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The role of Fyn kinase in mediating microglial pro-inflammatory responses in Parkinson's disease

Nikhil S. Panicker
Iowa State University

The role of Fyn kinase in mediating microglial pro-inflammatory responses in Parkinson's disease

by

Nikhil S. Panicker

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Program of Study Committee:
Anumantha Kanthasamy, Major Professor
Arthi Kanthasamy
Cathy Miller
Guru Rao
Douglas Jones
Amy Andreotti

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GENERAL ABSTRACT

Disparate lines of evidence including *in vitro* cell culture, *ex vivo* brain slice culture, and virtually every *in vivo* model system have clearly implicated microglia activation and neuroinflammation in the pathophysiology of the dopaminergic neuronal cell death in Parkinson's disease (PD). The signaling pathways that lead to this chronic activation are still being elucidated. We show herein, the role of the non-receptor Src family tyrosine kinase Fyn in mediating pro-inflammatory signaling in microglia cells in response to various inflammogens. Our results from cell and animal models as well as postmortem brain tissues conclusively demonstrate that Fyn is preferentially activated in microglia post-stimulation with either Lipopolysaccharide (LPS) or Tumor necrosis factor alpha (TNFα). Activated Fyn then tyrosine-phosphorylates the known pro-inflammatory kinase PKCδ, mediating PKCδ-dependent activation of the NF-κB pathway, leading to pro-inflammatory cytokine and nitrite production. Both $Fyn^{-/-}$ and $PKC\delta^{-/-}$ mice were remarkably resistant to LPS-mediated neuroinflammation, as well as neuroinflammation and dopaminergic neuronal loss induced by the Parkinsonian toxicant 6-hydroxydopamine (6-OHDA).

Activation of the NLRP3 inflammasome, mediated by fibrillar amyloid-β, the major component of Alzheimer's disease-associated senile plaques, has recently been shown to contribute to disease progression. Thus, we sought to validate whether the aggregated form of the PD-associated protein α-synuclein could activate the NLRP3 inflammasome within microglia. Our results conclusively demonstrate that α-synuclein can elicit the NF-κB-dependent induction of the inflammasome components pro-IL-1β and NLRP3, as well as the Caspase-1- and ASC-dependent processing of pro-IL-1β to mature IL-1β. Remarkably, Fyn kinase was shown to contribute to both processes, via PKCδ-dependent NF-κB pathway

activation to prime the NLRP3 inflammasome as well as to the uptake of α-synuclein into the cell, which leads to the assembly and activation of the inflammasome complex. Lastly, we demonstrate activation of the NLRP3 inflammasome in various α-synuclein-overexpressing PD model systems, as well as in *post-mortem* PD patient tissues. Fyn also contributes to microglial ASC speck formation in the adenoviral α-synuclein overexpression system. Overall, we identify Fyn kinase as a key upstream regulator of the microglia-mediated chronic neuroinflammatory cascade that is central to the pathophysiological process of nigral dopaminergic degeneration in PD.

CHAPTER 1. GENERAL INTRODUCTION

Thesis Layout & Organization

The alternative format was chosen for this thesis and consists of manuscripts that have been published, or are being prepared for submission. The dissertation contains a general introduction, three research papers and a conclusions/future directions section that briefly discusses the overall findings from all chapters, and charts out where the research story will be taken next. The references for each manuscript chapter are listed at the end of that specific section. References pertaining to the background and literature review as well as those used in general conclusion section are listed at the end of the dissertation. The introduction section under Chapter 1 provides a background and overview of Parkinson's disease (PD). The Background and Literature Review-I section covers current evidence implicating a pathogenic role for reactive microgliosis in mediating progressive dopaminergic neuron loss in Parkinson's disease. It also introduces the non-receptor tyrosine kinase Fyn and serine threonine kinase protein kinase C delta (PKCδ) and discusses their roles in pro-inflammatory signaling in immune cells, thus providing an overview of the research objectives pertaining to Chapter 2. The Background and Literature Review-II pertains to Chapters 3 and 4 and provides an introduction to inflammasomes and their roles in neurodegenerative and neuroimmune diseases. The manuscript from Chapter 2 was recently published in the *Journal of Neuroscience*. It studies the roles of the Fyn-PKCδ axis in mediating pro-inflammatory responses in microglia in *in-vitro* and *in-vivo* models of PD. Chapter 3 explores how aggregated α-synuclein, the major component of PD-associated Lewy bodies and a gene linked to the development of familial PD, can prime and activate the NLRP3 inflammasome in a Fyn dependent manner. Chapter 4 studies how Fyn contributes to

microgliosis and inflammasome activation in α-synuclein mediated *in-vivo* PD models as well as in *post-mortem* PD brain tissues. Chapters 3 and 4 are in the process of being submitted for publication.

All of the research described by the author in this thesis was performed during the course of his doctoral studies at Iowa State University under the guidance of Dr. Anumantha G. Kanthasamy.

Introduction

First described as the shaking palsy by James Parkinson in 1817, Parkinson's Disease is a progressive neurodegenerative disorder characterized by the onset of motor deficits- resting tremors, bradykinesia (or slowness of movement), akinesia (or absence of movement), rigidity and postural instability. Recently, various non-motor symptoms have also been linked to PD development. These include olfactory deficits, constipation, and sleep disorders. In many cases, these symptoms can precede motor deficits by several years. PD is the second most-prevalent neurodegener-ative disorder in the world, with over a million currently affected in the United States alone (von Bohlen und Halbach et al., 2004).

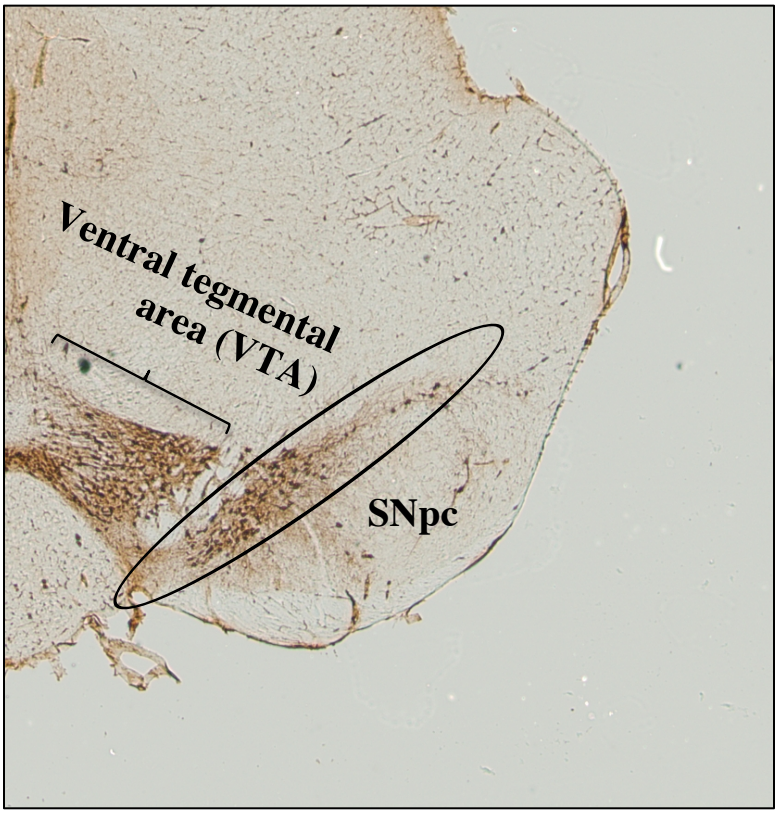

Figure 1. Murine coronal ventral-midbrain section stained for the dopaminergic neuronal marker tyrosine hydroxylase (TH) via 3,3'-diaminobenzidine (DAB) immunohistochemistry

Idiopathic PD has a median onset of 60 years, and the occurrence frequency increases with age. The best-characterized feature of PD is the selective death of melanized dopaminergic neurons in the substantia nigra (SN) pars compacta (pc) of the brain, which project to the caudate putamen. This results in the depletion of dopamine in this region in PD patients. By the time the symptoms are manifested, more than 60% of the dopaminergic neurons have

already been lost, along with 80% of the striatal dopamine content (Dauer and Przedborski, 2003). The death of neurons in other regions, such as the ventral tegmental area (VTA), has also been documented. The mouse SNpc and VTA are demonstrated in Figure 1.

In PD genetics nomenclature, 18 chromosomal regions, which have been assigned a 'PARK' status, have been found to be associated with PD. However, only 6 of these regions code for genes that are conclusively responsible for monogenic PD (whereby a mutation in the gene is sufficient to cause PD). Among these 6 genes, mutations in SNCA, the gene that codes for the α-synuclein protein (PARK1 and 4), and LRRK2 (PARK8) are responsible for autosomal-dominant PD (AD–PD) forms, whereas mutations in Parkin (PARK2), PINK1 (PARK6), DJ-1 (PARK7), and ATP13A2 (PARK9) result in a form of autosomal recessive PD (AR-PD) (Klein and Westenberger, 2012).

The major neuropathological hallmark of sporadic PD is the presence of aggregated α-synuclein containing intracytoplasmic Lewy Bodies. The factors that are associated with PD associated neurodegeneration include mitochondrial dysfunction, proteasomal impairment and excessive reactive oxidative species (ROS) production(Jenner and Olanow, 2006; Levy et al., 2009; Olanow, 2007; Przedborski, 2005). The emerging consensus suggests that PD is a complex, heterogeneous disease with multiple genetic as well as environmental causative factors. The fact that multiple interacting factors appear to contribute to PD progression makes therapeutic intervention an immense challenge. Most of the drugs and therapies that are prescribed for PD patients today, including L-DOPA administration and deep brain stimulation, only alleviate PD symptoms and have no effect in slowing the progression of the disease.

Reactive microgliosis is another prominent feature of PD; loss of dopaminergic neurons within the SN is accompanied by a concomitant increase in the number of microglial cells found in this region, as well as by an increase in the activation of these cells (Bartels and Leenders, 2007; Mosley et al., 2006; Whitton, 2007). Chronic activation of microglia has been shown to contribute to dopaminergic neuronal death. Activated microglia produce ROS and nitrite, as well as pro-inflammatory mediators that can directly effect neuron death, and/or bring about further glial activation, perpetuating the inflammatory response. Figure 2 shows Iba-1 positive microglial cell within the SNpc, apposed against a TH positive dopaminergic neuron.

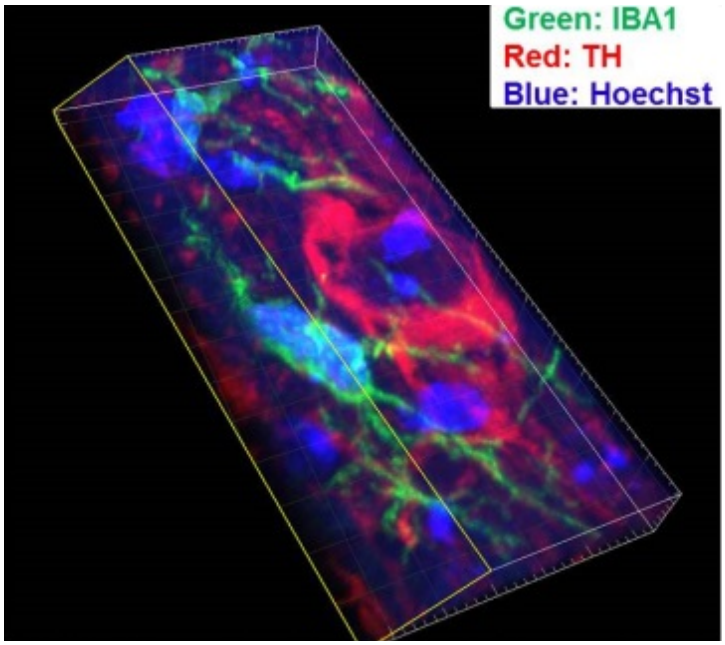

Figure 2. Confocal Z-stack maximal projection image of dopaminergic neuron and microglial cell in the SNpc of a 6-OHDA injected mouse.

The current consensus on the progression of PD is that is arises from the complex interactions between the environmental and genetic processes, which in tandem cause the progressive loss of dopaminergic neurons over time. A 'multiple hit' hypothesis for disease onset has been postulated, wherein multiple causative factors can accumulate over decades, resulting in what is described as idiopathic PD (Carvey et al., 2006).

Several genes associated with Parkinson's Disease have multifactorial roles, specifically with regards to neuroinflammation, that may contribute to PD pathology; fibrilar α-synuclein can cause apoptosis (Cookson, 2009), and in its aggregated form, can also

potently activate microglial cells, leading to the production of proinflammatory cytokines and chemokines (Kim et al., 2013a; Lee et al., 2010; Su et al., 2008). LRRK2, PINK1 and DJ-1 have also been implicated to play a role in the process of autophagy, required for proper degradation and turnover of various proteins and/or organelles within the cell. LRRK2 has been shown to promote microglial pro-inflammatory responses (Moehle et al., 2012). PINK1 deficiency enhances microglial pro-inflammatory signaling in brain slices, including the activation of the NF-κB pathway (Kim et al., 2013b), and the loss of DJ-1 promotes pro-inflammatory signaling in microglia and astrocytes due to hyperactivation of STAT1 (Kim et al., 2013c).

Hence, neuroinflammation may be a promising target to control the progression of PD, as well as other neurodegenerative disorders; exacerbated neuroinflammation has been demonstrated in every model of PD, and non-steroidal anti-inflammatory drug intake has been linked to a lower risk of acquiring PD (Bower et al., 2006; Chen et al., 2003). Therefore, identification of novel signaling pathways that play a role in the hyperactivation of microglia may serve as an attractive drug target to prevent the progression of PD.

Background and Literature Review – I

Neuroinflammation and an introduction to Fyn and PKCδ signaling

Inflammatory responses need to be tightly regulated; on the one hand, they must effectively deal with invading pathogens, killing and clearing them as well as dead or dying cell debris, which if left uncleared, would prevent healing and serve as sites for infection. At the same time, the responses themselves produce potentially toxic factors that can elicit significant pathology (Glass et al., 2010). Neuroinflammation is an example of the latter process gone awry within the central nervous system (CNS). An exaggerated neuroinflammatory component has been consistently associated with most neurodegenerative disorders, such as Alzheimer's disease, Amyotrophic lateral sclerosis, Huntington's disease and PD (Block et al., 2007; Minghetti, 2005). The hallmark of neuroinflammation is the persistent hyperactivation of the microglia and astrocytes at the site of neurodegeneration. In many of these diseases, this hyperactivation is mediated by sterile inflammogens in the form of aggregated or misfolded proteins or peptides, as indicated in Table 1. Previously, the major role of reactive gliosis accompanying neurodegeneration was believed to be phagocytic clearance of dead and dying neurons, and thought to be a generally supportive process to maintain homeostasis within the central nervous system (CNS). One major reason for this line of thought is because microglia have been demonstrated to play a role in establishment of synapses within the developing brain, and contribute to neuronal health by the production of various trophic factors (Aarum et al., 2003; Walton et al., 2006). Microglia also serve as the major neuroimmune response to infection within the central nervous system. In many cases post acute brain injury or infection, neuroinflammatory responses are transient, and subside upon clearance of the offending substance. However, in neurodegenerative disorders

such as PD, the neuroinflammatory responses are chronic and progressive, and are demonstrated in most animal models of the disease, such as the MPTP, 6 –OHDA and LPS models (Panicker et al., 2015) as well as in post mortem PD patient tissues (Imamura et al., 2003).

TABLE 1: Proteins that are known sterile inflammogens in major neurodegenerative diseases

NAME	DESCRIPTION/ FUNCTION	DISEASE
Amyloid-β	36-43 amino acid peptides yielded upon amyloid precursor protein cleavage, are a major component of amyloid plaques. Function unknown.	Alzheimer's Disease
Tau	Microtubule-associated protein. Mediates axonal microtubule stabilization. Hyperphosphorylation of Tau can result in the formation of pathological Tau aggregates.	Alzheimer's Disease
α-synuclein	Presynaptic protein. Wild-type α-synuclein is neuroprotective, mediates PKCδ downregulation in neurons. Aggregated α-synuclein is the major component of Lewy bodies, Mutations in the SNCA gene can cause Autosomal dominant PD.	Parkinson's Disease
Matrix metalloproteinase-3 (MMP-3)	Degradation of extracellular matrix components. Released by stressed dopaminergic neurons in PD models.	Parkinson's Disease
Prion neurotoxic peptide ($PrP_{106-126}$)	Non-infectious PrP^{c} assists in synaptic long term potentiation. Infectious form PrP^{sc} seeds aggregation of non-infectious PrP^{c} to PrP^{sc}.	Prion disease
Huntingtin	Its function is unclear. May play a role in vesicle and organelle trafficking as well as endocytosis. Mutant huntingtin has abnormally long polyglutamine tracts at its N terminal, promoting the formation of inclusions within neurons.	Huntington's disease
Superoxide dismutase 1 (SOD1)	Catalyzes conversion of superoxide radicals to H_2O_2 and molecular oxygen. Mutant forms amplify ROS production from neurons and glia.	Amyotrophic lateral sclerosis

Microglial cells and their role in the CNS

Microglia are the resident macrophagic cells of the CNS. Rio Hortega was the first to observe their phagocytic properties and suggest that they could function similarly to macrophages. This was later corroborated by Hickey and Kimura (Hickey and Kimura, 1988), when they demonstrated that perivascular microglia are antigen presenting cells that express high levels of MHC class II molecules. Contrary to popular opinion, microglial cells do not arise from the bone-marrow hematopoietic stem cells. Utilizing studies carried out with parabiotic mice (which are surgically connected and have a common blood circulatory system) suggested that postnatal microglial cells are maintained in the CNS independently of circulating monocytes. They were found to arise from primitive myeloid progenitors, which arise from the yolk sac (Ginhoux et al., 2013). Microglia comprise 10-15% of the total cells in the brain, and their density varies with different areas of the brain – the hippocampus and ventral midbrain, including the SN, show highest concentrations of microglia. Microglial activation is our body's first response to neuronal injury in both acute and chronic neurodegenerative states. In a normal adult brain, microglia typically exist in their normal or 'resting' state, which is consistently identifiable by a ramified morphology. They secrete anti-inflammatory and neurotrophic factors which promote neuronal survival and plasticity (Carson, 2002). Recent studies have demonstrated that microglia are highly dynamic and mobile; constantly scanning and surveying their external microenvironment for signs of any kind of neuronal injury (Nimmerjahn et al., 2005). Upon encountering a potential pathogen or neuroinflammatory agent, these cells undergo a rapid transformation to an amoeboid form, or 'activated' state, with concurrent upregulation of various cell surface proteins as well as cytokine and chemokine receptors. Transcriptional activation of response genes typically

occurs rapidly in microglia. Activated microglia can produce a plethora of secreted factors that can alter the environment at the site of neuronal injury or cell death. 'Natural' activation of microglia, which is transient and highly regulated, typically facilitates a microenvironment that is conducive to neuron survival and restoration of homeostasis (Glezer et al., 2007; Napoli and Neumann, 2010; Simard and Rivest, 2007). To achieve that goal, microglia produce anti-inflammatory factors such as BDNF, GDNF, IL-10 and TGFβ1, which can actually prevent neuronal apoptosis and effect neuronal differentiation of existing pools of neuronal progenitor cells. Microglia have also been shown to protect neurons from excitotoxicity by upregulating GLT-1, a glutamate uptake protein under conditions where astrocytic glutamate uptake is impaired (Persson et al., 2005; Shaked et al., 2005). Additionally, microglia are solely responsible for the phagocytic clearance of dead cells from the site of injury. They can also participate in the regeneration of synapses by facilitating synapse removal at the site of injury. The stripping of synapses and clearance of local cell debris provides an environment that engenders neurogenesis and the migration of neuroprogenitor cells in the brain. Microglia can also inhibit local neurogenesis by providing a local pro-inflammatory environment (Butovsky et al., 2006). Their activation and secretion of trophic factors at the site of injury for infection was thought to facilitate a return to homeostasis (Kreutzberg, 1996).

Microglial activation in neurodegenerative disorders

Unregulated, elevated and sustained levels of microglial activation leads to microglial hyperactivation, which is the predominant state of microglia at the site of neuron loss in case of neurodegenerative disorders. Hyperactivation of microglia is distinct from the transient

microglial activation, both morphologically as well as in regards to the secretory factors produced. Several lines of evidence, from cell culture, co-culture, animal models, post mortem tissue analyses and genetic linkage analysis conclusively demonstrates that sustained neuroinflammation negatively affects neuron health, and in fact contributes to neuron death. Activated microglia change their morphology from ramified or branched to amoeboid. Activated microglia release ROS, nitrite and various other pro-inflammatory mediators and cytokines that can be toxic over time (Aschner et al., 1999; Glass et al., 2010).

Clinical evidence of neuroinflammation playing a critical role in the progression of PD

Substantiation for the critical role of neuroinflammation and microglial hyperactivation in PD comes from several sources; Post mortem analysis of PD brains clearly indicates amoeboid microglia around the regions of melanized dopaminergic neuron loss. PD Patients also display upregulated levels of pro-inflammatory cytokines in their cerebrospinal fluid (Banati et al., 1998; McGeer et al., 1988; Vawter et al., 1996; Whitton, 2007). Increased levels of many of these cytokines, such as TNFα, IL-1β and IFNγ has also directly demonstrated within the PD brain itself (Hirsch et al., 2005; Hunot et al., 1997; McGeer et al., 1988; Mogi et al., 2000). Apart from nigral inflammation, excessive inflammation in the striatum and basal ganglia has also been demonstrated via PET imaging. New evidence also suggests that innate immune responses may not be the sole signaling mechanisms that go awry in PD; Animal PD models, and more importantly, post-mortem PD tissue analysis also revealed the entry of CD8+ and CD4 + cells into the SN. Infiltrating T cells can serve as neuroprotective as well as neurotoxic agents, depending on the pre-existing stimuli within the brain. Post mortem analysis of the heroin addicts who accidentally injected MPTP and

developed rapid onset Parkinsonism also demonstrated massive microgliosis (Langston et al., 1999). Adenoviral overexpression of IL-1β in the SN of mice was discovered to cause progressive dopaminergic neurodegeneration and a concurrent loss of motor function (Ferrari et al., 2006). Several genetic linkage studies in PD patient and age-matched control groups have identified single nucleotide polymorphisms (SNPs) that are linked to PD development. Some of the SNPs are found within the promoter regions of pro-inflammatory genes (Hakansson et al., 2005; Kruger et al., 2000; Nishimura et al., 2001; Nishimura et al., 2000). Lastly, as mentioned in the introduction section, regular NSAID use is associated with a reduced risk for developing PD.

Evidence for neuroinflammation in animal models of PD

Neuroinflammation has consistently been demonstrated as being a fundamental hallmark of every PD mouse model:

1. The MPTP model

An acute regimen of the Parkinsonian toxicant MPTP (4 injections at 18 mg/kg, spaced out every 2 hours) has widely been used to evoke a neuroinflammatory response, evidenced by an induction of pro-inflammatory proteins with the SN and the Striatum, and concurrent change in microglial morphology from ramified to amoeboid. (Hirsch and Hunot, 2009; Sriram et al., 2006; Wu et al., 2002; Wu et al., 2003). Preventing microglial hyperactivation by preventing microglial ROS generation via NAPDH complex inhibition, as well as inhibiting microglial NOS2 and NF-κB function can be greatly neuroprotective in this model (Du et al., 2001; Ghosh et al., 2002; Ghosh et al., 2007).

2. The 6-OHDA model

Before the development of the MPTP model, the 6-OHDA model was widely utilized to elicit neurodegeneration within the SN. Despite its usage, which began in the 1960s, evidence of neuroinflammation in this model was only recently demonstrated with unequivocal clarity (Stott and Barker, 2014; Virgone-Carlotta et al., 2013).

3. AAV-Synuclein overexpression model

Recently developed models of PD include the AAV-SYN model, which entails delivery of an adeno-associated virus overexpressing human α-synuclein into the SN or the striatum. Green fluorescent protein overexpressing adeno-associated virus(AAV-GFP) is used as the control for these experiments. Several studies have demonstrated a clear role of microgliosis, including increased pro-inflammatory cytokine production, and IgG deposition within the SN (Chung et al., 2009; Theodore et al., 2008). AAV mediated GFP overexpression in the SNpc dopaminergic neurons is demonstrated in Figure 3.

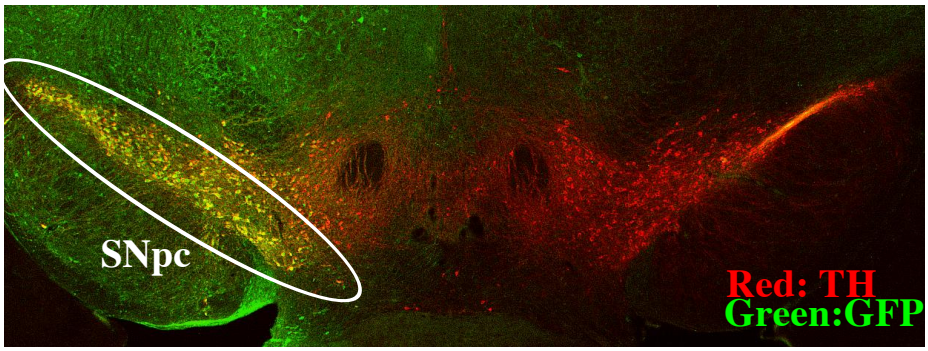

Figure 3. AAV-mediated GFP overexpression in the SNpc dopaminergic neurons. The AAV-GFP construct was stereotactically injected into the mouse SNpc and targeted overexpression validated by double IHC for GFP and TH.

4. The Mitopark model

The recently developed Mitopark model entails the selective knockdown of the mitochondrial transcription factor TFAM in dopaminergic neurons. This results in a

progressive, age dependent loss of dopaminergic neurons that begins from 12 weeks onwards. Recent evidence of neuroinflammation in the MitoPark mice were recently demonstrated by our group (Langley et al., unpublished).

5. Lipopolysaccharide (LPS) based models of PD

Possibly the strongest evidence for the role of inflammation in PD, comes from various animal models that utilize LPS as a Parkinsonian neurotoxin. LPS is a component of the cell wall of gram negative bacteria. It is a potent inflammogen, acting as a pathogen associated molecular pattern (PAMP) that evokes an innate inflammatory response from immune cells. Intranigral injection of LPS is sufficient to elicit both a neuroinflammatory response, as well as dopaminergic neuron death in the absence of any other neurotoxic agent. Intraperitoneal administration of LPS is also able to elicit a rapid, yet sustained neuroinflammatory response (Castano et al., 1998, 2002; Herrera et al., 2000; Hsieh et al., 2002; Qin et al., 2007). The LPS model is distinct from all the aforementioned models, since the other models elicit a microglial response by first bringing about dopaminergic neuron loss or stress, whereas LPS selectively brings about microglial activation and subsequent neuron death. LPS can also act in concert with MPTP and other mitochondrial toxicants including rotenone, paraquat and manganese to amplify dopaminergic cell death (Gao et al., 2003a, b; Zhang et al., 2010), indicating that PD associated environmental hazards can contribute to PD associated neuroinflammation and neuron loss.

Signaling mechanisms that regulate microglial hyperactivation in Parkinson's disease

Although the LPS model clearly demonstrates that microgliosis can initiate and contribute to dopaminergic neuronal loss, the clinical significance of using LPS is limited, since PD does

not involve CNS bacterial infection. However, in the past few years, it has become evident that misfolded proteins within the CNS can elicit a sterile inflammatory response in microglial cells. Exploring the cell signaling mechanisms that regulate microgliosis is an active area of investigation. α-synuclein, the 14 kDa protein which was found to be associated with AD PD as well as a major component of PD associated Lewy bodies was found to activate microglia in its aggregated, nitrated and oxidized form (Reynolds et al., 2008; Zhang et al., 2005), α-synuclein is taken up by microglia, which is followed by the production of ROS and various pro-inflammatory cytokines, and this neuroinflammatory response has been shown to be complicit in the cell death that occurs in these models. Even though it has been conclusively demonstrated that pathogenic forms of α-synuclein can lead to MAP kinase activation and nuclear p65-NF-κB translocation, the signaling pathway through which these classical inflammatory signaling pathways were activated in microglia post synuclein stimulation is poorly characterized. The aggregated α-synuclein mediated pro-inflammatory cytokine induction was only recently shown to be Myd-88 dependent (Daniele et al., 2015). The receptor that mediates α-synuclein uptake and is responsible pro-inflammatory signaling has not been conclusively identified, although circumstantial evidence in various studies has identified TLR2 (Kim et al., 2013a), TLR4 (Fellner et al.,

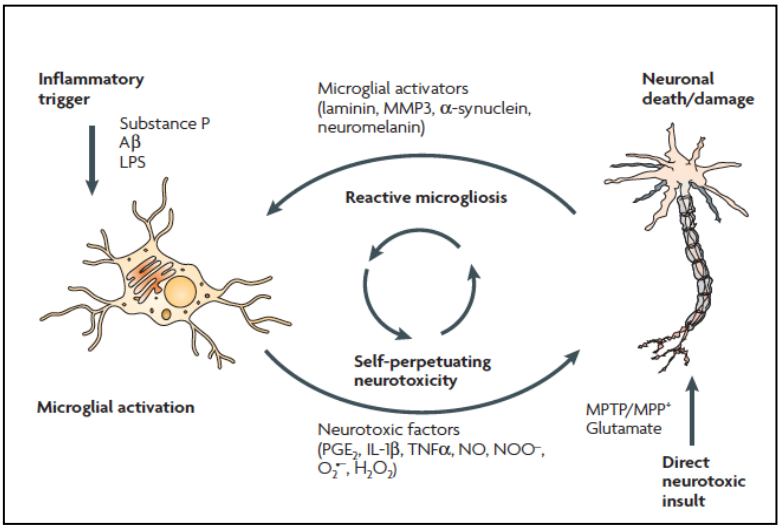

Figure 4. Sterile inflammation and neurodegeneration: A mutually amplifying, self-sustaining process.

2013) CD36 (Su et al., 2008), And the gamma chain subunit of Fc receptors (Cao et al., 2012).

Apart from modified forms of α-synuclein, the neuromelanin found in melanized dopaminergic neuronal cells can be taken up my microglia and activate them (Wilms et al., 2003; Zecca et al., 2008). Neuron secreted Matrix Metalloprotease 3 (MMP-3) MMP-3, which activates microglia via protease-activated receptors (Kim et al., 2007). Lastly, PD associated environmental toxicants such as the pesticides rotenone and paraquat (Bonneh-Barkay et al., 2005; Gao et al., 2002) were shown to elicit neuronal death through microglial ROS generation.

The current consensus of the role that neuroinflammation plays in PD is summarized in Figure 4, (adapted from Block et al., 2007), which demonstrates that neuronal damage, wrought by environmental toxicants or mutations, effect the release of misfolded proteins from dead or dying dopaminergic neurons. These misfolded proteins can activate resident brain microglia, bringing about the release of ROS, nitrite and pro-inflammatory cytokines, which can further exacerbate neuronal health. In summary, sterile neuroinflammation plays a significant role in the development of PD, but the signaling mechanisms that underlie this process are poorly understood and characterized. Discovering novel signaling nodes could identify potential targets for developing therapies for PD.

Fyn kinase

Fyn is a member of the Src family kinase (SFK) family, which comprises of proto-oncogenes that play key roles in cell morphology, motility, proliferation, and survival. v-Src (a viral protein) is encoded by the chicken oncogene of the Rous sarcoma virus, and Src (the

cellular homologue) is encoded by a physiological gene, the first of the proto-oncogenes. From the N- to C-terminus, as demonstrated in Scheme 2, Src kinases contain an N-terminal 14-carbon myristoyl group, a unique segment, an SH3 domain, an SH2 domain, a protein–tyrosine kinase domain, and a C-terminal regulatory tail (Roskoski, 2004). Src activity is regulated by a well-studied post-translational mechanism - tyrosine phosphorylation. The phosphorylation of different domains is responsible for the control of the kinase activity of this family of enzymes, bringing about a change in their conformation to activated or inhibited states (Boggon and Eck, 2004). The key sites that were identified to undergo these events were - Y529, which is 6 residues upstream of the C-terminus and Y417 in the kinase domain. Phospho-Y-529 binds intramolecularly with the Src SH2 domain, keeping the kinase in a closed and inactive conformation. Y417, which occurs in the activation loop, is sequestered and is not a substrate for phosphorylation by another kinase. When phospho-Y-529 dissociates or is displaced from the SH2 domain, Y417 can be phosphorylated, usually by another Src kinase molecule. The phosphorylation of Y417 residue is a key to several signaling events, activating Src kinases and enabling them to achieve high levels of kinase activity.

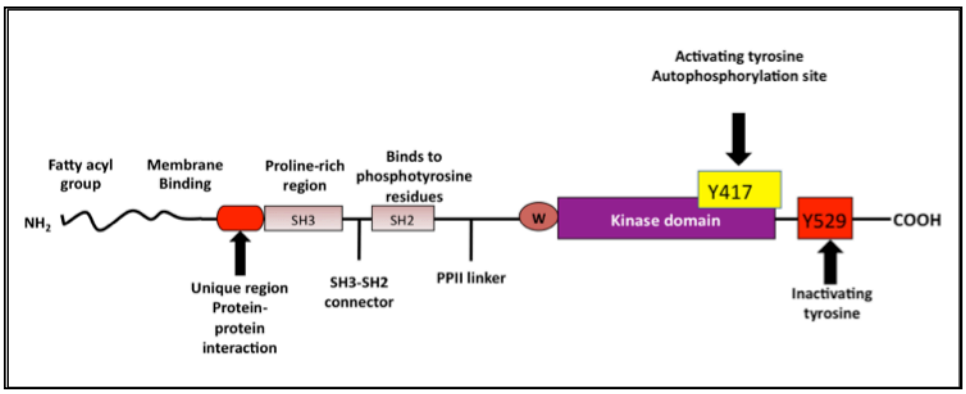

Figure 5. Domain structure of Fyn kinase.

The switch between phosphorylation dependent activation and inactivation of Src kinases is controlled by several cellular signaling molecules. Csk (C-terminal src kinase) and

Chk (Csk homologous kinase) are two tyrosine kinases that are known to phosphorylate Y529 – the inhibitory tyrosine of SFK (Chong et al., 2005; Cole et al., 2003). Csk forms an inhibitory complex with Src through non-covalent interactions regardless of the phosphorylation state of Src (Chong et al., 2005).

Signal transduction involving SFKs are initiated by ligand mediated as well as ligand-independent pathways. Non-ligand mediated activation of SFKs involve several ROS species such as H_2O_2 and nitric oxide (Meurer et al., 2005; Yan and Berton, 1996), diamide (Nakamura et al., 1993), oxidized low-density lipoproteins (Maschberger et al., 2000), alkylating agents such as iodoacetamide (Devary et al., 1992), heavy metal ions such as mercury and arsenic (Soto-Pena and Vega, 2008) and X-rays (Kharbanda et al., 1994). Ligand mediated pathways such as coupling of T-cell receptors, Fc receptors and cytokine receptors have been shown to activate SFKs (Gilfillan and Rivera, 2009; Salmond et al., 2009; Smith-Garvin et al., 2009). Fyn's putative role in Alzheimer's disease associated neurodegeneration has been a subject of intense scientific investigation for the past decade. Fyn kinase mediates the accumulation and redistribution of amyloid-β in the lipid rafts. This mechanism explained the accumulation of amyloid-β and tau and the key mechanism to amyloid-β derived diffusible ligand mediated neuronal death (Williamson et al., 2008). Fyn has also been shown to phosphorylate α-synuclein at tyrosine residue 125 (Nakamura et al., 2001). However, the physiological or pathological implications of this phosphorylation and PD are yet to be investigated. It was recently demonstrated that amyloid-β oligomers bound to post-synaptic prion protein in neurons, activating Fyn and Fyn-mediated tyrosine phosphorylation of the NR2B subunit of the NMDA receptor, leading to cell death (Um et al., 2012). Fyn was also shown to directly phosphorylate Tau at tyrosine residue 18,

promoting its disassociation from microtubules and the formation of Tau fibrils (Lee et al., 2004). Recently, we published a study that identified Fyn as the Src kinase that phosphorylates PKCδ at Y311 upon oxidative stress administration in dopaminergic neuronal cells, leaving it susceptible to caspase-3 mediated proteolytic cleavage (Saminathan et al., 2011).

Role of Fyn in immune signaling

Early studies demonstrated that thymic Fyn plays a crucial role in mounting signaling responses in T cells in response to stimulation with antibodies raised against the TCR co-receptor CD3 as well as mitogens (Appleby et al., 1992; Cooke et al., 1991). In mast cells, Fyn was found to associate with the FcεRI receptor, and mediate degranulation and pro-inflammatory cytokine production (Furumoto et al., 2005). Subsequent research in this area showed that Fyn activation downstream of FcεRI activation fed into the JNK pathway, and NF-κB pathway activation, leading to the production of the pro-inflammatory cytokines IL-6 and TNF-α, as well as the secretion of arachidonic acid. Interestingly, in a gene expression array used in the same study to identify transcripts whose levels were lower in stimulated $Fyn^{-/-}$ mast cells, the pro-IL-1β transcript was identified (Gomez et al., 2005). IL-1β is a cytokine associated with activation of inflammasomes, which will be the subject of the subsequent literature review. The hypersensitivity of Lyn deficient mast cells to stimulation was attributable to increased levels of Fyn mRNA and Fyn signaling (Hernandez-Hansen et al., 2005). With respect to mast cell degranulation, Fyn was shown to mediate Gab2 and Akt phosphorylation, feeding into PKCδ mediated degranulation (Parravicini et al., 2002). The anti-inflammatory activities of delphinidin, an anthocyanidin found in red wine and berries,

were attributed to its inhibition of Fyn kinase directly (Hwang et al., 2009). GPRC5B-associated Fyn contributed to NF-κB activation in inflammatory signaling in adipocytes, leading to increased obesity and inflammation in murine adipose tissue (Kim et al., 2012). More recently, Fyn was also shown to mediate NF-κB pathway activation downstream of NKG2D and CD137 activation in natural killer cells, utilizing a signaling mechanism dependent on ADAP (Rajasekaran et al., 2013). The role of Fyn in neuroinflammation has been an active area of investigation, and studies have shown that CD36 associated Fyn and Lyn contribute to amyloid-β induced ROS generation and (Moore et al., 2002). Fyn was also shown to regulate microglial migration via phosphorylation of p130Cas (Stuart et al., 2007). However, these studies were largely performed using peritoneal macrophages as substitutes for microglial cells. Microglia and macrophages have different origins and disparate gene expression profiles (Hickman et al., 2013). Recent work also demonstrated that CD36 contributes to pro-inflammatory cytokines in BV2 microglia post activation by neurotoxic prion protein fragment, and associates with Fyn to mediate its pro-inflammatory actions. However, the authors of this study did not directly show interaction of Fyn and CD36, (activation of Fyn was prevented using neutralizing antibody to CD36, providing circumstantial evidence for interaction) Moreover, the authors did not chemically or genetically inhibit/ reduce Fyn levels to show what role it might play in the process. Also, activation of Fyn was by a blot for activated Src family kinases, which does not distinguish between Fyn and other Src family kinases (Kouadir et al., 2012).

Protein kinase C delta (PKCδ)

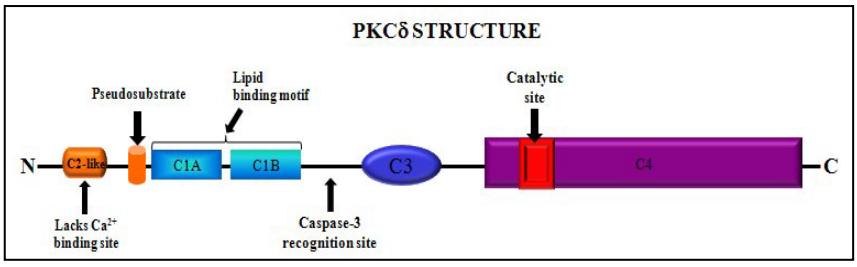

Figure 6. Domain structure of PKCδ

PKCδ was discovered by Geschwendt in 1986 and cloned from the rat brain cDNA library (Gschwendt et al., 1986). Classified under the AGC kinase family, twelve distinct PKC isoforms have been identified and grouped into three well-characterized sub-families. Based on their modes of activation, PKCs are classified as classical or conventional PKCs (α, βI, βII and γ; cPKCs), novel PKCs (δ, θ, ε, η ; nPKC), and atypical PKCs (ζ and λ (mouse)/ τ(human); aPKCs (Corbalan-Garcia and Gomez-Fernandez, 2006).

All PKCs are composed of an N-terminal regulatory domain and C-terminal catalytic domain that is connected at the variable region - V3. cPKCs contain four conserved regions (C1-C4) and five variable regions (V1-V5). The structure of PKCδ is depicted in Scheme 1. The cPKCs are dependant on intracellular calcium concentrations and are activated by diacylglycerol (DAG), while nPKCs are activated by DAG and are calcium independent. The aPKCs don't require either calcium or phospholipids for their activity (Kanthasamy et al., 2003). PKCδ does not possess the C2-region that binds Ca^{2+} and therefore is calcium-independent for its activity (Pappa et al., 1998). PKCδ has been implicated in cell differentiation, secretion and apoptosis. We have shown PKCδ is expressed in the rat and mouse substantia nigra and striatum (Yang et al., 2004; Zhang et al., 2007). PKCδ is activated by DAG binding to the regulatory domain and by translocation to the cell membrane and other sub-cellular organelles. Several apoptotic stimuli activate PKCδ in

different cell types by post-translational modifications such as phosphorylation at serine/threonine and tyrosine residues. Ser-643, Thr-505, and Ser-662 are known to be phosphorylated, and influence PKCδ kinase activity (Kanthasamy et al., 2003).

Proteolytic cleavage of PKCδ by caspase-3 in non-neuronal cell types in response to several apoptotic stimuli has been identified (Emoto et al., 1995; Ghayur et al., 1996; Koriyama et al., 1999; Leverrier et al., 2002; Sitailo et al., 2006). Studies from our group demonstrated that PKCδ is proteolytically cleaved by caspase-3 in response to numerous apoptotic stimuli in neuronal models to yield a 41 kD catalytically active and a 38 kD regulatory fragment. This results in increased activity of the kinase. We have also shown that prevention of the proteolytic activation of PKCδ rescues the dopaminergic neurons from apoptosis (Kitazawa et al., 2003; Kitazawa et al., 2005; Kitazawa et al., 2002; Latchoumycandane et al., 2005; Sun et al., 2005). The pro-apoptotic role of PKCδ in experimental PD models is well-established finding and understanding the mechanisms of activation of this kinase presents with a novel molecular candidate in the intervention of PD progression. Tyrosine phosphorylation of PKCδ and caspase-3 dependant cleavage during apoptotic stimuli are the key determinants of its pro-apoptotic function. Moreover, preincubation of the neuronal cells with both, a broad specific kinase inhibitor, and a Src kinase inhibitor, prevented Y311 phosphorylation and cleavage of PKCδ, thereby rescuing the cells from apoptosis (Kaul et al., 2005).

Role of PKCδ in immune signaling

PKCδ has been found to contribute to pro-inflammatory signaling in several cell types; In monocytes, LPS treatment caused the cytosol to membrane translocation of PKCδ .

Pharmacological inhibition of PKCδ by Rottlerin, a chemical inhibitor, attenuated the production of TNFα and IL-1β via diminishing the DNA binding activity of the transcription factor AP-1 (Kontny et al., 2000). *Staphylococcus aureus* peptidoglycan mediated nitrite production in macrophages was diminished by inhibition of PKCδ (Bhatt et al., 2011). A mechanistic investigation into how PKCδ might mediate its pro-inflammatory activities showed that it interacted with the TLR adaptor protein TIRAP via its TIR domain (Kubo-Murai et al., 2007). PKCδ$^{-/-}$ macrophages showed diminished cytokine and nitrite levels when stimulated with LPS/IFNγ, with or without co-infection with *Leishmania major* (Guler et al., 2011). It was also shown to mediate the LPS mediated induction of the sepsis effector soluble fms-like tyrosine kinase-1 receptor in macrophages (Lee et al., 2008). Genetic knockdown of PKCδ via sh-RNA resulted in reduced oxidized LDL (OxLDL) uptake and the intracellular accumulation of cholesterol in THP-1 monocyte derived macrophages as well as primary macrophages, leading to CD36 upregulation and foam cell formation (Lin et al., 2012). In vascular smooth muscle cells, PKCδ interacted with the p65 subunit of the NF-κB subunit, and mediated S536 phosphorylation (Ren et al., 2014). In dendritic cells, it was found to contribute to antigen presentation and activation (Majewski et al., 2007). PKCδ mediates Cd11b expression and MMP release in eosinophils (Langlois et al., 2009). It also mediates the activation of the NAPDH oxidase and the ROS production in response to COS-7 cell stimulation (Cheng et al., 2007). In a model of sepsis, a PKCδ inhibitor peptide treated mice had reduced levels of several pro-inflammatory chemokines in lung and blood samples. There was also a concurrently diminished infiltration of inflammatory cells into the lungs, and alleviated pulmonary edema (Kilpatrick et al., 2011). PKCδ inhibited the anti-apoptotic signaling mediated by TNFα in neutrophils (Kilpatrick et al., 2002). In human bronchial

epithelial cells, PKCδ was shown to participate in NF-κB activation and IL-8 release (Cummings et al., 2004). PKCδ deficient mice that were exposed to asbestos showed diminished accumulation of several pro-inflammatory cytokines in their lung fluid, as well as a reduced infiltration of immune cells within the lung (Shukla et al., 2007). Specifically with regards to neuroinflammation, chemical inhibition of PKCδ prevented NF-κB activation and nitrite generation in microglia (Kim et al., 2005). PKCδ has been shown to mediate neuroinflammatory responses to LPS and IFNγ in microglial cells (Shen et al., 2005).

Background and Literature Review – II

Inflammasomes and their role in neurodegenerative diseases

A major role of the innate immune system is to recognize tissue damage that occurs as the result of an injury, requiring clearance of cell debris and dead cells. Invading pathogens need to be eliminated and cleared. The innate immune system does this by the means of receptors that recognize both, modified self-molecules, as well as foreign molecules (Kato et al., 2011; Kawai and Akira, 2011). Inflammasomes are a crucial arm of the innate immune system. First described by Tschopp in the early 2000s, inflammasomes are large, cytosolic multimeric assembly platforms that effect the activation of pro-inflammatory caspases such as Caspase-1. These complexes comprise of an inflammasome sensor receptor, the adaptor protein ASC, and Caspase-1, which upon receiving the appropriate stimulus cleaves itself into the active p20 form, which in turn cleaves inflammatory pro-cytokines into their mature secretable form. The 2 major cytokines that are released upon inflammasome activation are IL-1β and IL-18 (Martinon et al., 2002). Concurrently, inflammasomes also bring about a rapid, pro-inflammatory form of cell death called pyroptosis (Miao et al., 2011).

Inflammasome biology is an extremely active area of research; as pattern recognition receptors such as cytosolic inflammasome receptors are identified, the cellular signaling processes that occur downstream of their activation are being elucidated. Although inflammasomes play a crucial role in mounting innate immune responses to potential pathogens, recent discoveries have shed light on how their signaling goes awry in a whole host of diseases such as atherosclerosis, type 2 diabetes, and Alzheimer's disease. Recent compelling evidence has suggested that inhibiting inflammasomes could protect against

many of the aforementioned diseases, which have an immune hyperactivation component.

