data provided early support for a role of LRRK2 in microglial activation, and potentially the response to αSyn. The role of LRRK2 in microglial responses to αSyn may also involve other signaling pathways with which LRRK2 has been associated. For example, more recent studies in microglia have proposed that LRRK2 is downstream of an ERK5-dependent response to interferon gamma (IFNγ) (Kuss et al., 2014). LRRK2 has also been implicated in ERK and c-Jun N-terminal kinase (JNK) pathways (Bravo-San Pedro et al., 2012; Chen et al., 2012; Zhu et al., 2013), Wnt signaling (Sancho et al., 2009; Berwick and Harvey, 2012), nuclear factor of activated T cells (NFAT) regulation (Liu et al., 2011; Greggio et al., 2012) and others (Yuan et al., 2011; Dzamko et al., 2012). Alternatively, LRRK2 may modulate αSyn-induced microgliosis in a yet unidentified pathway (Fig. 2, Point 3, "unidentified receptor"). In addition to the ligand-receptor model for αSyn-induced activation of microglia, one must also consider the possibility that the internalization and glial accumulation of pathologic αSyn, regardless of its conformation or aggregation state, may itself represent a challenge or stress to microglia resulting in their activation in a non-receptor-mediated fashion. This responsiveness, as well, is yet another opportunity for mutations in LRRK2 to dysregulate microglial behavior in PD-relevant ways. While we are at the early stages of dissecting its non-autonomous contribution to the neurodegeneration that occurs in PD, the potential for LRRK2-dependent regulation of microgliosis and neuroinflammation is a critically important area of current investigation.

Independent of the ability of αSyn to simulate microglia, these cells have a well-established potential for the degradation of secreted, aggregation-prone proteins in the brain (reviewed in (Fu et al., 2014). Given the numerous links between LRRK2 and the ALP degradation pathway summarized in the present review, it is entirely possible that pathogenic mutations in LRRK2 impact the ability of microglia to internalize and/or degrade neuronally secreted αSyn (Points 6 and 7, Fig. 2). The links between αSyn and autophagy are in fact many-fold, as conditional KO of the autophagy protein