Structure of major inflammasome components

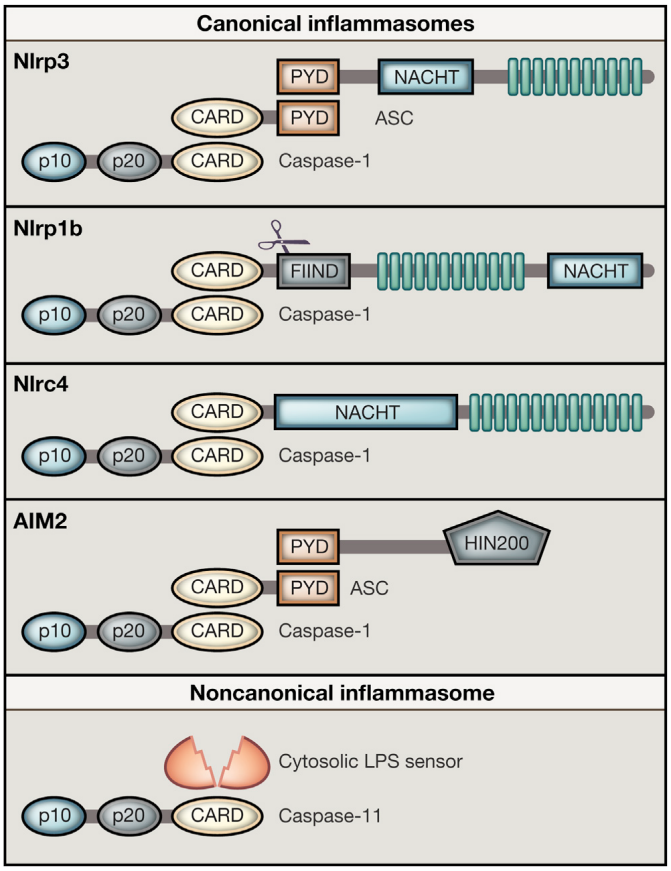

Figure 7. The major studied inflammasomes.

The components of the major studied inflammasomes are shown in Figure 7, (adapted from Lamkanfi and Dixit, 2014).

1. Cytosolic receptors

Inflammasome receptors belong to the class of pattern recognition receptors that reside in the cytoplasm, and include the nucleotide binding domain and Leucine-rich repeat containing receptors (NLRs) (Takeuchi and Akira, 2010) and the AIM-2 like receptors (ALRs). NLRs, such as NLRP3, NLRP1b and NLRC4 contain a NACHT domain and variable number of Leucine-rich repeat domains (LRR). NLRP3 and AIM2 contain pyrin domains, through which they interact with the pyrin domains of ASC. The NACHT domains possess ATPase activity, and may contribute to the oligomerization of inflammasome complexes, and the LRRs may facilitate ligand interaction. NLRP1b and NLRP3 have Caspase activation and recruitment domains (CARD), through which they can directly activate Caspase-1. However, the presence of ASC can stabilize and increase the efficiency of these inflammasomes (Mariathasan et al., 2004; Proell et al., 2013). The NLRP1

inflammasome has a function to find domain (FIIND), and seems to require auto-proteolysis within this domain in order to become active (D'Osualdo et al., 2011; Finger et al., 2012).

2. The adaptor molecule ASC

ASC is an adaptor protein encoded by the PYCARD gene. It interacts with the cytosolic inflammasome receptors via its pyrin domain. This interaction facilitates the condensation of the cytosolic ASC into large specks, which mostly comprise of multimers of ASC dimers (Fernandes-Alnemri et al., 2007). In ASC overexpressing cells, the pool of ASC coalesces into a single large speck following inflammasome activation and assembly.

3. Caspase-1

Caspase-1 is recruited to the inflammasome assembly via ASC, by the means of its CARD. Two monomers of Caspase-1 are brought close to each other, and this results in the self-cleavage and consequent activation of Caspase-1. Active Caspase-1 cleaves the pro-cytokines pro-IL-1β and pro-IL-18 into their mature forms, following which they are secreted from the cell (Gu et al., 1997; Thornberry et al., 1992).

Caspase-11 can also bring about the cleavage of pro-IL-1β through non-canonical inflammasome activation, and it is activated by a hitherto unknown receptor.

The major studied inflammasomes are:

1. The NLRC4 inflammasome

Expressed mainly in cells that are hematopoietic in origin, NLRC4 can directly activate Caspase-1 through its CARD. Deletion of the LRR domain results in excessive Caspase-1 activation, indicating that the LRR may have an inhibitory function to prevent hyperactivation of the inflammasome (Poyet et al., 2001). The NLRC4 inflammasome has been found to play a role in host response to microbial infection. Infection of murine

macrophages with *Shigella* brings about dual activation of both the NLRC4 and NLRP3 inflammasomes (Suzuki et al., 2007; Willingham et al., 2007), showing that multiple NLRs might respond to the same microorganism, but recognize disparate bacterial products. The NLRC4 mediated response to these pathogens is dependent on ASC, evidenced by the finding that ASC deficient macrophages produce significantly less IL-1β and IL-18. However, ASC is not required for NLRC4 dependent pyroptotic cell death. The major bacterial activator of the NLRC4 inflammasome was shown to be flagellin (Franchi et al., 2006; Miao et al., 2006). However, responses to *Shigella* were found to be NLRC4 dependent even though *Shigella* lacks flagellin, but these apparent discrepancies can be explained by the fact that certain rod protein components in microorganisms can serve as activators of the NLRC4 inflammasome (Miao et al., 2010). The posttranslational modifications that govern NLRC4 activation are largely unstudied. One notable exception is the study that showed how NLRC4 serine 533 phosphorylation mediated by PKCδ is strongly required for NLRC4 inflammasome activation (Qu et al., 2012).

2. The NLRP1 inflammasome

NLRP1 is expressed on adaptive immune cells as well as other cell types, including neurons. The first discovered activator of the NLRP1 inflammasome thus far is the major toxin from *Bacillus anthracis*, Lethal toxin (LT). This discovery stemmed from the observation that certain strains of mice with polymoprhisms for the NLRP1b gene were more susceptible to LT (Boyden and Dietrich, 2006). This lead to the discovery of the NLRP1b inflammasome, which is a membrane-associated inflammasome that mediates the LT induced production of IL-1β and IL-18. NLRP1 has a CARD domain through which it can recruit and activate Caspase-1 without ASC (Nour et al., 2009), although studies have shown

that ASC presence can increase the efficiency of the inflammasome (Faustin et al., 2007). The NLRP1 inflammasome is unique in its requirement for muramyl di-peptide for its assembly. Its assembly was also found to require K+ efflux from the cell and lysosomal cathepsin B release, and these requirements for activation are shared by the NLRP3 inflammasome as well.

3. The AIM2 inflammasome

This is the only inflammasome described here that does not comprise of a NLR as its cytosolic receptor. AIM2 was initially identified in a tumor screen for melanoma (DeYoung et al., 1997). It was later identified as an inflammasome receptor (Hornung and Latz, 2010). AIM2 contains an N terminal pyrin domain, which is utilized for interactions with ASC, and a C terminal HIN200 domain. The latter binds to double stranded DNA. AIM2 inflammasome activation occurs through DNA binding, and the ds-DNA- AIM2 complex then recruits ASC, and Caspase-1. The AIM2 inflammasome does not sense the origin of DNA as self or non-self, but instead detects the presence of cytosolic DNA to become activated. Owing to these properties, this inflammasome was thought to play a role in the response to microorganisms that invade the cells. In favor of this hypothesis, $Aim1^{-/-}$ macrophages display diminished IL-1β and IL-18 production in response to vaccinia and mouse cytomegalovirus (Fernandes-Alnemri et al., 2010; Rathinam et al., 2010).

4. The NLRP2 inflammasome

NLRP2 is a NLR that was found to inhibit the NF-κB pathway and subsequent TNFα production in macrophages, whilst simultaneously contributing to the production of IL-1β through ASC (Bruey et al., 2004; Conti et al., 2005). Intriguingly, the expression of NLRP2 itself was found to be mediated by p65- NF-κB, in what might constitute a negative feedback

loop that limits NF-κB activation (Fontalba et al., 2007). Even though limited studies exist on this inflammasome, it may have clinical relevance; a functional and unique NLRP2 inflammasome was discovered in astrocytes, which will be discussed in the subsequent section.

5. The NLRP3 inflammasome

The NLRP3 inflammasome is the best-characterized and most widely studied inflammasome. It is unique among other inflammasomes, in that the basal levels of NLRP3 are low in 'resting' cells such as macrophages or microglia. Activation of this inflammasome is a two-step mechanism,

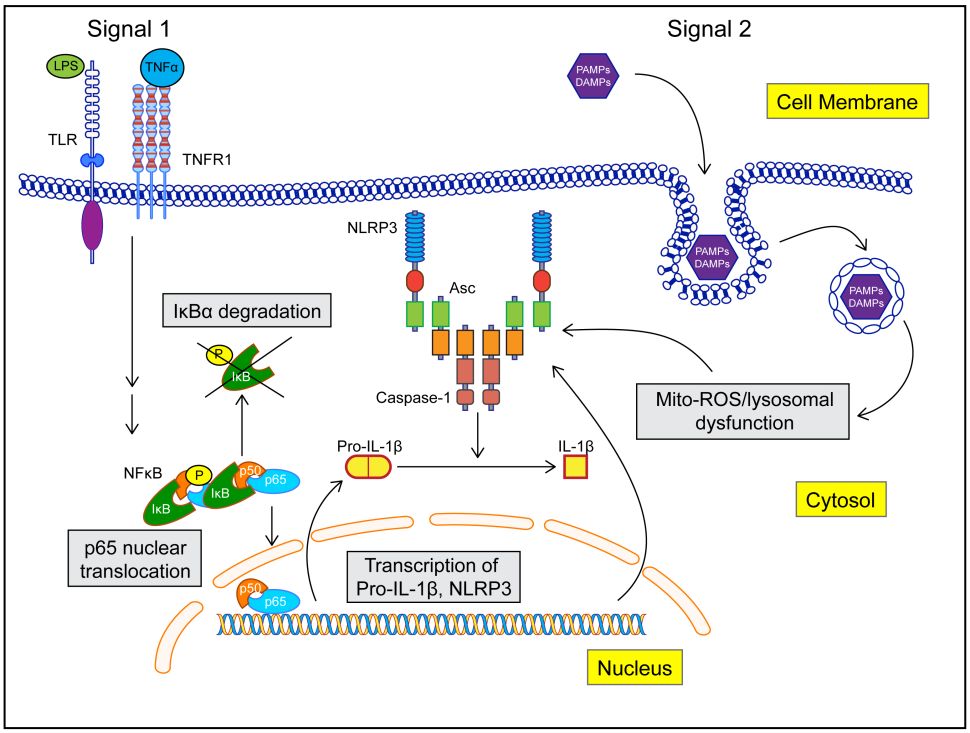

Figure 8. Dual signal activation mechanism of the NLRP3 inflammasome.

and is summarized in Figure 8. It is hypothesized that this two-step activation mechanism provides a checkpoint to prevent uncontrolled release of IL-1β. The first step, priming, is required to increase the levels of pro-IL-1β and NLRP3. The TLR ligand LPS and the TNFR1 ligand TNFα are adept at mediating this induction via activation of the NF-κB pathway (Bauernfeind et al., 2009). The nature of the second step, which induces the

assembly of the inflammasome, is strongly contested. However, the generally accepted consensus is that mitochondrial dysfunction and mito-ROS generation, along with lysosomal disruption, play a role in the process. K+ efflux is also important for optimal activation to occur. The NLRP3 inflammasome is activated by a huge variety of molecules, calling into question whether it really is a receptor at all, or if it reacts to changes in the cytosol as a result of mitochondrial or lysosomal disruption. In support of this notion, mitochondrial toxicants can bring about activation of NLRP3 inflammasome dependent responses in macrophages (Zhou et al., 2011). Many of the early discovered activators of the NLRP3 inflammasome were non-self in origin. Crystalline and particulate substances in particular, can contribute to the sterile inflammation mediated by the NLRP3 inflammasome. Examples of such substances include asbestos and silica (Cassel et al., 2008; Dostert et al., 2008; Hornung et al., 2008). Of note, NLRP3 deficient mice are protected from asbestos and silica induced neutrophil infiltration, pro-inflammatory cytokine generation and cell death (Hornung et al., 2008). Metal alloy particles from prosthetic joints were also found to activate the NLRP3 inflammasome (Caicedo et al., 2009). The NLRP3 inflammasome is also activated in response to several microorganism pathogens. *Staphylococcus aureus* hemolysin (Craven et al., 2009; Munoz-Planillo et al., 2009), listerialysin from *Listeria monocytogenes* (Meixenberger et al., 2010) and tetanolysin-O from *Clostritidium tetani* (Chu et al., 2009) have all been demonstrated to elicit NLRP3 inflammasome activation, as have ds-RNA molecules from the influenza virus (Ichinohe et al., 2009). The NLRP3 inflammasome is thought to play a supportive role during DNA virus infection (Delaloye et al., 2009; Muruve et al., 2008). Fungal toxins and the malarial toxin Hemozoin also elicit the activity of the NLRP3 inflammasome, probably through the activation of the tyrosine kinase

SYK (Joly et al., 2009; Shio et al., 2009). The NLRP3 inflammasome can also be activated by many self-moieties, which are generally crystalline or aggregated, and are associated with cell death, danger, and inflammation. Extracellular ATP, which is probably mitochondrial in origin, is a danger associated molecular pattern (DAMP), which acts as a potent danger signal to activate the NLRP3 inflammasome (Iyer et al., 2009). This activation is dependent on the K+ efflux mediated by the P2X7 receptor. Uric acid, a byproduct of purine biogenesis forms crystalline urate crystals when deposited into the extracellular space. Chronic urate crystal deposition in the joints is a hallmark of the disease gout. This deposition is accompanied by the secretion of various pro-inflammatory cytokines, including IL-1β and IL-18 (Martinon, 2010). When phagocytosed by macrophages, the crystals activate the NLRP3 inflammasome, which contributes to disease pathology. In strong evidence of the role that NLRP3 plays in the disease process, patients who suffered from acute gout showed symptomatic relief in clinical trials when treated with Anakinra, which is an interleukin-1 receptor antagonist. Another well studied activator of the NLRP3 inflammasome that has great clinical relevance is crystalline cholesterol; It was observed that cholesterol crystal deposition contributed to macrophage infiltration, chronic inflammation and plaque development but did so to a lesser extent under conditions of NLRP3 inflammasome inactivation (Duewell et al., 2010). Hyaluronan is a component of the extra-cellular matrix and is also found in the bacterial cell walls. In its polymeric form, it can activate the NLRP3 inflammasome (Yamasaki et al., 2009). During traumatic injuries, a plethora of inflammasome activators such as ATP, hyaluronan and uric acid can be released which can, in concert, bring about massive inflammation via NLRP3 inflammasome activation (Gasse et al., 2009; Idzko et al., 2007; Muller et al., 2011; Nakae et al., 2003; Riteau et al., 2010).

Cryopyrin-associated periodic syndromes (CAPS) are a set of autoimmune diseases that are used to describe three phenotypes depending on the severity of the disease; familial cold autoinflammatory syndrome (FCAS), the mildest phenotype, Muckle-Wells syndrome (MWS) with intermediate severe disease phenotype, and neonatal-onset multisystem inflammatory disease (NOMID), which is the severest form of the disease. CAPS is an autosomal dominant condition, caused by a gain of function mutation in the NLRP3 gene, leading to the uncontrolled production of IL-1β in immune cells. In the past few years, several drugs to treat this disease have been effectively utilized (Yu and Leslie, 2011). In 2008, it was discovered for the first time that the aggregated form of the senile plaque associated protein amyloid-β could activate the NLRP3 inflammasome. This was the first time a misfolded protein was demonstrated to activate the NLRP3 inflammasome, and was the first link that inflammasomes may play a role in the pathogenesis of neurodegenerative disorders (Halle et al., 2008). This topic will be covered in more detail in the subsequent section.

6. Non-canonical inflammasomes

Activation of the non-canonical inflammasomes is primarily mediated by Caspase-11. This was protein was shown to mediate the activation of other Caspases, contributing to apoptosis (Kang et al., 2000). Caspase-11 was subsequently demonstrated to be essential for Caspase-1 activation and IL-1β production upon infection with *Escherichia coli*, *Citrobacter rodentium* or *Vibrio cholerae*, but was found to not affect the activation of Caspase-1 downstream of typical NLRP3 activators such as ATP and urate crystals (Kayagaki et al., 2011). Incidentally, this was also the study that demonstrated that Caspase1$^{-/-}$ mice inherently lacked Caspase-11 as well, necessitating the use of Caspase-11$^{-/-}$ macrophages as negative

controls for inflammasome activation in many studies, including ours. It was later discovered that the non-canonical activation of this inflammasome occurred independent of TLR4, leading to the current consensus that a cytosolic receptor mediates Caspase-11 activation to elicit non-canonical inflammasome activation (Kayagaki et al., 2013).

7. The NLRP6 inflammasome

Although included in this section, current evidence has not yet conclusively identified the protein NLRP6 as being part of an inflammasome complex; evidence needs to be provided showing that it associates with procaspase-1 directly and mediates the processing of pro-IL-18 and/or pro-IL-1β. NLRP6 was found to associate with ASC and effect NF-κB and Caspase-1 activation in a model system that utilized overexpression of the protein to validate the findings (Grenier et al., 2002). Recent studies have expounded a role for this inflammasome in colitis; NLRP6$^{-/-}$ mice are more susceptible to colitis in response to colitis inducing agents (Chen et al., 2011; Elinav et al., 2011; Normand et al., 2011). In fact, one study showed that NLRP6$^{-/-}$ mice have spontaneous colitis and inflammatory cell recruitment, as well as amplified inflammatory responses to dextran sodium sulfate (DSS), an inducer of colitis like symptoms, as well as expanded pathobiont gut microbiota. This points to a role of the NLRP6 inflammasome in serving as a checkpoint to prevent gut inflammation (Elinav et al., 2011). Related studies also showed that NLRP6 is expressed in myofibroblasts, and its absence triggered colitis and tumorigenesis, accompanied by dysregulated regeneration of the colonic mucosa However, IL-1β levels were not significantly altered. (Normand et al., 2011) NLRP6 deficiency enhanced MAP kinase and NF-κB pathways downstream of TLR activation, leading to increased levels of cytokine and chemokine production (Nigam and Narula, 1990).

Role of inflammasomes in neurodegenerative disorders

1. The NLRC4 inflammasome

As mentioned previously, NLRC4 mainly recognizes bacterial pathogen associated molecular patterns (PAMPs). This makes the NLRC4 inflammasome the primary CNS responder to bacterial infections of the brain that cause meningitis and encephalitis (Jamilloux et al., 2013; Wu et al., 2010). As in peripheral bacterial infections, the NLRC4 inflammasome senses the presence of flagellin, as well as PrgJ (Miao et al., 2006; Miao et al., 2010). Recent evidence suggests, however, that this sensing may be mediated indirectly; neuronal apoptosis inhibitory proteins (NAIPs) first detect the bacterial proteins, and may then subsequently interact with NLRC4 to mediate inflammasome activation (Kofoed and Vance, 2011; Zhao et al., 2011).

2. The NLRP1 inflammasome

Interaction studies carried out on the NLRP1 inflammasome showed that the anti-apoptotic proteins BCL-2 and BCL-X could bind to and subsequently inhibit NLRP1 activation (Bruey et al., 2007). The NLRP1 inflammasome was subsequently shown to be activated in neurons (de Rivero Vaccari et al., 2009; de Rivero Vaccari et al., 2008; Silverman et al., 2009). Recent studies have demonstrated that neuronal NLRP1 mediates pyroptosis in response to β-amyloid treatment (Tan et al., 2014). It was also demonstrated that there was a massive upregulation of NLRP1 containing neurons in AD brains versus controls, and that the functional NLRP1 inflammasome in these cells could activate Caspase-1 and Caspase-6, and the processing of IL-1β in neurons (Kaushal et al., 2015). Hence, activation of the NLRP1 inflammasomes in neurons may identify a novel cell death pathway that mediates neuronal loss in AD. Circumstantial evidence for this hypothesis comes from

studies that have linked NLRP1 variants with AD development (Pontillo et al., 2012). NLRP1 might also contribute to the pathogenesis of stroke, evidenced by the finding that administering neutralizing antibodies against NLRP1b was found to be protective in a stroke model (Abulafia et al., 2009). Middle cerebral artery occlusion in rats induces NLRP1 expression (Frederick Lo et al., 2008), and increased NLRP1b in the cerebrospinal fluid is associated with a poorer prognosis in patients with traumatic brain injuries (Adamczak et al., 2012).

3. The AIM2 inflammasome

The AIM2 inflammasome has not been studied in the central nervous system. However, it is hypothesized that it might play a role in CNS infections (Walsh et al., 2014), since within the peripheral immune system, it binds to ds-DNA from *Listeria monocytogenes*, which can cause meningitis (Wu et al., 2010).

4. The NLRP2 inflammasome

Stimulation of astrocytes with ATP resulted in the activation of the NLRP2 inflammasome, the activation of which was inhibited by the pannexin inhibitor probenecid and the P2X7 receptor antagonist Brilliant Blue G (Minkiewicz et al., 2013). The clinical significance of the activation of this inflammasome is not known.

5. The NLRP3 inflammasome

a. Role in brain infections- Activation of the NLRP3 inflammasome can have disparate consequences inside or outside the CNS; in a mouse model of *Streptococcus pneumoniae* induced pneumonia, the NLRP3 response had a beneficial effect and was required for clearance of the pathogen (McNeela et al., 2010). However, in a meningitis model that utilized the same pathogen, activation of the NLRP3

inflammasome and pro-inflammatory cytokine secretion had a detrimental effect (Hoegen et al., 2011; Mitchell et al., 2012). In response to the Japanese Encephalitis Virus (JEV) and West Nile Virus (WNV) infections, activation of the NLRP3 inflammasome is protective (Kaushik et al., 2012; Kumar et al., 2013; Ramos et al., 2012).

b. Role in sterile brain inflammatory responses: Unlike its role in CNS infections, activation of the NLRP3 inflammasome in sterile inflammatory responses within the CNS has mostly been shown to be detrimental. In mouse models of experimental autoimmune encephalitis (EAE), activation of the NLRP3 inflammasome was demonstrated to be critical to disease progression (Gris et al., 2010; Inoue et al., 2012). Inhibiting the action of the NLRP3 inflammasome prevented T-cell infiltration into the CNS. Treatment regimen with a small molecular inhibitor of the NLRP3 inflammasome, MCC950, was found to be protective in an EAE mouse model (Coll et al., 2015).

Many neurodegenerative disorders are propagated by misfolded proteins which can activate the innate immune system (Glass et al., 2010). As mentioned previously, fibrilar amyloid-β was the first misfolded/aggregated protein shown to activate the NLRP3 inflammasome. In a subsequent study, it was shown that NLRP3 and Caspase-1 deficient mice were massively protected against the Alzheimer's Disease (AD) pathology, when crossed to the APP/PS1 transgenic mice. The same study also demonstrated increased Caspase-1 and IL-1β levels under APP/PS1 and AD conditions (Heneka et al., 2013). Misfolded prion protein fibrils were also shown to activate the NLRP3 inflammasome (Hafner-Bratkovic et al., 2012). In the field of PD biology, the aggregated form of α-synuclein was shown to effect the

production of IL-1β in monocytes (Codolo et al., 2013). However, this study used monocytes as cell culture model system, which, unlike microglia and macrophages, have constitutively activated Caspase-1 (Netea et al., 2009), and hence any stimulus that primes the NLRP3 inflammasome will also bring about inflammasome activation, without the need for an intervening signal-2. Thus, we question the relevance of using a model system that does not take into consideration all the factors that engender inflammasome activation. Moreover, there was no direct evidence, either by specific chemical inhibition, or by genetic knockdown of NLRP3 specifically being involved. There is some evidence to suggest that inflammasomes may play a role in-vivo. Overexpression of α-synuclein within the SN effected increased IL-1β levels in the striatum. However, inflammasomes were not directly implicated (Chung et al., 2009). Lastly, increased levels of the cytokine IL-1β have been found in PD brain and CSF levels (Mogi et al., 1996; Mogi et al., 2000). Recently, NLRP3$^{-/-}$ mice were demonstrated to be resistant to MPTP induced dopaminergic neuronal cell loss and serum IL-1β secretion, but this protection was attributed to the inherent inhibitive effect dopamine has on NLRP3 inflammasome activity (Yan et al., 2015). Hence, to conclude, activation of the NLRP3 inflammasome may play a role in the development of several neurodegenerative disorders that have a sterile inflammatory component to them.

Inflammasomes: Beyond the role of IL-1β

IL-1β is a versatile cytokine; it can elicit a proinflammatory response from immune cells such as microglia and macrophages (Basu et al., 2002; Jayaraman et al., 2013), as well as from astrocytes (John et al., 2004). It can also directly elicit cell death in neurons through p38 MAP kinase activation (Wang et al., 2005). However, the significance of NLRP3

inflammasome activation may extend beyond IL-1β processing and secretion. It was recently demonstrated that upon NLRP3 inflammasome activation, oligomeric forms of the NLRP3 inflammasome were released from the cell, where they were able to activate Caspase-1 in non-activated cells extracellularly. Oligomeric ASC particles were found in the serum of patients with CAPS (Baroja-Mazo et al., 2014). In a study that was simultaneously published, specks of ASC released by cells could take up and seed the nucleation of soluble ASC in unactivated cells, thereby contributing to inflammasome activation and the spreading of inflammation in a prionid manner (Franklin et al., 2014).

CHAPTER 2. FYN KINASE REGULATES MICROGLIAL NEUROINFLAMMATORY RESPONSES IN CELL CULTURE AND ANIMAL MODELS OF PARKINSON'S DISEASE[1]

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Nikhil Panicker[2], Hariharan Saminathan[2], Huajun Jin[2], Matthew Neal[2], Dilshan S. Harishchandra[2], Richard Gordon[2], Kavin Kanthasamy[3], Vivek Lawana[2], Souvarish Sarkar[2], Jie Luo[2], Vellareddy Anantharam[2], Anumantha G. Kanthasamy[2] and Arthi Kanthasamy[2,4]

Abstract

Sustained neuroinflammation mediated by resident microglia is recognized as a key pathophysiological contributor to many neurodegenerative diseases, including Parkinson's disease (PD), but the key molecular signaling events regulating persistent microglial activation have yet to be clearly defined. In the present study, we examined the role of Fyn, a non-receptor tyrosine kinase in microglial activation and neuroinflammatory mechanisms in cell culture and animal models of PD. The well-characterized inflammogens lipopolysaccharide (LPS) and tumor necrosis factor alpha (TNFα) rapidly activated Fyn kinase in microglia. Immunocytochemical studies revealed that activated Fyn preferentially localized to the microglial plasma membrane periphery and the nucleus. Furthermore,

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[2] Parkinson's Disorders Research Program, Iowa Center for Advanced Neurotoxicology, Department of Biomedical Sciences, Iowa State University, Ames, Iowa 50011.
[3] Present address: Baylor College of Medicine, Houston, Texas 77005.
[4] Author for correspondence.
Author Contributions: N.P., A.G.K., and A.K. designed research; N.P., H.S., H.J., M.N., D.S.H., R.G., K.K., V.L., S.S., and J.L. performed research; N.P., H.J., and A.K. analyzed data; N.P., H.J., V.A., A.G.K., and A.K. wrote the paper.

activated Fyn phosphorylated PKCδ at tyrosine residue 311, contributing to an inflammogen-induced increase in its kinase activity. Notably, the Fyn-PKCδ signaling axis further activated the LPS- and TNFα-induced MAP kinase phosphorylation and activation of the NFκB pathway, implying that Fyn is a major upstream regulator of the pro-inflammatory signaling. Functional studies in microglia isolated from wild type ($Fyn^{+/+}$) and Fyn knockout ($Fyn^{-/-}$) mice revealed that Fyn is required for the pro-inflammatory responses, including cytokine release as well as iNOS activation. Interestingly, a prolonged inflammatory insult induced Fyn transcript and protein expression, indicating that Fyn is upregulated during chronic inflammatory conditions. Importantly, *in vivo* studies using MPTP, LPS, or 6-OHDA models revealed a greater attenuation of neuroinflammatory responses in $Fyn^{-/-}$ and PKCδ $^{-/-}$ mice when compared to wild-type mice. Collectively, our data demonstrate that Fyn is a major upstream signaling mediator of microglial neuroinflammatory processes in PD.

Introduction

Parkinson's disease (PD) is a highly prevalent neurodegenerative disorder and is mainly characterized by the loss of dopaminergic neurons in the substantia nigra (SN) of the ventral midbrain region. Extra nigral lesions and non-motor deficits have recently been recognized (Chaudhuri et al., 2006; Bohnen et al., 2014). Although the etiopathogenesis of PD is not known, both environmental insults and genetic defects have been implicated in its onset. Mutations in seven disparate genes have been linked to Parkinsonism, which clinically resembles PD with varying onset and disease progression. Additionally, 19 other genes have been postulated to have a disease-causing role (Puschmann, 2013).

The pathophysiology of PD is complex and multifactorial, with mitochondrial dysfunction, oxidative stress, apoptosis and proteasomal dysfunction, being identified among others as potential disease mechanisms underlying nigrostriatal dopaminergic neuronal degeneration (Przedborski, 2005; Jenner and Olanow, 2006; Olanow, 2007; Levy et al., 2009). Recently, a wealth of data from cell culture, animal models and post-mortem analyses of human PD brains have established chronic, sustained microglia-mediated neuroinflammation as being a major event in the delayed and progressive loss of dopaminergic neurons within the SN (Imamura et al., 2003; Block et al., 2007; Glass et al., 2010; Tansey and Goldberg, 2010). As the macrophagic cells of the central nervous system (CNS), microglia comprise a major component of the brain's innate immune system. Under 'normal' physiological conditions, they produce anti-inflammatory and neurotrophic factors to promote neuronal survival and plasticity (Carson, 2002). However, when they encounter a potential pathogen, a dead or dying neuron or neurotoxic stress, they switch to an 'activated' phenotype, producing pro-inflammatory cytokines and chemokines, reactive nigtrogen species, and reactive oxygen species. Activated microglia may also directly contribute to cell death by phagocytizing dopaminergic neurons (Barcia et al., 2012; Virgone-Carlotta et al., 2013). Thus, the pathophysiology of PD is accompanied by a sustained pro-inflammatory microglial response that contributes to neuron death, thereby exacerbating disease progression.

Fyn, a member of the Src family of kinases, is a non-receptor tyrosine kinase expressed in the brain. The kinase has been shown to play a role in amyloid-mediated apoptosis in cortical neurons (Lambert et al., 1998), astrocyte migration (Dey et al., 2008) and oligodendrocyte differentiation (Sperber et al., 2001). In the peripheral immune system,

Fyn plays a role in mast cell and B/T cell activation (Palacios and Weiss, 2004; Gomez et al., 2005a). Fyn was shown to mediate pro-inflammatory mediator production in mast cells, macrophages, basophils as well as in natural killer cells (Rajasekaran et al., 2013). Fyn was shown to be activated following fibrillar β-amyloid peptide engagement of its receptor CD36, contributing to activation and migration of primary murine peritoneal macrophages (Moore et al., 2002; Stuart et al., 2007), and in BV2 microglial cells stimulated with the neurotoxic fragment of prion protein (Kouadir et al., 2012). Recently, we have identified a pro-apoptotic Fyn/PKCδ-mediated signaling pathway that contributes to oxidative stress-induced cell death in dopaminergic neurons (Kaul et al., 2005; Saminathan et al., 2011). However, the role of Fyn in microglial activation and neuroinflammation has never been studied in PD. Therefore, we sought to characterize the role of the Fyn-PKCδ signaling pathway in microglial activation and neuroinflammation in cell culture and animal models of PD. The results from these comprehensive studies reveal that Fyn kinase plays a key role in microglial activation and sustained neuroinflammation in the nigral dopaminergic system.

Materials and Methods

Chemicals and reagents

Dulbecco's modified Eagle's medium/F-12 (DMEM/F-12), ascorbic acid, RPMI, fetal bovine serum (FBS), L-glutamine, Hoechst nuclear stain, penicillin, streptomycin and other cell culture reagents were purchased from Invitrogen (Gaithersburg, MD). Recombinant TNFα was purchased from Peprotech (Rocky Hill, NJ), and LPS (*E. coli* 0111:B4, Endotoxin content 6.6000000 EU/mg) and 6-OHDA were purchased from Sigma (St. Louis, MO). The mouse Fyn antibody was purchased from Thermo Scientific (Waltham,

MA). Antibodies for rabbit Fyn, PKCδ, p-Y311 PKCδ, IκBα, Lamin-B, NOS2 (iNOS) and mouse Tubulin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies against rabbit p-Src family kinase Y416 (p-Y416 SFK), native p65, p-p38 MAP kinase, native p38 MAP kinase, p-p44/42 MAP kinase (p-ERK) and native p44/42 MAP kinase (ERK) were purchased from Cell Signaling (Beverly, MA). The $gp91^{phox}$ antibody was purchased from BD Biosciences (San Jose, CA). The mouse GFAP antibody was purchased from Millipore (Billerica, MA). The TH antibody was purchased from Chemicon (Temecula, CA). Mouse M2 FLAG and β-actin antibodies, as well as the rabbit β-actin antibody were purchased from Sigma. Rabbit and goat Iba-1 antibodies were purchased from Wako Chemicals (Richmond, VA) and Abcam (Cambridge, MA), respectively. The goat TNFα antibody was purchased from R&D Systems (Minneapolis, MN). ^{32}P-ATP was purchased from Perkin Elmer (Boston, MA) and the histone substrate from Sigma. The Bradford protein assay kit was purchased from Bio-Rad Laboratories (Hercules, CA). FLAG-tagged human WT Fyn and Y417A mutant Fyn constructs were obtained as described previously (Kaspar and Jaiswal, 2011).

Animal studies

The $Fyn^{-/-}$ and $PKC\delta^{-/-}$ mice used in these studies were bred in our animal facility. $Fyn^{-/-}$ mice were originally obtained from Dr. Dorit Ron's laboratory at the University of California, San Francisco and are available from Jackson Laboratory (stock number 002271). $PKC\delta^{-/-}$ mice were obtained originally from Dr. Keiichi Nakayama's laboratory (Division of Cell Biology, Department of Molecular and Cellular Biology, Medical Institute of Bioregulation, Kyushu University, Fukuoka, Japan). Wild type ($Fyn^{+/+}$ and $PKC\delta^{+/+}$), $PKC\delta^{-/-}$

and Fyn$^{-/-}$ mice were housed under standard conditions of constant temperature (22 ± 1°C), humidity (relative, 30%), and a 12-h light cycle with food and water provided *ad libitum*. Six- to eight-week-old male mice were used for all studies. The well-characterized acute MPTP mouse model of PD (Wu et al., 2003; Przedborski et al., 2004; Kim et al., 2007; Hu et al., 2008) was primarily used for neuroinflammation studies. The mice from the MPTP treatment group received 4 intraperitoneal (i.p.) injections of MPTP-HCl (18 mg/kg free-base) dissolved in saline at 2-h intervals. Mice were sacrificed 3 h after the last injection. The nigral neuroinflammatory response was also studied using the systemic LPS injection model (Qin et al., 2007), which induces chronic neuroinflammation and progressive dopaminergic degeneration in mice. A single injection of LPS (5 mg/kg, i.p.) was delivered to wild type, Fyn$^{-/-}$ and PKCδ$^{-/-}$ mice. Mice were sacrificed 24 to 48 h later. Control groups for both MPTP and LPS received equivolume injections of saline. We injected 2 μL of 6-OHDA, diluted at a concentration of 5 μg/μL in 0.02% ascorbic acid, into the left striatum (0.2 μL/min) using the Angle 2 stereotaxic apparatus (Leica Biosystems, St. Louis, MO). The coordinates, relative to bregma were: 0.7 mm anteroposterior, 2 mm lateral, and 2.4 mm ventral. The contralateral side was either not injected or injected with 2 μL of 0.02% ascorbic acid diluted in sterile PBS as a negative control. All animal procedures were approved by the Iowa State University Institutional Animal Care and Use Committee (IACUC).

Primary microglial cultures and treatments

Primary microglial cultures were prepared from wild type, Fyn$^{-/-}$ and PKCδ$^{-/-}$ postnatal day 1 (P1) mouse pups as described previously (Gordon et al., 2011). Briefly, mouse brains were harvested, meninges removed, and then placed in DMEM-F12 supplemented with 10%

heat-inactivated FBS, 50 U/mL penicillin, 50 µg/mL streptomycin, 2 mM L-glutamine, 100 µM non-essential amino acids, and 2 mM sodium pyruvate. Brain tissues were then incubated in 0.25% Trypsin-EDTA for 30 min with gentle agitation. The trypsin reaction was stopped by adding double the volume of DMEM/F12 complete medium and then washing brain tissues three times. Tissues were then triturated gently to prepare a single cell suspension, which was then passed through a 70-µm nylon mesh cell strainer to remove tissue debris and aggregates. The cell suspension was then made up in DMEM/F12 complete medium and seeded into T-75 flasks, which were incubated in humidified 5% CO_2 at 37°C. The medium was changed after five to six days and the mixed glial cells were grown to confluence. Microglial cells were separated from confluent mixed glial cultures by differential adherence and magnetic separation to >97% purity, and then were allowed to recover for 48 h after plating. Primary microglia were treated in DMEM/F12 complete medium containing 2% FBS. For signaling experiments, the protocol employed by Stuart (2007) was utilized with a small modification. For this, the primary microglial cells were kept in 2% DMEM/F12 complete medium for 5 h at 37ºC prior to treatment. The microglial cells were treated with 100-200 ng/mL LPS and 10-30 ng/mL TNFα for durations sampled at pre-specified time points. We selected the LPS doses used in this study based on previous studies in which stimulation of cultured primary mouse microglia with 100 and 200 ng/ml LPS resulted in significant microglial activation (Haynes et al., 2006; Crotti et al., 2014; Lee et al., 2014).

siRNAs and transfections of microglia

Transient transfections of primary microglia with Fyn promoter reporter were performed using Lipofectamine LTX & Plus Reagent according to the manufacturer's protocol. Primary microglia were plated at 0.75 x 10^6 cells/well in 12-well plates one day before transfection. We transiently transfected 3 μg of Fyn promoter construct. Cells were treated 24 h after transfection with or without 200 ng/mL of LPS for 12 h and then lysed. Luciferase activity was measured using a Dual-luciferase assay kit (Promega) on a Synergy 2 multi-mode microplate reader (BioTek). Firefly luciferase luminescence values were used to normalize Renilla luciferase luminescence values. The pre-designed, on-target plus SMART pool Fyn siRNA (a combination of four siRNAs, Cat. No. LQ-040112-00-0002) and scrambled siRNA (Cat. No. D-001210-03-05) were purchased from Dharmacon (Lafayette, CO). We carried out siRNA transfections in primary mouse microglial cells with Lipofectamine 3000 reagent according to the manufacturer's protocol. Briefly, primary microglia were plated at 2 x 10^6 cells/well in 6-well plates one day before transfection. For each well, 300 pmol of Fyn siRNA pool (75 pmol each) or an equal amount of scrambled siRNA mixed with 5 µl of Lipofectamine 3000 were added to the cells. Seventy-two hours after the initial transfection, cells were analyzed by Western blotting to confirm the extent of Fyn knockdown or treated with LPS (200 ng/mL) for 24 further hours, after which cytokine content was analyzed by Luminex bioassay.

Transfection of BV2 microglia with WT Fyn-FLAG, Y417A Fyn-FLAG and Empty Vector pcDNA3.1 constructs was performed by using the AMAXA Nucleofector Kit. Briefly, BV2 cells were resuspended in transfection buffer (Solution 1: 400 µM ATP-disodium (Sigma A7699), 600 µM $MgCl_2$-$6H_2O$ in water; Solution 2: 100 µM KH_2PO_4, 20

μM $NaHCO_3$, 5 μM glucose in water) to a final concentration of 3 x 10^6 cells per 100 μL and mixed with the respective vector; 5 μg of vector DNA was used per transfection.

Immunohistochemistry and immunofluorescence studies

Immunohistochemistry was performed on sections from the substantia nigra and other brain regions of interest as described previously (Jin et al., 2011b; Ghosh et al., 2013). Briefly, mice were anesthetized with a mixture of 100 mg/kg ketamine and 10 mg/kg xylazine and then perfused transcardially with freshly prepared 4% paraformaldehyde (PFA). Extracted brains were post-fixed in 4% PFA for 48 h and 30-μm sections were cut using a freezing microtome (Leica Microsystems). Antigen retrieval was performed in citrate buffer (10 mM sodium citrate, pH 8.5) for 30 min at 90°C. Sections were then washed several times in PBS and blocked with PBS containing 2% BSA, 0.2% Triton X-100 and 0.05% Tween 20 for 1 h at room temperature. Sections were then incubated with primary antibodies overnight at 4°C and washed 7 times in PBS on a Belly Dancer Shaker (SPI supplies). The sections were incubated with Alexa dye-conjugated secondary antibodies for 75 min at room temperature and their cell nuclei were stained with Hoechst dye. Sections were mounted on slides using Prolong antifade gold mounting medium (Invitrogen) according to the manufacturer's instructions. Samples were visualized using an inverted fluorescence microscope (Nikon TE-2000U) and images were captured using a Spot digital camera (Diagnostic Instruments Inc).

Immunofluorescence studies in primary microglia were performed according to previously published protocols with some modifications (Gordon et al., 2011). Briefly, microglial cells were grown on poly-D-lysine-coated coverslips and treated 48 h later. At the

end of treatments, cells were fixed with 4% PFA, washed in PBS and incubated in blocking buffer (PBS containing 2% BSA, 0.5% Triton X-100 and 0.05% Tween 20) for 1 h at room temperature. The coverslips were then incubated overnight at 4°C with respective primary antibodies diluted in PBS containing 2% BSA. Samples were then washed several times in PBS and incubated with Alexa 488 and 555 dye-conjugated secondary antibodies. The nuclei were labeled with Hoechst stain (10 μg/mL) and coverslips were mounted with Fluoromount medium (Sigma Aldrich) on glass slides for visualization. Quantification of the number of microglial/astroglial cells obtained post-separation was accomplished using JACoP, a downloadable ImageJ plugin from Fabrice P. Cordelières. Original Hoechst or antibody TIFF files were converted into 8-bit black-and-white images, and a colocalization image was generated. Counting of Hoechst-positive and Iba-1+Hoechst-positive cells was done using the Cell counter function of the default 'Analyze' plugin in ImageJ.

Confocal imaging and Z stack image acquisition and analysis

Confocal imaging was performed at the Iowa State University Microscopy Facility, using a Leica DMIRE2 confocal microscope with the 63X and 43X oil objectives and Leica Confocal Software. One optical series covered 11-13 optical slices of 0.5-μm thickness each. Microglial neuronal contact identification and quantification were performed by counting the number of colocalizations of the two markers, with TH marked red by anti 555 and Iba-1 marked green by anti 488 using the methodology described by Barcia and colleagues (Barcia et al., 2012). The Imaris software was used to analyze the Z stack images for contact identification. The surface reconstruction wizard in the Imaris software was used to make 3-

D reconstructions of stacks for easier viewing of microglial-dopaminergic contacts and surface topology.

qRT-PCR

RNA isolation from primary microglial cells and brain tissue samples was performed using the Absolutely RNA Miniprep Kit, and then 1 µg total of isolated RNA was used for reverse transcription with the AffinityScript qPCR cDNA synthesis system (Agilent Technologies) according to the manufacturer's instructions. Quantitative SYBR Green PCR assays for gene expression were performed using the RT² SYBR Green Master Mix with pre-validated primers (SABiosciences qPCR assay system). Catalog numbers of the primers were Fyn - PPM04015A, pro-IL1β - PPM03109E, TNFα-PPM03113G. The mouse 18S rRNA gene (catalog number - PPM57735E) was used as the housekeeping gene for normalization. For each primer, the amount of template providing maximum efficiency without inhibiting the PCR reaction was determined during initial optimization experiments. For all experiments, dissociation curves were generated to ensure a single peak was obtained at the right melting temperature without non-specific amplicons. The fold change in gene expression was determined by the $\Delta\Delta C_t$ method using the threshold cycle (C_t) value for the housekeeping gene and the respective target gene of interest in each sample.

Western blotting

Brain tissue and microglial cell lysates were prepared using modified RIPA buffer and were normalized for equal amounts of protein using the Bradford protein assay kit. Equal amounts of protein (12 to 25 μg for cell lysates and 30-40 μg for tissue lysates) were loaded

for each sample and separated on either 12% or 15% SDS-PAGE gels depending on the molecular weight of the target protein. After separation, proteins were transferred to a nitrocellulose membrane and the nonspecific binding sites were blocked for 1 h using a blocking buffer specifically formulated for fluorescent Western blotting (Rockland Immunochemicals). Membranes were then probed with the respective primary antibodies for 3 h at room temperature or overnight at 4°C. After incubation, the membranes were washed 7 times with PBS containing 0.05% Tween 20, and then Secondary IR-680-conjugated anti-mouse (1:10,000, goat anti-mouse, Molecular Probes) and IR-800 conjugated anti rabbit (1:10,000, goat anti-rabbit, Rockland) were used for antibody detection with the Odyssey IR imaging system (LiCor). Membranes were visualized on the Odyssey infrared imaging system. Antibodies for β-actin and Tubulin were used as loading controls.

Co-immunoprecipitation studies

We adopted an immunoprecipitation (IP) protocol with slight modifications from Gao and colleagues (Gao et al., 2011). Cell lysates were prepared in TNE buffer (10 mM Tris-HCl at pH 7.5, 1% Nonidet P-40, 0.15 M NaCl, 1 mM EDTA, and 1:100 protease inhibitor cocktail) and centrifuged at 17,400g for 40 min at 4°C. The supernatant protein concentration was measured and normalized between samples. Approximately 50 µL of the sample containing 20 µg protein was used as input. For immunoprecipitation analysis, 1 mg of protein in 400 µL TNE buffer was used. Next, 10 µL (2 µg) of Fyn rabbit polyclonal antibody was added to the lysates, and the samples were set on an orbital shaker overnight at 4°C. The next day, protein G Sepharose beads were spun down at 17,400g for 5 min and the ethanol supernatant was replaced with an equal volume of the lysis buffer. The Protein G

Sepharose slurry was washed once and 50 µL was added to each sample. The samples were set on an orbital shaker overnight at 4°C. Protein G beads were collected by centrifugation at 2000g for 5 min and were washed four times with TNE buffer. The bound proteins were eluted by boiling in 2X protein-loading dye for 5 min. Immunoblots were performed on 12 % SDS-PAGE gels as described for Western blotting.

Nuclear and cytoplasmic fractionation

Nuclear and cytoplasmic fractions were performed using the NE-PER Kit (Thermo Scientific) as previously described (Jin et al., 2011a; Jin et al., 2014). Briefly, 5 X 10^6 cells were treated with LPS or TNFα for 15 min. CER1 reagent (200 µL) was used for each sample to extract the cytoplasmic fraction, and 50 µL of NER reagent was used to extract the nuclear fraction. Tubulin or β-actin was used as a cytosolic fraction marker. Lamin B was used as a nuclear fraction marker.

Fyn kinase assays

Cell pellets were washed with ice-cold PBS and resuspended in lysis buffer (25 mM HEPES at pH 7.5, 20 mM β-glycerophosphate, 0.1 mM sodium orthovanadate, 0.1% Triton X-100, 0.3 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM dithiothreitol, 10 mM NaF, and 4 µg/mL each of aprotinin and leupeptin) (Kaul et al., 2005). Next, 50 µg of crude protein was incubated with 150 mM Fyn kinase substrate (Biomol), 100 mCi of [γ-^{32}P] ATP, Src-Mn-ATP cocktail and Src reaction buffer (Millipore) for 10 min at 30°C with agitation. To bring about precipitation the Fyn kinase substrate peptide, 20 mL of 40% trichloroacetic acid was added. 25 µL of the mixture was then spotted onto a P81 phosphocellulose square, and 5

min after spotting, the squares were washed five times in 0.75% phosphoric acid in PBS with a final wash step in acetone. The squares were transferred into a scintillation vial and the CPMA counts were read in a liquid scintillation system after adding 5 mL of scintillation cocktail to each vial.

PKCδ kinase assays

PKCδ IP kinase activity assays were performed as described previously (Anantharam et al., 2002; Latchoumycandane et al., 2011; Harischandra et al., 2014) with some modifications for microglial cells. Briefly, primary microglial cells were collected after treatments, washed in ice-cold PBS and resuspended in a mild RIPA lysis buffer containing protease and phosphatase inhibitor cocktail (Pierce Biotechnology). The cells were placed on ice for 20 min to allow for complete lysis and then centrifuged at 16,200g for 45 min. The supernatant protein concentration was determined using the Bradford protein assay kit. Samples were normalized to a uniform total protein concentration of 2 μg/mL, and then 200 μg of total protein in a 250 μL reaction volume was immunoprecipitated overnight at 4°C using 5 μg of PKCδ antibody. The next day, protein-A agarose beads (Sigma-Aldrich) were incubated for 1 h at room temperature. The protein A-bound antibody complexes were collected and washed 3 times in 2X kinase assay buffer (40 mM Tris, pH 7.4, 20 mM $MgCl_2$, 20 μM ATP, and 2.5 mM $CaCl_2$), and then resuspended in the same buffer. The kinase reaction was started by adding 40 μL of the reaction buffer containing 0.4 mg of histone H1, 50 μg/mL phosphatidylserine, 4 μM dioleoylglycerol, and 10 μCi of [γ-^{32}P] ATP at 3000 Ci/mM to the immunoprecipitated samples. The samples were then incubated for 10 min at 30°C and the kinase reaction was stopped by adding 2X SDS loading buffer and boiling for 5

min. Proteins were separated on a 15% SDS-PAGE gel and the phosphorylated histone bands were imaged using a Fujifilm FLA 5000 imager. Image analysis and band quantification were performed using ImageJ.

Nitric oxide detection

Nitric oxide production by primary microglia was measured indirectly by quantification of nitrite in the supernatant using the Griess reagent (Sigma Aldrich). Microglia were plated in poly-D-lysine-coated 96-well plates at 1 x 10^5 cells/well. Cells were treated with 100 ng/mL of LPS for 24 h and after 100 µL of supernatant was collected from each well, an equal volume of the Griess reagent was added. The samples were incubated on a plate shaker at room temperature for 15 min until a stable color was obtained. The absorbance at 540 nm was measured using a Synergy 2 multi-mode microplate reader (BioTek Instruments) and the nitrite concentration was determined from a sodium nitrite standard curve.

Multiplex cytokine Luminex immunoassays

Primary microglia obtained from wild type, PKCδ$^{-/-}$ and Fyn$^{-/-}$ mice were seeded in poly-D-lysine-coated 96-well plates at 1 x 10^5 cells/well. The cells were treated for 24 h with 100-200 ng/mL LPS or 10 ng/mL TNFα. After treatment, 50 μL of supernatant from each well was collected and frozen at -80°C. The levels of cytokines and chemokines in the supernatants were determined using the Luminex bead-based immunoassay platform (Vignali 2000) and pre-validated multiplex kits (Milliplex mouse cytokine panel – Millipore) according to the manufacturer's instructions.

DAB immunostaining and grading of microglial morphology

Iba-1 diaminobenzidine (DAB) immunostaining was performed on striatal and substantia nigral sections as described previously (Ghosh et al., 2010). Briefly, mice were perfused with 4% PFA and brains were post-fixed with PFA for 48 h before storage in 30% sucrose. Fixed brains were embedded in O.C.T compound (Tissue-Tek) and stored frozen at -80°C until the frozen blocks were sliced into 30-μm coronal sections using a cryostat. Sections were probed with the primary antibodies overnight at 4°C and then incubated with biotinylated anti-rabbit secondary antibody. The sections were then treated with Avidin peroxidase (Vectastain ABC Elite kit). The DAB reagent was used for producing the brown colored stain. Grading of microglial morphology was performed as described elsewhere (Lastres-Becker et al., 2012). For microglial grading, images were sharpened in ImageJ so the morphology could be more clearly visualized. The cell counter function in the 'Analyze' plugin was used to count the number of Type A, B, C and D microglia in the ventral midbrain sections.

Data analysis

Data analysis was performed using Prism 4.0 (GraphPad Software, San Diego, CA). The data was initially analyzed using one-way ANOVA and Bonferroni's post-test to compare the means of treatment groups. Differences of $p<0.05$ were considered statistically significant. Student's t-test was used when comparing two groups.

Results

Fyn and PKCδ are differentially expressed in primary astrocytes and microglia

Primary mouse microglia were prepared as described in our recent publication using a magnetic separation method, which enables us to obtain a high-yield pure fraction of microglia from mixed glial cultures (Gordon et al., 2011). Iba-1 and GFAP immunocytochemistry confirmed that the microglial fraction obtained after magnetic separation was devoid of astrocytes (Fig. 1A). Quantification of Hoechst co-localized Iba-1-positive microglia and GFAP-positive astrocytes using the ImageJ plugin JACoP revealed a microglial population that was ~97% pure post-separation (Figs. 1B-C). Immunoblotting analysis revealed that microglia-enriched fractions expressed significantly more Fyn (60 kDa) and PKCδ (76 kDa) than did astrocyte-enriched (microglia-depleted) fractions (Figs. 1D-E). The differential expression of both Fyn and PKCδ in microglia compared to astrocytes prompted us to study the roles these proteins may play in microglial pro-inflammatory signaling.

Fyn kinase is rapidly activated in microglial cells and in the ventral midbrain following inflammogen stimulation

Our initial experiment to determine whether the non-receptor tyrosine kinase Fyn plays a role in regulating neuroinflammatory responses in PD was carried out in BV2 microglial cells, which are widely used *in-vitro* models of neuroinflammation (Henn et al., 2009; Gao et al., 2011; Kim et al., 2013b). We treated BV2 cells with 1 μg/mL LPS for 10-60 min and measured Fyn activity using an *in-vitro* kinase assay (Saminathan et al., 2011). A kinase reaction mixture containing ^{32}P-ATP and a Fyn-specific peptide substrate were added

to whole cell lysates. LPS stimulation of BV2 microglia rapidly induced Fyn activity as early as 10 min post-LPS stimulation (Fig. 2A), and maximal activity was attained 30 min post-LPS stimulation. In addition to the Fyn kinase activity assay, we also determined the phosphorylation status of the Y416 residue in its activation loop domain, by utilizing the phospho Y416 Src family kinase (p-Y416 SFK) antibody, which recognizes activated Src family kinases. This antibody has been used extensively to demonstrate Fyn kinase activation (Larson et al.; Um et al.; Wake et al., 2011; Kouadir et al., 2012). Our immunoblotting analysis of LPS-treated BV2 lysates using the p-Y416 SFK antibody revealed LPS-induced SFK activation (Fig. 2B). To further confirm inflammogen-induced Fyn activation in BV2 microglia, we transiently transfected BV2 cells with FLAG-tagged WT-Fyn and Y417A-Fyn (activation loop mutant) constructs. We then performed immunoprecipitation studies in LPS-treated transfected BV2 cells. We pulled down Fyn from FLAG-tagged WT-Fyn and Y417A-Fyn transfected, LPS-treated BV2 cells and immunoblotted for p-Y416 SFK levels. A strong p-Y416 SFK signal was detected in the LPS-treated WT-Fyn-FLAG-transfected cells, but not in the LPS-treated Y417A-Fyn-transfected cells (Figs. 2C-D).

Next, we extended our studies to primary microglia derived from both wild-type and Fyn-deficient ($Fyn^{-/-}$) mice. These were treated with 200 ng/mL LPS for 0-30 min. In line with the analyses of BV2 cells, stimulation of the primary microglia from $Fyn^{+/+}$ mice rapidly increased the levels of p-Y416 SFK (Fig. 2E). Interestingly, p-Y416 SFK was not detected in LPS-treated $Fyn^{-/-}$ microglia, suggesting that LPS preferentially induces Fyn phosphorylation in microglia over other Src family kinases. Treatment of wild-type and Fyn-deficient microglia with TNFα also yielded similar results. Both 10 ng/mL and 30 ng/mL TNFα treatments induced similar levels of p-Y416 SFK in wild-type, but not in Fyn-deficient

microglia (Fig. 2F). Pretreatment of wild-type microglia with either the TLR (Toll-like Receptor) antagonist IAXO-101 or the TNFα signaling antagonist Etanercept significantly attenuated both LPS- and TNFα-mediated Fyn activation, respectively (Fig. 2G). We also examined subcellular localization of activated Fyn following LPS stimulation. The Iba-1/p-Y416 SFK double-immunocytochemical analysis showed that LPS treatment dramatically increased p-Y416 Fyn levels in WT primary microglia (Fig. 2H). Active Fyn seems to be preferentially expressed at the periphery of the microglia, possibly allowing it to become activated quickly in response to a pro-inflammatory stimulus. Additionally, activated Fyn was also found in the nucleus of LPS-treated primary microglia. Next, we wanted to confirm that LPS-treatment would activate Fyn in the substantia nigra of mice. Knowing that a single intraperitoneal LPS injection elicits microglial cell activation in the substantia nigra (Qin et al., 2007), we challenged $Fyn^{+/+}$ and $Fyn^{-/-}$ mice with 5 mg/kg LPS or sterile PBS vehicle intraperitoneally for 3 h. Immunoblot analysis of ventral midbrain lysates revealed that LPS significantly increased p-Y416 SFK levels in wild-type compared to saline control, whereas LPS failed to increase p-Y416 SFK levels in the $Fyn^{-/-}$ ventral midbrain lysates.(Fig. 2I). These studies indicate that stimulating microglia with inflammatory stimuli rapidly activates Fyn kinase in both cell culture and animal models of neuroinflammation.

Fyn contributes to LPS- and TNFα-induced tyrosine phosphorylation and activation of PKCδ in primary microglia

It has been shown that Src family kinases, including Fyn, phosphorylate PKCδ at residue Y311 in platelets and in immortalized dopaminergic neuronal cells (Steinberg, 2004; Saminathan et al., 2011). Therefore, we investigated if Fyn-PKCd signaling regulates

microglial pro-inflammatory responses using primary microglia cultures from wild-type, Fyn$^{-/-}$ and PKCδ$^{-/-}$ mice. Stimulation with LPS induced a rapid and time-dependent increase in p-Y311 PKCδ in wild-type microglia. In contrast, LPS failed to increase Y311 phosphorylation of PKCδ in the Fyn$^{-/-}$ microglia (Figs. 3A-B). Similarly, TNFα stimulation of microglia also increased PKCδ Y311 phosphorylation in wild-type, but not in Fyn-deficient primary microglia (Figs. 3C-D). As expected, immunoblot analysis did not detect any LPS-induced phosphorylation of Y311 PKCδ in PKCδ$^{-/-}$ microglia. To confirm further that Fyn mediates the activation of PKCδ in activated microglia, we measured PKCδ kinase activity in wild-type and Fyn$^{-/-}$ microglia. An *in-vitro* PKCδ kinase assay showed that LPS rapidly increased PKCδ kinase activity in wild-type microglia; however, LPS-induced PKCδ kinase activity was significantly less in Fyn$^{-/-}$ microglia (Fig. 3E). To further confirm the Fyn-PKCδ interaction, we performed co-immunoprecipitation studies in BV2 cells transfected with the WT-Fyn-FLAG construct. As shown in Figs. 3F-G from the co-immunoprecipitation results, co-IP analysis of WT-Fyn-FLAG transfected lysates revealed that Fyn and PKCδ interact during LPS stimulation. Taken together with the PKCδ kinase activity results, these data reveal that Fyn kinase mediates LPS- and TNFα-induced activation of PKCδ in primary microglia.

The Fyn-PKCδ signaling axis mediates MAP kinase activation in microglial cells

We next examined whether the Fyn-PKCδ signaling axis plays a role in mediating activation of the MAP kinase pathway, a key hallmark of neuroinflammatory signaling in microglia. MAP kinases are important regulators of pro-inflammatory cytokine synthesis in microglial cells (Koistinaho and Koistinaho, 2002; Tansey and Goldberg, 2010). For this

purpose, we treated wild-type, Fyn$^{-/-}$, and PKCδ$^{-/-}$ microglia with LPS for 15, 30 and 45 min each and determined MAPK activation. The LPS treatment significantly increased the phosphorylation of p38 and p44/42 (p-ERK) kinases in wild-type microglia (Figs. 4A-B), with LPS-induced phosphorylation peaking at 15 min and decreasing thereafter. In contrast, LPS-induced phosphorylation of p38 and p44/42 (p-ERK) was significantly reduced in Fyn$^{-/-}$ and PKCd$^{-/-}$ primary microglia. Similar results were obtained with TNFα treatment of wild-type, Fyn$^{-/-}$ and PKCδ$^{-/-}$ microglia (Figs. 4C-D). These results suggest that Fyn-PKCδ signaling is an important upstream regulator of MAP kinases in microglia during both LPS and TNFα stimulation.

Fyn contributes to inflammogen-mediated NFκB pathway activation in microglial cells

Pro-inflammatory signaling mediated by both LPS and TNFα converges at the NFκB pathway. Activation of NFκB signaling during the pro-inflammatory process is characterized by the phosphorylation and subsequent degradation of the inhibitory protein IκBα, after which the NFκB p65-p50 heterodimer enters the nucleus, leading to the transcription of various pro-inflammatory genes (Hayden and Ghosh, 2004). To elucidate whether the Fyn mediates the nuclear translocation and activation of NFκB signaling in activated microglia, primary microglia obtained from wild-type and Fyn$^{-/-}$ microglia were treated with LPS for 15-45 min. Whole cell lysates were prepared and probed for IκBα. LPS treatment induced a greater degradation of IκBα in wild-type microglia than in Fyn$^{-/-}$ microglia at the 15 minute time point, followed by the resynthesis of IκBα 30 and 45 minutes post stimulation in the WT cells. Resynthesis of IκBα in Fyn$^{-/-}$ microglia was almost completely abrogated, indicating diminished NFκB activation (Figs. 5A-B). Next, we investigated the role of Fyn in

the nuclear translocation of the p65 component of the NFκB complex in response to LPS and TNFα treatments. Nuclear and cytoplasmic fractions were prepared from WT and Fyn$^{-/-}$ microglia treated with LPS or TNFα for 15 min before being assessed for p65 content. Immunoblotting revealed lesser nuclear translocation of p65 in LPS- and TNFα-treated Fyn$^{-/-}$ microglia than in wild-type microglia (Figs. 5C-D). These results were further supported by Iba-1/p65 double-immunocytochemistry showing strong LPS-induced nuclear translocation of p65 in wild-type, but not in the Fyn$^{-/-}$ microglia (Fig. 5E). Together, these results clearly suggest that Fyn kinase regulates NFκB activation in microglial cells.

LPS- or TNFα-induced pro-inflammatory cytokine production is suppressed in Fyn/ PKCδ deficient microglia

Next, we determined whether Fyn-PKCδ signaling axis regulates microglia-mediated pro-inflammatory mediator production. After treating wild-type, PKCδ$^{-/-}$ and Fyn$^{-/-}$ microglial cultures with LPS or TNFα, we utilized multiplexed immunoassays to quantify inflammogen-induced cytokine secretion. We observed significant production of the cytokines IL-6, IL-12p70, and TNFα from wild-type microglia treated with LPS (Fig. 6A). However, the production of these cytokines was significantly dampened in Fyn- and PKCδ-deficient microglia, providing evidence for the hypothesis that attenuated pro-inflammatory signaling in Fyn$^{-/-}$ and PKCδ$^{-/-}$ microglia suppresses pro-inflammatory mediator production. When we knocked down Fyn expression in wild-type primary microglia by Fyn-specific siRNA (Fig. 6B), diminished amounts of the pro-inflammatory cytokines IL-6 and TNFα were produced in response to LPS treatment (Fig. 6C). Next, treatment of wild-type and Fyn$^{-/-}$ microglia with TNFα yielded similar results, with the Fyn$^{-/-}$ microglia showing reduced IL-

6 and TNFα production (Fig. 6D). Western blot analysis also demonstrated that Fyn-deficient microglia produced less TNFα relative to wild-type microglia (Fig. 6E). To further confirm the role of Fyn in pro-inflammatory cytokine production, we expressed Fyn wild-type (WT-Fyn-FLAG) or activation loop mutant (kinase deficient Fyn kinase, Y417A Fyn-FLAG) in BV2 microglial cells (Fig. 6F). Following the transfection, BV2 cells transfected WT-Fyn-FLAG, Y417A Fyn-FLAG or empty vector constructs were treated with 1 μg/mL LPS for 24 h. Luminex immunoassay of cell supernatants revealed that overexpressing wild-type Fyn augmented pro-inflammatory cytokine release, whereas overexpressing the inactive Y417A Fyn mutant suppressed the production of IL-6 and IL-12 (Figs. 6F-G).

Fyn/PKCδ regulates the induction of neuroinflammatory markers iNOS and gp91^{phox} in microglia during LPS stimulation

We further assessed whether Fyn alters the induction of iNOS and gp91^{phox}, which are key pro-inflammatory responses of microglial activation following LPS treatment. Treatment with LPS induced a stronger nitrite response from wild-type microglia than from Fyn$^{-/-}$ microglia (Fig. 7A). This was further confirmed by immunostaining and immunoblotting for iNOS, the enzyme that mediates nitrite production. There was a greater induction of iNOS in Fyn wild-type microglia relative to Fyn$^{-/-}$ microglia (Figs. 7B-D). We also determined the expression of other key neuroinflammatory markers, including gp91^{phox} and Iba-1, in response to LPS stimulation. We, as well as other groups, have previously shown increased expression of the NADPH oxidase component gp91^{phox} and Iba-1 following LPS stimulation of primary microglia (Gao et al., 2011; Gordon et al., 2011)**.** Western blot analysis revealed that LPS increased expression of both gp91^{phox} and Iba-1 in wild-type, but not in Fyn$^{-/-}$ or

PKCδ$^{-/-}$ microglia (Figs. 7E-F). Collectively, these data indicate that Fyn-PKCδ signaling plays a major pro-inflammatory role in microglial cells.

Fyn$^{-/-}$ and PKCδ$^{-/-}$ mice are resistant to LPS- and MPTP-induced neuroinflammatory responses

To extend our findings from isolated primary microglia to *in vivo* animal models of neuroinflammation, we first used the LPS model, which has previously been used to evoke neuroinflammatory responses *in vivo* (Choi et al., 2007; Qin et al., 2007). Wild-type (PKCδ$^{+/+}$ and Fyn$^{+/+}$), PKCδ$^{-/-}$ and Fyn$^{-/-}$ mice were injected with 5 mg/kg LPS or PBS and were sacrificed 3 h later. Striatal mRNA contents of the pro-inflammatory cytokines pro-IL-1β and TNFα were determined by qRT-PCR. The levels of cytokine induction were almost identical in both wild-type groups, and we thus pooled the results. Systemic LPS administration strongly increased the levels of pro-IL-1β and TNFα transcripts in wild-type striata, but not in Fyn$^{-/-}$ and PKCδ$^{-/-}$ striata (Fig. 8A). To further establish the role of Fyn relevant to PD-associated neuroinflammation, we used the well-known Parkinsonian toxicant MPTP. We subjected wild-type and Fyn$^{-/-}$ mice to an acute MPTP regimen (4 × 18 mg/kg, 2 h apart) and collected their brains for immunohistochemical analysis 24 h after the final MPTP injection. This acute MPTP model has been widely adopted for studying the neuroinflammatory response in the nigrostriatal pathway because maximal microglial activation occurs 24-48 h after the MPTP challenge (Wu et al., 2002; Wu et al., 2003; Sriram et al., 2006; Hirsch and Hunot, 2009). Following the MPTP challenge, successive 30-μm ventral midbrain sections from Fyn$^{+/+}$ and Fyn$^{-/-}$ mice were stained for the microglial marker Iba-1, and then microglial morphology was quantified using a recently well-established

morphometric rating scale as discussed by others (Lastres-Becker et al., 2012). Representations of Type A-D microglial phenotype are provided in Fig. 8B. Treating $Fyn^{+/+}$ mice with the acute MPTP regimen increased Iba-1 expression and discernibly shifted microglial morphology from its typical ramified state to its more amoeboid, activated morphology. After MPTP administration, significantly fewer Type A and more Type B and C microglia were observed in the $Fyn^{+/+}$ SN, but this shift in microglial morphology was not apparent in the $Fyn^{-/-}$ mice (Figs. 8C-D). We also determined the induction of the NADPH oxidase component $gp91^{phox}$ in MPTP animal model of neuroinflammation. Immunoblotting analysis revealed that MPTP increased expression of $gp91^{phox}$ in WT but not in $Fyn^{-/-}$ ventral midbrain tissues (Figs. 8E-F). Overall, these results confirm that our *in-vitro* data translate well to animal models of neuroinflammation.

$Fyn^{-/-}$ and $PKC\delta^{-/-}$ mice are protected against 6-OHDA-induced nigrostriatal dopaminergic neuronal deficits and microgliosis

The 6-OHDA mouse model has recently been shown to elicit a neuroinflammatory response and neurodegeneration in the nigrostriatal dopaminergic system (Stott and Barker, 2014). While studying the role of Fyn in dopamine D1 receptor agonist-induced redistribution of NMDA receptor subunits, it was serendipitously discovered that $Fyn^{-/-}$ mice were remarkably intransigent to 6-OHDA-induced behavioral deficits and striatal TH loss (Dunah et al., 2004). $Fyn^{+/+}$ and $Fyn^{-/-}$ mice, injected unilaterally with 6-OHDA (Fig. 9B) were sacrificed 9 days post-treatment, since mice at this treatment stage concurrently exhibit fewer striatal dopaminergic terminals, significantly fewer TH-positive cells in the SN, and microgliosis within the SN (Stott and Barker, 2014). $Fyn^{-/-}$ mice were more resistant to 6-

OHDA-induced striatal nerve terminal degeneration relative to $Fyn^{+/+}$ mice (Figs. 9A and C). We also show in our studies that 6-OHDA induced massive gliosis coupled with dopaminergic neuronal loss (Fig. 9D). However, $Fyn^{-/-}$ mice show both greater survival of nigral dopaminergic neurons and a diminished neuroinflammatory microglial response.

In the next set of *in vivo* experiments, we checked whether $PKC\delta^{-/-}$ mice were also resistant to 6-OHDA-induced nigral microgliosis and dopaminergic neuronal loss. $PKC\delta^{+/+}$ and $PKC\delta^{-/-}$ mice were injected unilaterally with 6-OHDA for 9 days, and DAB-TH immunostaining was performed on striatal sections as described above (Fig. 10B). Similar to $Fyn^{-/-}$ mice, $PKC\delta^{-/-}$ mice showed reduced striatal TH loss following 6-OHDA treatment (Figs. 10A and C). We also assessed nigral microgliosis by double-staining ventral midbrain sections for TH and Iba-1. As shown in Fig. 10D-E, $PKC\delta^{+/+}$ mice showed less TH-positive neuronal staining in the SN along with significantly more microgliosis on the ipsilateral side than on the contralateral side; however, the $PKC\delta^{-/-}$ mice showed a marked resistance to 6-OHDA-induced nigral TH loss as well as microgliosis. Thus, results from both Fyn and PKCδ knockout models of 6-OHDA neurotoxicity confirm the role of the Fyn-PKCδ signaling axis in a neuroinflammatory response in the nigrostriatal dopaminergic system.

Diminished 6-OHDA-induced glial neuronal contact formation in the $Fyn^{-/-}$ substantia nigra

Recently, it was demonstrated that treating mice with MPTP rapidly increased the number of microglial-neuronal appositions, termed gliapses (Barcia et al., 2012). These contacts preceded neuronal phagocytosis by the microglia. Similar appositions between microglia and dopaminergic neurons were demonstrated in the 6-OHDA model, with

evidence suggesting that microglial cells actually phagocytized neurons (Virgone-Carlotta et al., 2013), which has been postulated to occur if the neurons are dysfunctional. Our confocal high magnification Z stack image analysis (Imaris software) revealed a sharply increased number of microglial-neuronal contacts formed in the $Fyn^{+/+}$ SN post-6-OHDA treatment as indicated by arrowheads (Figs. 11A, B and F). The 3-D reconstructions of the respective stacks demonstrating contacts between dopaminergic neurons and microglia are shown adjacent to the original images (Fig. 11B). The number of gliapses per SN dopaminergic neuron was dramatically reduced in the 6-OHDA-injected $Fyn^{-/-}$ mice (Figs. 11C, D, and F). Typical contacts formed between microglial processes and dopaminergic neuronal cell bodies (termed Process-Body, or Pr-B contacts), and those formed between the microglial cell body and the dopaminergic neuronal cell body (Body-Body, or B-B contacts), are shown in Fig. 11E. Image analysis involving optical slices through the Z plane allowed us to both easily count gliapses and visualize actual engulfment events. Representative (Figs. 11B and D) gliapses between a dopaminergic neuron and a microglial cell in the SN of 6-OHDA-injected $Fyn^{+/+}$ and $Fyn^{-/-}$ mice reveal a conspicuous reduction in the number of gliapses per neuron. Collectively, our confocal imaging results demonstrate Fyn plays a key role in activation of microglial morphological changes *in vivo* during inflammatory insults in nigrostriatal system.

Prolonged inflammogen stimulation effects Fyn induction upon microglial activation

Thus far, our results demonstrated that short-term treatment of microglial cells with LPS and TNFα brings about an increase in Fyn activity, but not its expression. Strikingly, we discovered that prolonged treatment (12-24 h) of microglia with LPS or TNFα actually resulted in increased Fyn expression, evidenced by Western blot and immunocytochemistry

(Fig. 12A-C). To confirm whether this is really due to induction of Fyn protein or increased protein stability, we performed qRT-PCR for Fyn mRNA expression in control and LPS-treated microglial cells. The result showed that treatment of microglia with LPS for 12 h brought about an increase in Fyn transcript levels (Fig. 12D). We also evaluated the effects of prolonged LPS treatment on Fyn promoter activity. For this, we transiently transfected primary microglia with a dual-luciferase Fyn reporter construct containing the 3.1 kb Fyn promoter fragment. LPS treatment significantly increased Fyn promoter activity (Fig. 12E), indicating strongly that Fyn is transcriptionally induced in microglial cells post-prolonged inflammogen administration. To further examine whether LPS upregulates Fyn mRNA expression, we injected wild-type mice with a single dose of LPS (5 mg/kg, i.p.) and evaluated the Fyn mRNA expression by qRT-PCR analysis. As shown in Fig. 12F, administration of LPS also induced Fyn transcript levels in the striatum. Together, these data suggest that prolonged LPS exposure induces Fyn gene upregulation in microglia, indicating that Fyn may have a sustained role in chronic neuroinflammatory processes.

Discussion

Evidence from experimental models and human PD post-mortem studies strongly implicates the microglial-mediated inflammatory response as a major driver in the progression of PD; however, the key upstream cell signaling mechanisms that govern the neuroinflammatory processes have yet to be elucidated. Our results obtained from both cell culture and animal models provide novel insight into the role of the Fyn-PKCδ signaling cascade in regulating microglia-mediated neuroinflammation as related to PD pathogenesis. We have demonstrated dual regulation of pro-neuroinflammatory responses in microglia

involving post-translational tyrosine phosphorylation of Fyn at its activation loop during the early stages of an inflammatory insult as well as transcriptional upregulation of Fyn upon prolonged exposure to pro-inflammatory stimuli. We have also showed that Fyn serves as a major upstream signaling molecule that works in concert with PKCδ to influence MAP kinase downstream and the NFκB pro-inflammatory cascade. Collectively, our study provides novel and significant insight into the pro-inflammatory function of Fyn-PKCδ signaling in PD models, and to the best of our knowledge, we are the first to discern this key signaling cascade that is relevant to microglia-mediated neuroinflammation in the nigrostriatal dopaminergic system.

We demonstrate that both the tyrosine kinase Fyn and the serine/threonine kinase PKCδ are differentially expressed in microglia and astrocytes (Fig. 1). No prior comparative data are available on Fyn and PKCδ expression in primary microglia. Although the roles of Src family kinases in TLR signaling are being identified, most studies have used peripheral immune and non-immune cells to determine Src kinase signaling. For example, multiple Src family kinases were activated by LPS in human lung microvascular endothelial cells (Gong et al., 2008). The activation of Src kinases mediated by TLR agonists depends on CD14, TLR2 and TLR4 (Reed-Geaghan et al., 2009), and Fyn has been shown to be associated with TLR2 in TLR2-overexpressing HEK293 cells (Finberg et al., 2012). Peritoneal macrophages have often been used as putative substitutes for brain microglia; Fyn contributes to CD36-mediated signaling responses upon $A\beta_{1-42}$ stimulation of macrophages (Moore et al., 2002). Of note, the authors reported unaltered LPS-induced MAP kinase activation in $Fyn^{-/-}$ peritoneal macrophages when compared to WT macrophages. These apparent discrepancies may be attributed to the inherent differences between the microglial and macrophage gene

expression profiles (Hickman et al., 2013). Many studies have used the p-Y416 src family kinase antibody as a direct indicator of Fyn activation, without using immunoprecipitation or $Fyn^{-/-}$ primary microglia as confirmatory tools to establish Fyn activation. In the present study, we demonstrate that Fyn is rapidly activated in primary microglia within 15-30 min of exposure to inflammogens (Figs. 2A-F). Immunoprecipitation studies and experiments with $Fyn^{-/-}$ microglia clearly confirmed that Fyn kinase is specifically activated during LPS and TNFα stimulation. LPS and TNFα activate microglia/macrophages via TLR4 and TNFα Receptor 1 (TNFR1) signaling, respectively (Olson and Miller, 2004; Parameswaran and Patial, 2010). Importantly, our study reveals that Fyn is a common signaling conduit in both TLR- and TNFR1-mediated signaling, since the TLR antagonist IAXO-101 and the TNFα signaling antagonist Etanercept attenuated Fyn activation (Fig. 2G). Immunocytochemistry analysis revealed that activated Fyn primarily localized to the microglial cell membrane. Although the functional relevance of this localization is not presently known, it is possible that movement of activated Fyn to the microglial membrane may regulate cell migration and cytokine release. Our results with the LPS mouse model provide *in vivo* evidence for rapid Fyn activation in the ventral midbrain region during inflammatory insults (Fig. 2I).

Our group has previously shown that PKCδ kinase proteolytic activation promotes oxidative stress-induced pro-apoptotic signaling pathways in dopaminergic neuronal cells (Kaul et al., 2003; Zhang et al., 2007; Jin et al., 2011a; Jin et al., 2011b). Recently, it was demonstrated that PKCδ is proteolytically cleaved by caspase-3 in LPS-treated BV2 cells (Burguillos et al., 2011). In the present study, we demonstrate that activated Fyn associates with PKCδ to phosphorylate the Y311 site, resulting in increased PKCδ kinase activity (Fig.

3). To the best of our knowledge, we are the first group to show the assembly of the Fyn-PKCδ signaling complex in microglial cells during pro-inflammatory conditions.

MAP kinase activation is necessary for cytokine production in various immune cell types, including microglia (El Benna et al., 1996; Koistinaho and Koistinaho, 2002). We demonstrate that Fyn-PKCδ signaling contributes to MAP kinase phosphorylation during microglial activation. Both LPS and TNFα stimulations rapidly activated the p38 and p-ERK MAP kinases in WT, but to a significantly lesser extent in the $Fyn^{-/-}$ and $PKC\delta^{-/-}$ microglia (Fig. 4), indicating that Fyn-PKCδ signaling lies upstream of MAP kinase in microglia. Given that p38 is a prominent MAP kinase associated with the inflammatory cascade, our results suggest that Fyn and PKCδ are key upstream regulators of the pro-inflammatory function of this kinase. The downstream events of MAP kinase activation include NFκB signaling, which plays a cardinal role in eliciting pro-inflammatory responses in microglia. Selective inhibition of NFκB signaling has also proved beneficial *in-vitro* as well as in an experimental mouse model of PD (Ghosh et al., 2007). We show here that IκBα degradation and p65-NFκB nuclear translocation were diminished in $Fyn^{-/-}$ microglia stimulated with LPS or TNFα (Fig. 5), lending credence to the hypothesis that upstream Fyn signaling contributes to NFκB pathway activation in microglia. To our knowledge, the role of Fyn signaling in NFκB-mediated pro-inflammatory signaling in microglia has never been explored. Fyn has been shown to contribute to anaphylaxis inducer DNP_{36}-HSA mediated NFκB activation in Mast cells (Gomez et al., 2005b). More recently, Fyn was shown to mediate the nuclear translocation of p65-NFκB downstream of NKG2D and CD137 activation in natural killer cells, utilizing a signaling mechanism dependent on ADAP (Rajasekaran et al., 2013). This

signaling pathway is almost certainly distinct from the Fyn-dependent microglial activation pathway, evidenced by the fact that ADAP$^{-/-}$ microglia display unaltered pro-inflammatory responses (Engelmann et al., 2013).

Classical activation of microglia by TLR and TNFR1 agonists produces pro-inflammatory cytokines and chemokines, which mediate the downstream effects of microglial activation. Recently, we showed that TNFα directly induces dopaminergic neuronal apoptosis (Gordon et al., 2012). In our present study, the induction of the cytokines IL-6, IL-12 and TNFα was all diminished in Fyn$^{-/-}$ and PKCδ$^{-/-}$ microglia in comparison to wild-type microglia (Fig. 6A). Consistently, genetic knockdown of Fyn via siRNA also resulted in diminished LPS-induced pro-inflammatory cytokine secretion (Figs. 6B-C). TNFα-mediated production of IL-6 and TNFα was also diminished in Fyn deficient microglia (Figs. 6D-E). Overexpressing the Fyn Y417A activation loop kinase deficient mutant construct in BV2 microglial cells also diminished LPS-stimulated cytokine production, implicating that the phosphorylation of tyrosine 417 is critical to pro-inflammatory function of Fyn (Figs. 6F-G). Furthermore, we showed that the LPS-induced expression of iNOS and secretion of nitrite were significantly attenuated in the Fyn$^{-/-}$ microglia (Figs. 7A-D). We and several other groups have reported increased expression of the NADPH oxidase component gp91^{phox}, as well as the microglial marker Iba-1 following pro-inflammatory stimulation of microglia (Gao et al., 2011; Gordon et al., 2011). We demonstrate herein that prolonged LPS stimulation brought about the induction of these neuroinflammatory markers in wild-type, but not in Fyn$^{-/-}$ and PKCδ$^{-/-}$ microglia (Figs. 7E-F).

We extended our *in-vitro* studies to well-characterized animal models of neuroinflammation, wherein a single intraperitoneal injection of LPS in mice increases

TNFα in the brain to levels that remain elevated long after serum TNFα levels have returned to normal (Qin et al., 2007). We utilized this model system to check for LPS-induced striatal pro-inflammatory cytokine induction in wild type, $Fyn^{-/-}$ and $PKC\delta^{-/-}$ mice. Strikingly, a single injection of LPS strongly increased WT striatal TNFα and pro-IL-1β mRNA levels; however, the induction of these cytokines was greatly diminished in $Fyn^{-/-}$ and $PKC\delta^{-/-}$ striata (Fig. 8A). In addition to the LPS model, we also determined the pro-inflammatory role of Fyn in the well-studied acute MPTP model of neuroinflammation. MPTP induced reactive microgliosis and increased $gp91^{phox}$ expression in the nigra of WT mice, but not in $Fyn^{-/-}$ mice (Figs. 8E-F). Interestingly, a quiescent ramified state of microglial morphology was observed in MPTP treated $Fyn^{-/-}$ mice, while more amoeboid activated microglia were noted in Fyn wild-type mice (Figs. 8C-D). In addition to the MPTP model, we further utilized the 6-OHDA-induced selective dopaminergic lesion model to validate that ablating Fyn or PKCδ confers resistance to nigrostriatal dopaminergic degeneration and microgliosis (Figs. 9-10). Taken together, our results indicate that the Fyn-PKCδ signaling axis plays an important role in mediating pro-inflammatory response in both cell culture and animal models of neuroinflammation.

Recent imaging studies have demonstrated the formation of glial-neuronal contacts, called gliapses, formed between dopaminergic neurons and microglia that precede neuron loss in the MPTP model (Barcia et al., 2012; Barcia et al., 2013). To determine whether Fyn plays a role in microglial-dopaminergic neuron contact formation, we adopted the 6-OHDA mouse model. The formation of gliapses was described recently in the 6-OHDA model (Virgone-Carlotta et al., 2013). Our results from high magnification confocal analysis revealed the formation of gliapses was almost completely blocked in 6-OHDA injected $Fyn^{-/-}$

mice (Fig. 11). The reduced number of gliapses correlated well with reduced dopaminergic neuronal loss following 6-OHDA administration to the $Fyn^{-/-}$ mice. Lastly, prolonged stimulation of microglial cells with inflammogens strongly elicited an induction in Fyn kinase expression levels (Fig. 12). The aggregated form of α-synuclein, the primary component of PD-associated Lewy bodies, can activate microglia by utilizing CD36- and TLR2-dependent pathways (Su et al., 2008; Kim et al., 2013a). Studies are underway in our lab to demonstrate the role that Fyn plays in aggregated α-synuclein-induced neuroinflammatory events.

We demonstrate that Fyn activation plays an upstream regulatory role in eliciting pro-inflammatory signaling following both acute and chronic states of microglia stimulation. We arrived at this conclusion based on various lines of experimental evidence from cell culture, primary culture and *in vivo* models utilizing both Fyn and PKCδ knockout mice. Our mechanistic studies revealed that Fyn serves as a major upstream regulator of pro-inflammatory signaling involving PKCδ, MAP kinase and the NFκB pathways. Thus, Fyn could be exploited as a potential signaling node in the development of novel anti-neuroinflammatory drug candidates for treating PD and other related neurodegenerative diseases with associated microglia-mediated pro-inflammatory processes.

References

Anantharam V, Kitazawa M, Wagner J, Kaul S, Kanthasamy AG (2002) Caspase-3-dependent proteolytic cleavage of protein kinase Cdelta is essential for oxidative stress-mediated dopaminergic cell death after exposure to methylcyclopentadienyl manganese

tricarbonyl. The Journal of neuroscience : the official journal of the Society for Neuroscience 22:1738-1751.

Barcia C, Ros CM, Annese V, Carrillo-de Sauvage MA, Ros-Bernal F, Gomez A, Yuste JE, Campuzano CM, de Pablos V, Fernandez-Villalba E, Herrero MT (2012) ROCK/Cdc42-mediated microglial motility and gliapse formation lead to phagocytosis of degenerating dopaminergic neurons in vivo. Sci Rep 2:809.

Barcia C, Ros CM, Ros-Bernal F, Gomez A, Annese V, Carrillo-de Sauvage MA, Yuste JE, Campuzano CM, de Pablos V, Fernandez-Villalba E, Herrero MT (2013) Persistent phagocytic characteristics of microglia in the substantia nigra of long-term Parkinsonian macaques. Journal of neuroimmunology 261:60-66.

Block ML, Zecca L, Hong JS (2007) Microglia-mediated neurotoxicity: uncovering the molecular mechanisms. Nat Rev Neurosci 8:57-69.

Bohnen NI, Frey KA, Studenski S, Kotagal V, Koeppe RA, Constantine GM, Scott PJ, Albin RL, Muller ML (2014) Extra-nigral pathological conditions are common in Parkinson's disease with freezing of gait: An in vivo positron emission tomography study. Movement disorders : official journal of the Movement Disorder Society 29:1118-1124.

Burguillos MA, Deierborg T, Kavanagh E, Persson A, Hajji N, Garcia-Quintanilla A, Cano J, Brundin P, Englund E, Venero JL, Joseph B (2011) Caspase signalling controls microglia activation and neurotoxicity. Nature 472:319-324.

Carson MJ (2002) Microglia as liaisons between the immune and central nervous systems: functional implications for multiple sclerosis. Glia 40:218-231.

Chaudhuri KR, Healy DG, Schapira AH (2006) Non-motor symptoms of Parkinson's disease: diagnosis and management. Lancet Neurol 5:235-245.

Choi HB, Ryu JK, Kim SU, McLarnon JG (2007) Modulation of the purinergic P2X7 receptor attenuates lipopolysaccharide-mediated microglial activation and neuronal damage in inflamed brain. The Journal of neuroscience : the official journal of the Society for Neuroscience 27:4957-4968.

Crotti A, Benner C, Kerman BE, Gosselin D, Lagier-Tourenne C, Zuccato C, Cattaneo E, Gage FH, Cleveland DW, Glass CK (2014) Mutant Huntingtin promotes autonomous microglia activation via myeloid lineage-determining factors. Nature neuroscience 17:513-521.

Dey N, Crosswell HE, De P, Parsons R, Peng Q, Su JD, Durden DL (2008) The protein phosphatase activity of PTEN regulates SRC family kinases and controls glioma migration. Cancer research 68:1862-1871.

Dunah AW, Sirianni AC, Fienberg AA, Bastia E, Schwarzschild MA, Standaert DG (2004) Dopamine D1-dependent trafficking of striatal N-methyl-D-aspartate glutamate receptors requires Fyn protein tyrosine kinase but not DARPP-32. Mol Pharmacol 65:121-129.

El Benna J, Faust RP, Johnson JL, Babior BM (1996) Phosphorylation of the respiratory burst oxidase subunit p47phox as determined by two-dimensional phosphopeptide mapping. Phosphorylation by protein kinase C, protein kinase A, and a mitogen-activated protein kinase. The Journal of biological chemistry 271:6374-6378.

Engelmann S, Togni M, Thielitz A, Reichardt P, Kliche S, Reinhold D, Schraven B, Reinhold A (2013) T cell-independent modulation of experimental autoimmune encephalomyelitis in ADAP-deficient mice. Journal of immunology 191:4950-4959.

Finberg RW, Yim C, Yan J, Cao LC, Mandell L, Kurt-Jones EA (2012) Phosphorylated Toll-Like Receptor 2 Interacts with Fyn and Cross-Talks with the Phosphorylation-Independent TLR2-Signaling Pathway. The Open Immunology Journal 5:36-45.

Gao HM, Zhou H, Zhang F, Wilson BC, Kam W, Hong JS (2011) HMGB1 acts on microglia Mac1 to mediate chronic neuroinflammation that drives progressive neurodegeneration. The Journal of neuroscience : the official journal of the Society for Neuroscience 31:1081-1092.

Ghosh A, Chandran K, Kalivendi SV, Joseph J, Antholine WE, Hillard CJ, Kanthasamy A, Kalyanaraman B (2010) Neuroprotection by a mitochondria-targeted drug in a Parkinson's disease model. Free Radic Biol Med 49:1674-1684.

Ghosh A, Roy A, Liu X, Kordower JH, Mufson EJ, Hartley DM, Ghosh S, Mosley RL, Gendelman HE, Pahan K (2007) Selective inhibition of NF-kappaB activation prevents

dopaminergic neuronal loss in a mouse model of Parkinson's disease. Proceedings of the National Academy of Sciences of the United States of America 104:18754-18759.

Ghosh A, Saminathan H, Kanthasamy A, Anantharam V, Jin H, Sondarva G, Harischandra DS, Qian Z, Rana A, Kanthasamy AG (2013) The peptidyl-prolyl isomerase Pin1 up-regulation and proapoptotic function in dopaminergic neurons: relevance to the pathogenesis of Parkinson disease. The Journal of biological chemistry 288:21955-21971.

Glass CK, Saijo K, Winner B, Marchetto MC, Gage FH (2010) Mechanisms underlying inflammation in neurodegeneration. Cell 140:918-934.

Gomez G, Gonzalez-Espinosa C, Odom S, Baez G, Cid ME, Ryan JJ, Rivera J (2005a) Impaired FceRI-Dependent Gene Expression and Defective Eicosanoid and Cytokine Production as a Consequence of Fyn Deficiency in Mast Cells. J Immunol 175.

Gomez G, Gonzalez-Espinosa C, Odom S, Baez G, Cid ME, Ryan JJ, Rivera J (2005b) Impaired FcepsilonRI-dependent gene expression and defective eicosanoid and cytokine production as a consequence of Fyn deficiency in mast cells. Journal of immunology 175:7602-7610.

Gong P, Angelini DJ, Yang S, Xia G, Cross AS, Mann D, Bannerman DD, Vogel SN, Goldblum SE (2008) TLR4 signaling is coupled to SRC family kinase activation, tyrosine phosphorylation of zonula adherens proteins, and opening of the paracellular pathway in human lung microvascular endothelia. The Journal of biological chemistry 283:13437-13449.

Gordon R, Anantharam V, Kanthasamy AG, Kanthasamy A (2012) Proteolytic activation of proapoptotic kinase protein kinase Cdelta by tumor necrosis factor alpha death receptor signaling in dopaminergic neurons during neuroinflammation. Journal of neuroinflammation 9:82.

Gordon R, Hogan CE, Neal ML, Anantharam V, Kanthasamy AG, Kanthasamy A (2011) A simple magnetic separation method for high-yield isolation of pure primary microglia. Journal of neuroscience methods 194:287-296.

Harischandra DS, Kondru N, Martin DP, Kanthasamy A, Jin H, Anantharam V, Kanthasamy AG (2014) Role of proteolytic activation of protein kinase Cdelta in the pathogenesis of prion disease. Prion 8:143-153.

Hayden MS, Ghosh S (2004) Signaling to NF-kappaB. Genes Dev 18:2195-2224.

Haynes SE, Hollopeter G, Yang G, Kurpius D, Dailey ME, Gan WB, Julius D (2006) The P2Y12 receptor regulates microglial activation by extracellular nucleotides. Nature neuroscience 9:1512-1519.

Henn A, Lund S, Hedtjarn M, Schrattenholz A, Porzgen P, Leist M (2009) The suitability of BV2 cells as alternative model system for primary microglia cultures or for animal experiments examining brain inflammation. ALTEX 26:83-94.

Hickman SE, Kingery ND, Ohsumi TK, Borowsky ML, Wang LC, Means TK, El Khoury J (2013) The microglial sensome revealed by direct RNA sequencing. Nature neuroscience 16:1896-1905.

Hirsch EC, Hunot S (2009) Neuroinflammation in Parkinson's disease: a target for neuroprotection? The Lancet Neurology 8:382-397.

Hu X, Zhang D, Pang H, Caudle WM, Li Y, Gao H, Liu Y, Qian L, Wilson B, Di Monte DA, Ali SF, Zhang J, Block ML, Hong JS (2008) Macrophage antigen complex-1 mediates reactive microgliosis and progressive dopaminergic neurodegeneration in the MPTP model of Parkinson's disease. J Immunol 181:7194-7204.

Imamura K, Hishikawa N, Sawada M, Nagatsu T, Yoshida M, Hashizume Y (2003) Distribution of major histocompatibility complex class II-positive microglia and cytokine profile of Parkinson's disease brains. Acta neuropathologica 106:518-526.

Jenner P, Olanow CW (2006) The pathogenesis of cell death in Parkinson's disease. Neurology 66:S24-36.

Jin H, Kanthasamy A, Anantharam V, Rana A, Kanthasamy AG (2011a) Transcriptional regulation of pro-apoptotic protein kinase Cdelta: implications for oxidative stress-induced neuronal cell death. The Journal of biological chemistry 286:19840-19859.

Jin H, Kanthasamy A, Ghosh A, Yang Y, Anantharam V, Kanthasamy AG (2011b) alpha-Synuclein negatively regulates protein kinase Cdelta expression to suppress apoptosis in dopaminergic neurons by reducing p300 histone acetyltransferase activity. The Journal of neuroscience : the official journal of the Society for Neuroscience 31:2035-2051.

Jin H, Kanthasamy A, Harischandra DS, Kondru N, Ghosh A, Panicker N, Anantharam V, Rana A, Kanthasamy AG (2014) Histone Hyperacetylation Up-regulates Protein Kinase Cdelta in Dopaminergic Neurons to Induce Cell Death: RELEVANCE TO EPIGENETIC MECHANISMS OF NEURODEGENERATION IN PARKINSON DISEASE. The Journal of biological chemistry 289:34743-34767.