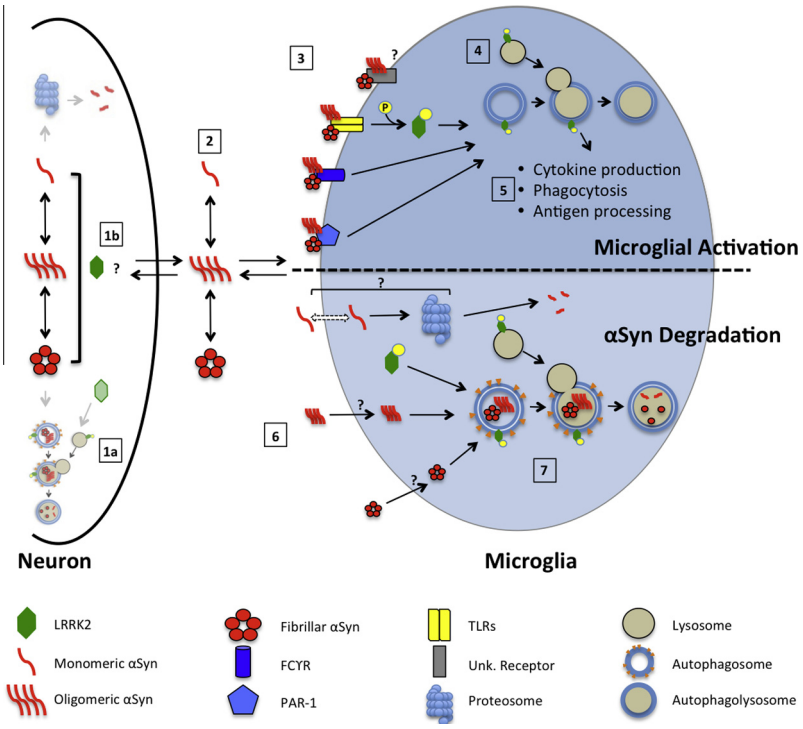

Fig. 2. Opportunities for pathogenic mutations in LRRK2 to dysregulate microglial function. In the neuron, αSyn may exist in an equilibrium between soluble (monomer, oligomer) and insoluble species (fibrillar). It could then be degraded by LRRK2-directed autophagy (1a) or be secreted into the extracellular milieu (scale of neuronal αSyn degradation pathways reduced for clarity). Neuronal αSyn is released via an unknown mechanism, and given the numerous ties between LRRK2 function and membrane dynamics, this process could be affected by LRRK2 (1b). As in the neuron, αSyn in the extracellular milieu may form higher order structures or remain as a monomer (2). Extracellular αSyn could then re-enter neurons, or interact with surrounding microglia. At the cell surface level, αSyn aggregates may activate different microglia receptors to initiate cell activation, where as monomeric αSyn may be inert. An activation of TLRs, or other as yet unidentified cell-surface receptors, would result in phosphorylation and membrane translocation of LRRK2 protein (3). LRRK2 recruited to ALP vesicles could assist in the microglial activation response (4), modulating processes including the production/secretion of cytokines, phagocytosis of αSyn, or antigen processing to promote immune tolerance (5). Independently, it is believed that αSyn species are internalized by microglia (by a yet unknown mechanism) (6), potentially by passive diffusion (monomer), or by more active or receptor-mediated means (oligomer/fibrillar). LRRK2 may then regulate the degradation of these species through a lysosomal pathway (7). Dysregulation of these LRRK2-implicated pathways by pathogenic PD-associated missense mutations could then result in neuroinflammation and accumulation of αSyn, respectively.

ATG7 in the murine substantia nigra resulted in an age-dependent loss of dopaminergic neurons and an accumulation of αSyn comparable to that seen in the later stages of PD (Hara et al., 2006; Komatsu et al., 2006). An independent study showed that inhibition of autophagy leads to the increased exocytosis of αSyn (Lee et al., 2013), which would likely increase the rate of disease progression. Directly related to this theme, our group recently showed that inhibition of endogenous LRRK2 kinase activity impaired the autophagic clearance of a disease-linked aggregate-prone protein in microglial cells (Schapansky et al., 2014). One could imagine that a deficiency in the ALP machinery, as seen in LRRK2 animal models (Herzig et al., 2012; Hinkle et al., 2012; Baptista et al., 2013; Miklavc et al., 2014), could hinder microglial clearance of αSyn. The elevation of αSyn protein and markers of autophagy in the kidneys of LRRK2 KO mice (Tong et al., 2010) certainly suggests that dysregulation of the LRRK2/ALP axis can lead to αSyn accumulation. The alteration of such processes and their role in PD pathogenesis is not limited to inherited mutations in LRRK2, but is likely impacted in normal aging (Terman and Brunk, 2005; Combaret et al., 2009; Barnett and Brewer, 2011; Cuervo and Macian, 2014), which is the single greatest risk factor in idiopathic PD (Hindle, 2010; Collier et al., 2011; Chinta et al., 2013).

OUTLOOK

Here we describe multiple opportunities for the αSyn protein to impinge upon microglial function and how these interactions may contribute to PD pathogenesis and disease progression. From a classic ligand-receptor model, pathologic αSyn species may be produced and secreted by neurons where they stimulate microgliosis, neuroinflammation, and hasten neuronal loss. In a more passive manner, microglia may serve as vigilant protectors, internalizing and degrading pathologic αSyn and attenuating propagation of synucleinopathy, and this homeostatic process may be altered through inherited LRRK2 mutations affecting microglial function, or simply through aging. It is noteworthy that distinct literatures support the potential involvement of LRRK2 in both capacities, and that these two models are not mutually exclusive for their relevance to PD pathogenesis nor the potential dysregulation by disease-linked mutations in LRRK2. It has been suggested that in the rodent brain that the density of microglia is highest in the

substantia nigra (Lawson et al., 1990). Given that this brain region is most vulnerable to αSyn deposition and neuron loss in PD, it is interesting to speculate that perhaps these cells have a greater intrinsic vulnerability that requires an unusually high density of microglial to buffer them from challenges, such as pathogenic αSyn. Clearly, there is much work to do, but present and emerging data continue to make the compelling case that non-cell autonomous pathways likely contribute to the etiology and progression of PD, even in the context of a neuronal protein such as αSyn.

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