Kaspar JW, Jaiswal AK (2011) Tyrosine phosphorylation controls nuclear export of Fyn, allowing Nrf2 activation of cytoprotective gene expression. FASEB journal : official publication of the Federation of American Societies for Experimental Biology 25:1076-1087.

Kaul S, Kanthasamy A, Kitazawa M, Anantharam V, Kanthasamy AG (2003) Caspase-3 dependent proteolytic activation of protein kinase Cdelta mediates and regulates 1-methyl-4-phenylpyridinium (MPP+)-induced apoptotic cell death in dopaminergic cells: relevance to oxidative stress in dopaminergic degeneration. European Journal of Neuroscience 18:1387-1401.

Kaul S, Anantharam V, Yang Y, Choi CJ, Kanthasamy A, Kanthasamy AG (2005) Tyrosine phosphorylation regulates the proteolytic activation of protein kinase Cdelta in dopaminergic neuronal cells. The Journal of biological chemistry 280:28721-28730.

Kim C, Ho DH, Suk JE, You S, Michael S, Kang J, Joong Lee S, Masliah E, Hwang D, Lee HJ, Lee SJ (2013a) Neuron-released oligomeric alpha-synuclein is an endogenous agonist of TLR2 for paracrine activation of microglia. Nature communications 4:1562.

Kim JH, Choi DJ, Jeong HK, Kim J, Kim DW, Choi SY, Park SM, Suh YH, Jou I, Joe EH (2013b) DJ-1 facilitates the interaction between STAT1 and its phosphatase, SHP-1, in brain microglia and astrocytes: A novel anti-inflammatory function of DJ-1. Neurobiology of disease 60:1-10.

Kim YS, Choi DH, Block ML, Lorenzl S, Yang L, Kim YJ, Sugama S, Cho BP, Hwang O, Browne SE, Kim SY, Hong JS, Beal MF, Joh TH (2007) A pivotal role of matrix metalloproteinase-3 activity in dopaminergic neuronal degeneration via microglial activation. FASEB J 21:179-187.

Koistinaho M, Koistinaho J (2002) Role of p38 and p44/42 mitogen-activated protein kinases in microglia. Glia 40:175-183.

Kouadir M, Yang L, Tan R, Shi F, Lu Y, Zhang S, Yin X, Zhou X, Zhao D (2012) CD36 Participates in PrP106–126-Induced Activation of Microglia. PLOS One 7.

Lambert MP, Barlow AK, Chromy BA, Edwards C, Freed R, Liosatos M, Morgan TE, Rozovsky I, Trommer B, Viola KL, Wals P, Zhang C, Finch CE, Krafft GA, Klein WL (1998) Diffusible, nonfibrillar ligands derived from Ab1–42 are potent central nervous system neurotoxins. Proc Natl Acad Sci USA 95.

Larson M, Sherman MA, Amar F, Nuvolone M, Schneider JA, Bennett DA, Aguzzi A, Lesne SE The complex PrP(c)-Fyn couples human oligomeric Abeta with pathological tau changes in Alzheimer's disease. The Journal of neuroscience : the official journal of the Society for Neuroscience 32:16857-16871a.

Lastres-Becker I, Ulusoy A, Innamorato NG, Sahin G, Rabano A, Kirik D, Cuadrado A (2012) alpha-Synuclein expression and Nrf2 deficiency cooperate to aggravate protein aggregation, neuronal death and inflammation in early-stage Parkinson's disease. Human molecular genetics 21:3173-3192.

Latchoumycandane C, Anantharam V, Jin H, Kanthasamy A, Kanthasamy A (2011) Dopaminergic neurotoxicant 6-OHDA induces oxidative damage through proteolytic activation of PKCdelta in cell culture and animal models of Parkinson's disease. Toxicology and applied pharmacology 256:314-323.

Lee S, Nam Y, Koo JY, Lim D, Park J, Ock J, Kim J, Suk K, Park SB (2014) A small molecule binding HMGB1 and HMGB2 inhibits microglia-mediated neuroinflammation. Nature chemical biology 10:1055-1060.

Levy OA, Malagelada C, Greene LA (2009) Cell death pathways in Parkinson's disease: proximal triggers, distal effectors, and final steps. Apoptosis 14:478-500.

Moore KJ, El Khoury J, Medeiros LA, Terada K, Geula C, Luster AD, Freeman MW (2002) A CD36-initiated signaling cascade mediates inflammatory effects of beta-amyloid. The Journal of biological chemistry 277:47373-47379.

Olanow CW (2007) The pathogenesis of cell death in Parkinson's disease--2007. Mov Disord 22 Suppl 17:S335-342.

Olson JK, Miller SD (2004) Microglia Initiate Central Nervous System Innate and Adaptive Immune Responses through Multiple TLRs. J Immunol 173:3916-3924.

Palacios EH, Weiss A (2004) Function of the Src-family kinases, Lck and Fyn, in T-cell development and activation. Oncogene 23:7990-8000.

Parameswaran N, Patial S (2010) Tumor Necrosis Factor-α Signaling in Macrophages. Crit Rev Eukaryot Gene Expr 20:87-103.

Przedborski S (2005) Pathogenesis of nigral cell death in Parkinson's disease. Parkinsonism & related disorders 11 Suppl 1:S3-7.

Przedborski S, Tieu K, Perier C, Vila M (2004) MPTP as a mitochondrial neurotoxic model of Parkinson's disease. J Bioenerg Biomembr 36:375-379.

Puschmann A (2013) Monogenic Parkinson's disease and parkinsonism: clinical phenotypes and frequencies of known mutations. Parkinsonism & related disorders 19:407-415.

Qin L, Wu X, Block ML, Liu Y, Breese GR, Hong JS, Knapp DJ, Crews FT (2007) Systemic LPS causes chronic neuroinflammation and progressive neurodegeneration. Glia 55:453-462.

Rajasekaran K, Kumar P, Schuldt KM, Peterson EJ, Vanhaesebroeck B, Dixit V, Thakar MS, Malarkannan S (2013) Signaling by Fyn-ADAP via the Carma1-Bcl-10-MAP3K7 signalosome exclusively regulates inflammatory cytokine production in NK cells. Nature immunology 14:1127-1136.

Reed-Geaghan EG, Savage JC, Hise AG, Landreth GE (2009) CD14 and toll-like receptors 2 and 4 are required for fibrillar A{beta}-stimulated microglial activation. The Journal of neuroscience : the official journal of the Society for Neuroscience 29:11982-11992.

Saminathan H, Asaithambi A, Anantharam V, Kanthasamy AG, Kanthasamy A (2011) Environmental neurotoxic pesticide dieldrin activates a non receptor tyrosine kinase to promote PKCdelta-mediated dopaminergic apoptosis in a dopaminergic neuronal cell model. Neurotoxicology 32:567-577.

Sperber BR, Boyle-Walsh EA, Engleka MJ, Gadue P, Peterson AC, Stein PL, Scherer SS, McMorris FA (2001) A Unique Role for Fyn in CNS Myelination. The Journal of neuroscience : the official journal of the Society for Neuroscience 21:2039-2047.

Sriram K, Matheson JM, Benkovic SA, Miller DB, Luster MI, O'Callaghan JP (2006) Deficiency of TNF receptors suppresses microglial activation and alters the susceptibility of

brain regions to MPTP-induced neurotoxicity: role of TNF-alpha. FASEB journal : official publication of the Federation of American Societies for Experimental Biology 20:670-682.

Steinberg SF (2004) Distinctive activation mechanisms and functions for protein kinase Cδ. Biochem J 384:449-459.

Stott SR, Barker RA (2014) Time course of dopamine neuron loss and glial response in the 6-OHDA striatal mouse model of Parkinson's disease. Eur J Neurosci 39:1042-1056.

Stuart LM, Bell SA, Stewart CR, Silver JM, Richard J, Goss JL, Tseng AA, Zhang A, El Khoury JB, Moore KJ (2007) CD36 signals to the actin cytoskeleton and regulates microglial migration via a p130Cas complex. The Journal of biological chemistry 282:27392-27401.

Su X, Maguire-Zeiss KA, Giuliano R, Prifti L, Venkatesh K, Federoff HJ (2008) Synuclein activates microglia in a model of Parkinson's disease. Neurobiology of aging 29:1690-1701.

Tansey MG, Goldberg MS (2010) Neuroinflammation in Parkinson's disease: its role in neuronal death and implications for therapeutic intervention. Neurobiology of disease 37:510-518.

Um JW, Nygaard HB, Heiss JK, Kostylev MA, Stagi M, Vortmeyer A, Wisniewski T, Gunther EC, Strittmatter SM Alzheimer amyloid-beta oligomer bound to postsynaptic prion protein activates Fyn to impair neurons. Nature neuroscience 15:1227-1235.

Virgone-Carlotta A, Uhlrich J, Akram MN, Ressnikoff D, Chretien F, Domenget C, Gherardi R, Despars G, Jurdic P, Honnorat J, Nataf S, Touret M (2013) Mapping and kinetics of microglia/neuron cell-to-cell contacts in the 6-OHDA murine model of Parkinson's disease. Glia 61:1645-1658.

Wake H, Lee PR, Fields RD (2011) Control of local protein synthesis and initial events in myelination by action potentials. Science 333:1647-1651.

Wu DC, Teismann P, Tieu K, Vila M, Jackson-Lewis V, Ischiropoulos H, Przedborski S (2003) NADPH oxidase mediates oxidative stress in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine model of Parkinson's disease. Proceedings of the National Academy of Sciences of the United States of America 100:6145-6150.

Wu DC, Jackson-Lewis V, Vila M, Tieu K, Teismann P, Vadseth C, Choi DK, Ischiropoulos H, Przedborski S (2002) Blockade of microglial activation is neuroprotective in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine mouse model of Parkinson disease. The Journal of neuroscience : the official journal of the Society for Neuroscience 22:1763-1771.

Zhang D, Anantharam V, Kanthasamy A, Kanthasamy AG (2007) Neuroprotective effect of protein kinase C delta inhibitor rottlerin in cell culture and animal models of Parkinson's disease. The Journal of pharmacology and experimental therapeutics 322:913-922.

Figures

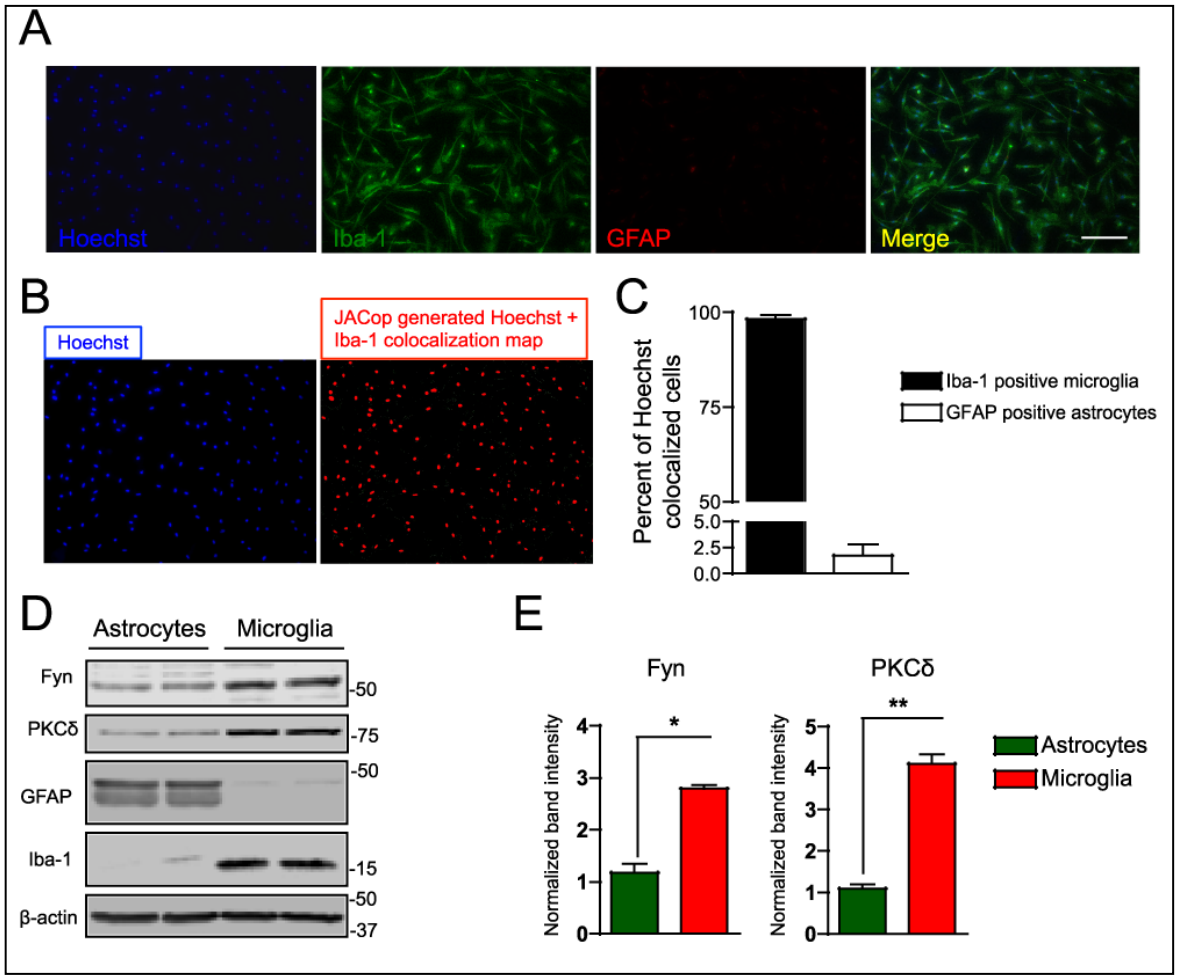

Figure 1: Fyn and PKCδ are differentially expressed in primary astrocytes and microglia. ***A***, Representative image from immunocytochemical analysis for the microglial marker Iba-1 and the astrocytic marker GFAP on both, the magnetically purified and pour-off fractions of cells obtained post-separation revealed almost no astrocytic contamination in the samples**.** Scale bar, 200 microns. ***B***, The colocalization image of Hoechst (nuclear stain) and Iba-1 images in 6 random image fields were obtained using the ImageJ plugin JACoP. ***C***, The number of Hoechst-positive and colocalization-positive cells were counted using ImageJ. The magnetically purified samples were >97% positive for microglial cells. ***D***, ***E***, Western

Blot analyses of the magnetically separated cells revealed that the microglial fraction expressed higher amounts of the non-receptor Src kinase Fyn and the serine threonine kinase PKCδ than did the astrocyte-rich pour-off fraction (*$p < 0.05$, **$p < 0.01$).

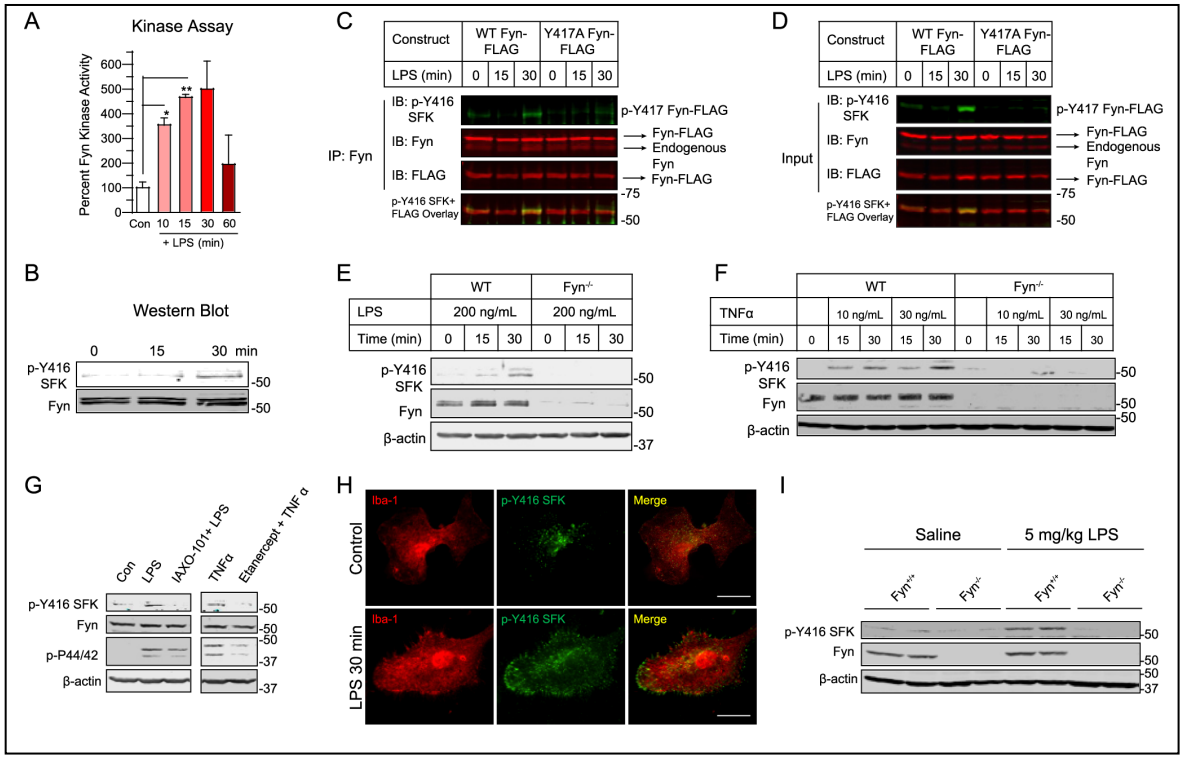

Figure 2. Fyn kinase is rapidly activated in microglial cells and in the ventral midbrain following inflammogen stimulation. ***A***, Fyn kinase assay shows that Fyn activity was highly induced in BV2 microglia treated with 1 μg/mL LPS for 10, 15 and 30 min. (*p < 0.05, **p < 0.01). ***B***, Immunoblots showing a concomitant rise in p-Y416 SFK levels in BV2 cell lysates post-LPS treatment. ***C***, ***D***, Immunoprecipitation studies revealed that $Fyn^{+/+}$ (WT Fyn), but not active loop tyrosine-mutant Fyn (Y417A Fyn), when overexpressed in BV2 microglia, was activated following LPS stimulation. ***E,*** Treatment of primary microglia with LPS and ***F,*** TNFα for 15 and 30 min increased p-Y416 SFK levels in primary microglia obtained from wild-type $Fyn^{+/+}$, but not $Fyn^{-/-}$ mice, identifying Fyn as the primary Src family kinase that was activated by inflammogen stimulation. ***G***, Pretreatment of primary microglia with the TLR-signaling antagonist IAXO-101 or the TNFα receptor decoy Etanercept abolished Fyn activation by LPS or TNFα stimulation (p-44/42 phosphorylation used as marker for early microglial activation) ***H***, Immunocytochemistry of LPS-treated WT primary

microglia showing that activated Fyn expression greatly increased and was localized preferentially to the membrane periphery of the microglial cell. Scale bar, 20 microns. ***I***, Immunoblots of ventral midbrain lysates showed that peripheral administration of the inflammogen LPS (5 mg/kg) increased p-Y416 SFK levels in $Fyn^{+/+}$, but not in $Fyn^{-/-}$ ventral midbrain tissues.

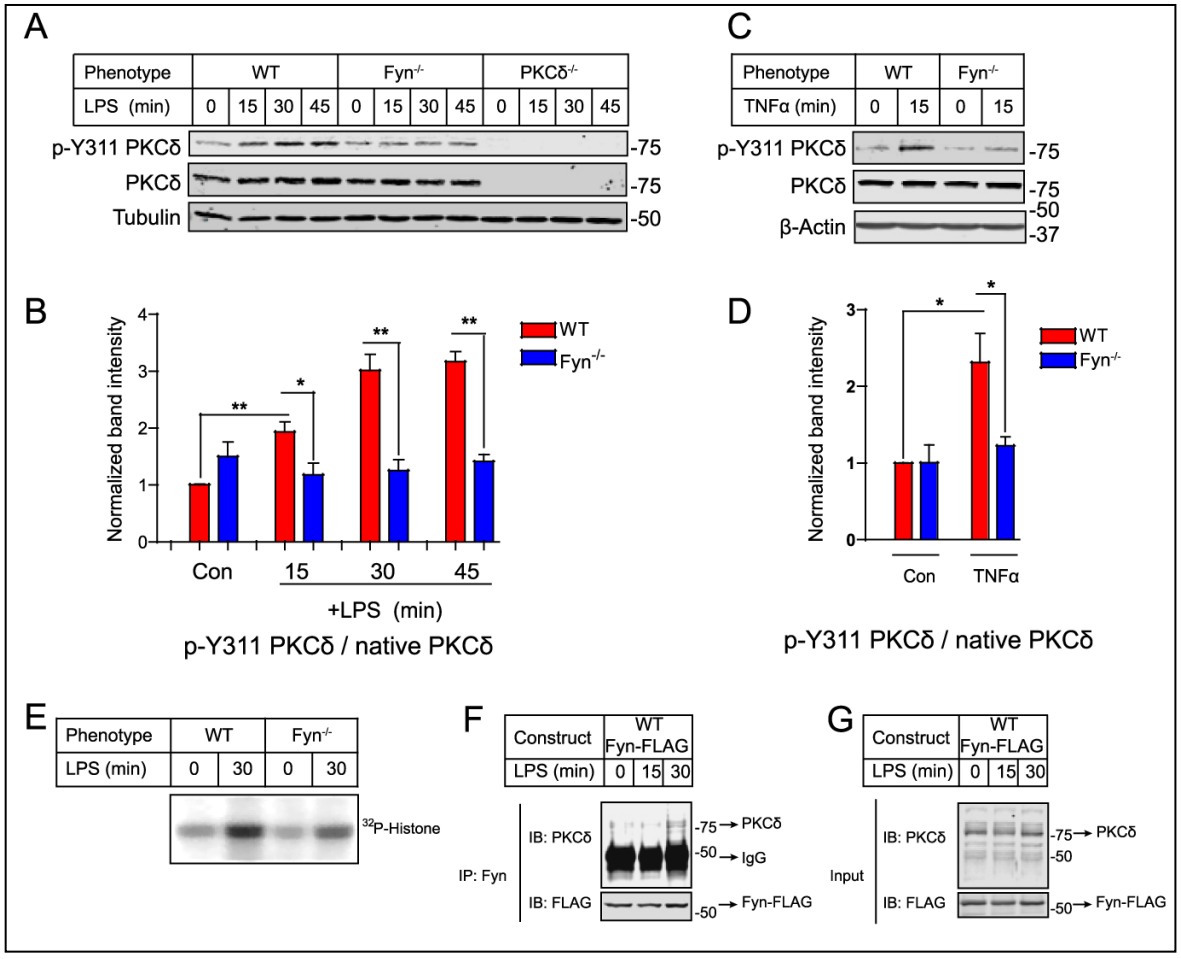

Figure 3. Fyn contributes to LPS- and TNFα-induced tyrosine phosphorylation and activation of PKCδ in primary microglia. Western blot analysis revealed that stimulation of microglia with LPS (***A***, ***B***) and TNFα (***C***, ***D***) induced a time-dependent increase in p-Y311 PKCδ levels in wild type but not Fyn$^{-/-}$ microglia (*p < 0.05, **p < 0.01). ***E***, LPS-induced PKCδ kinase activity was reduced in Fyn$^{-/-}$ microglial lysates in contrast to wild type lysates, as measured by PKCδ kinase assay. ***F***, ***G***, Co-immunoprecipitation studies showed that LPS stimulation elicited a physical interaction between Fyn and PKCδ in WT Fyn-transfected BV2 microglial cells.

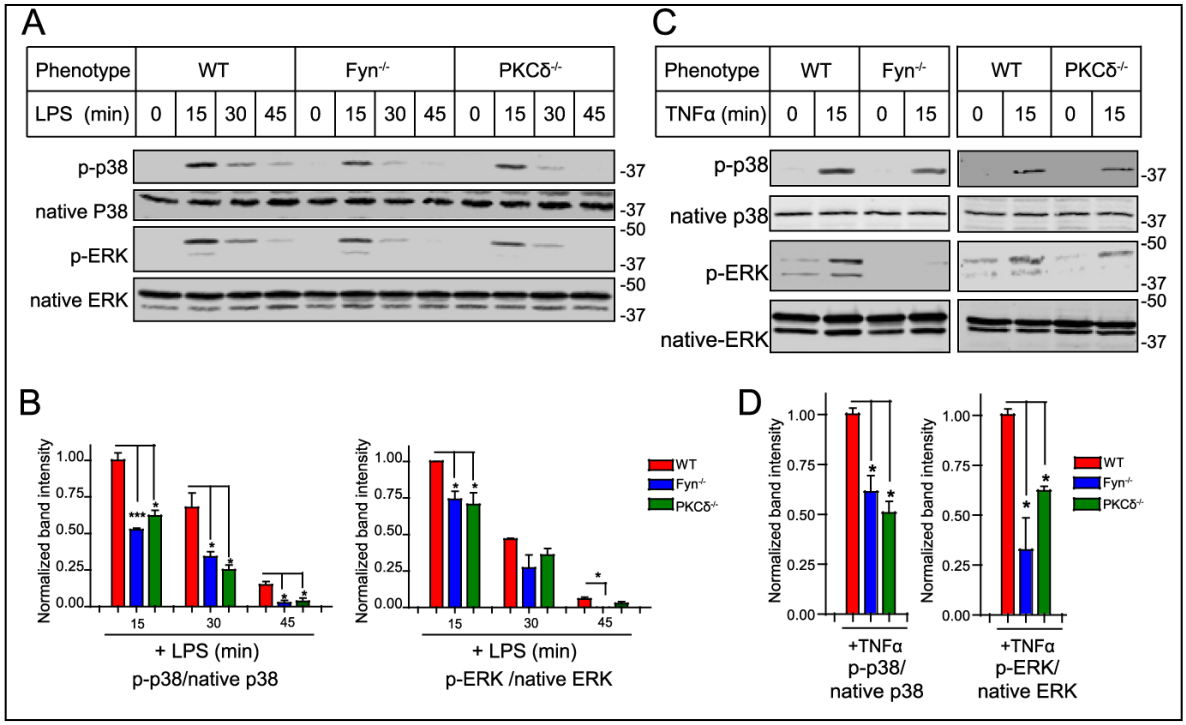

Figure 4. The Fyn–PKCδ signaling axis mediates MAP kinase activation in microglial cells. ***A***, ***B,*** Immunoblot analysis demonstrated diminished LPS-induced p38 and p44/42 (p-ERK) phosphorylation in Fyn$^{-/-}$ and PKCδ$^{-/-}$ microglia (*$p < 0.05$, ***$p < 0.001$). ***C, D,*** Diminished TNFα-induced p38 and p44/42 (p-ERK) phosphorylation in Fyn$^{-/-}$ and PKCδ$^{-/-}$ microglia (*$p < 0.05$).

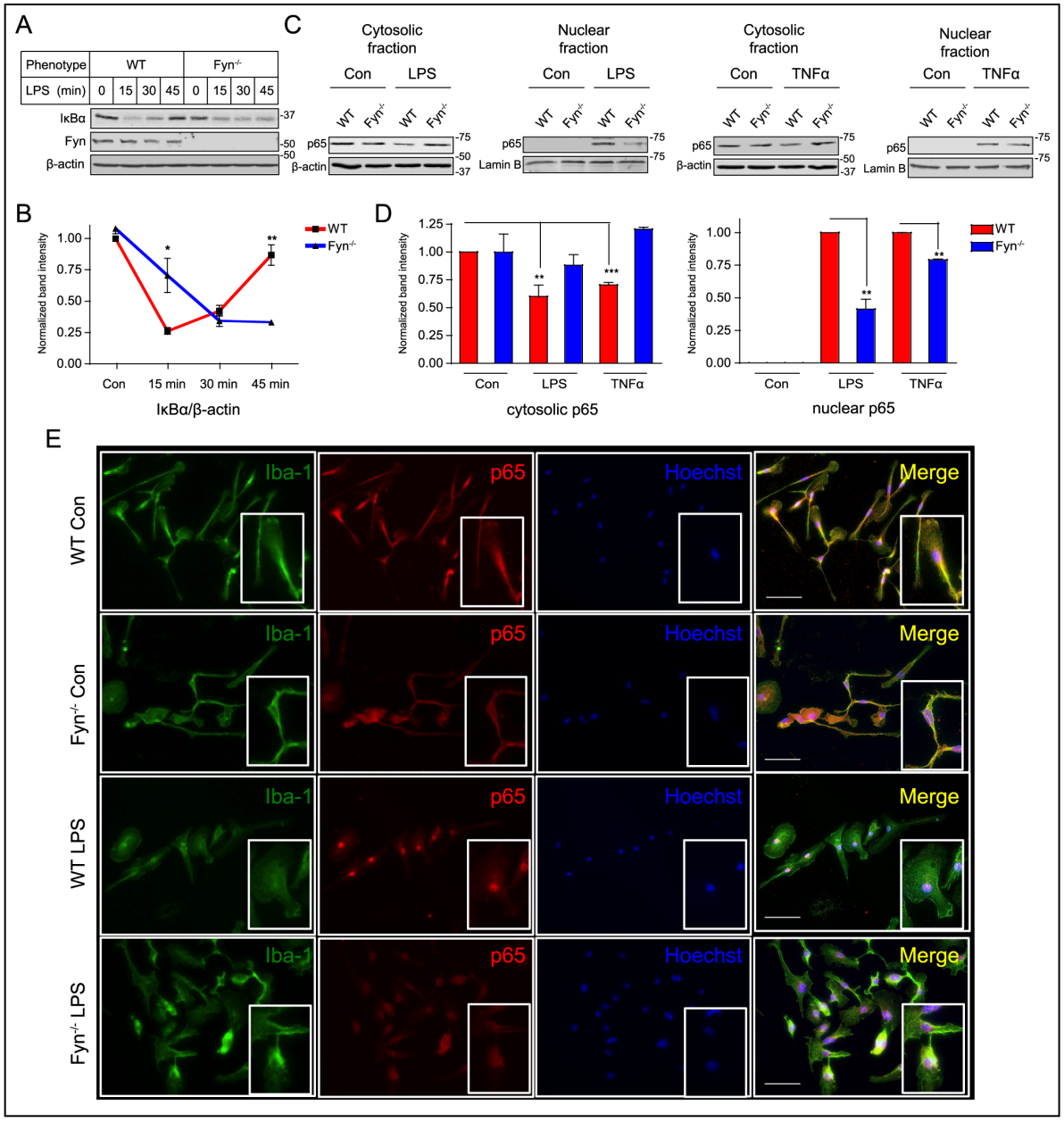

Figure 5. Fyn contributes to inflammogen-mediated NFκB pathway activation in microglial cells. ***A***, ***B***, Immunoblot analyses of whole cell lysates of wild-type and Fyn$^{-/-}$ microglia treated with LPS for 15-45 min revealed reduced IκBα degradation in Fyn$^{-/-}$ microglia at 15 min, and attenuated IκBα resynthesis at 30 and 45 min (*$p < 0.05$, **$p < 0.01$). ***C***, ***D***, Cytosolic and nuclear fractionation of LPS- and TNFα-treated wild type and Fyn$^{-/-}$ microglia revealed diminished nuclear translocation of the p65 subunit of the NFκB

complex in the Fyn$^{-/-}$ microglia (**p < 0.01, ***p < 0.001). ***E***, Immunocytochemistry also showed reduced nuclear p65 in LPS-treated Fyn$^{-/-}$ microglia. Scale bar, 50 microns.

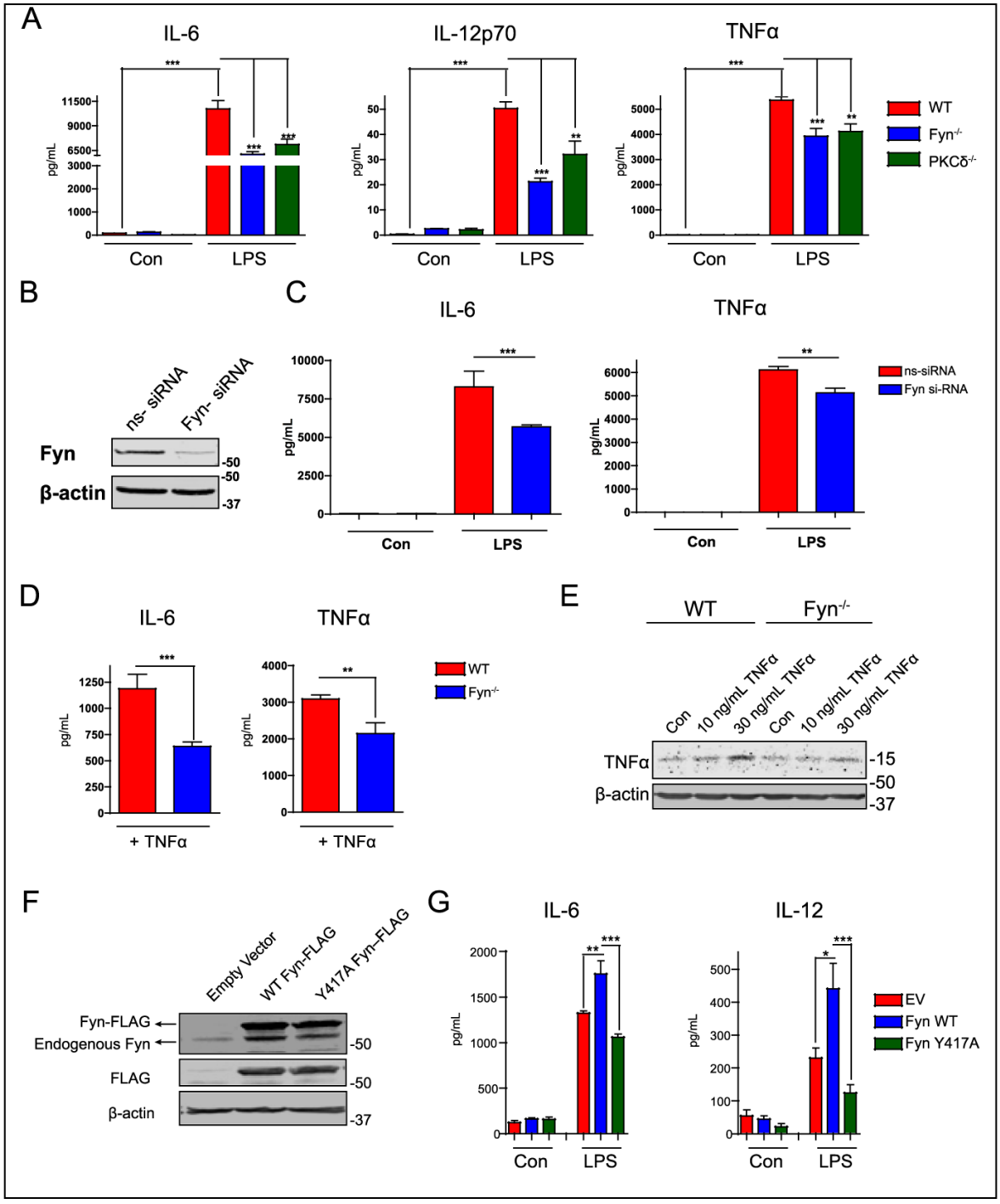

Figure 6. LPS- or TNFα-induced proinflammatory cytokine production is suppressed in Fyn/PKCδ deficient microglia. ***A***, Luminex analyses of supernatants from LPS-treated wild-type, PKCδ$^{-/-}$ and Fyn$^{-/-}$ microglia revealed reduced secretion of the pro-inflammatory cytokines IL-6, IL-12 and TNFα (**p < 0.01, ***p < 0.001). ***B***, Wild-type primary

microglia were transfected with non-targeting and Fyn-specific siRNA for 72 hours. Knockdown of Fyn was evaluated by Western blot. ***C***, Fyn depleted microglia demonstrated diminished IL-6 and TNFα secretion in response to LPS stimulation (**p < 0.01, ***p < 0.001). ***D***, TNFα stimulation of $Fyn^{-/-}$ microglia reduced IL-6 and TNFα production in contrast to wild-type microglia. (**p < 0.01, ***p < 0.001). ***E***, Immunoblots showing reduced TNFα levels in Fyn-deficient microglia after TNFα stimulation in contrast to wild-type microglia. ***F***, ***G***, Overexpressing the FLAG-tagged activation loop tyrosine mutant of Fyn in BV2 microglia attenuated IL-6 and IL-12 production when the cells were treated with LPS, as shown by Luminex cytokine analysis (*p < 0.05, **p < 0.01 and ***p < 0.001).

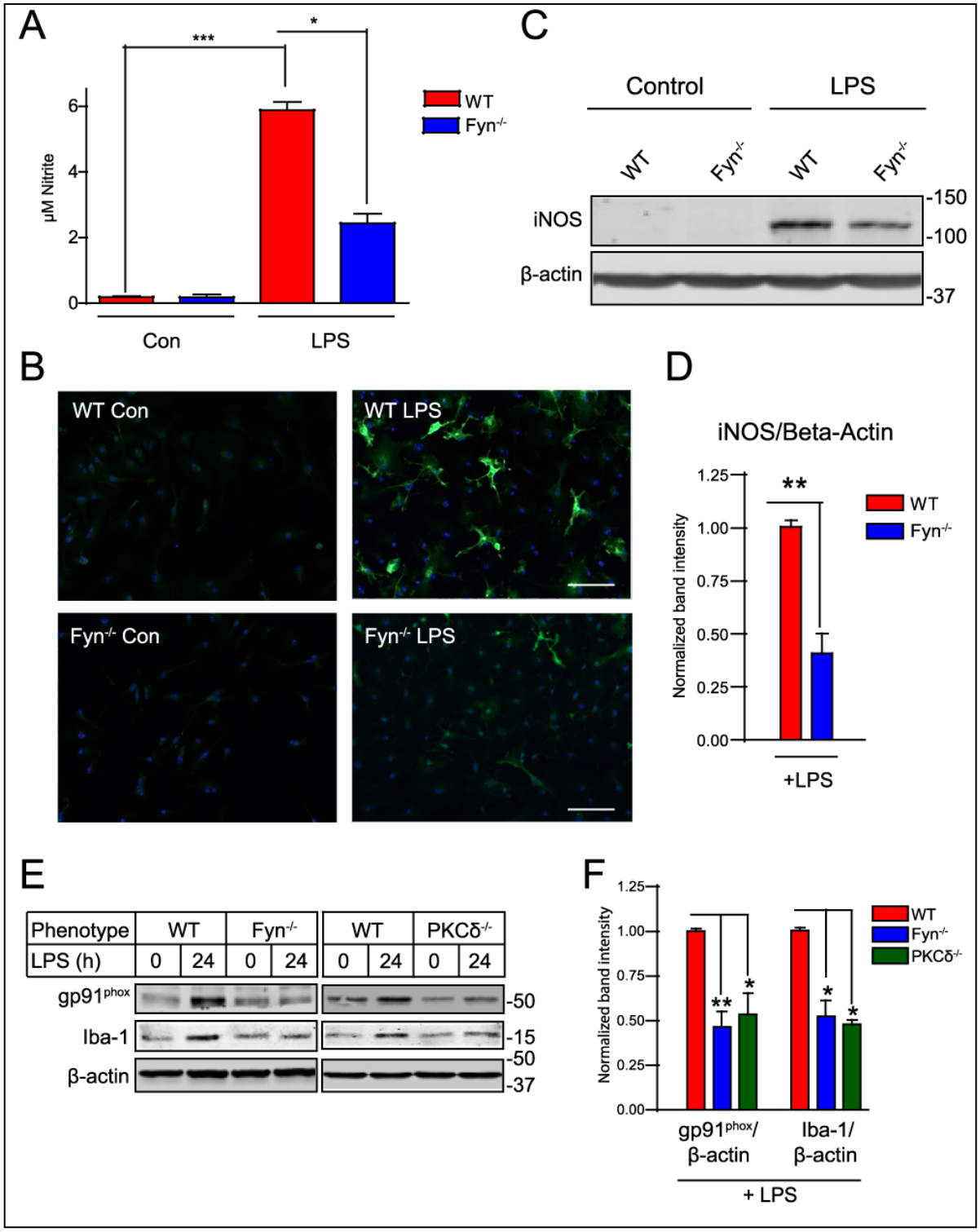

Figure 7. Fyn plays a role in LPS-induced iNOS expression, nitrite production and neuroinflammatory marker expression. ***A***, Griess nitrite measurement assay demonstrated

that LPS-induced nitrite production was reduced in $Fyn^{-/-}$ microglia (*$p < 0.05$, ***$p < 0.001$). ***B***, ***C***, ***D***, Diminished iNOS expression in LPS-treated $Fyn^{-/-}$ microglia (**$p < 0.01$). Scale bar, 100 microns. ***E***, ***F***, Reduced $gp91^{phox}$ and Iba-1 expression in LPS-treated $Fyn^{-/-}$ and $PKC\delta^{-/-}$ microglia, as shown by immunoblotting analysis (*$p < 0.05$, **$p < 0.01$).

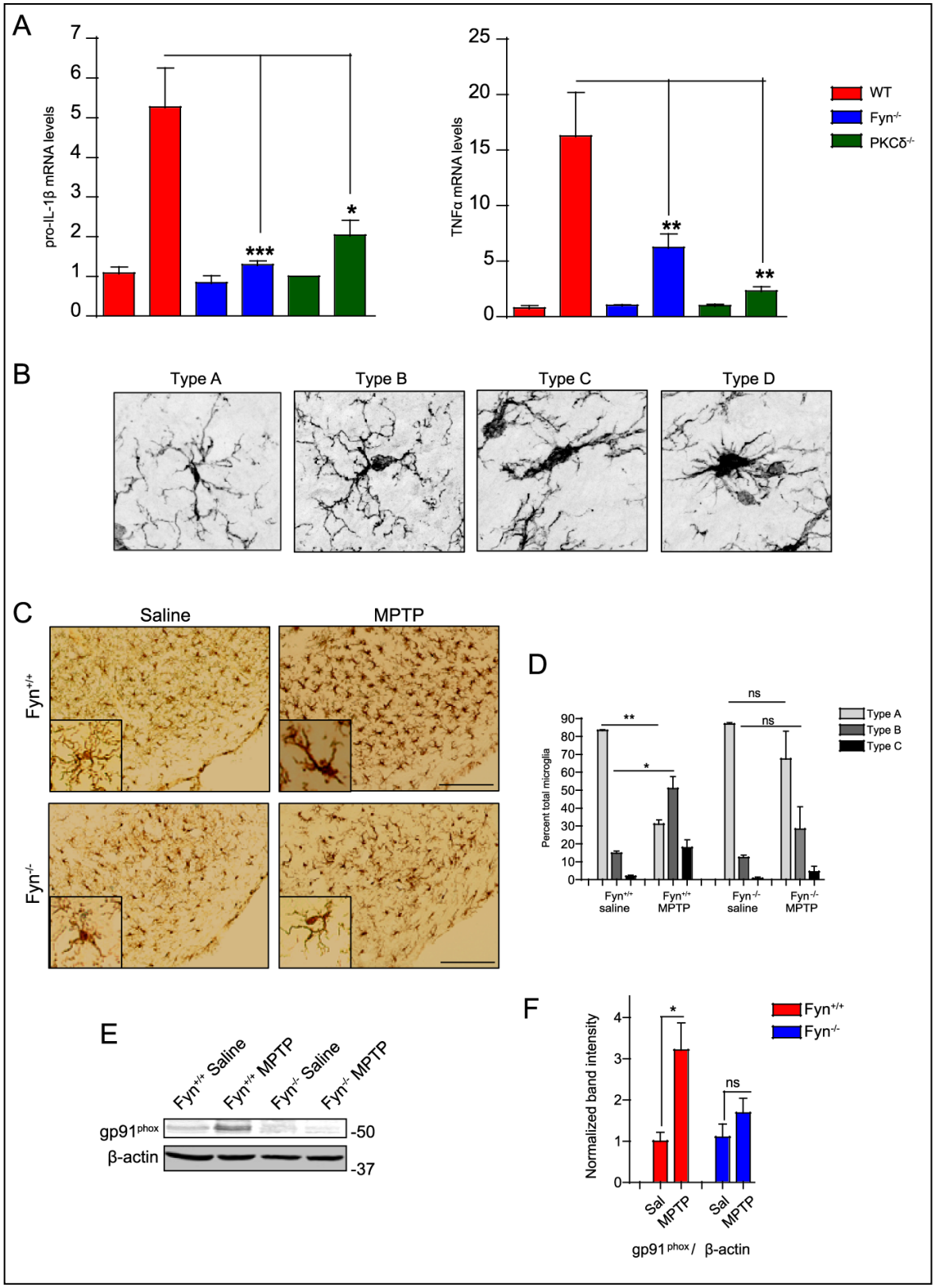

Figure 8. Fyn$^{-/-}$ and PKCδ$^{-/-}$ mice are resistant to LPS- and MPTP-induced neuroinflammatory responses. ***A***, Wild-type, PKCδ$^{-/-}$ and Fyn$^{-/-}$ mice were injected intraperitoneally with 5 mg/kg LPS for 3 h. Striatal cytokine mRNA levels, assessed by q-RT PCR, showed significantly reduced induction of pro-IL-1β and TNFα mRNA levels in PKCδ$^{-/-}$ and Fyn$^{-/-}$ mice in contrast to wild-type mice (*$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$). ***B***, The transitional stages of microglial activation, from ramified (inactivated, type A) to amoeboid (activated, types B, C and D), are shown by representative images. ***C***, ***D***, Iba-1-DAB immunohistochemistry in MPTP-injected Fyn$^{-/-}$ and wild-type ventral midbrain sections demonstrated nigral microgliosis, assessed by quantification of microglial morphology, in the WT, but not the Fyn$^{-/-}$ sections. Scale bar, 75 microns (*$p < 0.05$, **$p < 0.01$). ***E***, ***F***, Fyn$^{-/-}$ mice showed diminished induction of the proinflammatory marker gp91^{phox} in ventral midbrain lysates following the acute MPTP regimen (*$p < 0.05$).

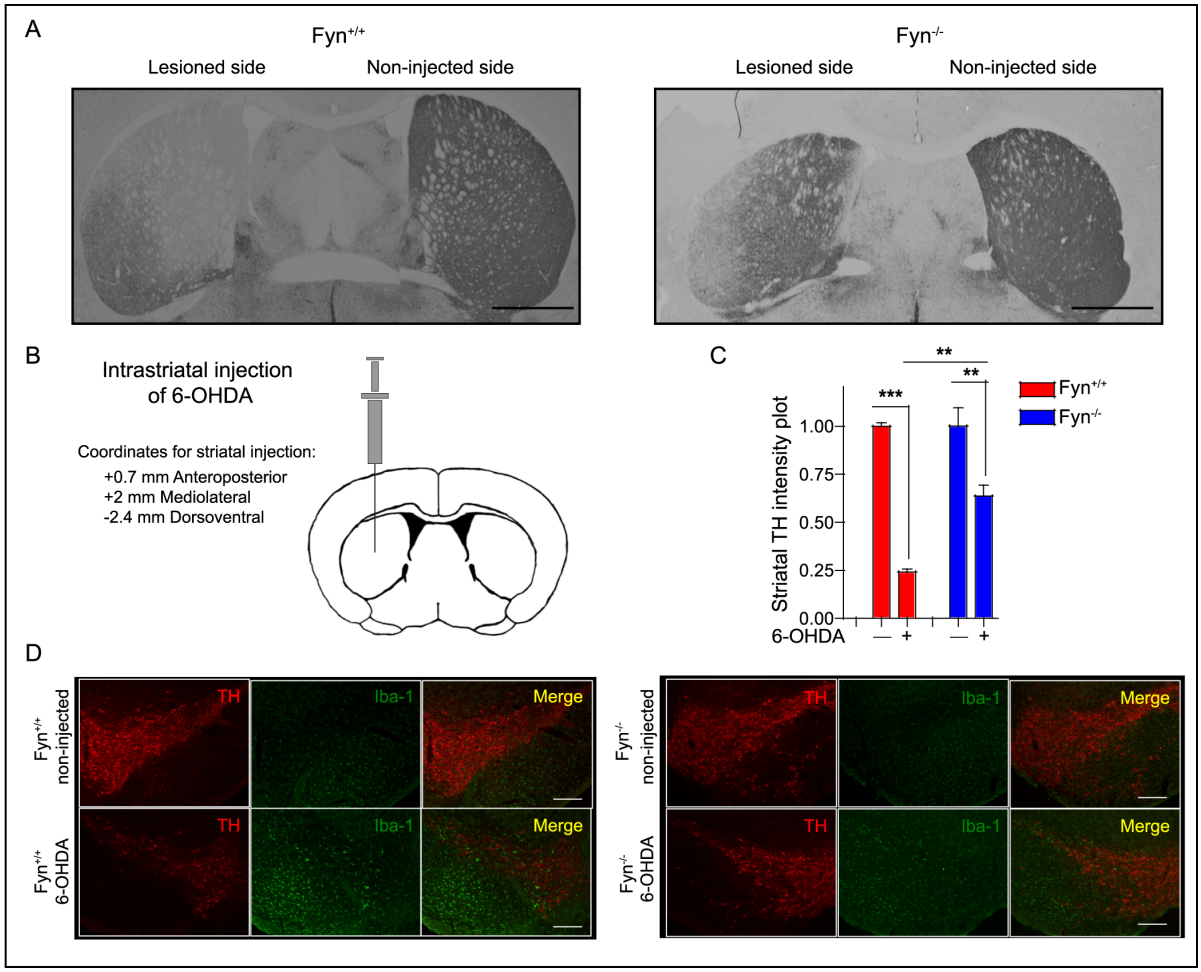

Figure 9. Fyn$^{-/-}$ mice are protected against 6-OHDA-induced nigrostriatal dopaminergic neuronal deficits and microgliosis. ***A***, TH-DAB immunohistochemistry in 6-OHDA-injected Fyn$^{-/-}$ and wild-type mouse striatal sections. Scale bar, 1000 microns. ***B***, Schematic diagram of a coronal section through the mouse striatum at the level of the injection. ***C***, Significant preservation of 6-OHDA-induced degeneration of dopaminergic terminals is seen in the Fyn$^{-/-}$ mice in contrast to wild-type mice (**p < 0.01, ***p < 0.001). ***D***, Immunofluorescence staining of 6-OHDA-injected Fyn$^{-/-}$ and wild-type ventral midbrain sections reveals diminished microgliosis and concomitant nigral neuroprotection in Fyn$^{-/-}$ mice after 6-OHDA administration, in contrast to the massive microgliosis and nigral dopaminergic neuronal death observed in the wild-type mice. Scale bar, 200 microns.

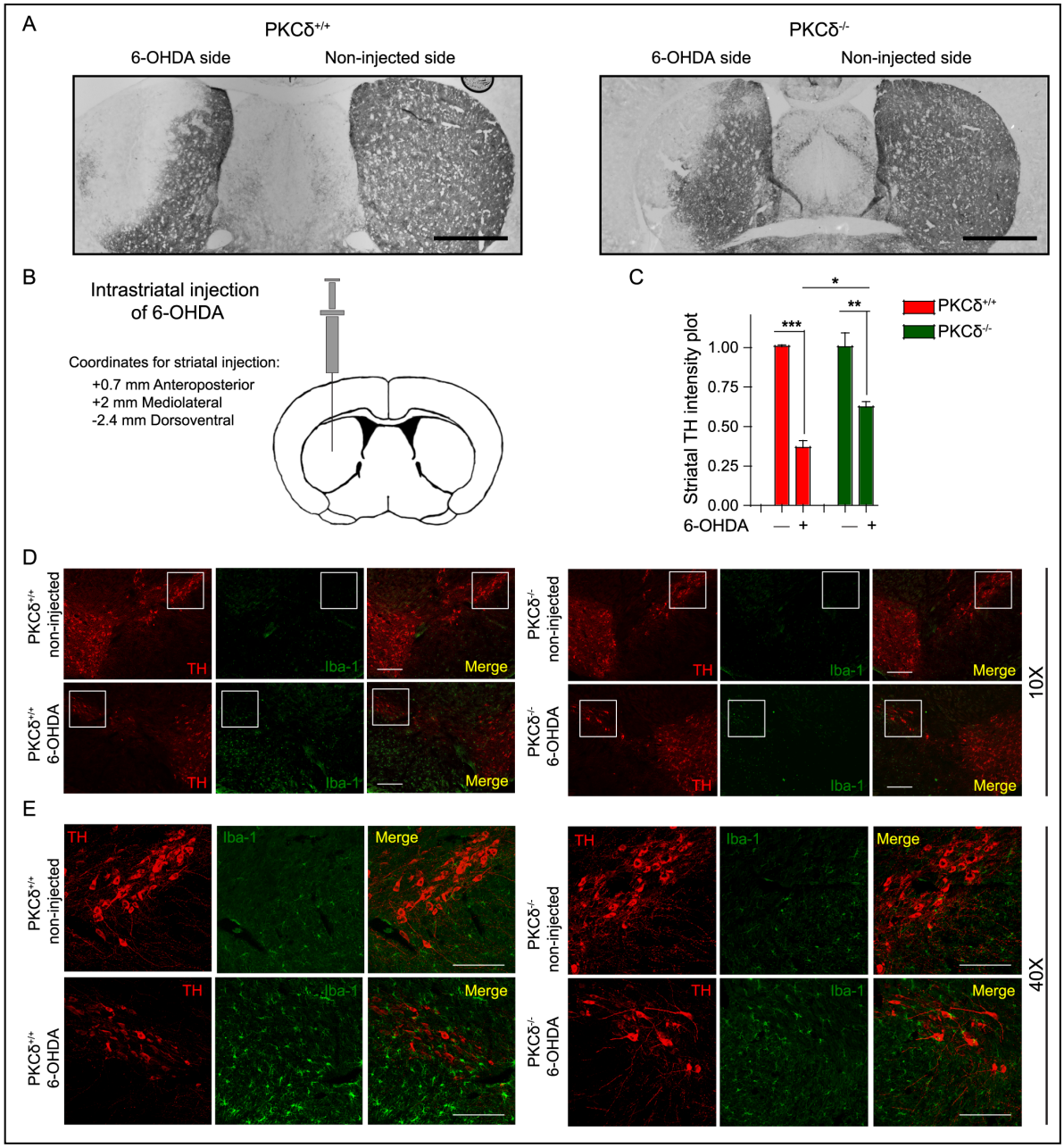

Figure 10. PKCδ$^{-/-}$ mice are resistant to 6-OHDA-induced nigrostriatal dopaminergic neuronal deficits and microgliosis. ***A***, TH-DAB immunohistochemistry in 6-OHDA-injected PKCδ$^{-/-}$ and wild-type mouse striatal sections. Scale bar, 1000 microns. ***B,*** Schematic diagram of a coronal section through the mouse striatum at the level of the injection. ***C,*** Significant preservation of dopaminergic terminals is seen in the 6-OHDA-treated PKCδ$^{-/-}$ mice in contrast to wild-type mice (*p < 0.05, **p < 0.01 and ***p < 0.001). ***D***,

Immunofluorescence staining of 6-OHDA-injected PKCδ$^{-/-}$ and wild-type ventral midbrain sections reveals reduced nigral TH degeneration and microgliosis in PKCδ$^{-/-}$ mice after 6-OHDA administration, in contrast to the wild-type mice. Scale bar, 200 microns. ***E***, High magnification image of 6-OHDA-injected PKCδ$^{-/-}$ and wild-type ventral midbrain sections. Scale bar, 50 microns.

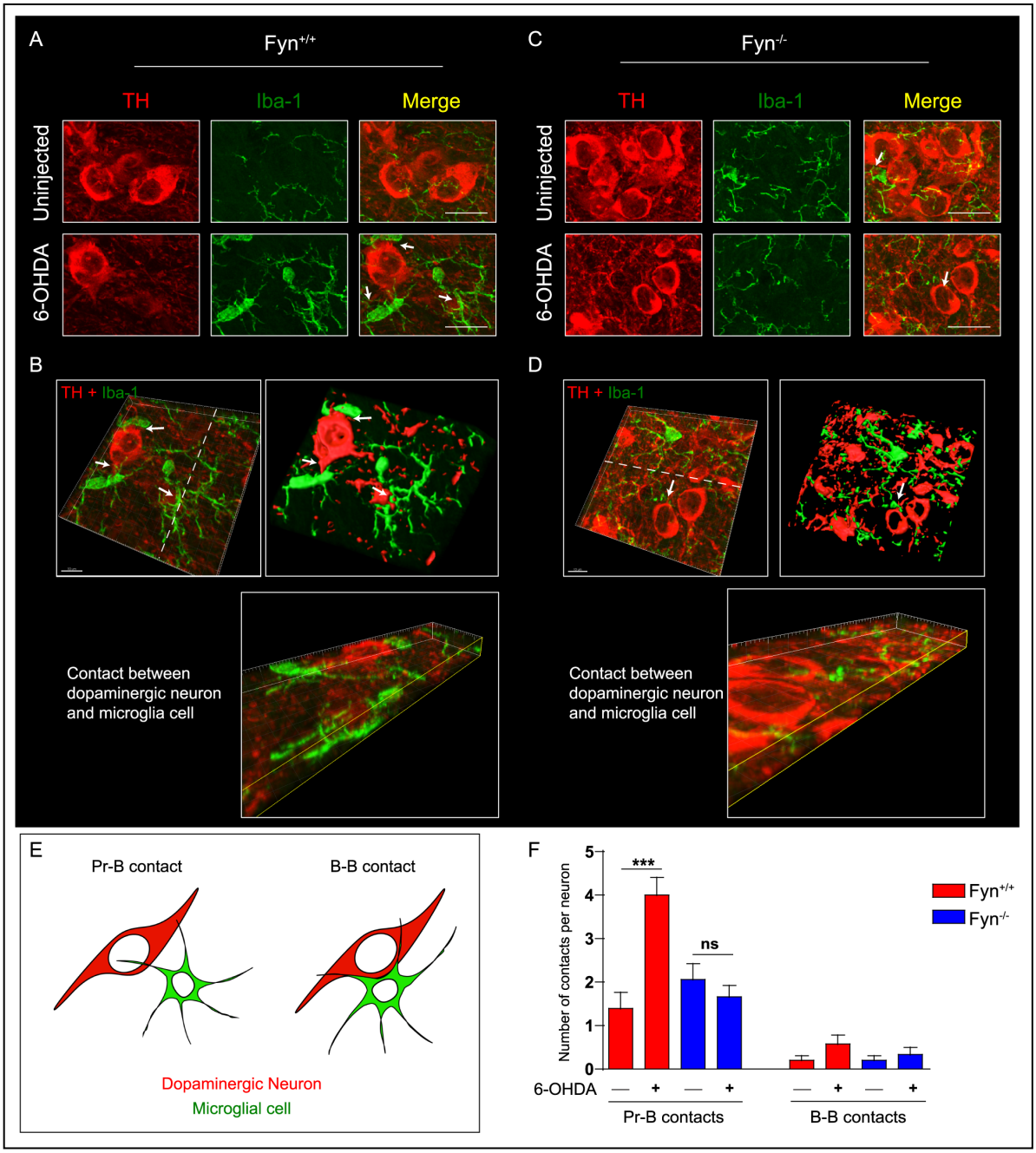

Figure 11. Diminished 6-OHDA-induced glial-neuronal contact (gliapse) formation in the Fyn$^{-/-}$ substantia nigra. ***A***, ***C***, Confocal Z stack maximum projection image analysis of ventral midbrain sections reveals a strongly increased number of microglial-neuronal contacts and appositions upon 6-OHDA treatment of Fyn$^{+/+}$ but not Fyn$^{-/-}$ mice. Scale bar, 12 microns ***B***, ***D***, Confocal Z stack images were rotated and optically sectioned along the Z

plane using Imaris software, allowing easy visualization of gliapse formation. Scale bar, 10 microns ***E***, Diagrams of Process-Body (Pr-B) and Body-Body (B-B) gliapses formed between dopaminergic neurons and microglia. ***F,*** Fyn ventral midbrain sections revealed significantly fewer gliapses formed per dopaminergic neuron in the SN (***$p < 0.001$).

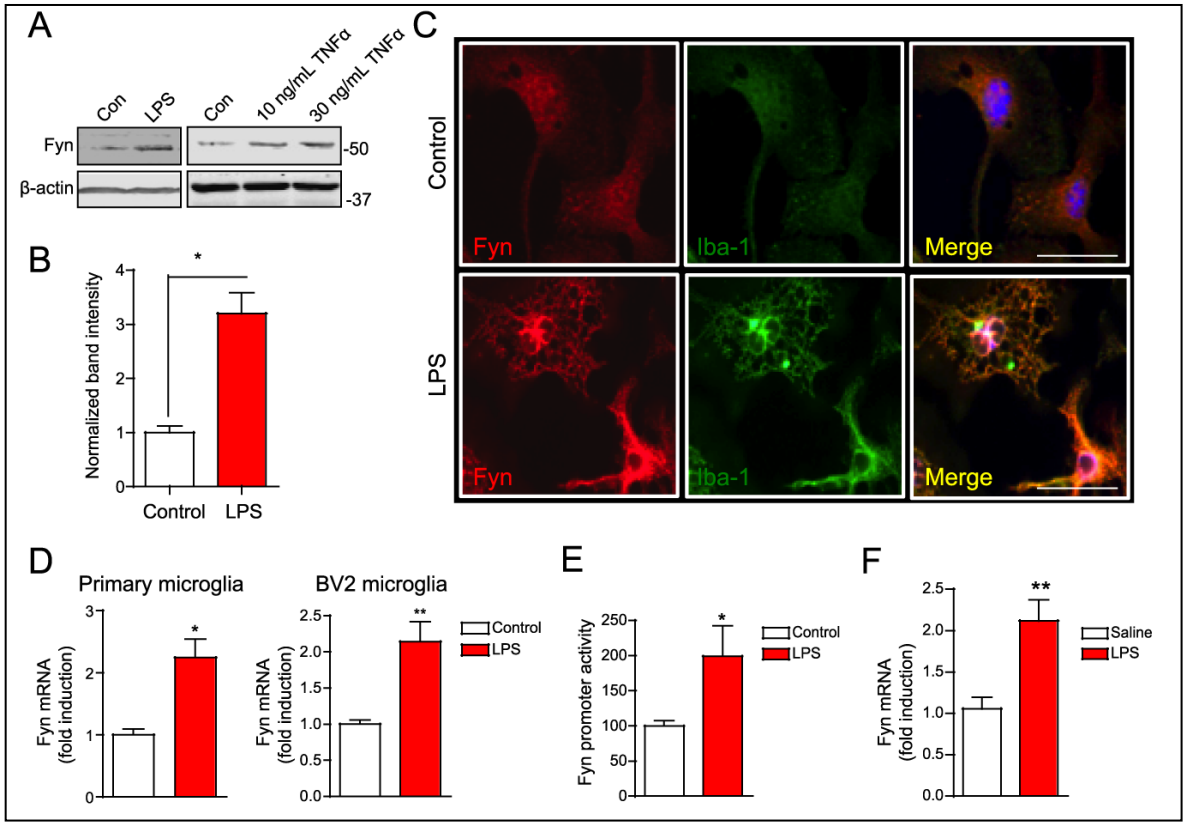

Figure 12. Prolonged inflammogen stimulation induces Fyn upon microglial activation. ***A***, ***B***, Stimulation of primary microglia with LPS for 12 h and TNFα for 24 h increased Fyn expression, as evidenced by Western blotting (*$p < 0.05$). ***C***, ICC analysis of Fyn expression. Scale bar, 20 microns. ***D***, q-RT PCR analysis of Fyn mRNA levels in LPS-stimulated primary microglia and BV2 microglia revealed induction of Fyn at the message level (*$p < 0.05$, **$p < 0.01$). ***E***, Induction of Fyn promoter activity in primary microglia following LPS activation of wild-type primary microglia (*$p < 0.05$). ***F,*** Increased striatal Fyn mRNA levels were seen in the $Fyn^{+/+}$ mice injected intraperitoneally with LPS (5 mg/kg) for 12 h, as assessed by q-RT PCR (**$p < 0.01$).

CHAPTER 3. FYN KINASE CONTRIBUTES TO HUMAN ALPHA-SYNUCLEIN-INDUCED PRIMING AND ACTIVATION OF THE NLRP3 INFLAMMASOME IN MICROGLIA, LEADING TO EXACERBATED STERILE INFLAMMATION

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Nikhil Panicker[1], Souvarish Sarkar[1], Hariharan Saminathan[1], Huajun Jin[1], Dilshan S. Harishchandra[1], Matthew Neal[1], Adhithiya Charli[1], Dharmin Rokad[1], Vellareddy Anantharam[1], Anumantha G. Kanthasamy[1] and Arthi Kanthasamy[1,2]

Abstract

Persistent neuroinflammation is recognized as a major pathophysiological contributor to the progression of Parkinson's disease (PD). Microglia, the resident macrophagic cells of the brain, mediate chronic neuroinflammation through the production of pro-inflammatory factors. Identifying novel molecular signaling events that perpetuate sustained microglial activation, which in turn contributes to progressive neurodegeneration in PD could potentially identify drug targets that halt its progression. Hyperactivation of the NLRP3 inflammasome, traditionally shown to be involved in the innate immune response to microbial pathogens and cellular stress, has recently been demonstrated to contribute to the pathology of Alzheimer's disease. However, its role in PD pathogenesis is yet to be established. The signaling mechanisms that govern microglial NLRP3 inflammasome

[1] Parkinson's Disease Research Program, Iowa Center for Advanced Neurotoxicology, Department of Biomedical Sciences, Iowa State University, Ames, Iowa 50011.
[2] Author of correspondence.
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signaling are poorly characterized. Herein, we show that aggregated human α-synuclein, the major component of Lewy bodies, can activate the inflammasome pathway in microglia through the activation of the non-receptor Src family kinase - Fyn. Aggregated α-synuclein treatment amplified LPS-induced priming of the NLRP3 inflammasome in murine primary microglia, synergistically promoting NLRP3 and pro-IL-1β induction, as well as subsequent IL-1β processing and Caspase-1 activation, culminating in the secretion of cleaved IL-1β into the supernatant. LPS primed $Fyn^{-/-}$ microglia showed diminished α-synuclein-induced NLRP3 inflammasome activation when compared to wild-type (WT) microglia. We then demonstrate that aggregated α-synuclein can mediate both priming and activation of the NLRP3 inflammasome independent of LPS stimulation. Fyn was found to be rapidly activated in microglial cells upon aggregated α-synuclein stimulation, and contributed to PKCδ activation and subsequent NF-κB activation, which induced pro-IL-1β and NLRP3 mRNA and protein levels. Strikingly, Fyn was also found to play a role in the import of α-synuclein into microglial cells, contributing to the generation of mitochondrial reactive oxygen species (MitoROS). We observed diminished production of IL-1β and other pro-inflammatory cytokines from Fyn-deficient microglia in response to aggregated α-synuclein stimulation. Taken together, our studies show for the first time that aggregated α-synuclein can prime and activate the NLRP3 inflammasome, and that Fyn contributes to both these processes.

Introduction

Parkinson's Disease (PD) is a neurodegenerative disorder characterized by progressive motor deficits and the death of dopaminergic neurons within the nigrostriatal

tract, which project from the Substantia Nigra (SN) to the striatum. Several causative factors contribute to PD-associated neurodegeneration, including mitochondrial dysfunction, oxidative stress and proteasomal impairment (Jenner and Olanow, 2006; Levy et al., 2009; Olanow, 2007; Przedborski, 2005). Currently, there is no cure for PD and existing treatments focus on controlling the symptoms, rather than preventing the progression of the disease (Jankovic and Aguilar, 2008).

Recently, sterile inflammation, mediated primarily by resident brain microglia and infiltrating monocytes, has been identified and gained traction as both an important abettor of neuron loss that contributes to the progressive nature of most neurodegenerative diseases, including PD, as well as an attractive drug target for neurodegenerative disease therapy. Various lines of evidence, including cell culture, animal models and post-mortem tissue analysis have implicated sustained neuroinflammation in being critical to PD progression (Block et al., 2007; Glass et al., 2010; Imamura et al., 2003; Tansey and Goldberg, 2010).

Inflammasomes are large, multimeric protein complexes that comprise of a pattern-recognition receptor such as the nucleotide-binding oligomerization domain (NOD) like receptors (NLRs), the adaptor protein ASC and Caspase-1. Inflammasome assembly is triggered by a variety of stimuli and culminates in the activation of Caspase-1, which then cleaves the inflammogen-inducible pro-Interleukin-1β (pro-IL-1β) to mature IL-1β (Latz et al., 2013; Walsh et al., 2014). The NLRP1b and NLRC4 inflammasomes can be activated independent of ASC (Lamkanfi and Dixit, 2014).

The NLRP3 inflammasome is the most widely studied mediator of Caspase-1 activation. Activation of this inflammasome is a two-step process; the first step, priming, involves the inflammogen-mediated induction of the proteins NLRP3 and pro-IL-1β by

activating the NF-κB pathway. The second step, known as activation, requires a second stimulus called a danger signal, which, through the disruption of cytosolic homeostasis, brings about the assembly of the inflammasome components into a functional complex, leading to the activation of Caspase-1 and subsequent Caspase-1-mediated cleavage of pro-IL-1β to mature IL-1β, which is secreted. This two-step mechanism is hypothesized to serve as a checkpoint to prevent unabated release of IL-1β. The NLRP3 inflammasome-associated hypersecretion of IL-1β is, in turn, linked to the pathology of various inflammatory diseases, including Alzheimer's disease (Heneka et al., 2013), diabetes (Lee et al., 2013) and atherosclerosis (Duewell et al., 2010; Sheedy et al., 2013). IL-1β has been shown to directly kill neurons and also increase pathological neuronal Tau phosphorylation (Li et al., 2003; Wang et al., 2005) and contribute to pro-inflammatory signaling in glial cells (Moynagh, 2005). Increased IL-1β and cleaved Caspase-1 levels have already been demonstrated in PD patient tissues, but these studies were published before it was demonstrated that IL-1β processing is mediated by inflammasome complexes (Mogi et al., 1996; Mogi et al., 2000).

The NLRP3 inflammasome, classically implicated in the response to cellular stress or microbial pathogens in peripheral immune cells, was first shown to be activated in microglia when fibrilar β-amyloid was used as a danger signal to activate them. This was also the first time that the NLRP3 inflammasome activation was shown to be mediated by a misfolded protein (Halle et al., 2008).

Fyn is a non-receptor Src family tyrosine kinase. Within the peripheral immune system, it was demonstrated to mediate T cell and mast cell activation (Gomez et al., 2005; Palacios and Weiss, 2004). Recent studies also demonstrate that it contributes to Natural Killer cell activation (Rajasekaran et al., 2013). It is expressed by most cells within the

Central Nervous System (CNS), and has been shown to mediate β-amyloid-induced apoptosis in cortical neurons (Lambert et al., 1998), as well as contribute to astrocytic migration (Dey et al., 2008) and the differentiation of oligodendrocytes (Sperber et al., 2001). It was also shown to contribute to the activation of murine macrophages and microglia in response to fibrilar β-amyloid (Moore et al., 2002; Stuart et al., 2007). Our group has demonstrated how activated Fyn contributes to oxidative stress-induced pro-apoptotic signaling in N27 dopaminergic neuronal cells, as well as in inflammogen-activated microglial cells in *in-vitro* and *in-vivo* models of PD via PKCδ tyrosine phosphorylation (Kaul et al., 2005; Panicker et al., 2015; Saminathan et al., 2011). To the best of our knowledge, no study exists on the role that Fyn plays in the activation of the NLRP3 inflammasome in any cell type. Although it was demonstrated that PKCδ plays a crucial role in the activation of the NLRC4 inflammasome via S533 phosphorylation activation (Qu et al., 2012), this is also the first study that characterizes the role that this protein plays in NLRP3 inflammasome activation.

The α-synuclein gene (SNCA), was the first gene that was linked to autosomal recessive PD (AR-PD). Missense point mutations or triplication of the α-synuclein gene can both cause autosomal dominant familial PD or PD-like conditions (Allen Reish and Standaert, 2015; Appel-Cresswell et al., 2013; Kruger et al., 1998; Lesage et al., 2013; Pasanen et al., 2014; Polymeropoulos et al., 1997; Zarranz et al., 2004). α-synuclein is a pre-synaptic protein, which is predominantly expressed by neurons throughout the brain. It has the predilection to form fibrils, which under pathogenic conditions may form aggregates of varying degrees of organization. It constitutes the major component of Lewy bodies, the neuropathological hallmark of PD. A fascinating theory that has gained support and credence

in recent times postulates that α-synuclein, in its pathogenic forms, can propagate from cell to cell in a prionic manner, seeding the aggregation of non-pathogenic α-synuclein to form pathogenic aggregates, which can propagate inflammation and cell death in a progressive manner (Luk et al., 2012a; Luk et al., 2012b; Volpicelli-Daley et al., 2011).

The signaling pathways through which α-synuclein mediates microglial activation have recently begun to be elucidated. It was demonstrated that recombinant endotoxin-free and aggregated α-synuclein was able to elicit the production of pro-inflammatory cytokines, including IL-1β in unprimed microglia, but the NLRP3 inflammasome was not selectively identified as the causative factor for this event (Boza-Serrano et al., 2014; Lee et al., 2010; Su et al., 2008). α-synuclein was also demonstrated to effect IL-1β production in monocytes (Codolo et al., 2013; Gustot et al., 2015). Though infiltrating monocytes contribute to the sterile inflammation in neurodegenerative diseases, they have constitutively activated Caspase-1, which enables them to produce Il-1β in response to a Toll-like receptor (TLR) ligand such as Lipopolysaccharide (LPS), without requiring an intervening danger signal step (Netea et al., 2009). Because of this, pathways identified using these systems may not have significance to resident microglial cells. AAV-mediated overexpression of synuclein in the SN was also demonstrated to result in elevated striatal cytokine production, including IL-1β and TNFα, but again, inflammasomes were not directly implicated (Chung et al., 2009). We demonstrate conclusively that aggregated α-synuclein is able to both prime and activate the NLRP3 inflammasome in microglia.

To the best of our knowledge, we are the first group to conclusively, through immunoprecipitation, demonstrate interaction between α-synuclein and its receptors TLR2 and CD36. We also demonstrate here that α-synuclein stimulation of microglia results in the

association of CD36 with the non-receptor Src family tyrosine kinase Fyn, which phosphorylates PKCδ and subsequently contributes to PKCδ-dependent priming of the NLRP3 inflammasome as well as PKCδ-independent α-synuclein import into microglia, leading to mitoROS generation and subsequent mitochondrial dysfunction.

Materials And Methods

Chemicals and reagents

Dulbecco's modified Eagle's medium/F-12 (DMEM/F-12), ascorbic acid, RPMI, fetal bovine serum (FBS), L-glutamine, Hoechst nuclear stain, penicillin, streptomycin and other cell culture reagents were purchased from Invitrogen (Gaithersburg, MD). L929 conditioned medium was a kind gift from Douglas Jones at Iowa State University. Recombinant TNFα was purchased from Peprotech (Rocky Hill, NJ), and LPS (E. coli 0111:B4, Endotoxin content 6.6000000 EU/mg) and 6-OHDA were purchased from Sigma (St. Louis, MO). SN50 and the rabbit antibody to human α-synuclein were obtained from EMD Millipore (Billerica, Massachusetts). The Mitotracker Red and MitoSOX dyes were obtained from Life Technologies (Grand Island, NY). Goat IL-1β, rat NLRP3, and goat CD-36 and TLR-2 antibodies were obtained from R & D systems. Mouse NLRP3, Mouse Caspase-1 and rabbit ASC antibodies were obtained from Adipogen. The mouse Fyn and GAPDH antibodies was purchased from Thermo Scientific (Waltham, MA). Antibodies for rabbit Fyn, PKCδ, p-Y311 PKCδ, IκBα, Lamin-B, NOS2 (iNOS) and mouse Tubulin and human α-synuclein were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies against rabbit p-Src family kinase Y416 (p-Y416 SFK), native p65, were purchased from Cell Signaling (Beverly, MA). The TH antibody was purchased from

Chemicon (Temecula, CA). Mouse M2 FLAG and β-actin antibodies, as well as the rabbit β-actin antibody were purchased from Sigma. Rabbit and goat Iba-1 antibodies were purchased from Wako Chemicals (Richmond, VA) and Abcam (Cambridge, MA), respectively. 32P-ATP was purchased from Perkin Elmer (Boston, MA) and the histone substrate from Sigma. The Bradford protein assay kit was purchased from Bio-Rad Laboratories (Hercules, CA). FLAG-tagged human Wild Type (WT) Fyn and Y417A mutant Fyn constructs were obtained as described previously (Kaspar and Jaiswal, 2011). The rat Caspase-11 antibody was purchased from Novus Biologicals. The WT and Caspase-$1^{-/-}$ microglial cell lines, as well as the ASC-CFP cell line were a gift from Dr. Douglas Golenbock at the University of Massachusetts. Femurs from WT, NLRP$3^{-/-}$, ASC$^{-/-}$, Caspase-$1^{-/-}$ and Caspase-$11^{-/-}$ mice were obtained from Dr. Jenny PY Ting at the University of North Carolina.

Human α-synuclein purification, aggregation and removal of endotoxin content

BL21 (DE3) cells transformed with a pT7-7 plasmid encoding WT human α-synuclein were freshly grown on an ampicillin agar plate; then a single colony was transferred to 10 mL of LB medium with 100 μg/mL ampicillin incubated overnight at 37°C with shaking (pre-culture). The next day, the pre-culture was used to inoculate 1L of LB/ampicillin medium. When the OD_{600} of the cultures reached 0.5, protein expression was induced with 1 mM isopropyl β-D-1-thiogalactopyranoside (Invitrogen), and the cells were further incubated at 37°C for 8 h before harvesting by centrifugation. Lysis was performed on ice, by resuspending the cell pellet in 10 mM Tris-HCl, pH 8.0, 1 mM EDTA, and 1 mM phenylmethylsulfonyl fluoride (PMSF) and ultrasonicated with 30 sec pulses followed by a 30 sec pause, for a total ultrasonication time of 2 min. Lysates were finally filtered

through 0.22 μm membranes and loaded onto a Bio-Rad UNO Q6 ion exchange column on BioLogic DuoFlow (Bio-Rad) system chromatography system. Fractions collected during elution with a salt gradient were assayed for the presence of α-synuclein protein by SDS-PAGE followed by Coomassie staining. Fractions containing α-synuclein were pooled, dialyzed against 10 mM HEPES, 50 mM NaCl, pH 7.4 and protein concentration determined by Bradford assay.

Primary microglial cultures and treatments

Primary microglial cultures were prepared from WT, $Fyn^{-/-}$ and $PKC\delta^{-/-}$ postnatal day 1 (P1) mouse pups as described previously with slight modifications (Gordon et al., 2011). Briefly, mouse brains were harvested, meninges removed and then placed in DMEM-F12 supplemented with 10% heat-inactivated FBS, 50 U/mL penicillin, 50 μg/mL streptomycin, 2mM L-glutamine, 100 μM nonessential amino acids and 2 mM sodium pyruvate. Brain tissues were then incubated in 0.25% trypsin-EDTA for 15 min with gentle agitation. The trypsin reaction was stopped by adding double the volume of DMEM/ F12 complete medium and then washing brain tissues three times. Tissues were then triturated gently to prepare a single-cell suspension, which was then passed through a 70 μm nylon mesh cell strainer to remove tissue debris and aggregates. The cell suspension was then made up in DMEM/F12 complete medium and seeded into T-75 flasks, which were incubated in humidified 5% CO_2 at 37°C. The medium was changed after 5–6 d, and the mixed glial cells were grown to confluence. Microglial cells were separated from confluent mixed glial cultures by differential adherence and magnetic separation to more than 97% purity and then were allowed to recover for 48 h after plating. Primary microglia were treated in DMEM/F12

complete medium containing 2% FBS. Microglia were primed with 1 μg/mL LPS for 3 h, is a dose and time point which has been used in several published studies (Halle et al., 2008). For signaling experiments, the protocol used by Stuart et. al. (Stuart et al., 2007) was used with a small modification. For this, the primary microglial cells were kept in 2% DMEM/F12 complete medium for 5 h at 37°C before treatment. The microglial cells were treated with 3.5-7 μg/mL aggregated α-synuclein, which is a similar dose used by several published articles (Boza-Serrano et al., 2014; Kim et al., 2013). For immunycytochemistry studies, microglia were obtained by the shake-off method as previously described (Gordon et al., 2011).

Primary BMDM macrophage and culture

A sterile blade was used to cut the bone epiphyses off from the femurs exposing the marrow cavity. A 10 mL syringe with a 30 ga ½ inch needle was filled with 10% Fetal Bovine Serum (FBS) containing DMEM. This medium was flushed through the bone and the marrow was collected a sterile 14 mL falcon tube. The marrow was centrifuged at 250g for 15 min at 4°C. Pellets were resuspended in bone marrow macrophage medium (DMEM medium containing 20% FBS, 30% L929 cell conditioned medium, Penicillin/Streptomycin, Sodium pyruvate). 15 X 10^6 cells were plated out in (15 mm x 150 mm) Petri dishes. Additional bone marrow macrophage medium was supplemented on day 3. The cells were kept at 37°C and 5% CO_2 for 6 d. Adherent differentiated macrophages were trypsinized on day 6 and used for experiments.

Immunoblotting

Lysates were prepared using modified RIPA buffer and were normalized for equal amounts of protein using the Bradford protein assay kit. Equal amounts of protein (6-10 μg for nuclear lysates and 10-25 μg for cell lysates) were loaded for each sample and separated on either 12% or 15% SDS-PAGE gels depending on the molecular weight of the target protein. After separation, proteins were transferred to a nitrocellulose membrane and the nonspecific binding sites were blocked for 1 h using a blocking buffer specifically formulated for fluorescent Western blotting (Rockland Immunochemicals). Membranes were then probed with the respective primary antibodies for 3 h at room temperature or overnight at 4°C. After incubation, the membranes were washed 7 times with PBS containing 0.05% Tween 20 and then Secondary IR-680-conjugated anti-mouse (1:10,000, goat anti-mouse, Molecular Probes) and IR-800 conjugated anti rabbit (1:10,000, goat anti-rabbit, Rockland) were used for antibody detection with the Odyssey IR imaging system (LiCor). Membranes were visualized on the Odyssey infrared imaging system. Antibodies for GAPDH, β-actin and Tubulin were used as loading controls. Antibodies against Lamin B were used as the loading control for nuclear lysates. Immunoblots with cell supernatants, 400 µL methanol and 100 µL chloroform were added to 400 µL of cell supernatants obtained post-treatment from microglial cells treated in 12 well plates. The samples were vortexed vigorously for 30 sec. They were then centrifuged at 13,000g for 5 min. The aqueous phase was removed using vacuum. 400 µL of methanol was added to each sample. The samples were vortexed vigorously. The samples were centrifuged at 13,000g for 5 min. Supernatant methanol was removed by vacuum. The pellets were dried for 5-10 min at 55°C and were subsequently

reconstituted in 32 µL of 1× SDS sample buffer + 8 µL β mercapthoethanol. The samples were then vortexed, boiled for 5 min and used for immunoblotting for IL-1β.

qPCR

The RNA extraction protocol was adapted and modified from published protocols (Seo et al., 2014). 2.5 X 10^6 microglia per treatment group obtained post separation were plated in a 6 well plate and after treatment RNA was extracted using the trizol chloroform extraction method. 1 μg of RNA was converted to cDNA using High Capacity cDNA Reverse Transcription Kit from Applied Biosystems (#4368814) following the manufacturer's protocol. Quantitative RT-PCR was performed on the following genes using SYBRGreen Mastermix from Qiagen (#208056)- NLRP3 forward- 'TGCTCTTCACTGCTATCAAGCCCT', NLRP3 reverse- 'ACAAGCCTTTGCTCCAGACCCTAT' (synthesized in Iowa State University DNA facility) & IL-1β-Qiagen QuantiTect Primer Assay (QT01048355). 18SrRNA (Qiagen catalog #PPM57735E) was used as the house keeping gene for all the qPCR experiments. No template controls (NTCs) and dissociation curves were obtained for every experiments to make sure there was no cross contamination.

Co-immunoprecipitation studies

5 X 10^6 primary microglia per treatment group were treated with α-synuclein or vehicle for 30 min. Lysates were prepared using the TNE buffer. Cell lysates were prepared in TNE buffer (10 mM Tris-HCl at pH 7.5, 1% Nonidet P-40, 0.15 M NaCl, 1 mM EDTA and 1:100 protease inhibitor mixture). Pellets were resuspended in TNE buffer and were kept on ice for 30 min. The lysates were then centrifuged at 17,400g for 35 min at 4°C. The

supernatant protein concentration was measured and normalized between samples. Approximately 50 μg protein was used as the input fraction. For immunoprecipitation analysis, 400-500 μg of protein per sample in 500 μL TNE buffer was used. 5 μg of goat polyclonal TLR2 or CD36 antibody were added to the lysates and the samples were set on an orbital shaker overnight at 4°C. The next day, protein G Sepharose beads were added to each sample. The samples were set on an orbital shaker overnight at 4°C. Protein G beads were collected by centrifugation at 2000g for 5 min and were washed four times with TNE buffer. The bound proteins were eluted by boiling in 2 protein loading dye for 5 min. Immunoblots were performed on 12% SDS-PAGE gels as described in the Immunoblots section.

Caspase-1 assay

WT and Fyn$^{-/-}$ microglial cells were plated out onto PDL-coated 96 well plates at 150,000 cells per well. Cells were treated with α-synuclein at the pre-specified doses for 2 h post LPS priming. The FLICA dye was added in PBS for 30 min at 37°C. The cells were washed in PBS 3 times post treatment and the fluorescence read as per the manufacturer's instructions.

Nuclear and cytoplasmic fractionation

Nuclear and cytoplasmic fractions were performed using the NE-PER Kit (Thermo Scientific) as previously described (Jin et al., 2011). Briefly, 5 X 10^6 cells were treated with α-synuclein for 30 min. CER1 reagent (150-200 µL) was used per sample to extract the cytoplasmic fraction and 45 µL of NER reagent was used to extract the nuclear fraction.

Nitric oxide detection

Nitric oxide production by primary microglia was measured indirectly by quantification of nitrite in the supernatant using the Griess reagent (Sigma Aldrich). Microglia were plated in poly-D-lysine-coated 96-well plates at 1 X 10^5 cells/well. Cells were treated with α-synuclein for 24 h post priming with LPS for 3 h and after 100 µL of supernatant was collected from each well, an equal volume of the Griess reagent was added. The samples were incubated on a plate shaker at room temperature for 15 min until a stable color was obtained. The absorbance at 540 nm was measured using a Synergy 2 multi-mode microplate reader (BioTek Instruments) and the nitrite concentration was determined from a sodium nitrite standard curve.

Multiplex cytokine Luminex immunoassays

Primary microglia obtained from WT, PKCδ$^{-/-}$ and Fyn$^{-/-}$ mice were seeded in poly-D-lysine-coated 96 well plates at 1 X 10^5 cells/well. The cells were treated with α-synuclein for 24 h. After treatment, 50 μL of supernatant from each well was collected and frozen at -80°C. The levels of cytokines and chemokines in the supernatants were determined using the Luminex bead-based immunoassay platform (Vignali 2000) and pre-validated multiplex kits (Milliplex mouse cytokine panel – Millipore) according to the manufacturer's instructions.

Transfections of primary microglia

The pre-designed, on-target plus SMART pool Fyn siRNA (a combination of four siRNAs, Cat. No. LQ-040112-00-0002) and scrambled siRNA (Cat. No. D-001210-03-05)

were purchased from Dharmacon (Lafayette, CO). We carried out siRNA transfections in primary mouse microglial cells with Lipofectamine 3000 reagent according to the manufacturer's protocol. Briefly, primary microglia were plated at 2 X 10^6 cells/well in 6-well plates one day before transfection. For each well, 300 pmol of Fyn siRNA pool (75 pmol each) or an equal amount of scrambled siRNA mixed with 5 μL of Lipofectamine 3000 were added to the cells. 72 h after the initial transfection, cells were treated with aggregated α-synuclein for 4 h. Lysates were prepared in modified RIPA buffer as mentioned in the Immunoblotting section. Lysates checked for the expression of pro-IL-1β and Fyn. Transfection of primary microglia with WT Fyn-FLAG, Y417A Fyn-FLAG plasmid constructs was also performed using 5 μL of Lipofectamine 3000, but this was left on for 48 h. FLAG immunoblots were performed to check for successful transfections.

Immunofluorescence

Immunofluorescence studies in primary microglia were performed according to previously published protocols with some modifications (Gordon et al., 2011). Briefly, cells were plated out onto poly-D-lysine-coated coverslips. At the end of treatments, cells were fixed with 4% PFA, washed in PBS and incubated in blocking buffer (PBS containing 1.5% BSA, 0.5% Triton X-100 and 0.05% Tween 20) for 1 h at room temperature. The coverslips were then incubated overnight at 4°C with respective primary antibodies diluted in PBS containing 1% BSA. Samples were then washed several times in PBS and incubated with Alexa 488 and 555 dye-conjugated secondary antibodies. The nuclei were labeled with Hoechst stain (10 μg/mL) and coverslips were mounted with Fluoromount medium (Sigma Aldrich) on glass slides for visualization. ASC-CFP fluorescence was imaged by assessing

the natural fluorescence using the CFP filter using the Leica DMIRE2 confocal microscope objectives.

Mitochondrial ROS measurement

Mitochondrial ROS generation was quantified using the MitoSOX red fluorescent indicator. WT and Fyn$^{-/-}$ microglia were plated out into coverslips at 150,000 cells per well. α-synuclein was added to the cells., The MitoSOX probe was added at the same time, to a final concentration of 1 μM. Fluorescence expressed by the generated mitochondrial superoxides were measured every hour for 0-12 hours using Cytation 3 Cell Imaging Multi-Mode Reader as per the manufacturer's instructions (BioTek- Winooski, VT).

Mitochondrial visualization

Microglia obtained via shale-off method were plated out onto PDL coated cover slips. After treatment under various paradigms, 300 μL of 166 nM CMXROS MitoTracker red dye diluted in serum-free DMEM/F12 media was added and incubated at 37°C for 13 min. After incubation, wells were gently washed with PBS 3-5 times and then fixed in 4% PFA for 30 min. The wells were washed with PBS 3 times. After this, immunocytochemistry for Iba-1 was performed as described in the immunofluorescence section.

Data analysis

Data analysis was performed using Prism 4.0 (GraphPad Software, San Diego, CA). The data was initially analyzed using one-way ANOVA and Bonferroni's post-test to

compare the means of treatment groups. Differences of $p<0.05$ were considered statistically significant. Student's t-test was used when comparing two groups

Results

Aggregated human α-synuclein acts as an efficient danger signal of the NLRP3 inflammasome, effecting IL-1β maturation and release

Previous studies have demonstrated that misfolded proteins can activate the NLRP3 inflammasome in microglia (Hafner-Bratkovic et al., 2012; Halle et al., 2008). Human α-synuclein was obtained and purified as described in the Methods section. Aggregation of α-synuclein was performed as previously described (Zhang et al., 2005). LPS-primed primary murine microglia were treated with α-synuclein for 24 h, with or without the pre-treatment of the pan-Caspase inhibitor ZVAD-FMK or the Caspase-1 specific inhibitor ZYVAD-FMK. α-synuclein treatment sharply induced IL-1β cleavage and secretion, which was reduced in the group pre-treated with the Caspase inhibitors (Figure 1A). There was no significant change in the secretion/production of TNFα in the α-synuclein treated cells and only a marginal reduction of TNFα in the Caspase-1 inhibitor pre-treated groups. Next, we treated LPS-primed bone marrow-derived macrophages (BMDMs) from WT, NLRP3$^{-/-}$, ASC$^{-/-}$, Caspase-1$^{-/-}$ and Caspase-11$^{-/-}$ mice with aggregated human α-synuclein for 24 h (Figure 1B). There was a dramatic reduction in the secretion of IL-1β from NLRP3, ASC and Caspase-1 deficient, but not Caspase-11 deficient macrophages (Caspase-11$^{-/-}$ macrophages were used as a control, since the Caspase-1$^{-/-}$ mouse line inherently also lacks Caspase-11). There was no change in the levels of supernatant TNFα in any of the genotypes. We also confirmed that none of these proteins played a role in the priming of the NLRP3 inflammasome by assessing

the levels of pro-IL-1β produced upon LPS treatment by immunoblot (Figure 1C). Next, we assessed the inflammasome activating ability of aggregated human α-synuclein by utilizing the ASC-CFP reporter cell line. These cells overexpress ASC tagged with cyan fluorescent protein (CFP). Upon the activation of the NLRP3 inflammasome, the ASC coalesces to form a single speck within each cell. The number of ASC positive specks can be counted and used as a readout of inflammasome activation. ASC-CFP cells were primed with LPS and treated with aggregated synuclein for 2 h. Endogenous CFP fluorescence was assessed. In the synuclein treated group, specks of ASC were observed, indicating inflammasome activation (Figure 1D). These results indicate that aggregated α-synuclein serves as an efficient danger signal to activate the NLRP3 inflammasome.

Fyn kinase contributes to LPS and TNFα mediated priming of the NLRP3 inflammasome

Recently, we described how inflammogen stimulation of microglia resulted in rapid Fyn activation, and Fyn-dependent NF-κB pathway activation, which contributed to LPS and TNFα-mediated cytokine production and pro-inflammatory signaling (Panicker et al., 2015). Accordingly, we assessed the role of Fyn in LPS and TNFα-mediated priming of the NLRP3 inflammasome. WT and $Fyn^{-/-}$ microglia were primed with various doses of the aforementioned inflammogens, and inflammasome priming was assessed by blotting for pro-IL-1β and NLRP3. As expected, LPS and TNFα elicited a dose dependent induction of pro-IL-1β and NLRP3 levels in WT microglia, but to a significantly lower extent in the $Fyn^{-/-}$ microglia (Figure 2A). We next treated WT and $Fyn^{-/-}$ mice with 5 mg/kg LPS for 24 h, and

checked for serum IL-1β via Luminex. The LPS-mediated serum IL-1β production in the Fyn$^{-/-}$ mice was strongly attenuated (Figure 2B).

α-synuclein amplifies LPS induced priming of the NLRP3 inflammasome in a Fyn dependent manner

The first step in the activation process of the NLRP3 inflammasome is priming, which entails the NF-κB-p65-mediated induction of pro-IL-1β and NLRP3, which occurs subsequent to the engagement of appropriate pro-inflammatory ligands or inflammogens to their respective receptors (Hayden and Ghosh, 2004). Having established that α-synuclein could act as an efficient danger signal of the inflammasome, we sought to elucidate the mechanisms through which it might activate the NLRP3 inflammasome. Immunoblot analysis of LPS primed, α-synuclein treated WT and Fyn$^{-/-}$ microglia revealed that synuclein treatment actually amplified the LPS mediated induction of pro-IL-1β and NLRP3 levels, and did so to a statistically lower extent in the Fyn$^{-/-}$ microglia (Fig 3A), leading us to hypothesize that aggregated α-synuclein directly potentiated LPS mediated priming by further activating the NF-κB pathway). The synuclein-mediated induction of pro-IL-1β and NLRP3 in microglia was abolished in cells pre-treated (post LPS priming and before α-synuclein treatment) with SN-50, an NF-κB inhibitor (Figure 3B, C). We next checked the induction of pro-IL-1β and NLRP3 mRNAs in LPS and LPS + α-synuclein treated WT and Fyn$^{-/-}$ microglia. α-synuclein treatment effected the induction of pro-IL-1β and NLRP3 mRNAs in the WT microglia, but the levels of the respective mRNA in the Fyn$^{-/-}$ microglia were consistently lower (Figure 3D). The well-utilized FLICA assay was then employed to assess the α-synuclein-mediated induction of Caspase-1 activity in primed WT and Fyn$^{-/-}$

microglia. We discovered that Caspase-1 activity was strongly induced in WT microglia, but not at all in Fyn deficient microglia, strengthening our posit that Fyn played a role in the inflammasome activation (Figure 3E). The α-synuclein-mediated secretion of IL-1β and various other pro-inflammatory cytokines was also diminished in $Fyn^{-/-}$ microglia (Figure 3F). NOS2 is the rate-limiting enzyme that mediates nitrite production in various immune cells including microglia. It has previously been shown to be induced in the APP/PS1 mice (Heneka et al., 2013). We show that α-synuclein treatment of LPS primed microglia also significantly elicited the induction of NOS2 and increase in supernatant nitrite levels in WT but did so to a lower extent in the $Fyn^{-/-}$ microglia (Figure 3G). Taken together, our results indicate that aggregated human synuclein amplifies LPS-induced priming, while simultaneously acting as a danger signal of the NLRP3 inflammasome, culminating in the release of mature IL-1β and nitrite, in a signaling pathway that utilizes Fyn kinase.

Aggregated human α-synuclein primes and activates the NLRP3 inflammasome, resulting in IL-1β processing and secretion

The current model of the NLRP3 inflammasome postulates a two-step mechanism, with a TLR or TNFR1 ligand acting as an initial signal to induce the expression of pro-IL-1β and NLRP3 proteins and a subsequent second signal that effects lysosomal rupture, mitochondrial ROS generation and/or K^+ efflux that acts as a danger signal, resulting in the assembly and activation of the inflammasome complex, and the processing and secretion of IL-1β into the cell supernatant. Since we observed that α-synuclein can act as an efficient danger signal of the inflammasome, and can also amplify LPS mediated induction of the NLRP3 and pro-IL-1β at the message and protein level, we wondered if it could activate the

NLRP3 inflammasome independent of an LPS-mediated priming step. Unprimed, immortalized WT and Caspase1$^{-/-}$ microglial cells were treated with aggregated α-synuclein for 4 h and the levels of pro-IL-1β and NLRP3 assessed via immunoblot. Aggregated α-synuclein effected the induction of pro-IL-1β and NLRP3 to equivalent levels in both cell types (Figure 4A). It also brought about the secretion of IL-1β in the WT, but to a strikingly lower extent, in the Caspase-1 deficient microglial cells. The secretion of IL-1β could also be inhibited by pre-treatment with Saracatinib, a Fyn inhibitor in a dose-dependent manner (Figure 4B). We next utilized primary bone marrow-derived macrophages from WT, NLRP3$^{-/-}$, ASC$^{-/-}$, Caspase-1$^{-/-}$ and Caspase-11$^{-/-}$ mice and checked for the ability of α-synuclein to induce NLRP3 inflammasome activation in them. As expected, we saw no difference in the ability of macrophages of all aforementioned genotypes to induce pro-IL-1β and import α-synuclein. However, the α-synuclein-mediated processing of Caspase-1 was almost completely absent in ASC$^{-/-}$, NLRP3$^{-/-}$ and Caspase-1$^{-/-}$ macrophages (Figure 4C). Supernatant analysis of unprimed macrophages treated with α-synuclein for 12 h showed that α-synuclein elicited robust IL-1β production from WT macrophages, and this production was severely diminished in the NLRP3$^{-/-}$, ASC$^{-/-}$ and Caspase-1$^{-/-}$ macrophages and was largely restored in the Caspase-11$^{-/-}$ macrophages (Figure 4D). The α-synuclein-mediated TNFα production was not statistically different in any of the cell types.

Aggregated α-synuclein treatment rapidly activates Fyn.

We then sought to elucidate the signaling mechanism through which α-synuclein activated the NLRP3 inflammasome in microglia. Various studies have indirectly implicated disparate receptors in binding to and mediating α-synuclein signaling; TLR2 was described to be

essential for α-synuclein induced pro-inflammatory signaling and to contribute to α-synuclein import into microglia (Kim et al., 2013). TLR4, CD36 and FcγR deficient microglia demonstrated attenuated neuroinflammatory responses in response to α-synuclein treatment (Fellner et al., 2013, Su et al., 2008, Cao et al., 2012). Misfolded α-synuclein was shown to interact with microglial TLR1/2 and mediate Myd-88 dependent pro-inflammatory signaling (Daniele et al., 2015). Since we had previously demonstrated early inflammogen-mediated activation of Fyn and PKCδ, we sought to link α-synuclein recognition by a microglial receptor to Fyn activation. Fyn has classically shown to be activated downstream of CD36 (Chen et al., 2008; Moore et al., 2002), and more recently, TLR2 in TLR2 overexpressing HEK293 cells (Finberg et al., 2012). We immunoprecipitated CD36 and TLR2 in control and α-synuclein-stimulated WT microglia and checked for interaction of these receptors with both α-synuclein as well as Fyn. Both CD36 and TLR2 interacted with α-synuclein but Fyn only interacted with CD36 in α-synuclein-stimulated microglia (Figure 5A). We also checked if Fyn activation was inducible upon early α-synuclein stimulation of primary microglia. α-synuclein treatment rapidly induced the active loop phosphorylation of Fyn. The p-Y416 Src family kinase (p-Y416 SFK) antibody was utilized to recognize activated Fyn, since this antibody recognizes all active Src family kinases, we checked for the synuclein-induced p-Y416 SFK levels in $Fyn^{-/-}$ microglia and did not see any discernable Src kinase activation (Figure 5B). We also overexpressed FLAG tagged WT and activation loop mutant Fyn (Y417A Fyn) in WT primary microglial cells and treated cells with aggregated α-synuclein for 15, 30 and 45 min. Whole cell lysates were probed for FLAG and p-SFKY416 antibodies. WT Fyn-FLAG transfected cells demonstrated a rapid induction of p-Y416 SFK levels, which was abolished in the Y417A Fyn-FLAG transfected groups. The FLAG and p-Y416

SFK levels were assessed using secondary antibodies in the red and green channels respectively. The FLAG and p-Y416 SFK bands perfectly co-localized in the WT-Fyn transfected cells, indicating that Fyn as the Src family kinase that was preferentially activated in the cells following aggregated α-synuclein stimulation (Figure 5C). Lastly, immunocytochemistry analysis showed that p-Y416 SFK levels rapidly increased in microglial cells stimulated with aggregated α-synuclein. Active Fyn is preferentially localized along the membrane periphery, which is to be expected since it is activated rapidly following association with membrane-bound CD36 (Figure 5D).

Fyn contributes to aggregated α-synuclein mediated NF-κB activation, contributing to priming of the NLRP3 inflammasome

Priming of the NLRP3 inflammasome involves activation of the NF-κB pathway downstream of TLR/TNFR1 engagement of their respective ligands. The NF-κB complex comprises of heterotrimeric complex - IκBα, p65 and p50. Upon inflammogen stimulation, IκBα is phosphorylated and rapidly degraded, allowing the nuclear entry of the p65-NF-κB subunit. p65 is a transcription factor that binds to the promoters of various pro-inflammatory cytokine genes as well as the NLRP3 gene, bringing about their transcription, leading to the production of pro-inflammatory cytokines/pre-cytokines. We have previously shown that Fyn contributes to LPS and TNFα-mediated NF-κB activation in microglia (Panicker et al., 2015). To assess the role of Fyn in α-synuclein-mediated priming of the NLRP3 inflammasome, we first treated WT and $Fyn^{-/-}$ microglia with aggregated α-synuclein for 30 min. We then prepared nuclear and cytosolic extracts and probed the nuclear extracts for p65. There was lesser α-synuclein-induced nuclear translocation of p65 in the $Fyn^{-/-}$ microglial

nuclear lysates (Figure 6A, B). To directly assess the role of Fyn in priming of the inflammasome, we treated WT and Fyn$^{-/-}$ microglial cells with α-synuclein for 45 min and evaluated mRNA levels of pro-IL-1β, NLRP3, as well as NLRC4 and AIM2. The α-synuclein-mediated induction of pro-IL-1β and NLRP3 mRNAs was significantly attenuated in the Fyn$^{-/-}$ microglia. Notably, α-synuclein treatment did not bring about an induction of NLRC4 and AIM2 inflammasome levels, demonstrating that the NLRP3 inflammasome was preferentially activated (Figure 6C). We next checked for the induction of NLRP3, pro-IL-1β and the levels of cleaved Caspase-1 in the Fyn$^{-/-}$ microglial lysates. WT and Fyn$^{-/-}$ microglia were treated with α-synuclein for 2, 4 and 6 h. Immunoblotting analysis revealed that pro-IL-1β, NLRP3 and cleaved Caspase-1 levels were significantly diminished in the Fyn$^{-/-}$ microglia (Figure 6D, E). We also knocked Fyn down using Fyn specific siRNA and observed diminished induction of pro-IL-1β in aggregated α-synuclein treated microglia (Figure 6F, G). Lastly, immunoblot and luminex analyses of α-synuclein treated microglial supernatants revealed diminished secretion of mature IL-1β as well as IL-12 from Fyn$^{-/-}$ cells (Figure 6H, I).

Fyn contributes to α-synuclein import into microglial cells, thereby contributing to NLRP3 inflammasome associated mitochondrial dysfunction

Although most existing studies agree on the steps that underlie priming of the NLRP3 inflammasome, the exact nature of the danger or activating signal is contested. The current consensus points to lysosomal dysfunction, mitochondrial reactive oxygen species generation and potassium efflux as possible molecular events that lead to the assembly of the NLRP3 inflammasome. Since we observed the interaction of Fyn with CD36, a receptor protein that

has been show to play a role in the uptake and aggregation seeding ability of fibrilar amyloid-β(Sheedy et al., 2013), we wondered whether Fyn might play a role in the uptake of synuclein into microglial cells as well. To check for this, human synuclein was added to WT and $Fyn^{-/-}$ microglia for various time points, the cells washed 3 times with PBS, then fixed and stained for human synuclein to observe intracellular human synuclein. The human α-synuclein taken up by microglia shows up as intracellular puncta. The number of puncta per cell per field were counted and quantified. Taken together, $Fyn^{-/-}$ microglia display diminished uptake of human α-synuclein (Figure 7A, B). We also quantified the uptake of human α-synuclein using immunoblot. WT and $Fyn^{-/-}$ microglia were treated with human α-synuclein for 15, 30 and 45 min. The cell supernatant was aspirated and the cells washed several times in PBS. Immunoblots of whole cell lysates for human α-synuclein indicated significantly attenuated uptake in the Fyn deficient cells (Figure 7C,D). Uptake of an inflammasome activator disrupts cellular homeostasis through several possible mechanisms. ROS generation, specifically from the mitochondria, has gained acceptance as a prime contributor to inflammasome activation (Zhou et al., 2011). We utilized the mitoSOX dye to quantify synuclein induced mitochondrial ROS generation in WT and $Fyn^{-/-}$ microglia. Treatment of cells with synuclein rapidly induced progressively increasing mitoROS generation in the WT microglia, but did so to a statistically lesser extent in the $Fyn^{-/-}$ microglia (Figure 7E). Mitochondrial dysfunction is also characterized by a change in morphology of mitochondria, changing from thread like to round. We quantified the synuclein-induced change in mitochondrial morphology in the WT and $Fyn^{-/-}$ microglia. 24 h post treatment, the microglia in the WT synuclein treated group demonstrated rounded

mitochondria, whereas there was no discernable change in mitochondrial morphology in the $Fyn^{-/-}$ microglia post synuclein treatment (Figure 7F, G,).

PKCδ contributes to aggregated α-synuclein mediated NF-κB activation but not α-synuclein import into microglia

We have previously demonstrated that upon activation following LPS and TNFα stimulation, Fyn associates with and tyrosine phosphorylates the serine threonine kinase PKCδ in microglial cells at residue Y311. We observed that α-synuclein also mediated an increase in p-Y311 PKCδ levels, but did not do so in the Fyn deficient microglia, suggesting a conserved pro-inflammatory signaling pathway downstream of inflammogen activation (Figure 8A). Upon checking nuclear lysates from aggregated α-synuclein treated $PKC\delta^{+/+}$ and $PKC\delta^{-/-}$ cells for p65, we observed diminished activation of the NF-κB pathway, evidenced by reduced nuclear p65 in the $PKC\delta^{-/-}$ nuclear fractions, reminiscent of the Fyn-PKCδ mediated signaling cascade downstream of LPS and TNFα activation (Figure 8B,C). The aggregated α-synuclein mediated upregulation of pro-IL-1β mRNA was also significantly attenuated in the PKCδ deficient microglia (Figure 8D), as was the synthesis of pro-IL-1β and NLRP3 proteins (Figure 8E) and the production of supernatant IL-1β (Figure 8F). We also wanted to check whether PKCδ played a role in α-synuclein import. We used whole cell lysates from $PKC\delta^{+/+}$ and $PKC\delta^{-/-}$ microglia treated with aggregated α-synuclein for 30 and 45 minutes. Upon probing for human α-synuclein, we saw no change between either genotype with respect to α-synuclein import (Figure 8G). These results suggest a bifurcation of Fyn dependent signaling, showing that Fyn activation feeds into the PKCδ

dependent NF-κB pathway activation and priming of the NLRP3 inflammasome, and the PKCδ independent import of α-synuclein.

Discussion

A wealth of evidence from cell culture, animal models and post-mortem analysis of brains from PD patients implicates hyperactivation of the innate immune system in the brain as being central and contributive to the progressive nature of PD as well as other neurodegenerative disorders. This immune reaction is primarily mediated by microglia, the macrophagic cells of the brain. Another recent and intriguing hypothesis suggests that misfolded α-synuclein can seed aggregation of WT α-synuclein in a prionic manner, propagating the spread of neuron death and concurrent inflammation (Luk et al., 2012a; Luk et al., 2012b; Volpicelli-Daley et al., 2011). Aggregated α-synuclein has previously been demonstrated to effect microglial pro-inflammatory signaling and inflammatory mediator production (Zhang et al., 2005). However, the signaling pathways that are utilized to activate microglial cells, and specifically the NLRP3 inflammasome in microglia, are poorly characterized. This article describes a novel signaling mechanism through which α-synuclein aggregates effect the activation of the non-receptor tyrosine kinase Fyn, which contributes to both NF-κB priming of the inflammasome as well as α-synuclein import in the microglial cells, thereby contributing to the secretion of IL-1β as well as other inflammatory mediators, such as nitrite. Inhibitors of the NLRP3 inflammasome and Fyn have been used with success to limit the progress of experimental autoimmune encephalitis and AD respectively (Coll et al., 2015; Kaufman et al., 2015). It is hence envisageable that similar inhibitors could be used to halt the progression of PD. Activation of the NLRP3 inflammasome is a two-step

mechanism - a 'priming' step that induces levels of pro-IL-1β and NLRP3, and a second activating step, that induces the assembly of the inflammasome. Misfolded proteins have been shown to activate LPS-primed inflammasomes in microglia (Hafner-Bratkovic et al., 2012; Halle et al., 2008). We first demonstrate that aggregated α-synuclein can elicit IL-1β production in microglia by activating the NLRP3 inflammasome, evidenced by the production of IL-1β and the formation of ASC specks in the ASC-CFP reporter cell line (Figure 1A-D). A recent paper indicated that commercially obtained aggregated α-synuclein couldn't elicit IL-1β production (Gustin et al., 2015), but this finding is refuted by multiple studies that have used recombinant endotoxin-free aggregated α-synuclein to elicit microglial IL-1β production (Boza-Serrano et al., 2014; Daniele et al., 2015; Lee et al., 2010), although direct involvement of the NLRP3 inflammasome in the aforementioned studies was not proven. We sought to identify the signaling pathways through which it might activate the inflammasome. We discovered that aggregated α-synuclein amplified the LPS-induced expression of the inflammasome components pro-IL-1β and NLRP3 at both the mRNA and protein levels and did so in an NF-κB dependent manner (Figure 3A-D). We have previously demonstrated the role of the non-receptor tyrosine kinase Fyn in contributing to NF-κB activation in response to diverse inflammogens (Panicker et al., 2015). In agreement with these findings, we show that LPS and TNFα treatments elicit a dose dependent induction in NLRP3 and pro-IL-1β proteins, but do so to a significantly lower extent in $Fyn^{-/-}$ microglia (Figure 2A). $Fyn^{-/-}$ mice also demonstrated diminished serum IL-1β responses in response to intraperitoneal LPS administration (Figure 2B). The LPS and LPS + α-synuclein induced pro-inflammatory signaling was significantly dampened in the $Fyn^{-/-}$ microglia (Figure 3A-D). The synuclein-mediated induction of Capsase-1 activity, as well as the secretion of pro-

inflammatory cytokines and nitrite was significantly diminished in the Fyn deficient microglia (Figure 3E-G). We then reasoned that α-synuclein could prime and activate the NLRP3 inflammasome by itself, without requiring an intervening LPS mediated priming step. The α-synuclein-mediated induction of pro-IL-1β and NLRP3 were not changed in immortalized microglial cells obtained from Caspase1$^{-/-}$ mice, when compared to WT microglial cells. However, the processing of IL-1β was greatly diminished in the Caspase-1 deficient cells, as evidenced by Luminex cytokine analysis of cell supernatant content (Figure 4A, B). Pre-treating cells with the Fyn inhibitor Saracatinib also reduced the production of IL-1β from the WT cells in a concentration dependent manner (Figure 4B). To prove the involvement of the NLRP3 inflammasome in this process, we showed that BMDMs from NLRP3$^{-/-}$, ASC$^{-/-}$ and Caspase-1$^{-/-}$ had negligible α-synuclein-induced Caspase-1 activation (Figure 4C). Furthermore, the α-synuclein-mediated secretion of IL-1β, but not TNFα, was greatly diminished in the cell supernatants obtained from these cells, but not from cells obtained from Caspase-11$^{-/-}$ mice (Figure 4D). We used Caspase-11$^{-/-}$ BMDMs in this study since Caspase-1$^{-/-}$ mice were developed using an S129 mouse background, and these mice inherently lack Caspase-11 expression. Caspase-11 has been demonstrated to participate in the activation of a non-canonical inflammasome. Microglia obtained from Fyn$^{+/+}$ mice (developed on an S129 background) and PKCδ$^{+/+}$ (developed on a C57BL6 background) had nearly identical IL-1β responses to aggregated α-synuclein. Although it has been shown in various studies to activate microglia, a direct biochemical interaction of synuclein with its receptors has yet to be demonstrated, with the exception of one that shows the interaction of TLR1/2 with synuclein, shown by Duolink immunocytochemistry (Daniele et al., 2015). We showed that synuclein could interact with the microglial receptors TLR2

and CD36 in untransfected, WT microglia. CD36 also interacted with Fyn following stimulation with α-synuclein aggregates (Figure 5A). There was a dramatic induction in Fyn activation post stimulation with α-synuclein aggregates(Figure 5B-D). In support of our hypothesis that Fyn plays a role in NLRP3 inflammasome priming, the α-synuclein-mediated nuclear translocation of p65-NF-κB and subsequent mRNA induction of IL-1β and NLRP3 were diminished in the $Fyn^{-/-}$ cells (Figure 6A-C). The aggregated α-synuclein-mediated induction of pro-IL-1β and NLRP3 proteins and the cleavage of Caspase-1 were also significantly lesser in the $Fyn^{-/-}$ microglial lysates (Figure 6D, E), as was the production of IL-1β and IL-12 (Figure 6H, I). Knockdown of Fyn expression via siRNA-reduced α-synuclein mediated induction of pro-IL-1β in WT microglia (Figure 6F, G). We also wondered whether Fyn activation might contribute to activation of the danger signal with regards to inflammasome activation. We first checked for the uptake of α-synuclein into WT and $Fyn^{-/-}$ microglia and discovered to our surprise that lesser amounts of α-synuclein were taken up by the $Fyn^{-/-}$ cells, as evidenced by ICC for as well as immunoblots for human α-synuclein (Figure 7A-D). Among the signaling events that contribute to the danger signal activation with context to NLRP3 inflammasome activation, excessive ROS generation has now been accepted as a cardinal contributor to the activation of the NLRP3 inflammasome in various models and it was demonstrated that dysfunctional mitochondria might be the source of these species (Tschopp and Schroder, 2010; Zhou et al., 2011). Aggregated α-synuclein treatment induced a rapid, progressive, induction of mitoROS generation in WT microglia, but did so in the $Fyn^{-/-}$ microglia to a lesser extent (Figure 7E). Excessive mitoROS generation leads to eventual change in mitochondrial morphology. This change was observed in the WT but not the $Fyn^{-/-}$ microglia (Figure 7F, G). We also observed Fyn dependent

PKCδ tyrosine phosphorylation in the cells following α-synuclein stimulation (Figure 8A), which is reminiscent of the microglial activation pathway downstream of LPS and TNFα (Panicker et al., 2015). PKCδ$^{-/-}$ microglia imported aggregated α-synuclein with equivalent efficiency as their wild-type PKCδ$^{+/+}$ counterparts (Figure 8G), but showed diminished α-synuclein mediated priming of the NLRP3 inflammasome, and diminished secretion of supernatant IL-1β (Figure 8B-F).

Mitochondrial dysfunction is one of the major causative factors identified to contribute to the progression of both sporadic and familial PD. However, most existing studies focus on the role of this process in dopaminergic neurons. Several of the PARK genes encode proteins that have mitochondrial functions. It is possible that the loss of function of these proteins, either through inherited mutations or post-translational inactivation, may contribute to mitochondrial dysfunction and consequent NLRP3 inflammasome hyperactivation in the microglia. There is evidence to support the notion that the NLRP3 inflammasome may have relevance in animal models of PD and clinical relevance as well. Ole Isaacson and colleagues reported an increase in IL-1β striatal levels using the A53T α-synuclein-AAV model in rats (although activation of the NLRP3 inflammasome was not looked at or discussed) (Chung et al., 2009). Overexpression of IL-1β in the mouse SN can directly bring about the death of dopaminergic neurons (Ferrari et al., 2006), and most recently, dopamine was shown to inhibit the NLRP3 inflammasome. MPTP treatment was utilized to induce a depletion of dopamine levels, which activated the NLRP3 inflammasome and subsequent NLRP3 dependent dopaminergic neuron loss (Yan et al., 2015). Our subsequent studies will utilize human α-synuclein overexpressing systems in Fyn$^{+/+}$ and Fyn$^{-/-}$ mice to show the role of Fyn in activating the microglial NLRP3 inflammasome *in-vivo*. It

would be intriguing to assess whether sterile inflammation mediated by α-synuclein aggregates could act cooperatively with dopamine depletion to mediate unabated inflammasome activation that might contribute to the progressive neurodegeneration that characterizes PD. Since ASC released from activated peripheral immune cells was demonstrated to seed ASC oligomerization in a prionid manner (Baroja-Mazo et al., 2014), it might also be intriguing to explore whether microglia-released ASC could contribute to the spread of sterile inflammatory responses in PD brains. Overall, as summarized in Figure 9, our mechanistic studies demonstrate how Fyn plays a role in both, α-synuclein priming as well as activation of the NLRP3 inflammasome in microglial cells, identifying Fyn as a potential therapeutic target for inhibiting the sterile inflammation and inflammation-dependent cell death in PD.

References

Allen Reish, H.E., and Standaert, D.G. (2015). Role of alpha-synuclein in inducing innate and adaptive immunity in Parkinson disease. J Parkinsons Dis *5*, 1-19.

Appel-Cresswell, S., Vilarino-Guell, C., Encarnacion, M., Sherman, H., Yu, I., Shah, B., Weir, D., Thompson, C., Szu-Tu, C., Trinh, J., *et al*. (2013). Alpha-synuclein p.H50Q, a novel pathogenic mutation for Parkinson's disease. Mov Disord *28*, 811-813.

Baroja-Mazo, A., Martin-Sanchez, F., Gomez, A.I., Martinez, C.M., Amores-Iniesta, J., Compan, V., Barbera-Cremades, M., Yague, J., Ruiz-Ortiz, E., Anton, J., *et al*. (2014). The NLRP3 inflammasome is released as a particulate danger signal that amplifies the inflammatory response. Nat Immunol *15*, 738-748.

Block, M.L., Zecca, L., and Hong, J.S. (2007). Microglia-mediated neurotoxicity: uncovering the molecular mechanisms. Nat Rev Neurosci *8*, 57-69.

Boza-Serrano, A., Reyes, J.F., Rey, N.L., Leffler, H., Bousset, L., Nilsson, U., Brundin, P., Venero, J.L., Burguillos, M.A., and Deierborg, T. (2014). The role of Galectin-3 in alpha-synuclein-induced microglial activation. Acta Neuropathol Commun *2*, 156.

Chen, K., Febbraio, M., Li, W., and Silverstein, R.L. (2008). A specific CD36-dependent signaling pathway is required for platelet activation by oxidized low-density lipoprotein. Circ Res *102*, 1512-1519.

Chung, C.Y., Koprich, J.B., Siddiqi, H., and Isacson, O. (2009). Dynamic changes in presynaptic and axonal transport proteins combined with striatal neuroinflammation precede dopaminergic neuronal loss in a rat model of AAV alpha-synucleinopathy. The Journal of neuroscience : the official journal of the Society for Neuroscience *29*, 3365-3373.

Codolo, G., Plotegher, N., Pozzobon, T., Brucale, M., Tessari, I., Bubacco, L., and de Bernard, M. (2013). Triggering of inflammasome by aggregated alpha-synuclein, an inflammatory response in synucleinopathies. PLOS One *8*, e55375.

Coll, R.C., Robertson, A.A., Chae, J.J., Higgins, S.C., Munoz-Planillo, R., Inserra, M.C., Vetter, I., Dungan, L.S., Monks, B.G., Stutz, A., *et al*. (2015). A small-molecule inhibitor of the NLRP3 inflammasome for the treatment of inflammatory diseases. Nat Med *21*, 248-255.

Daniele, S.G., Beraud, D., Davenport, C., Cheng, K., Yin, H., and Maguire-Zeiss, K.A. (2015). Activation of MyD88-dependent TLR1/2 signaling by misfolded alpha-synuclein, a protein linked to neurodegenerative disorders. Sci Signal *8*, ra45.

Dey, N., Crosswell, H.E., De, P., Parsons, R., Peng, Q., Su, J.D., and Durden, D.L. (2008). The protein phosphatase activity of PTEN regulates SRC family kinases and controls glioma migration. Cancer research *68*, 1862-1871.

Duewell, P., Kono, H., Rayner, K.J., Sirois, C.M., Vladimer, G., Bauernfeind, F.G., Abela, G.S., Franchi, L., Nunez, G., Schnurr, M.*, et al*. (2010). NLRP3 inflammasomes are required for atherogenesis and activated by cholesterol crystals. Nature *464*, 1357-1361.

Fellner, L., Irschick, R., Schanda, K., Reindl, M., Klimaschewski, L., Poewe, W., Wenning, G.K., and Stefanova, N. (2013). Toll-like receptor 4 is required for alpha-synuclein dependent activation of microglia and astroglia. Glia *61*, 349-360.

Ferrari, C.C., Pott Godoy, M.C., Tarelli, R., Chertoff, M., Depino, A.M., and Pitossi, F.J. (2006). Progressive neurodegeneration and motor disabilities induced by chronic expression of IL-1beta in the substantia nigra. Neurobiology of disease *24*, 183-193.

Finberg, R.W., Yim, C., Yan, J., Cao, L.C., Mandell, L., and Kurt-Jones, E.A. (2012). Phosphorylated Toll-Like Receptor 2 Interacts with Fyn and Cross-Talks with the Phosphorylation-Independent TLR2-Signaling Pathway. The Open Immunology Journal *5*, 36-45.

Glass, C.K., Saijo, K., Winner, B., Marchetto, M.C., and Gage, F.H. (2010). Mechanisms underlying inflammation in neurodegeneration. Cell *140*, 918-934.

Gomez, G., Gonzalez-Espinosa, C., Odom, S., Baez, G., Cid, M.E., Ryan, J.J., and Rivera, J. (2005). Impaired FceRI-Dependent Gene Expression and Defective Eicosanoid and Cytokine Production as a Consequence of Fyn Deficiency in Mast Cells. J Immunol *175*.

Gordon, R., Hogan, C.E., Neal, M.L., Anantharam, V., Kanthasamy, A.G., and Kanthasamy, A. (2011). A simple magnetic separation method for high-yield isolation of pure primary microglia. Journal of neuroscience methods *194*, 287-296.

Gustin, A., Kirchmeyer, M., Koncina, E., Felten, P., Losciuto, S., Heurtaux, T., Tardivel, A., Heuschling, P., and Dostert, C. (2015). NLRP3 Inflammasome Is Expressed and Functional in Mouse Brain Microglia but Not in Astrocytes. PLOS One *10*, e0130624.

Gustot, A., Gallea, J.I., Sarroukh, R., Celej, M.S., Ruysschaert, J.M., and Raussens, V. (2015). Amyloid fibrils are the molecular trigger of inflammation in Parkinson's disease. Biochem J *471*, 323-333.

Hafner-Bratkovic, I., Bencina, M., Fitzgerald, K.A., Golenbock, D., and Jerala, R. (2012). NLRP3 inflammasome activation in macrophage cell lines by prion protein fibrils as the source of IL-1beta and neuronal toxicity. Cellular and molecular life sciences : CMLS *69*, 4215-4228.

Halle, A., Hornung, V., Petzold, G.C., Stewart, C.R., Monks, B.G., Reinheckel, T., Fitzgerald, K.A., Latz, E., Moore, K.J., and Golenbock, D.T. (2008). The NALP3 inflammasome is involved in the innate immune response to amyloid-beta. Nat Immunol *9*, 857-865.

Hayden, M.S., and Ghosh, S. (2004). Signaling to NF-kappaB. Genes Dev *18*, 2195-2224.

Heneka, M.T., Kummer, M.P., Stutz, A., Delekate, A., Schwartz, S., Vieira-Saecker, A., Griep, A., Axt, D., Remus, A., Tzeng, T.C., *et al*. (2013). NLRP3 is activated in Alzheimer's disease and contributes to pathology in APP/PS1 mice. Nature *493*, 674-678.

Imamura, K., Hishikawa, N., Sawada, M., Nagatsu, T., Yoshida, M., and Hashizume, Y. (2003). Distribution of major histocompatibility complex class II-positive microglia and cytokine profile of Parkinson's disease brains. Acta neuropathologica *106*, 518-526.

Jankovic, J., and Aguilar, L.G. (2008). Current approaches to the treatment of Parkinson's disease. Neuropsychiatr Dis Treat *4*, 743-757.

Jenner, P., and Olanow, C.W. (2006). The pathogenesis of cell death in Parkinson's disease. Neurology *66*, S24-36.

Jin, H., Kanthasamy, A., Anantharam, V., Rana, A., and Kanthasamy, A.G. (2011). Transcriptional regulation of pro-apoptotic protein kinase Cdelta: implications for oxidative stress-induced neuronal cell death. The Journal of biological chemistry *286*, 19840-19859.

Kaspar, J.W., and Jaiswal, A.K. (2011). Tyrosine phosphorylation controls nuclear export of Fyn, allowing Nrf2 activation of cytoprotective gene expression. FASEB journal : official publication of the Federation of American Societies for Experimental Biology *25*, 1076-1087.

Kaufman, A.C., Salazar, S.V., Haas, L.T., Yang, J., Kostylev, M.A., Jeng, A.T., Robinson, S.A., Gunther, E.C., van Dyck, C.H., Nygaard, H.B., *et al*. (2015). Fyn inhibition rescues established memory and synapse loss in Alzheimer mice. Ann Neurol *77*, 953-971.

Kaul, S., Anantharam, V., Yang, Y., Choi, C.J., Kanthasamy, A., and Kanthasamy, A.G. (2005). Tyrosine phosphorylation regulates the proteolytic activation of protein kinase Cdelta in dopaminergic neuronal cells. The Journal of biological chemistry *280*, 28721-28730.

Kim, C., Ho, D.H., Suk, J.E., You, S., Michael, S., Kang, J., Joong Lee, S., Masliah, E., Hwang, D., Lee, H.J., *et al*. (2013). Neuron-released oligomeric alpha-synuclein is an endogenous agonist of TLR2 for paracrine activation of microglia. Nature communications *4*, 1562.

Kruger, R., Kuhn, W., Muller, T., Woitalla, D., Graeber, M., Kosel, S., Przuntek, H., Epplen, J.T., Schols, L., and Riess, O. (1998). Ala30Pro mutation in the gene encoding alpha-synuclein in Parkinson's disease. Nat Genet *18*, 106-108.

Lambert, M.P., Barlow, A.K., Chromy, B.A., Edwards, C., Freed, R., Liosatos, M., Morgan, T.E., Rozovsky, I., Trommer, B., Viola, K.L., *et al*. (1998). Diffusible, nonfibrillar ligands derived from Ab1–42 are potent
central nervous system neurotoxins. Proc Natl Acad Sci USA *95*.

Lamkanfi, M., and Dixit, V.M. (2014). Mechanisms and functions of inflammasomes. Cell *157*, 1013-1022.

Latz, E., Xiao, T.S., and Stutz, A. (2013). Activation and regulation of the inflammasomes. Nat Rev Immunol *13*, 397-411.

Lee, E.J., Woo, M.S., Moon, P.G., Baek, M.C., Choi, I.Y., Kim, W.K., Junn, E., and Kim, H.S. (2010). Alpha-synuclein activates microglia by inducing the expressions of matrix metalloproteinases and the subsequent activation of protease-activated receptor-1. J Immunol *185*, 615-623.

Lee, H.M., Kim, J.J., Kim, H.J., Shong, M., Ku, B.J., and Jo, E.K. (2013). Upregulated NLRP3 inflammasome activation in patients with type 2 diabetes. Diabetes *62*, 194-204.

Lesage, S., Anheim, M., Letournel, F., Bousset, L., Honore, A., Rozas, N., Pieri, L., Madiona, K., Durr, A., Melki, R., *et al*. (2013). G51D alpha-synuclein mutation causes a novel parkinsonian-pyramidal syndrome. Ann Neurol *73*, 459-471.

Levy, O.A., Malagelada, C., and Greene, L.A. (2009). Cell death pathways in Parkinson's disease: proximal triggers, distal effectors, and final steps. Apoptosis *14*, 478-500.

Li, Y., Liu, L., Barger, S.W., and Griffin, W.S. (2003). Interleukin-1 mediates pathological effects of microglia on tau phosphorylation and on synaptophysin synthesis in cortical neurons through a p38-MAPK pathway. The Journal of neuroscience : the official journal of the Society for Neuroscience *23*, 1605-1611.

Luk, K.C., Kehm, V., Carroll, J., Zhang, B., O'Brien, P., Trojanowski, J.Q., and Lee, V.M. (2012a). Pathological alpha-synuclein transmission initiates Parkinson-like neurodegeneration in nontransgenic mice. Science *338*, 949-953.

Luk, K.C., Kehm, V.M., Zhang, B., O'Brien, P., Trojanowski, J.Q., and Lee, V.M. (2012b). Intracerebral inoculation of pathological alpha-synuclein initiates a rapidly progressive neurodegenerative alpha-synucleinopathy in mice. J Exp Med *209*, 975-986.

Mogi, M., Harada, M., Narabayashi, H., Inagaki, H., Minami, M., and Nagatsu, T. (1996). Interleukin (IL)-1 beta, IL-2, IL-4, IL-6 and transforming growth factor-alpha levels are elevated in ventricular cerebrospinal fluid in juvenile parkinsonism and Parkinson's disease. Neurosci Lett *211*, 13-16.

Mogi, M., Togari, A., Kondo, T., Mizuno, Y., Komure, O., Kuno, S., Ichinose, H., and Nagatsu, T. (2000). Caspase activities and tumor necrosis factor receptor R1 (p55) level are

elevated in the substantia nigra from parkinsonian brain. J Neural Transm (Vienna) *107*, 335-341.

Moore, K.J., El Khoury, J., Medeiros, L.A., Terada, K., Geula, C., Luster, A.D., and Freeman, M.W. (2002). A CD36-initiated signaling cascade mediates inflammatory effects of beta-amyloid. The Journal of biological chemistry *277*, 47373-47379.

Moynagh, P.N. (2005). The interleukin-1 signalling pathway in astrocytes: a key contributor to inflammation in the brain. J Anat *207*, 265-269.

Netea, M.G., Nold-Petry, C.A., Nold, M.F., Joosten, L.A., Opitz, B., van der Meer, J.H., van de Veerdonk, F.L., Ferwerda, G., Heinhuis, B., Devesa, I., *et al*. (2009). Differential requirement for the activation of the inflammasome for processing and release of IL-1beta in monocytes and macrophages. Blood *113*, 2324-2335.

Olanow, C.W. (2007). The pathogenesis of cell death in Parkinson's disease--2007. Mov Disord *22 Suppl 17*, S335-342.

Palacios, E.H., and Weiss, A. (2004). Function of the Src-family kinases, Lck and Fyn, in T-cell development and activation. Oncogene *23*, 7990-8000.

Panicker, N., Saminathan, H., Jin, H., Neal, M., Harischandra, D.S., Gordon, R., Kanthasamy, K., Lawana, V., Sarkar, S., Luo, J., *et al*. (2015). Fyn Kinase Regulates Microglial Neuroinflammatory Responses in Cell Culture and Animal Models of Parkinson's

Disease. The Journal of neuroscience : the official journal of the Society for Neuroscience *35*, 10058-10077.

Pasanen, P., Myllykangas, L., Siitonen, M., Raunio, A., Kaakkola, S., Lyytinen, J., Tienari, P.J., Poyhonen, M., and Paetau, A. (2014). Novel alpha-synuclein mutation A53E associated with atypical multiple system atrophy and Parkinson's disease-type pathology. Neurobiology of aging *35*, 2180 e2181-2185.

Polymeropoulos, M.H., Lavedan, C., Leroy, E., Ide, S.E., Dehejia, A., Dutra, A., Pike, B., Root, H., Rubenstein, J., Boyer, R., *et al*. (1997). Mutation in the alpha-synuclein gene identified in families with Parkinson's disease. Science *276*, 2045-2047.

Przedborski, S. (2005). Pathogenesis of nigral cell death in Parkinson's disease. Parkinsonism & related disorders *11 Suppl 1*, S3-7.

Qu, Y., Misaghi, S., Izrael-Tomasevic, A., Newton, K., Gilmour, L.L., Lamkanfi, M., Louie, S., Kayagaki, N., Liu, J., Komuves, L., *et al*. (2012). Phosphorylation of NLRC4 is critical for inflammasome activation. Nature *490*, 539-542.

Rajasekaran, K., Kumar, P., Schuldt, K.M., Peterson, E.J., Vanhaesebroeck, B., Dixit, V., Thakar, M.S., and Malarkannan, S. (2013). Signaling by Fyn-ADAP via the Carma1-Bcl-10-MAP3K7 signalosome exclusively regulates inflammatory cytokine production in NK cells. Nature immunology *14*, 1127-1136.

Saminathan, H., Asaithambi, A., Anantharam, V., Kanthasamy, A.G., and Kanthasamy, A. (2011). Environmental neurotoxic pesticide dieldrin activates a non receptor tyrosine kinase to promote PKCdelta-mediated dopaminergic apoptosis in a dopaminergic neuronal cell model. Neurotoxicology *32*, 567-577.

Seo, J., Ottesen, E.W., and Singh, R.N. (2014). Antisense methods to modulate pre-mRNA splicing. Methods Mol Biol *1126*, 271-283.

Sheedy, F.J., Grebe, A., Rayner, K.J., Kalantari, P., Ramkhelawon, B., Carpenter, S.B., Becker, C.E., Ediriweera, H.N., Mullick, A.E., Golenbock, D.T., *et al.* (2013). CD36 coordinates NLRP3 inflammasome activation by facilitating intracellular nucleation of soluble ligands into particulate ligands in sterile inflammation. Nat Immunol *14*, 812-820.

Sperber, B.R., Boyle-Walsh, E.A., Engleka, M.J., Gadue, P., Peterson, A.C., Stein, P.L., Scherer, S.S., and McMorris, F.A. (2001). A Unique Role for Fyn in CNS Myelination. The Journal of neuroscience : the official journal of the Society for Neuroscience *21*, 2039-2047.

Stuart, L.M., Bell, S.A., Stewart, C.R., Silver, J.M., Richard, J., Goss, J.L., Tseng, A.A., Zhang, A., El Khoury, J.B., and Moore, K.J. (2007). CD36 signals to the actin cytoskeleton and regulates microglial migration via a p130Cas complex. The Journal of biological chemistry *282*, 27392-27401.

Su, X., Maguire-Zeiss, K.A., Giuliano, R., Prifti, L., Venkatesh, K., and Federoff, H.J. (2008). Synuclein activates microglia in a model of Parkinson's disease. Neurobiology of aging *29*, 1690-1701.

Tansey, M.G., and Goldberg, M.S. (2010). Neuroinflammation in Parkinson's disease: its role in neuronal death and implications for therapeutic intervention. Neurobiology of disease *37*, 510-518.

Tschopp, J., and Schroder, K. (2010). NLRP3 inflammasome activation: The convergence of multiple signalling pathways on ROS production? Nat Rev Immunol *10*, 210-215.

Volpicelli-Daley, L.A., Luk, K.C., Patel, T.P., Tanik, S.A., Riddle, D.M., Stieber, A., Meaney, D.F., Trojanowski, J.Q., and Lee, V.M. (2011). Exogenous alpha-synuclein fibrils induce Lewy body pathology leading to synaptic dysfunction and neuron death. Neuron *72*, 57-71.

Walsh, J.G., Muruve, D.A., and Power, C. (2014). Inflammasomes in the CNS. Nat Rev Neurosci *15*, 84-97.

Wang, X.J., Kong, K.M., Qi, W.L., Ye, W.L., and Song, P.S. (2005). Interleukin-1 beta induction of neuron apoptosis depends on p38 mitogen-activated protein kinase activity after spinal cord injury. Acta Pharmacol Sin *26*, 934-942.

Yan, Y., Jiang, W., Liu, L., Wang, X., Ding, C., Tian, Z., and Zhou, R. (2015). Dopamine controls systemic inflammation through inhibition of NLRP3 inflammasome. Cell *160*, 62-73.

Zarranz, J.J., Alegre, J., Gomez-Esteban, J.C., Lezcano, E., Ros, R., Ampuero, I., Vidal, L., Hoenicka, J., Rodriguez, O., Atares, B., *et al*. (2004). The new mutation, E46K, of alpha-synuclein causes Parkinson and Lewy body dementia. Ann Neurol *55*, 164-173.

Zhang, W., Wang, T., Pei, Z., Miller, D.S., Wu, X., Block, M.L., Wilson, B., Zhou, Y., Hong, J.S., and Zhang, J. (2005). Aggregated alpha-synuclein activates microglia: a process leading to disease progression in Parkinson's disease. FASEB journal : official publication of the Federation of American Societies for Experimental Biology *19*, 533-542.

Zhou, R., Yazdi, A.S., Menu, P., and Tschopp, J. (2011). A role for mitochondria in NLRP3 inflammasome activation. Nature *469*, 221-225.

Figures

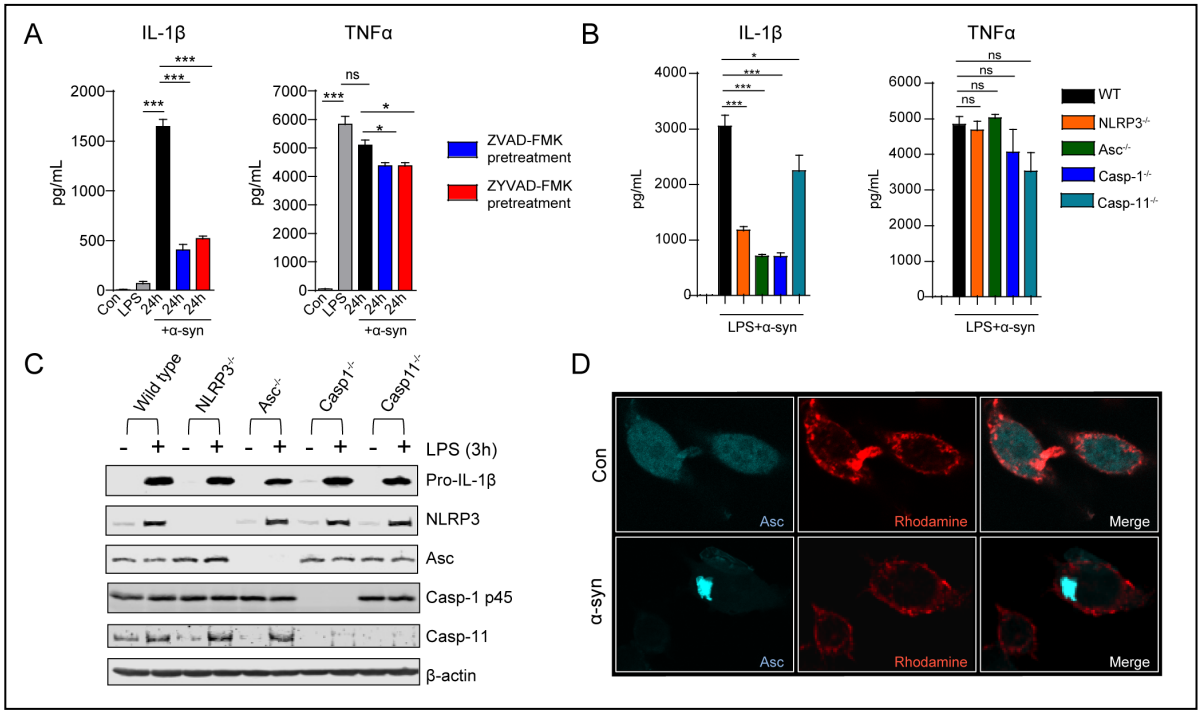

Figure 1. Aggregated α-synuclein acts as a danger signal to elicit NLRP3 inflammasome dependent IL-1β processing in LPS primed microglia. ***A*,** Aggregated α-synuclein was able to elicit significant IL-1β, but not TNFα production from LPS primed microglia. Pretreatment of the cells with pan-Caspase and Caspase-1 specific inhibitors post priming but before α-synuclein treatment strongly attenuated the production of IL-1β, but minimally affected TNFα production. ***B*,** The α-synuclein-mediated production of IL-1β was largely mediated by the NLRP3 inflammasome, Supernatant cytokine analysis from LPS primed, α-synuclein treated WT, NLRP3$^{-/-}$, ASC$^{-/-}$, Caspase-1$^{-/-}$ and Caspase-11$^{-/-}$ BMDMs revealed strongly diminished IL-1β, but not TNFα production from the NLRP3$^{-/-}$, ASC$^{-/-}$ and Caspase-1$^{-/-}$, but minimally affected IL-1β production from the Caspase-11$^{-/-}$ cells, indicating that the canonical activation of the NLRP3 inflammasome was primarily responsible for the IL-1β production in response to aggregated α-synuclein. ***C*,** No discernable changes in the LPS

induced pro- IL-1β levels in WT, NLRP3$^{-/-}$, ASC$^{-/-}$, Caspase-1$^{-/-}$ and Caspase-11$^{-/-}$ BMDMs. ***D*,** Aggregated α-synuclein treatment induced speck formation in the ASC-CFP reporter cell line.

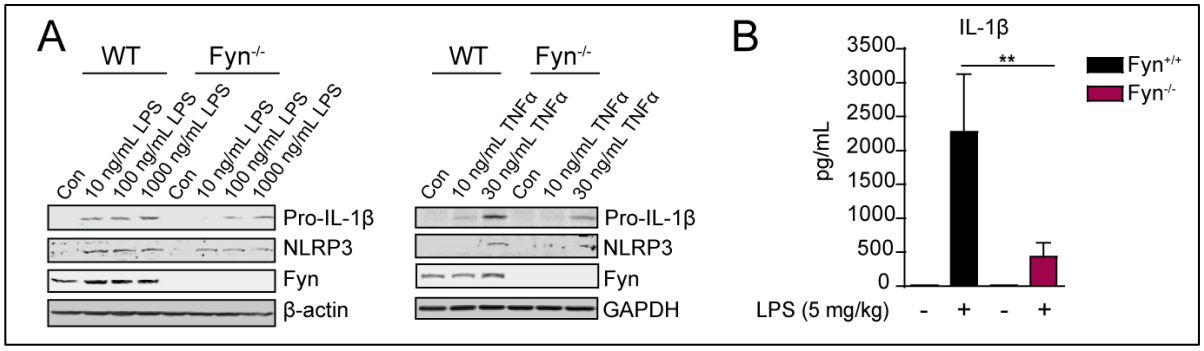

Figure 2. Fyn kinase contributes to the priming of the NLRP3 inflammasome in response to diverse inflammogens. ***A*****,** WT and Fyn$^{-/-}$ microglia were treated with various doses of LPS (10. 100 and 1000 ng/mL) and TNFα (10 and 30 ng/mL). Both inflammogens elicited a dose-dependent induction of pro-IL-1β and NLRP3 in WT microglia, but did so to a significantly lesser extent in the Fyn$^{-/-}$ microglia. ***B*****,** Fyn$^{-/-}$ mice treated with LPS (5mg/kg) for 24 hours showed diminished serum secretion of IL-1β when compared to Fyn$^{+/+}$ mice.

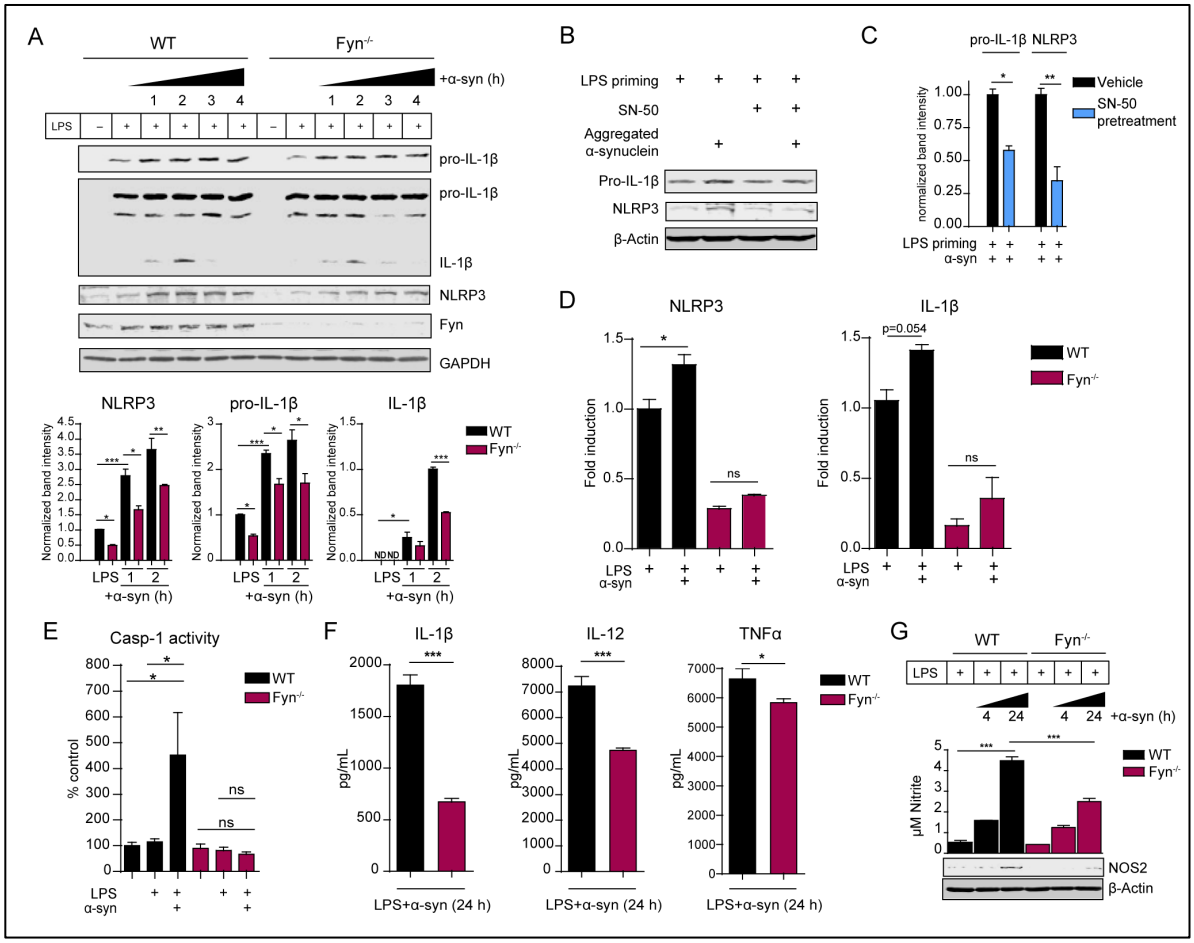

Figure 3. Aggregated α-synuclein amplifies LPS induced priming of the NLRP3 inflammasome and induces Caspase-1 activation, and pro-inflammatory cytokine and nitrite release in a Fyn dependent manner. ***A***, Aggregated α-synuclein potentiated the LPS mediated induction of pro-IL-1β and NLRP3, but did so to a lesser extent in the Fyn deficient microglia. ***B, C,*** Treatment of microglial cells post priming and pre α-synuclein treatment with the NF-κB inhibitor SN-50 prevented the induction of pro-IL-1β and NLRP3 proteins ***D***, α-synuclein treatment also increased the induction of pro-IL-1β and NLRP3 mRNAs in LPS treated WT, but not Fyn$^{-/-}$ microglial cells. ***E***, The FLICA assay revealed strongly increased Caspase-1 activation in α-synuclein treated WT but not Fyn$^{-/-}$ microglia. ***F***, LPS primed α-synuclein treated WT microglia produced higher amounts of pro-inflammatory

cytokines than Fyn deficient microglia. ***G,*** α-synuclein treatment induced the production of supernatant nitrite and NOS2 expression, but did so to a significantly lesser extent in the Fyn deficient microglia.

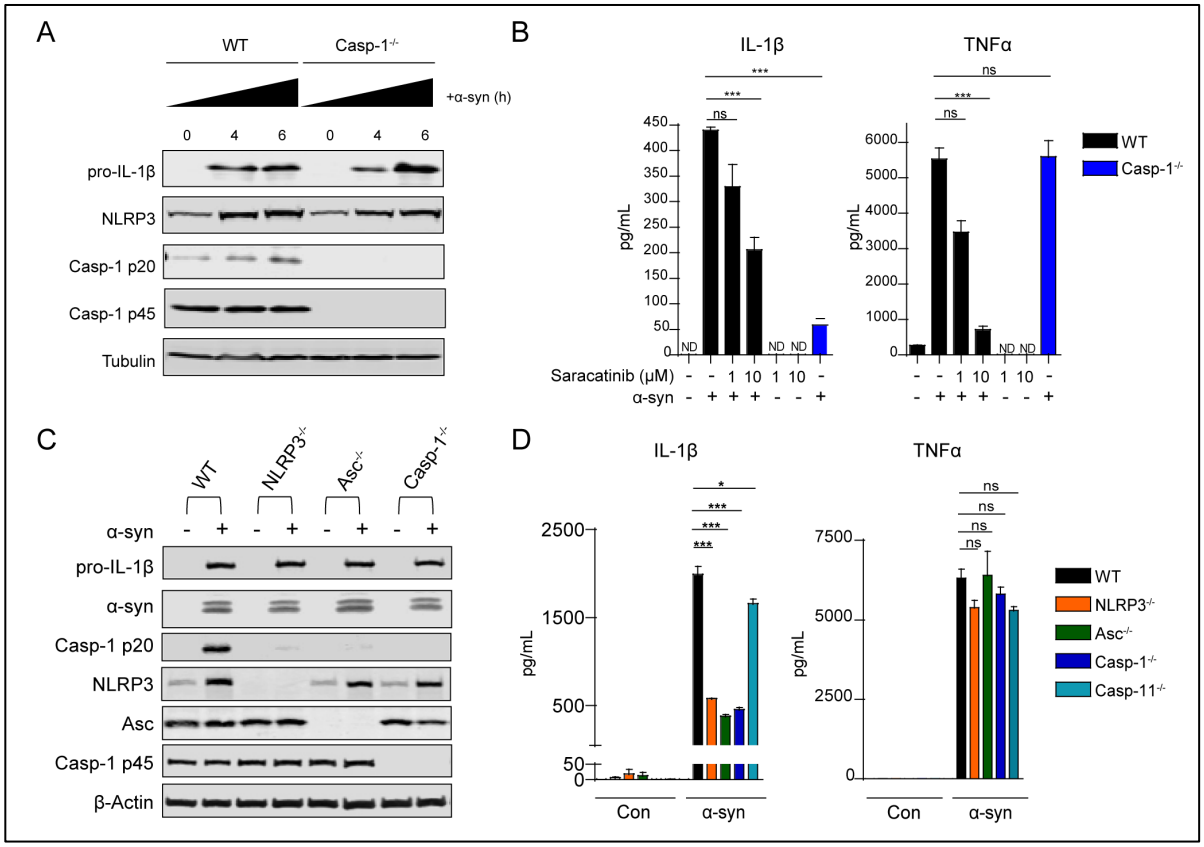

Figure 4. Aggregated α-synuclein can prime and activate the NLRP3 inflammasome to mediate IL-1β production. ***A,*** Aggregated α-synuclein elicited Caspase-1 independent induction of pro-IL-1β and NLRP3 levels, as evidenced by immunoblot analysis from synuclein treated WT and Caspase-1$^{-/-}$ microglial cell lysates. ***B,*** α-synuclein treatment induced the Caspase-1-dependent production of IL-1β from microglial cells, which was also inhibited with pretreatment of the Fyn inhibitor Saracatinib in a dose dependent manner. ***C,*** NLRP3$^{-/-}$, ASC$^{-/-}$ and Caspase-1$^{-/-}$ BMDMs demonstrated equable α-synuclein mediated induction of pro-IL-1β and uptake of α-synuclein, but almost completely attenuated Caspase-1 cleavage. ***D,*** The aggregated α-synuclein mediated production of IL-1β, but not TNFα was strongly attenuated in NLRP3$^{-/-}$, ASC$^{-/-}$, and Caspase-1$^{-/-}$, but minimally affected in Caspase-11$^{-/-}$ BMDM supernatants, indicating that α-synuclein is able to both prime, as well as activate the NLRP3 inflammasome primarily through its canonical activation.

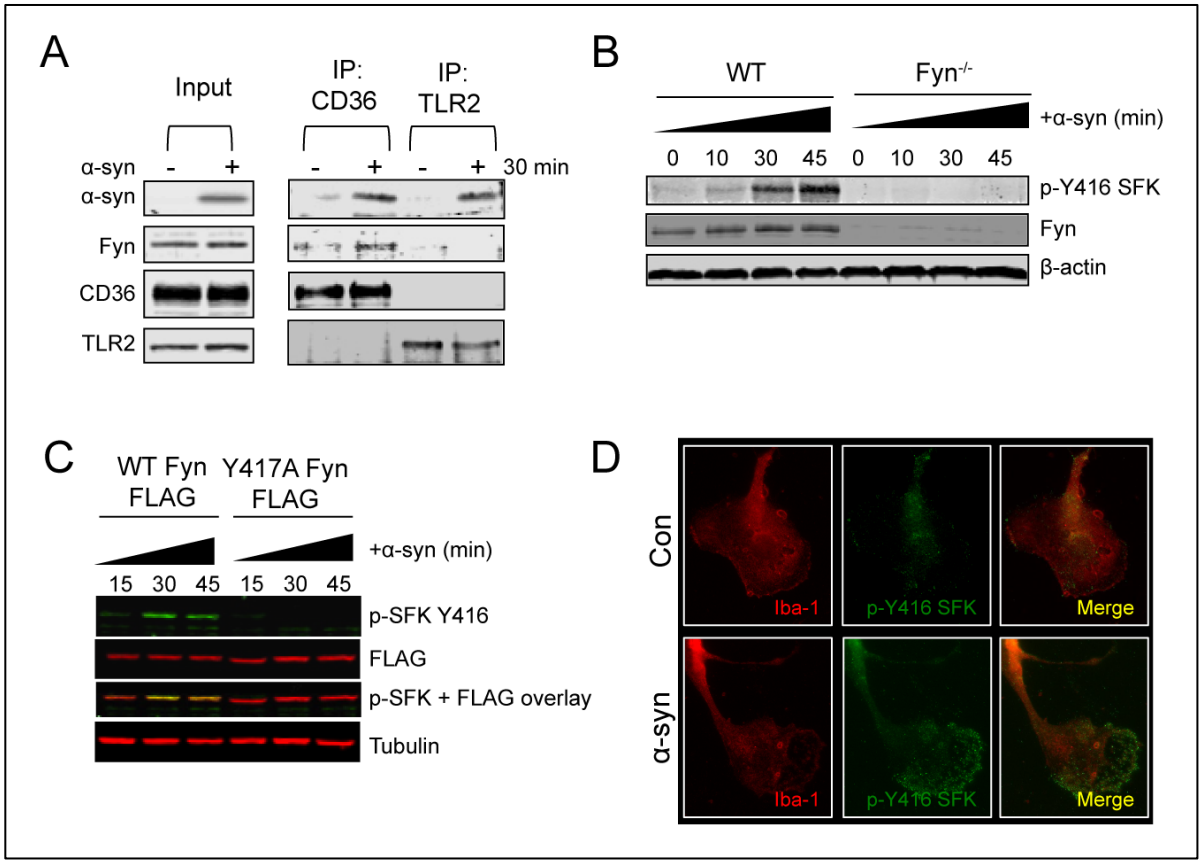

Figure 5. CD36 associated Fyn is rapidly activated following α-synuclein stimulation. ***A*****,** Upon its treatment to microglial cells, human α-synuclein associates with TLR-2 and CD36, as evidenced by co-immunoprecipitation analysis. Upon α-synuclein treatment, Fyn associates with CD36, but not TLR2. ***B*****,** Immunoblot analysis of aggregated α-synuclein treated WT and Fyn$^{-/-}$ microglial lysates reveals a rapid induction of Src family kinase activation the WT, but not Fyn$^{-/-}$ microglia. ***C***, Whole cell lysate analysis from α-synuclein treated wild type (WT) and activation loop deficient (Y417A) Fyn-FLAG transfected cells revealed a perfect overlap between the pY416-SFK and FLAG bands in the 30 and 45 min α-synuclein treated WT Fyn –FLAG transfected samples, and a complete absence of pY416-SFK activation in the Fyn activation loop mutant transfected samples ***D***,

Immunocytochemistry analysis reveals a rapid increase in pY416-SFK levels in α-synuclein treated Iba-1 positive WT microglial cells.

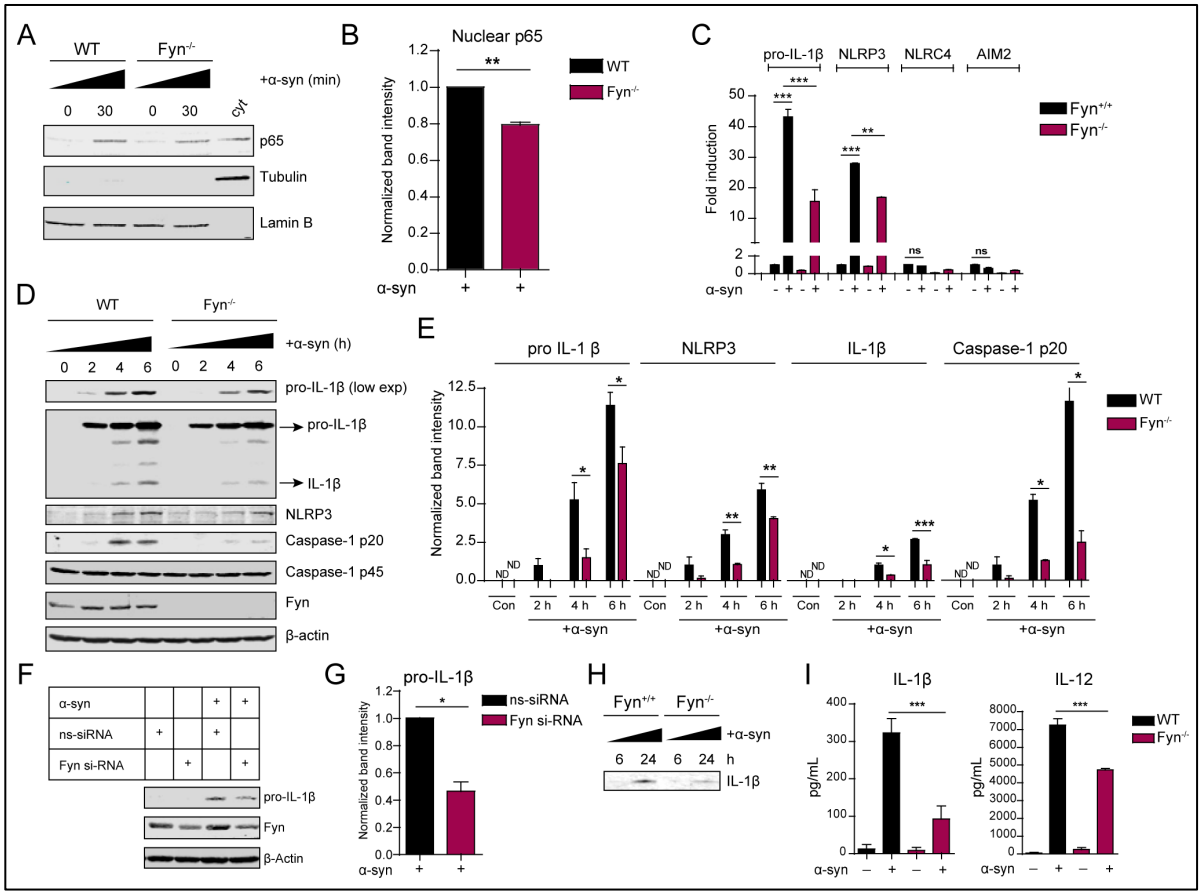

Figure 6. Fyn contributes to α-synuclein mediated priming of the NLRP3 inflammasome, resulting in diminished IL-1β and other pro-inflammatory cytokine production. ***A, B,*** Diminished α-synuclein induced nuclear translocation of NF-κB-p65 in the Fyn$^{-/-}$ microglial cells. ***C***, Diminished induction of pro-IL-1β and NLRP3 mRNA levels in the Fyn deficient microglial upon α-synuclein treatment ***D, E***, Reduced induction of pro-IL-1β and NLRP3 protein levels, as well as Caspase -1 and IL-1β cleavage in Fyn$^{-/-}$ microglia. ***F, G***, Knocking down Fyn using si-RNA reduces the α-synuclein mediated induction of pro-IL-1β in primary WT microglia. ***H, I,*** Reduced supernatant IL-1β and other pro-inflammatory cytokine production from α-synuclein treated Fyn$^{-/-}$ microglia.

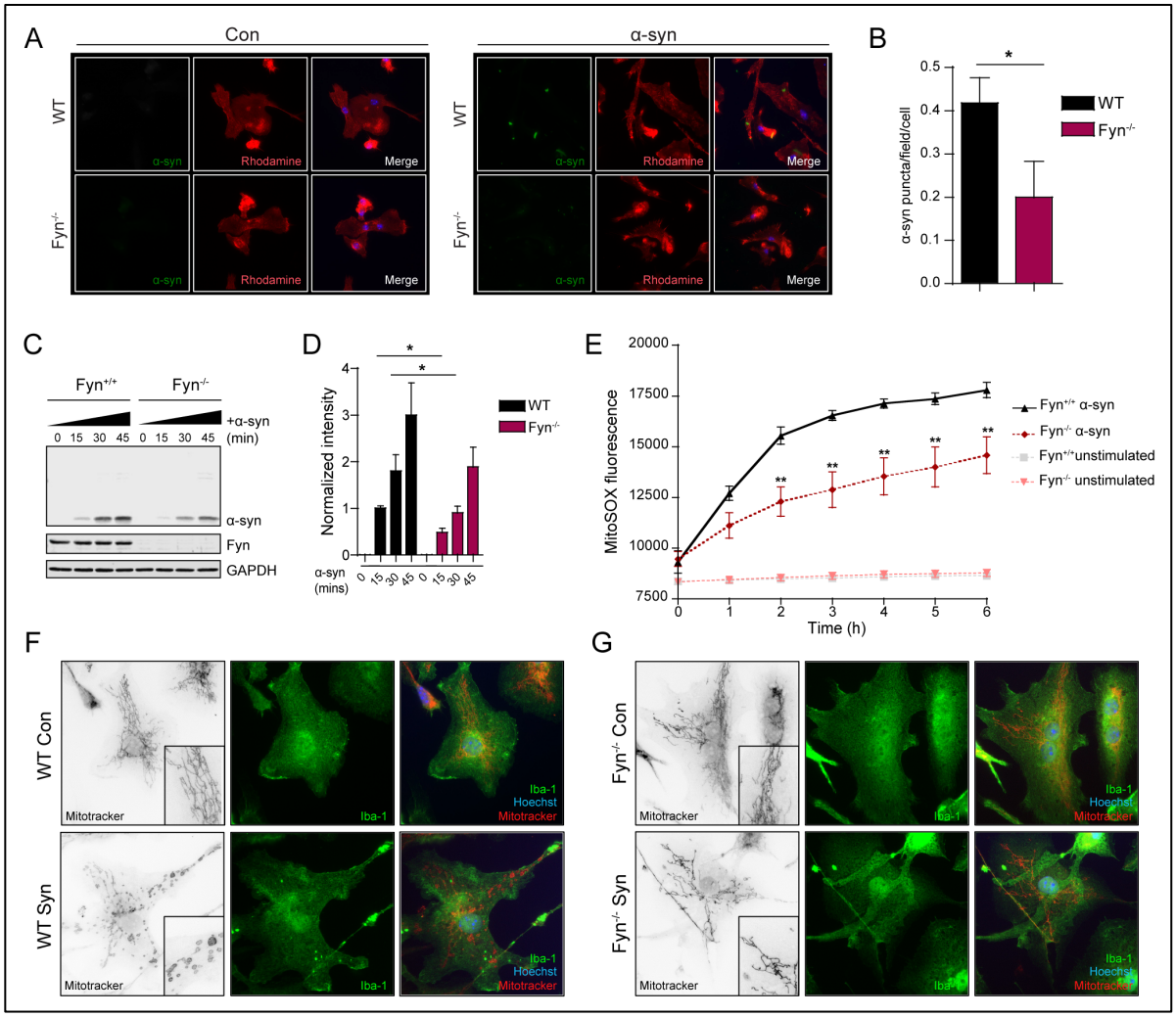

Figure 7. Fyn contributes to aggregated α-synuclein uptake into microglial cells, resulting in the mitochondrial ROS generation. ***A, B,*** Immunocytochemistry (for human α-synuclein) revealed diminished uptake of the protein in the Fyn deficient microglia. ***C, D***, Immunoblot analysis also reveals that human α-synuclein is taken up at lesser levels into Fyn$^{-/-}$ microglia ***E,*** Diminished mitoROS generation from α-synuclein treated Fyn$^{-/-}$ microglia. ***F, G,*** Diminished mitochondrial morphology deficits observed in the aggregated α-synuclein treated Fyn$^{-/-}$ microglia.

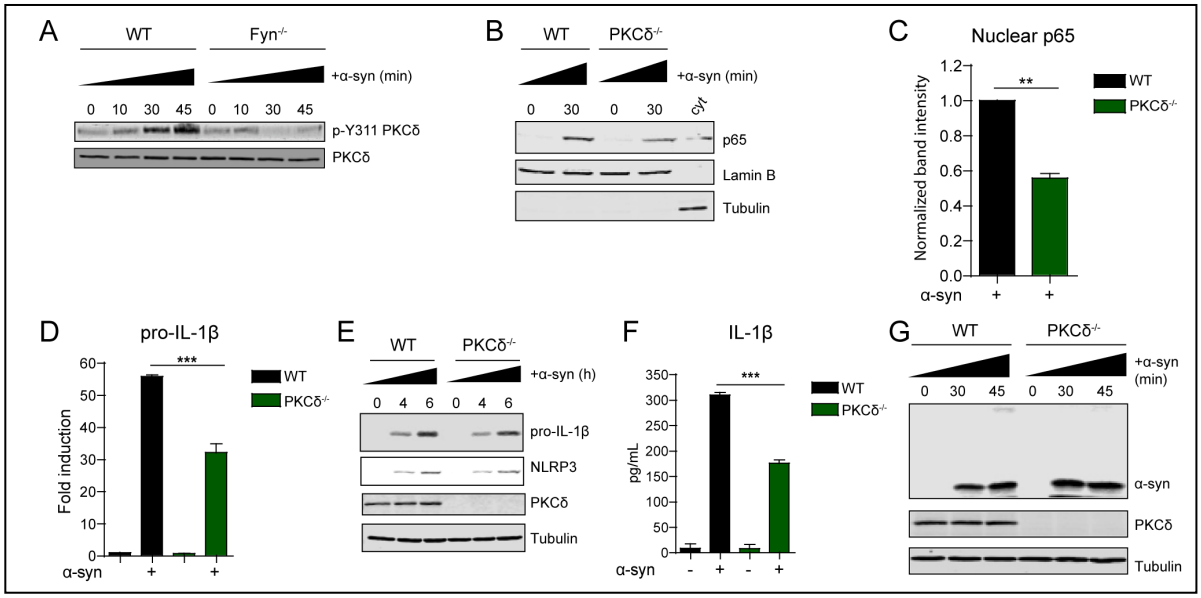

Figure 8. α-synuclein treatment brings about Fyn mediated PKCδ activation, which contributes to aggregated α-synuclein mediated priming of the NLRP3 inflammasome, but not to α-synuclein import into microglia. ***A,*** Immunoblot analysis of aggregated α-synuclein treated WT and Fyn$^{-/-}$ microglial lysates reveals a rapid induction of pY311-PKCδ levels in the WT, but not Fyn$^{-/-}$ microglia. ***B, C,*** Reduced aggregated α-synuclein mediated p65 nuclear translocation seen in PKCδ$^{-/-}$ microglia. ***D,*** Attenuated α-synuclein induced pro-IL-1β mRNA induction in PKCδ deficient microglia. ***E,*** Reduced induction of pro-IL-1β and NLRP3 proteins, and ***F,*** secretion of IL-1β from PKCδ$^{-/-}$ microglia. ***G,*** No change in the import of aggregated α-synuclein import observed between PKCδ$^{+/+}$ and PKCδ$^{-/-}$ microglia.

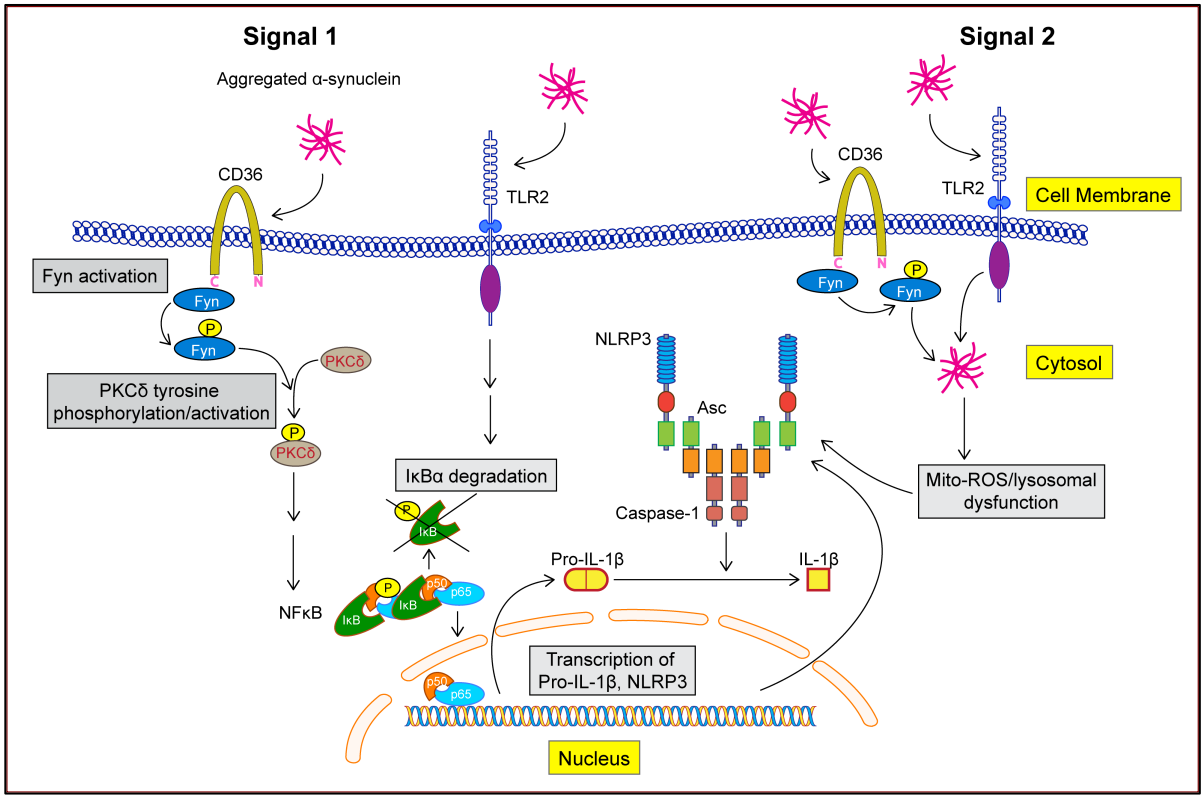

Figure 9. Aggregated α-synuclein mediated NLRP3 inflammasome activation pathway

Aggregated α-synuclein binds to the receptors TLR-2 and CD36 on microglial cells. CD36 recruits Fyn kinase, which in turn is activated and tyrosine phosphorylates PKCδ at Y311, leading to increased PKCδ dependent activation of the NF-κB pathway. p65 translocates to the nucleus and brings about the induction of pro-IL-1β and NLRP3 mRNAs. Aggregated α-synuclein is also taken up by the microglia, following which it brings about mitochondrial dysfunction mediated activation of the NLRP3 inflammasome. Fyn, but not PKCδ contributes to this process as well.

CHAPTER 4. HUMAN ALPHA-SYNUCLEIN ACTIVATES THE NLRP3 INFLAMMASOME IN A FYN-DEPENDENT MANNER IN ANIMAL MODELS OF PARKINSON'S DISEASE

A paper to be submitted to *Cell Reports*.

Nikhil Panicker[1], Matthew Neal[1], Dilshan S. Harishchandra[1], Souvarish Sarkar[1], Huajun Jin[1], Vellareddy Anantharam[1], Anumantha G. Kanthasamy[1] and Arthi Kanthasamy[1,2]

Abstract

Parkinson's disease (PD) is an age related neurodegenerative disorder characterized by the progressive degeneration of dopaminergic neurons within the substantia nigra (SN) and the concurrent development of motor deficits. It has now been accepted that the loss of dopaminergic neurons is accompanied and exacerbated by excessive microgliosis. The NLRP3 inflammasome is a multimeric protein complex of the cytosolic pattern recognition receptor NLRP3, the adaptor protein ASC, and Caspase-1, which upon assembly, mediates the autoproteolytic activation of Caspase-1 and the subsequent Caspase-1 mediated processing of pro-IL-1β to mature IL-1β. Hyperactivation of this complex by fibrilar β-amyloid was previously demonstrated to contribute to the pathogenesis of Alzheimer's disease (AD). We have shown that the aggregated PD associated protein α-synuclein can prime and activate the NLRP3 inflammasome in microglia *in-vitro* in a signaling pathway dependent on the non-receptor Src family tyrosine kinase Fyn. We wanted to investigate

[1] Parkinson's Disorders Research Program, Iowa Center for Advanced Neurotoxicology, Department of Biomedical Sciences, Iowa State University, Ames, Iowa 50011.
[2] Author of correspondence.
Author Contributions: N.P., A.G.K., and A.K. designed research; N.P., M.N., D.S.H., S.S., and H.J. performed research; N.P., H.J., and A.K. analyzed data; N.P., H.J., V.A., A.G.K., and A.K. wrote the paper.

whether the inflammasome could be activated in *in-vivo* models of PD involving α-synuclein aggregation or overexpression. We first demonstrate the activation of the inflammasome in the A53T human α-synuclein overexpression model and in ventral midbrain tissue lysates from PD patients. We then utilized the AAV-SYN overexpression model in $Fyn^{+/+}$ and $Fyn^{-/-}$ mice to check for the role that Fyn plays in mediating microgliosis and inflammasome activation *in-vivo*. Both, the α-synuclein induced microgliosis, and ASC speck formation in the microglial cells was diminished in the $Fyn^{-/-}$ mice. Lastly, we also demonstrate the induction of Fyn within the microglia upon intrastriatal aggregated α-synuclein administration and in PD tissues. Taken together, our results suggest that Fyn could mediate inflammasome microgliosis and inflammasome activation in Parkinson's Disease, contributing to the progression of the disease.

Introduction

Parkinson's Disease (PD) is a progressive neurodegenerative disorder characterized by the selective death of dopaminergic neurons of the Substantia nigra (SN) and the subsequent development of severe motor deficits. α-synuclein is a protein whose dysfunction is intimately associated with both idiopathic as well as genetically inherited PD. Point mutations and triplications of the SNCA gene, which codes for the α-synuclein protein, have been demonstrated to cause autosomal dominant PD (Allen Reish and Standaert, 2015; Appel-Cresswell et al., 2013; Kruger et al., 1998; Lesage et al., 2013; Pasanen et al., 2014; Polymeropoulos et al., 1997; Zarranz et al., 2004). Aggregated α-synuclein is also the major component of Lewy bodies, the neuropathological hallmark of idiopathic PD. Rodent models of PD are essential tools to both, identify and elucidate, the mechanisms though which the

disease progresses and also to identify potential therapeutic drug targets whose activation or inhibition could prevent or halt its advance. There are several models to study the disease, most of which perfectly mimic its most cardinal pathological hallmark, i.e. the death of dopaminergic neurons within the SN, which project to the striatum of the brain and mediate motor control. However, idiopathic PD is a progressive, age related disease, which occurs over decades and involves complicated non-motor deficits, the development of Lewy bodies, as well as non-dopaminergic neuronal cell death. The first rodent model system used to study PD utilized the unilateral intrastriatal injection of 6-hydroxydopamine (Ungerstedt, 1968). This treatment induced the rapid degeneration of dopaminergic neurons within the SN pars compacta region (SNpc), as well as a turning asymmetry towards the ipsilateral side. Even though this model is nearly 50 years old, it is still commonly used to study PD. Another model that was subsequently popularized was the MPTP (1-methyl, 4-phenyl, 1,2,3,6-tetrahydropyridine) model, which was developed after it was discovered that addicts who had injected themselves with MPTP, mistakenly believing it to be the analgesic 1-methyl-4-phenyl-4-propionoxypiperidine (MPPP) were admitted to the hospital with PD-like symptoms that were responsive to treatment with the traditional PD drug -levadopa/carbidopa. The offending substance was rapidly identified to be MPTP. Two studies that detailed how MPTP causes Parkinsonism in humans and primates rapidly followed (Burns et al., 1983; Langston et al., 1983). The MPTP model also effects the death of SN dopaminergic neurons, exacerbated neuroinflammation and motor deficits. Both the aforementioned models suffered from similar drawbacks; neither model was progressive (both treatment regimens induced cell death over days in rodent models, whereas PD occurs over decades in their human counterparts) nor displayed the formation of Lewy bodies. The

recently developed MitoPark model attempts to alleviate some of these problems by selectively knocking out the mitochondrial transcription factor TFAM from dopaminergic neurons. The resultant mice develop an age related progressive loss of dopaminergic neurons and development of motor deficits (Ekstrand et al., 2007). However, this model utilizes the mutation of a protein that has not been linked to genetically inherited PD development, and has "lewy body" like inclusions that lack α-synuclein, prompting several researchers to question the significance of using such a system. Newer models of PD utilize mice that globally overexpress pathogenic forms of α-synuclein and have been used by several groups, but often display the inverse phenotype to the ones seen with the MPTP and 6-OHDA models - the formation of synuclein-positive lewy body like structures, progressive development of motor deficits but the absence of dopaminergic neuronal loss (Giasson et al., 2002; Lee et al., 2002). In light of these considerations, it was recently suggested that the Adeno-associated Viruse (AAV) mediated α-synuclein (SYN) overexpression model of PD, wherein AAVs coding for α-synuclein, or GFP (as a control) are injected into the SNpc in mice, might prove to be a superior model when compared to classical PD models (Lindgren et al., 2012). This model has been recently shown to elicit progressive dopaminergic neuronal loss, as well as the concurrent development of α-synuclein-containing inclusions within the dopaminergic neurons (Decressac et al., 2012a; Decressac et al., 2012b). Inflammasomes are large multimeric protein complexes comprising of the cytosolic pattern recognition receptors, the adaptor protein ASC and Caspase-1. These complexes, when activated, mediate the production of the pro-inflammatory cytokines IL-1β and IL-18. The NLRP3 inflammasome is the best studied and characterized of the inflammasomes and has recently been implicated in the development of Alzheimer's disease (AD) (Heneka et al., 2013). There is limited

evidence to suggest that the NLRP3 inflammasome might be activated in PD neuropathology using *in vivo* model systems; the MPTP-induced dopaminergic neuronal degradation was diminished in NLRP3$^{-/-}$ mice (Yan et al., 2015). Viral vector-mediated overexpression of IL-1β brought about dopaminergic neurodegeneration (Ferrari et al., 2006). Since we have previously demonstrated that aggregated α-synuclein could activate the Fyn-dependent NLRP3 inflammasome activation in microglial cells (*in-vitro*), we wanted to check if this finding also held true in PD model systems. To this effect, we utilized various models of synuclein-mediated PD, such as the A53T mice as well as the well-characterized AAV-SYN model system in Fyn$^{+/+}$ and Fyn$^{-/-}$ mice. We discovered that viral overexpression of human α-synuclein, as well as global overexpression of A53T α-synuclein could elicit the activation of the NLRP3 inflammasome. In the AAV-SYN model, Fyn$^{-/-}$ mice showed reduced microgliosis and inflammasome activation.

Materials And Methods

Chemicals and Reagents

Green Fluorescent Protein (GFP) and Human α-synuclein overexpressing adeno-associated viruses (AAV-GFP, 0.95 X 10^{13} viral particles per mL and AAV-SYN, 1 X 10^{13} viral particles per mL) were obtained from The University of North Carolina Viral Vector Core. Both viral vectors were of the AAV-5 serotype and coded for GFP and human α-synuclein respectively under a chicken β-actin promoter. Antibodies to rabbit ASC and Caspase-1 were purchased from Adipogen. Antibodies to rabbit and goat Iba-1 were obtained from Wako and Abcam respectively. Antibodies to goat IL-1β were obtained from R & D systems. The mouse antibodies for tubulin and human α-synuclein were purchased from

Santa Cruz. The rabbit antibody to human α-synuclein and mouse antibody to tyrosine hydroxylase (TH) were purchased from EMD Millipore (Billerica, Massachusetts). PD patient and age matched ventral midbrain sections were obtained from Dr. Asgar Zaheer at the University of Iowa.

A53T mice

The human α-synuclein A53T overexpressing mice were obtained from Jackson laboratories (B6.Cg-Tg(Prnp-SNCA*A53T)23Mkle/J). These mice and their littermate controls were housed under standard conditions of constant temperature (22 ± 1°C), humidity (relative, 30%), and a 12-h light cycle with food and water provided ad libitum. These mice were sacrificed at 4 months of age and their tissues used for various studies.

Animal studies

The $Fyn^{+/+}$ and $Fyn^{-/-}$ mice used in these studies were bred in our animal facility. $Fyn^{-/-}$ mice were originally obtained from Dr. Dorit Ron's laboratory at the University of California, San Francisco and are available from Jackson Laboratory (stock number 002271). The mice were housed under standard conditions of constant temperature (22 ± 1°C), humidity (relative, 30%) and a 12-h light cycle with food and water provided *ad libitum*. Six- to eight-week-old male mice were used for all studies.

Stereotactic injection of the AAV-SYN and AAV-GFP viral particles

Mice were anesthetized with xylazine ketamine. Paw pinch was used to establish profound anesthesia. An incision was made at the back of the head and the area was

disinfected using povidone-iodine. Mice were mounted on the Angle 2 stereotaxic apparatus (Leica Biosystems, St. Louis, MO). The bregma was found and the injection into the SN subsequently performed. The coordinates, relative to bregma were: anteroposterior (AP) -3.1 mm and mediolateral (ML) -1.2 mm relative to bregma, and dorsoventral (DV) -4.0. 0.2 mL of AAV-GFP or AAV-SYN solution was injected per min. The needle was left in for 5 min to ensure no backflow. The needle was retracted at the rate of 1 mm per min. Intradermal injections of lactated ringer's solution was administered post injection to assist the recovery of the mice.

Stereotactic injection of recombinant human aggregated α-synuclein into mice

Mice were anesthetized as previously described. The mice were injected with 4 mL of aggregated α-synuclein or vehicle. The coordinates, relative to bregma were: 0.7 mm anteroposterior, 2 mm lateral and 2.4 mm ventral.

Immunoblotting

Brain tissue lysates were prepared using modified RIPA buffer and were normalized for equal amounts of protein using the Bradford protein assay kit. Equal amounts of protein (30-40 μg) were loaded for each sample and separated on either 12% or 15% SDS-PAGE gels depending on the molecular weight of the target protein. After separation, proteins were transferred to a nitrocellulose membrane and the nonspecific binding sites were blocked for 1 h using a blocking buffer specifically formulated for fluorescent Western blotting (Rockland Immunochemicals). Membranes were then probed with the respective primary antibodies for 3 h at room temperature or overnight at 4°C. After incubation, the membranes were washed 7

times with PBS containing 0.05% Tween 20, and then Secondary IR-680-conjugated anti-mouse (1:10,000, goat anti-mouse, Molecular Probes) and IR-800 conjugated anti rabbit (1:10,000, goat anti-rabbit, Rockland) were used for antibody detection with the Odyssey IR imaging system (LiCor). Membranes were visualized on the Odyssey infrared imaging system. Antibodies for GAPDH, β-actin and Tubulin were used as loading controls.

Immunohistochemistry

Immunohistochemistry (IHC) was performed on sections from the SN and other brain regions of interest as described previously (Ghosh et al., 2013; Jin et al., 2011). Briefly, mice were anesthetized with a mixture of 100 mg/kg ketamine and 10 mg/kg xylazine and then perfused transcardially with freshly prepared 4% paraformaldehyde (PFA). Extracted brains were post-fixed in 4% PFA for 48 h and 30-μm sections were cut using a freezing microtome (Leica Microsystems). Antigen retrieval was performed in citrate buffer (10 mM sodium citrate, pH 8.5) for 30 min at 90°C. Sections were then washed several times in PBS and blocked with PBS containing 2% BSA, 0.2% Triton X-100 and 0.05% Tween 20 for 1 h at room temperature. Sections were then incubated with primary antibodies overnight at 4°C and washed 7 times in PBS on a Belly Dancer Shaker (SPI supplies). The sections were incubated with Alexa 488, 555 and 633 dye-conjugated secondary antibodies for 75 min at room temperature and their cell nuclei were stained with Hoechst dye. Sections were mounted on slides using Prolong antifade gold mounting medium (Invitrogen) according to the manufacturer's instructions. Samples were visualized using an inverted fluorescence microscope (Nikon TE-2000U) and images were captured using a Spot digital camera (Diagnostic Instruments Inc). Samples were then washed several times in PBS and incubated

with Alexa 488 and 555 dye-conjugated secondary antibodies. The nuclei were labeled with Hoechst stain (10 μg/mL) and coverslips were mounted with Fluoromount medium (Sigma Aldrich) on glass slides for visualization.

Confocal imaging and Z stack image acquisition and analysis

Confocal imaging was performed at the Iowa State University Microscopy Facility, also using the Leica DMIRE2 confocal microscope with the 63X and 40X oil objectives and Leica Confocal Software. One optical series covered 11-13 optical slices of 0.5 μm thickness each. The Imaris software was used to analyze the Z stack images. The surface reconstruction wizard in the Imaris software was used to make 3D reconstructions of stacks for easier viewing of microglial-dopaminergic contacts. IHC on human sections was performed as described above, but with modifications to the protocol. Antigen retrieval was carried overnight in citrate buffer (10 mM sodium citrate, pH 8.5) at 4°C prior to the 90°C step. The autofluorescence eliminator reagent (Chemicon) was used to eliminate autofluoroscence in the sections.

Data analysis

Data analysis was performed using Prism 4.0 (GraphPad Software, San Diego, CA). The data was initially analyzed using one-way ANOVA and Bonferroni's post-test to compare the means of treatment groups. Differences of $p<0.05$ were considered statistically significant. Student's t-test was used when comparing two groups.

Results

Activation of the NLRP3 inflammasome in the A53T model and in post-mortem PD brain lysates

NLRP3 inflammasome has previously been conclusively demonstrated in the APP/PS-1 AD model, as well as in post-mortem AD brains (Heneka et al., 2013). The major readout of inflammasome activation in the aforementioned study was increased levels of cleaved Caspase-1. Accordingly, 4 month-old A53T and littermate control striatal lysates were assessed for cleaved Caspase-1 levels. There was a striking increase in the levels of cleaved Caspase-1 in the A53T striatal lysates, indicating that α-synuclein aggregation/overexpression is able to elicit inflammasome activation *in-vivo* (Fig. 1A). We also checked PD patient and age-matched control patient nigral tissue lysates for Caspase-1 and IL-1β. Both, Caspase-1 and IL-1β levels were significantly increased in the human PD lysates (Fig. 1B). Although both Caspase-1 and IL-1β levels were previously shown to be elevated in PD brain and CSF samples, these discoveries were made before the concept of the inflammasome was postulated (Mogi et al., 1996; Mogi et al., 2000). Data from the A53T model and from PD patient lysates provide evidence that there may be *in-vivo* activation of the inflammasome.

AAV-mediated targeted expression of α-synuclein in the ventral midbrain can effect the death of dopaminergic neurons in the SN of mice in a Fyn dependent manner.

We stereotaxically injected AAV-GFP and AAV-SYN into the $Fyn^{+/+}$ and $Fyn^{-/-}$ SNpc. 45 d later, the mice were sacrificed and brains collected, fixed and sectioned. To ensure that our injections were precise and no non-specific areas were targeted, we stained

the 30 μM thick sections with antibodies for GFP/human synuclein and tyrosine hydroxylase (TH), a dopaminergic neuronal marker in separate channels. The SNpc was perfectly targeted by the injected by the AAV injections, with a large degree of green and red channel overlap seen on the left hand side of the sections (Fig. 2A). Higher magnification images of the injected side were taken to greatly appreciate the degree of SNpc dopaminergic neuronal targeting (Fig. 2B). The AAV-SYN injected side in the WT mice showed a modest decrease of TH positive neurons 45 d post-injection when compared with the AAV-GFP injected animals, but there was no such loss in the $Fyn^{-/-}$ mice (Fig. 2A, B).

AAV-SYN overexpression in the SNpc elicits massive microgliosis and microglial-dopaminergic neuronal gliapse formation in WT, but not the $Fyn^{-/-}$ mice

Sections from the above study were also stained with antibodies against Iba-1 and TH to demonstrate microgliosis, specifically around the SN. This was done because in PD cases, the greatest degree of microgliosis is observed within the SNpc itself, contributing to a local loss of dopaminergic neurons (Bartels and Leenders, 2007; Mosley et al., 2006; Whitton, 2010). IHC analysis revealed that overexpression of human-α synuclein, but not GFP, induced massive microgliosis within the SN, specifically in the area of maximal dopaminergic neuronal loss in the WT mice, supporting the hypothesis that the hyperactivation of the microglal inflammatory response might contribute to the loss of dopaminergic neuronal loss. No increased microgliosis was observed in the $Fyn^{-/-}$ mouse SN sections (Fig. 3A). Recently, it was shown that MPTP intoxication rapidly increased the number of microglial-neuronal appositions, called gliapses. The formation of these contacts was rapidly followed by microglial phagocytosis of neurons, and inhibiting the formation of

these contacts proved to be neuroprotective in the MPTP model (Barcia et al., 2012). Similar results were obtained using the 6-OHDA model (Virgone-Carlotta et al., 2013). The Fyn-dependent formation of microglial-neuronal contacts was an area we explored using the 6-OHDA model (Panicker et al., 2015). Confocal maximal projection Z stack images were used to make 3D reconstructions to visualize the microglial-dopaminergic neuronal contact formation with more clarity. As seen in Figure 3B, AAV-SYN overexpression induced a massive increase in the microglial cells within the SNpc, and the number of gliapses formed per neuron. The $Fyn^{-/-}$, mice on the other hand, demonstrated diminished microgliosis and fewer gliapses (Fig. 3B).

Fyn kinase contributes to microglial inflammasome activation in the AAV-SYN mouse model of Parkinson's disease

Activation of the NLRP3 inflammasome in PD models is still a dynamic area of investigation and has not been conclusively demonstrated yet. However, there is some evidence to suggest that it may be activated under certain conditions; the transgenic db/db diabetic cell line, when subjected to a regimen with the Parkinsonian toxicant MPTP results in activation of the NLRP3 inflammasome and exacerbates neuroinflammation and neurodegeneration (Wang et al., 2014). Moreover, synuclein AAV overexpression in the nigra results in a significant production of IL-1β and TNFα in a rat model of synucleinopathy, even though the role of the inflammasome in this process was not conclusively proven (Chung et al., 2009). A recent study showed that $NLRP3^{-/-}$ mice were resistant to MPTP-induced TH-positive cell loss (Yan et al., 2015). One of the means to validate activation of the inflammasome *in vivo* is the formation of ASC specks in the

microglia. This was done in the APP/PS1 mice AD model (Heneka et al., 2013). Accordingly, to assess whether microglial inflammasome activation could be driven by human α-synuclein overexpression *in vivo*, we stained sections from this study with antibodies to ASC and Iba-1. The number of microglial cells that showed ASC specks per field were counted and quantified. AAV-SYN-injected WT, but not $Fyn^{-/-}$ mice demonstrated a significant increase in the number of ASC speck-positive microglia, showing that the AAV-SYN model effected the Fyn-dependent activation of the inflammasome in microglia (Fig. 4A, B).

Intrastriatal injection of α-synuclein aggregates effects microgliosis with concurrent upregulation of microglial Fyn

We have previously demonstrated that prolonged exposure to the inflammogens - LPS and TNFα results in the upregulation of Fyn kinase in microglial cells (Panicker et al., 2015). We wanted to observe whether these results could be extended to *in-vivo* models of microgliosis. Stereotactic injections of aggregated α-synuclein have previously been demonstrated to elicit pro-inflammatory responses (Couch et al., 2011). We injected $Fyn^{+/+}$ mice with 4 μg of α-synuclein protein in the striatum. Coronal brain sections from these mice were then stained for Iba-1 to mark microglia as well as Fyn. We saw that on the injected side, there was a dramatic shift of microglial morphology from ramified to amoeboid, indicating microglial activation, along with increased Fyn expression within the Iba-1-positive microglia (Fig. 5).

Microglial upregulation of Fyn in post-mortem PD brain sections

To provide a clinically relevant role for Fyn kinase in PD pathology, we stained human PD and age-matched control ventral midbrain sections with antibodies to Fyn and Iba-1. We observed that there was a strikingly increased expression of Fyn within the Iba-1-positive microglia in the PD brain sections providing clinical significance to our *in-vitro* and *in-vivo* findings (Fig. 6).

Discussion

PD is a devastating neurodegenerative disorder characterized by the loss of dopamine-producing neurons within the SNpc. It has now been universally accepted that this loss of neurons is accompanied by an excessive neuroinflammatory response, which contributes to the progressive nature of the disease. *In-vivo* models of Parkinsonian dysfunction are able to recapitulate various facets of PD with varying degrees of success; both the well utilized and characterized MPTP and 6-OHDA models can bring about the selective death of dopaminergic neurons within the SNpc, along with a concomitant neuroinflammatory response, but have been criticized for not being able to recapitulate the finer neuropathological hallmarks of PD, vis-à-vis the formation of Lewy bodies, a progressive age-dependent loss of neurons, etc. Among the new generation of PD models developed, the A53T human α-synuclein overexpressing mice and the AAV-SYN model have several advantages over the aforementioned classical models, such as the development of proteinaceous inclusions and progressive dopaminergic neuron loss (Decressac et al., 2012a; Decressac et al., 2012b). Using the AAV-SYN model is also of significance to us because it evokes a marked pro-inflammatory response. Previously we demonstrated how

aggregated human α-synuclein mediated the Fyn kinase-dependent priming and activation of the NLRP3 inflammasome in microglia. We wanted to assess whether inflammasome could be activated in the AAV-SYN model, and whether Fyn contributed to this response. We first provide evidence for the posit that inflammasomes might be activated in the A53T model and in PD post-mortem tissues, both of which showed greater levels of cleaved Caspase-1 than the control littermate/age-matched human control brain lysates respectively (Fig. 1A, B). We next stereotactically injected AAV-SYN or AAV-GFP constructs into $Fyn^{+/+}$ and $Fyn^{-/-}$ mice. The SNpc was targeted perfectly, with the TH-positive dopaminergic neurons on the injected side in both genotypes expressing human α-synuclein or GFP respectively (Fig. 2A, B). Just 45 d post-injection of the AAV-SYN, but not the GFP-SYN constructs, $Fyn^{+/+}$ mice displayed some loss of dopaminergic neurons on the injected side. The $Fyn^{-/-}$ mice showed no such loss (Fig. 2A). PD is characterized by microgliosis within the SN. To check for nigral microgliosis, we stained sections for TH (as a dopaminergic neuron marker) and Iba-1 (as a marker of microglia). There was massive microgliosis observed in the AAV-SYN injected $Fyn^{+/+}$ mice, and a complete absence of the same in the $Fyn^{-/-}$ mice. AAV-GFP overexpression did not seem to elicit a microglial inflammatory response in either the $Fyn^{+/+}$ or $Fyn^{-/-}$ mice (Fig. 3A, B). ASC oligomerization and speck formation are classical hallmarks of inflammasome activation in microglial cells, which have previously been demonstrated in AD models (Heneka et al., 2013). There is evidence to support the notion that the NLRP3 inflammasome may have relevance in animal models of PD and clinical relevance as well. Ole Isaacson and colleagues reported an increase in IL-1β striatal levels using the A53T α-synuclein-AAV model in rats (although activation of the NLRP3 inflammasome was not looked at or discussed) (Chung et al., 2009). Overexpression of IL-1β in the mouse SN can

directly bring about the death of dopaminergic neurons (Ferrari et al., 2006), and most recently, dopamine was shown to inhibit the NLRP3 inflammasome. MPTP treatment was utilized to induce a depletion of dopamine levels, which activated the NLRP3 inflammasome and subsequent NLRP3-dependent dopaminergic neuron loss (Yan et al., 2015). We assessed the ASC speck formation within microglia in the AAV-SYN and AAV-GFP injected $Fyn^{+/+}$ and $Fyn^{-/-}$ mice. There was an induction of ASC specks in the WT mice injected with AAV-SYN, but no such change in the $Fyn^{-/-}$ mice microglia (Fig. 4A, B). This supports our hypothesis that α-synuclein overexpression might contribute to inflammasome activation *in vivo*, and that Fyn might mediate this process. We also recently demonstrated that prolonged exposure of microglial cells to various inflammogens upregulated the levels of Fyn kinase (rather than just increasing kinase activity). To test whether this might occur in *in-vivo* models, we injected WT and $Fyn^{-/-}$ mice with aggregated α-synuclein (4 μg) for 4 d in the striatum. α-synuclein injection elicited a distinct shift in the microglial morphology from ramified (unactivated) to amoeboid (activated), along with a concomitant increase in Fyn expression in the Iba-1-positive microglia (Figure 5). We also assessed the expression of Fyn in human PD and age-matched control ventral midbrain sections. We observed upregulated expression of Fyn in the Iba-1-positive microglia within the SN, providing clinical relevance for our findings (Figure 6). It would be intriguing to assess whether sterile inflammation mediated by α-synuclein aggregates could act cooperatively with dopamine depletion to mediate unabated inflammasome activation that might contribute to the progressive neurodegeneration that characterizes PD. Since ASC released from activated peripheral immune cells was demonstrated to seed ASC oligomerization in a prionid manner (Baroja-

Mazo et al., 2014), it might also be intriguing to explore whether microglia released ASC could contribute to the spread of sterile inflammatory responses in PD brains.

References

Allen Reish, H.E., and Standaert, D.G. (2015). Role of alpha-synuclein in inducing innate and adaptive immunity in Parkinson disease. J Parkinsons Dis *5*, 1-19.

Appel-Cresswell, S., Vilarino-Guell, C., Encarnacion, M., Sherman, H., Yu, I., Shah, B., Weir, D., Thompson, C., Szu-Tu, C., Trinh, J., *et al*. (2013). Alpha-synuclein p.H50Q, a novel pathogenic mutation for Parkinson's disease. Mov Disord *28*, 811-813.

Barcia, C., Ros, C.M., Annese, V., Carrillo-de Sauvage, M.A., Ros-Bernal, F., Gomez, A., Yuste, J.E., Campuzano, C.M., de Pablos, V., Fernandez-Villalba, E., *et al*. (2012). ROCK/Cdc42-mediated microglial motility and gliapse formation lead to phagocytosis of degenerating dopaminergic neurons in vivo. Sci Rep *2*, 809.

Baroja-Mazo, A., Martin-Sanchez, F., Gomez, A.I., Martinez, C.M., Amores-Iniesta, J., Compan, V., Barbera-Cremades, M., Yague, J., Ruiz-Ortiz, E., Anton, J., *et al*. (2014). The NLRP3 inflammasome is released as a particulate danger signal that amplifies the inflammatory response. Nat Immunol *15*, 738-748.

Bartels, A.L., and Leenders, K.L. (2007). Neuroinflammation in the pathophysiology of Parkinson's disease: evidence from animal models to human in vivo studies with [11C]-PK11195 PET. Mov Disord *22*, 1852-1856.

Burns, R.S., Chiueh, C.C., Markey, S.P., Ebert, M.H., Jacobowitz, D.M., and Kopin, I.J. (1983). A primate model of parkinsonism: selective destruction of dopaminergic neurons in the pars compacta of the substantia nigra by N-methyl-4-phenyl-1,2,3,6-tetrahydropyridine. Proceedings of the National Academy of Sciences of the United States of America *80*, 4546-4550.

Chung, C.Y., Koprich, J.B., Siddiqi, H., and Isacson, O. (2009). Dynamic changes in presynaptic and axonal transport proteins combined with striatal neuroinflammation precede dopaminergic neuronal loss in a rat model of AAV alpha-synucleinopathy. The Journal of neuroscience : the official journal of the Society for Neuroscience *29*, 3365-3373.

Couch, Y., Alvarez-Erviti, L., Sibson, N.R., Wood, M.J., and Anthony, D.C. (2011). The acute inflammatory response to intranigral alpha-synuclein differs significantly from intranigral lipopolysaccharide and is exacerbated by peripheral inflammation. Journal of neuroinflammation *8*, 166.

Decressac, M., Mattsson, B., and Bjorklund, A. (2012a). Comparison of the behavioural and histological characteristics of the 6-OHDA and alpha-synuclein rat models of Parkinson's disease. Exp Neurol *235*, 306-315.

Decressac, M., Mattsson, B., Lundblad, M., Weikop, P., and Bjorklund, A. (2012b). Progressive neurodegenerative and behavioural changes induced by AAV-mediated overexpression of alpha-synuclein in midbrain dopamine neurons. Neurobiology of disease *45*, 939-953.

Ekstrand, M.I., Terzioglu, M., Galter, D., Zhu, S., Hofstetter, C., Lindqvist, E., Thams, S., Bergstrand, A., Hansson, F.S., Trifunovic, A., *et al*. (2007). Progressive parkinsonism in mice with respiratory-chain-deficient dopamine neurons. Proceedings of the National Academy of Sciences of the United States of America *104*, 1325-1330.

Ferrari, C.C., Pott Godoy, M.C., Tarelli, R., Chertoff, M., Depino, A.M., and Pitossi, F.J. (2006). Progressive neurodegeneration and motor disabilities induced by chronic expression of IL-1beta in the substantia nigra. Neurobiology of disease *24*, 183-193.

Ghosh, A., Saminathan, H., Kanthasamy, A., Anantharam, V., Jin, H., Sondarva, G., Harischandra, D.S., Qian, Z., Rana, A., and Kanthasamy, A.G. (2013). The peptidyl-prolyl isomerase Pin1 up-regulation and proapoptotic function in dopaminergic neurons: relevance to the pathogenesis of Parkinson disease. The Journal of biological chemistry *288*, 21955-21971.

Giasson, B.I., Duda, J.E., Quinn, S.M., Zhang, B., Trojanowski, J.Q., and Lee, V.M. (2002). Neuronal alpha-synucleinopathy with severe movement disorder in mice expressing A53T human alpha-synuclein. Neuron *34*, 521-533.

Heneka, M.T., Kummer, M.P., Stutz, A., Delekate, A., Schwartz, S., Vieira-Saecker, A., Griep, A., Axt, D., Remus, A., Tzeng, T.C., *et al*. (2013). NLRP3 is activated in Alzheimer's disease and contributes to pathology in APP/PS1 mice. Nature *493*, 674-678.

Jin, H., Kanthasamy, A., Ghosh, A., Yang, Y., Anantharam, V., and Kanthasamy, A.G. (2011). alpha-Synuclein negatively regulates protein kinase Cdelta expression to suppress apoptosis in dopaminergic neurons by reducing p300 histone acetyltransferase activity. The Journal of neuroscience : the official journal of the Society for Neuroscience *31*, 2035-2051.

Kruger, R., Kuhn, W., Muller, T., Woitalla, D., Graeber, M., Kosel, S., Przuntek, H., Epplen, J.T., Schols, L., and Riess, O. (1998). Ala30Pro mutation in the gene encoding alpha-synuclein in Parkinson's disease. Nat Genet *18*, 106-108.

Langston, J.W., Ballard, P., Tetrud, J.W., and Irwin, I. (1983). Chronic Parkinsonism in humans due to a product of meperidine-analog synthesis. Science *219*, 979-980.

Lee, M.K., Stirling, W., Xu, Y., Xu, X., Qui, D., Mandir, A.S., Dawson, T.M., Copeland, N.G., Jenkins, N.A., and Price, D.L. (2002). Human alpha-synuclein-harboring familial Parkinson's disease-linked Ala-53 --> Thr mutation causes neurodegenerative disease with alpha-synuclein aggregation in transgenic mice. Proceedings of the National Academy of Sciences of the United States of America *99*, 8968-8973.

Lesage, S., Anheim, M., Letournel, F., Bousset, L., Honore, A., Rozas, N., Pieri, L., Madiona, K., Durr, A., Melki, R., *et al*. (2013). G51D alpha-synuclein mutation causes a novel parkinsonian-pyramidal syndrome. Ann Neurol *73*, 459-471.

Lindgren, H.S., Lelos, M.J., and Dunnett, S.B. (2012). Do alpha-synuclein vector injections provide a better model of Parkinson's disease than the classic 6-hydroxydopamine model? Exp Neurol *237*, 36-42.

Mogi, M., Harada, M., Narabayashi, H., Inagaki, H., Minami, M., and Nagatsu, T. (1996). Interleukin (IL)-1 beta, IL-2, IL-4, IL-6 and transforming growth factor-alpha levels are elevated in ventricular cerebrospinal fluid in juvenile parkinsonism and Parkinson's disease. Neurosci Lett *211*, 13-16.

Mogi, M., Togari, A., Kondo, T., Mizuno, Y., Komure, O., Kuno, S., Ichinose, H., and Nagatsu, T. (2000). Caspase activities and tumor necrosis factor receptor R1 (p55) level are elevated in the substantia nigra from parkinsonian brain. J Neural Transm (Vienna) *107*, 335-341.

Mosley, R.L., Benner, E.J., Kadiu, I., Thomas, M., Boska, M.D., Hasan, K., Laurie, C., and Gendelman, H.E. (2006). Neuroinflammation, Oxidative Stress and the Pathogenesis of Parkinson's Disease. Clin Neurosci Res *6*, 261-281.

Panicker, N., Saminathan, H., Jin, H., Neal, M., Harischandra, D.S., Gordon, R., Kanthasamy, K., Lawana, V., Sarkar, S., Luo, J., *et al*. (2015). Fyn Kinase Regulates Microglial Neuroinflammatory Responses in Cell Culture and Animal Models of Parkinson's Disease. The Journal of neuroscience : the official journal of the Society for Neuroscience *35*, 10058-10077.

Pasanen, P., Myllykangas, L., Siitonen, M., Raunio, A., Kaakkola, S., Lyytinen, J., Tienari, P.J., Poyhonen, M., and Paetau, A. (2014). Novel alpha-synuclein mutation A53E associated with atypical multiple system atrophy and Parkinson's disease-type pathology. Neurobiology of aging *35*, 2180 e2181-2185.

Polymeropoulos, M.H., Lavedan, C., Leroy, E., Ide, S.E., Dehejia, A., Dutra, A., Pike, B., Root, H., Rubenstein, J., Boyer, R., *et al*. (1997). Mutation in the alpha-synuclein gene identified in families with Parkinson's disease. Science *276*, 2045-2047.

Ungerstedt, U. (1968). 6-Hydroxy-dopamine induced degeneration of central monoamine neurons. Eur J Pharmacol *5*, 107-110.

Virgone-Carlotta, A., Uhlrich, J., Akram, M.N., Ressnikoff, D., Chretien, F., Domenget, C., Gherardi, R., Despars, G., Jurdic, P., Honnorat, J., *et al*. (2013). Mapping and kinetics of microglia/neuron cell-to-cell contacts in the 6-OHDA murine model of Parkinson's disease. Glia *61*, 1645-1658.

Wang, L., Zhai, Y.Q., Xu, L.L., Qiao, C., Sun, X.L., Ding, J.H., Lu, M., and Hu, G. (2014). Metabolic inflammation exacerbates dopaminergic neuronal degeneration in response to acute MPTP challenge in type 2 diabetes mice. Exp Neurol *251*, 22-29.

Whitton, P.S. (2010). Neuroinflammation and the prospects for anti-inflammatory treatment of Parkinson's disease. Curr Opin Investig Drugs *11*, 788-794.

Yan, Y., Jiang, W., Liu, L., Wang, X., Ding, C., Tian, Z., and Zhou, R. (2015). Dopamine controls systemic inflammation through inhibition of NLRP3 inflammasome. Cell *160*, 62-73.

Zarranz, J.J., Alegre, J., Gomez-Esteban, J.C., Lezcano, E., Ros, R., Ampuero, I., Vidal, L., Hoenicka, J., Rodriguez, O., Atares, B., *et al*. (2004). The new mutation, E46K, of alpha-synuclein causes Parkinson and Lewy body dementia. Ann Neurol *55*, 164-173.

Figures

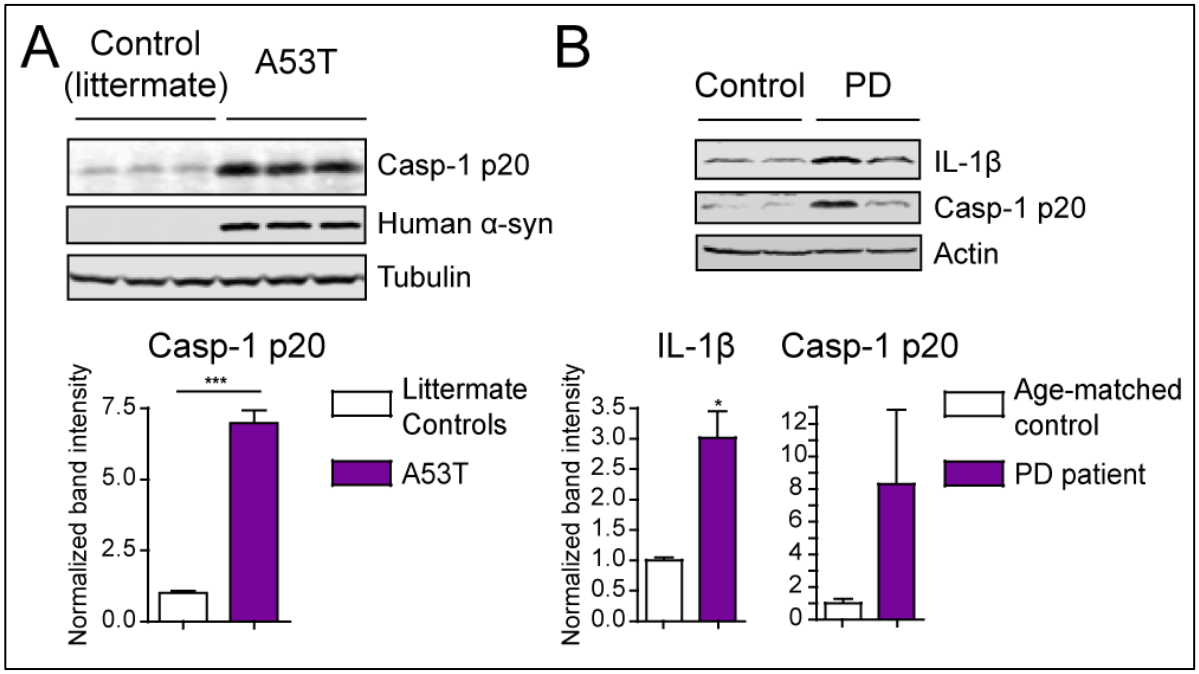

Figure 1. Inflammasome activation is elicited in A53T human α-synuclein overexpressing mice and in PD ventral midbrain lysates. ***A,*** Immunoblot analysis of 4 month old A53T striatal lysates revealed significant increase in the levels of cleaved Caspase-1 levels, when compared to littermate controls. ***B***, Immunoblot analysis of PD nigral tissue lysates revealed significantly increased IL-1β and Caspase-1 p20 levels when compared to age –matched control nigral lysates.

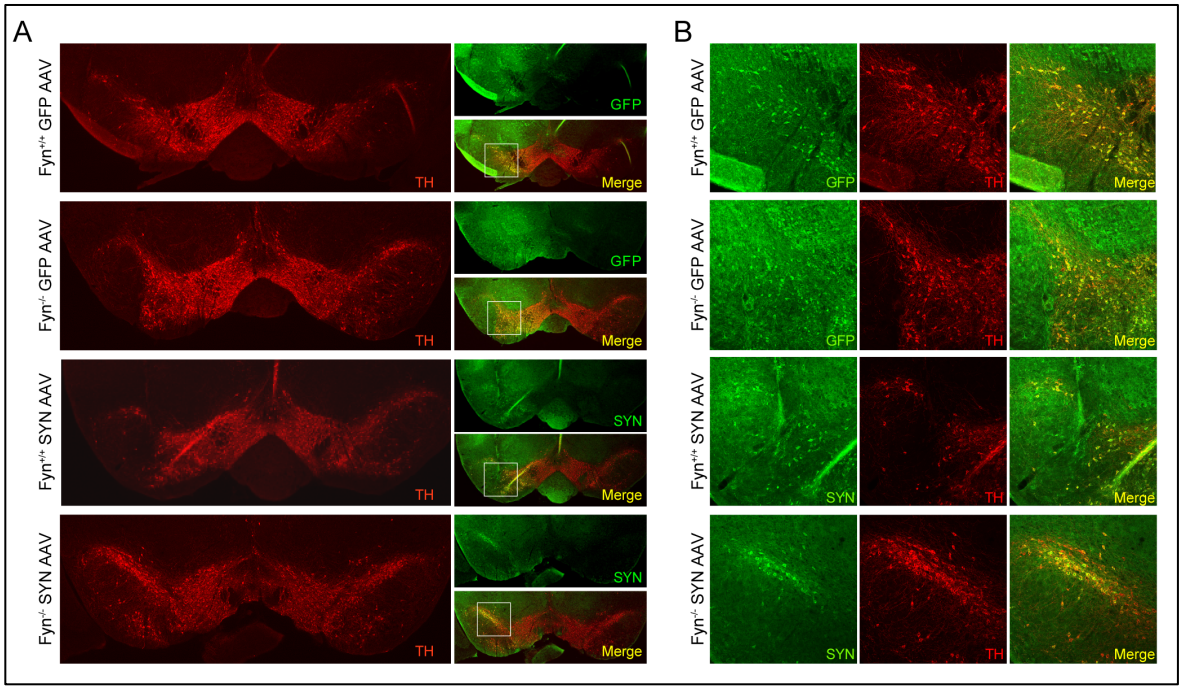

Figure 2. Viral mediated targeted overexpression of human synuclein and GFP in the SNpc of Fyn$^{+/+}$ and Fyn$^{-/-}$ mice. ***A***, Fyn$^{+/+}$ and Fyn$^{-/-}$ mice were intranigrally injected with AAVs encoding GFP and human α-synuclein. Coronal brain sections were stained for GFP/human α-synuclein and TH. ***B***, Higher magnification images of the injection sites reveal that the dopaminergic neurons were well targeted, with a large degree of overlap between the red (for TH) and green (for GFP or human α-synuclein) channels.

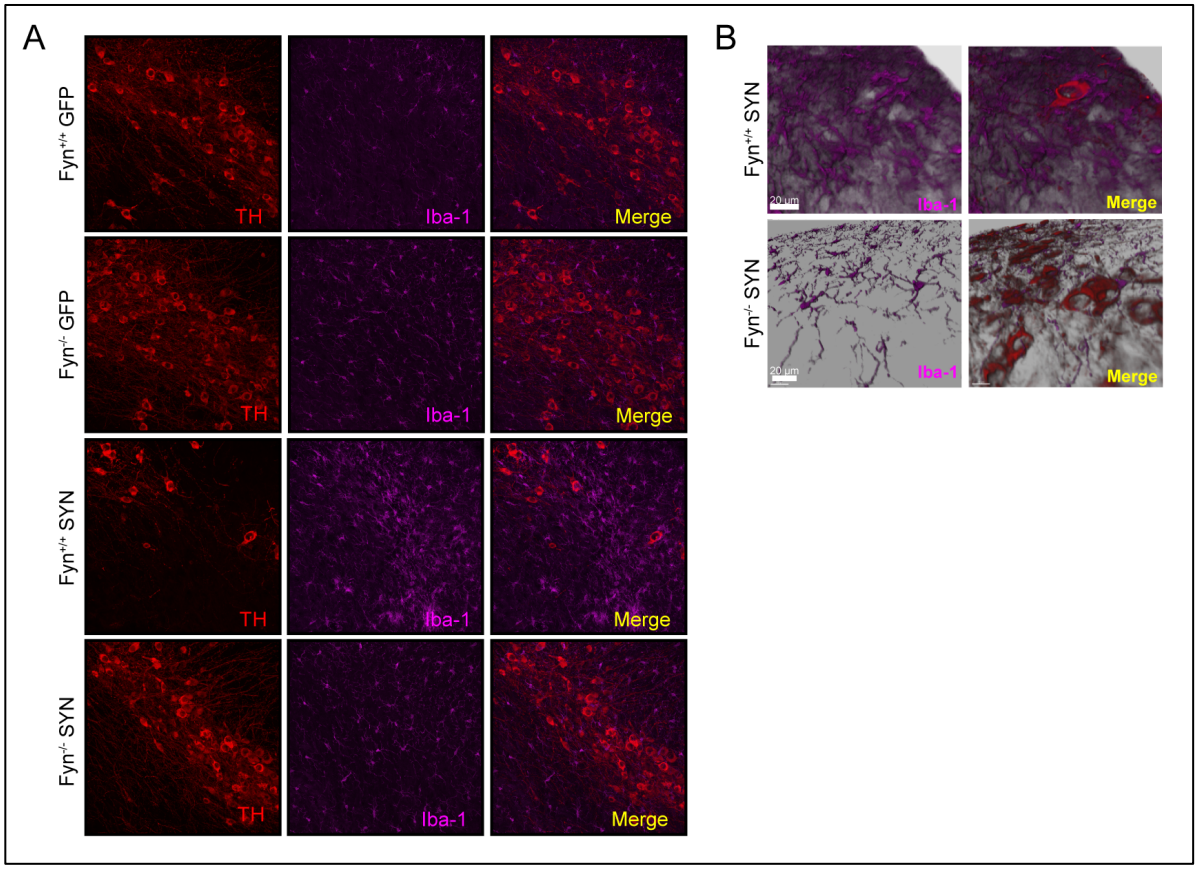

Figure 3. Diminished microgliosis in Fyn deficient mice using the AAV-SYN PD model. ***A,*** Massive microgliosis was observed within the SN in the $Fyn^{+/+}$ mice injected with the AAV-SYN construct, but not the $Fyn^{-/-}$ mice. AAV-GFP overexpression did not induce microgliosis. ***B,*** 3-D reconstruction of the Z-stack images in the ventral midbrain of AAV-SYN injected WT and $Fyn^{-/-}$ reveals the disparity of the microglial response/ neuron between the genotypes.

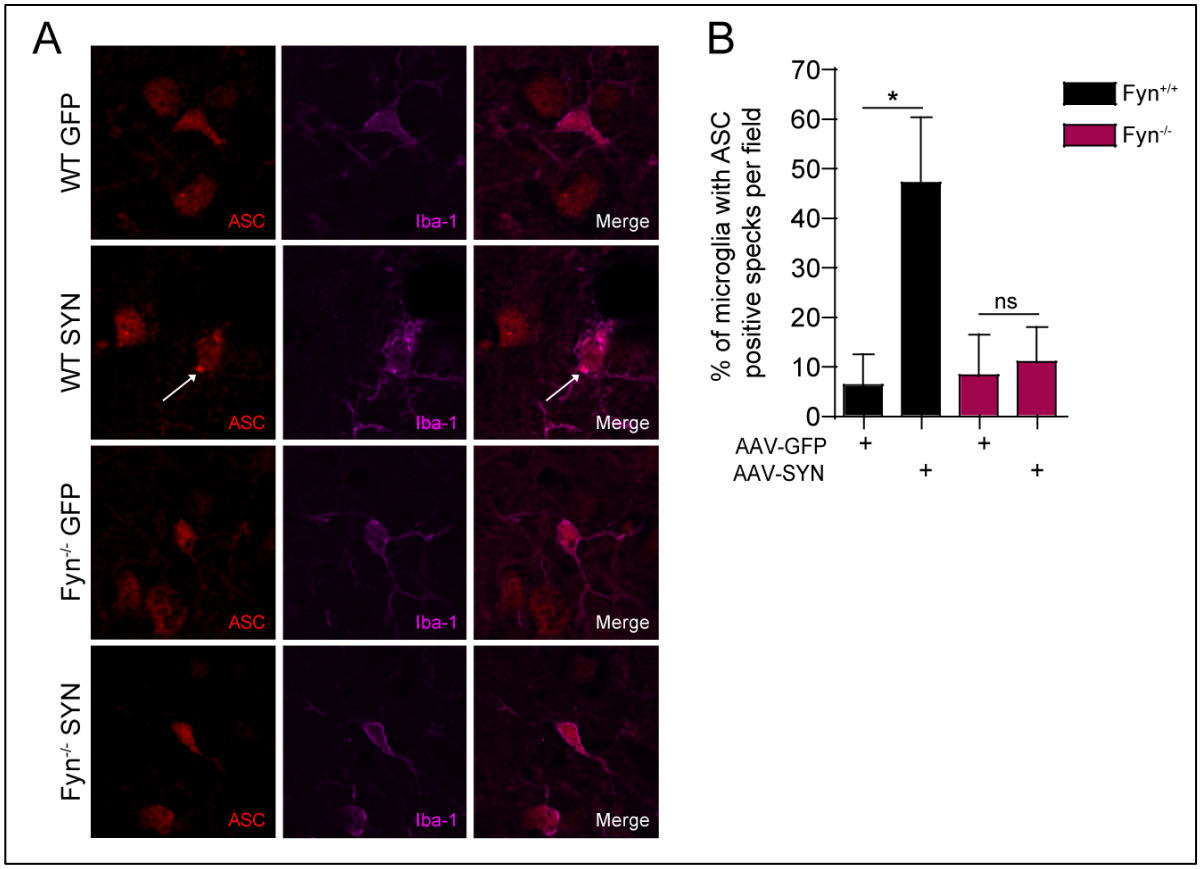

Figure 4. Fyn contributes to microglial inflammasome activation in the AAV-SYN PD model. ***A,*** Formation of ASC specks within the microglial cells in the $Fyn^{+/+}$, but not $Fyn^{-/-}$ AAV-SYN injected ventral midbrain sections. ***B***, Quantification of the microglial population positive for ASC specks upon AAV-GFP or AAV-SYN injection in $Fyn^{+/+}$ and $Fyn^{-/-}$ ventral midbrain sections.

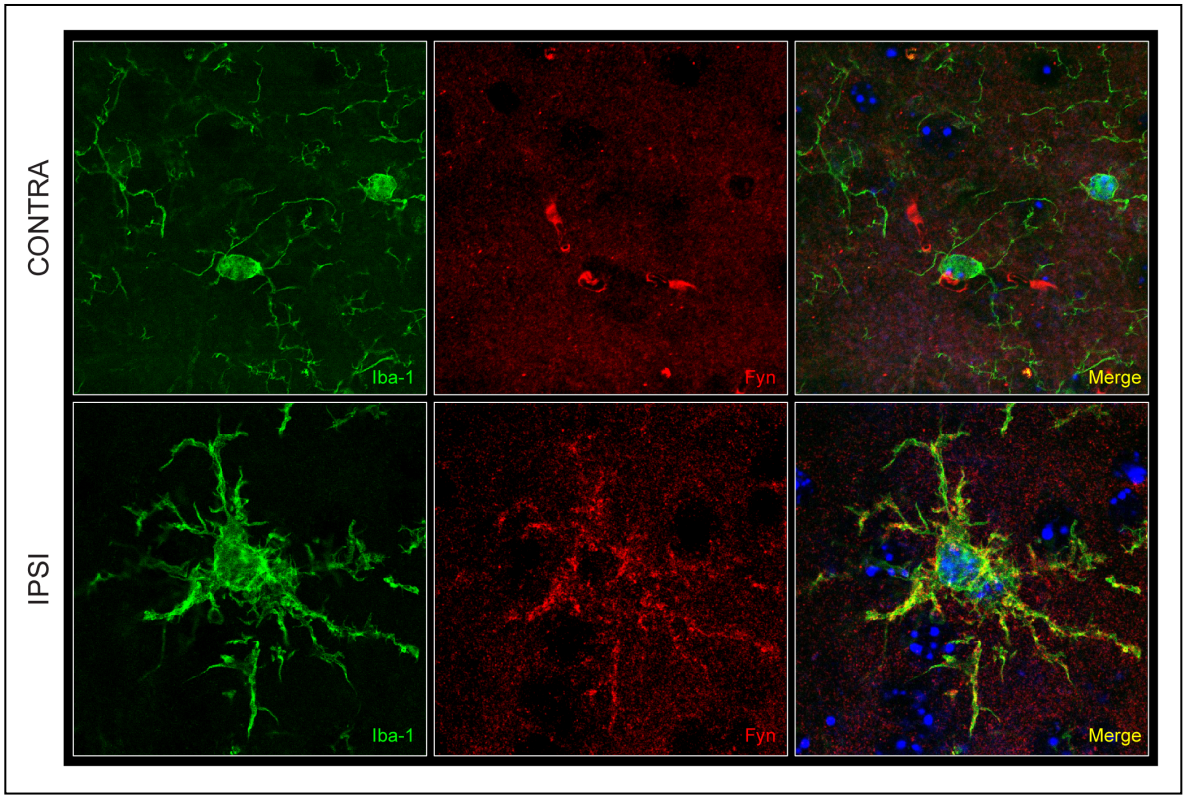

Figure 5. Intrastriatal injection of aggregated α-synuclein elicits Fyn protein induction within the Iba-1 positive microglia. Aggregated α-synuclein intrastriatal injection results in the activation of microglia, as seen by the shift in microglial morphology from ramified to amoeboid, along with a concurrent induction of Fyn within the microglia.

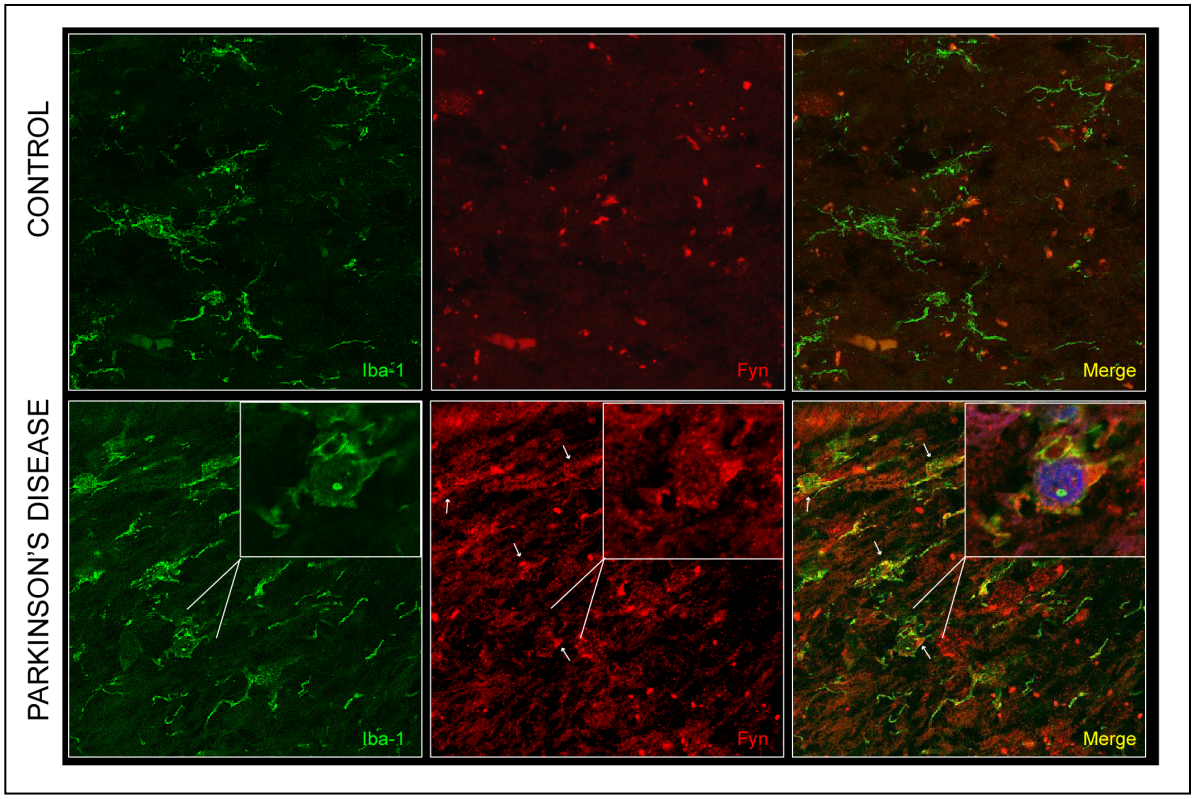

Figure 6. Microglial Fyn is upregulated in PD patient brains over age-matched control brains. PD patient and age-matched control ventral midbrain sections were stained for Iba-1 and Fyn. PD patient brains display more Iba-1 expression, and increased Fyn expression within the Iba-1, indicating microgliosis and microglial Fyn upregulation.

CHAPTER 5. GENERAL CONCLUSION AND FUTURE DIRECTIONS

This section presents a general overview of the results and findings described in the thesis, with special emphasis on future directions and overall implications of these findings for the pathogenesis and progression of Parkinson's disease. The major findings pertaining to each research manuscript and their specific implications are covered in the 'results and discussion' sections of the relevant chapters.

Fyn and Fyn-dependent PKCδ activation contribute to pro-inflammatory responses in microglia

The primary finding from Chapter 2 of the thesis is that the non-receptor Src family tyrosine kinase Fyn is rapidly activated following stimulation of microglia with both TLR and TNFR1 ligands. The activated Fyn then tyrosine-phosphorylates PKCδ, which in turn contributes to the activation of the MAP kinase and NF-κB pathways and the subsequent production of pro-inflammatory mediators in microglia. Although we have demonstrated conclusively that the NF-κB activation is diminished in $Fyn^{-/-}$ and $PKC\delta^{-/-}$ microglia (Panicker et al., 2015) (Panicker et al., 2015, Gordon et al., submitted), future studies will identify what substrates PKCδ phosphorylates to mediate pro-inflammatory responses. Other studies involving peripheral immune cells offer clues as to what these substrates might be. It was shown that PKCδ not only phosphorylates the p47 subunit of the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase complex, which mediates production of ROS from immune cells (Cheng et al., 2007), but also the p65 subunit of the NF-κB complex in vascular smooth muscle cells (Ren et al., 2014). PKCδ also phosphorylates PKD1 at residue S744 (Asaithambi et al., 2011; Doppler and Storz, 2007). PKD1 is an indispensable

component of the Myd-88 dependent pro-inflammatory signaling pathway downstream of TLR ligands (Park et al., 2009). A wealth of data has emerged showing how PKCδ is cleaved by Caspase-3, contributing to the pro-apoptotic pathway in dopaminergic neurons (Anantharam et al., 2002; Kitazawa et al., 2003; Yang et al., 2004). PKCδ is cleaved into a regulatory fragment (RF) and a catalytic fragment (CF), the latter of which enters the nucleus and induces apoptosis. Interestingly, PKCδ can be cleaved in microglial cells where the CF contributes to NF-κB activation (Burguillos et al., 2011). In summary, PKCδ is activated by Fyn-mediated tyrosine phosphorylation at Y311, which contributes to an increase in its kinase activity. Activated PKCδ contributes to NF-κB activation in a variety of ways, which will be explored in future studies.

Fyn- and PKCδ-deficient mice are resistant to neuroinflammation and neurodegeneration in PD models

In Chapter 2, we showed that $Fyn^{-/-}$ and $PKC\delta^{-/-}$ mice display attenuated pro-inflammatory striatal cytokine responses upon LPS treatment. They also exhibit less microgliosis and neuron death in the 6-OHDA PD model. To build upon these studies, we next utilized known Fyn inhibitor compounds and their analogues to test whether they have anti-inflammatory effects in various PD mouse models. Recently, the Fyn inhibitor Saracatinib was shown to prevent microgliosis in an AD mouse model (Kaufman et al., 2015). In addition, rosmarinic acid was demonstrated to be a Fyn inhibitor (Jelic et al., 2007). We intend to assess the ability of this compound and its analogues to inhibit Fyn *in vitro* and also to prevent microglia activation in PD mouse models. Next, to prove that microglial Fyn contributes to PD dopaminergic neuronal death, we will selectively knock out Fyn in the

microglial cells. To accomplish this, we will utilize the CX_3CR1 Cre-ER mice, available from Jackson laboratories. Microglia express high amounts of the chemokine receptor CX_3CR1, but the other resident cells of the CNS, the neurons and astrocytes do not. Investigators have made use of the CX_3CR1 gene promoter, replacing the gene downstream of the promoter with the gene for Cre recombinase fused to a mutant estrogen ligand binding domain (ER) that requires the presence of tamoxifen to be activated. These mice will be crossed with $Fyn^{fl/fl}$ mice, which have the Fyn gene flanked by two loxP sites. Upon administration of tamoxifen, the microglial cells express Cre recombinase, which then catalyzes the site-specific recombination between the loxP sites, effectively knocking out Fyn. Peripheral immune cells that express Fyn will also undergo Fyn knockout, but these cells have short half-lives and are replaced within 4 weeks, whereas microglial cells are long-lived and are not replaced. Hence, 4 weeks post-tamoxifen injection, only the resident microglial cells have no Fyn expression. This strategy was utilized to selectively knock out the protein TAK1 from microglia (Goldmann et al., 2013). Conditional microglial-specific Fyn knockout mice will be subjected to PD-related inflammogens including LPS, 6-OHDA, and MPTP to further characterize downstream signaling-associated neuroinflammatory processes in PD. Additional characterization of Fyn-dependent proinflammatory signaling will eventually yield novel disease-modifying strategies for slowing or halting the progression of neurodegenerative processes underlying Parkinson's disease.

Fyn contributes to α-synuclein-mediated priming and activation of the NLRP3 inflammasome

Chapter 3 demonstrates that Fyn is rapidly activated by aggregated α-synuclein, the major component of PD-associated Lewy bodies, and mediates PKCδ Y311 phosphorylation, which in turn feeds into NF-κB pathway activation. Fyn also contributes to the uptake of α-synuclein into microglial cells, which in turn acts as a danger signal, causing the release of MitoROS to activate the NLRP3 inflammasome. We predict that the ability of Fyn to regulate α-synuclein entry into microglial cells occurs in tandem with the membrane-bound receptor CD36. We also think that CD36 regulates Fyn activity, and that the inhibition/knockdown of CD36 prevents Fyn-mediated priming and activation of the NLRP3 inflammasome. To evaluate this hypothesis, we cultured primary microglia extracted from $CD36^{-/-}$ mice (Jackson Laboratories) and checked for the aggregated α-synuclein-mediated induction of NLRP3 and IL-1β proteins, as well as the uptake of aggregated α-synuclein. We also reduced CD36 expression/activity either by knocking down the expression of CD36 via siRNA or by using CD36 inhibitor peptides. Usage of CD36 inhibitors has been described previously (Angin et al., 2012; Kuda et al., 2013).

Alpha-synuclein aggregates activate the NLRP3 inflammasome in a Fyn dependent manner in PD mouse models

In Chapter 4, we utilized the A53T model, showing that these mice have hugely elevated levels of cleaved or activated Caspase-1. Lysates from control and PD brain tissues also showed elevated Caspase-1 levels under PD conditions. We then used the AAV-SYN model to show diminished microgliosis, ASC speck formation and TH neuronal loss in Fyn-

deficient mice. As mentioned previously, the pre-formed fibril (PFF) model of α-synuclein spread has gained support, whereby aggregated forms of α-synuclein can seed aggregation and propagate in a prionic manner. Astrogliosis, but not microgliosis, has been demonstrated in this model (Luk et al., 2012a; Luk et al., 2012b; Volpicelli-Daley et al., 2011). As our next logical step, we injected WT and $Fyn^{-/-}$ mice with α-synuclein PFFs and sacrificed them at various time points to check for microgliosis, ASC speck formation and Caspase-1 activation (via *in-vivo* FLICA assay). Some mice were also used to make tissue lysates to check for increased cleaved Caspase-1 levels. Since we demonstrated that prolonged exposure to inflammogens results in the induction of Fyn expression in microglial cells *in vivo* and *in vitro*, we anticipated the microglial upregulation of Fyn in this model. We assessed this by performing IHC for Iba-1 and Fyn. We also stereotaxically injected WT and microglial specific conditional Fyn knockout animals with the AAV-GFP and AAV-SYN constructs and checked for microgliosis, inflammasome activation and neurodegeneration at various time points.

To conclusively prove that the hyperactivation of the NLRP3 inflammasome contributes to α-synuclein-induced pathology in PD models, we will either have to breed the A53T mice to $NLRP3^{-/-}$, $ASC^{-/-}$ and $Caspase\text{-}1^{-/-}$ mice, or stereotaxically inject shRNAs to these proteins within the ventral midbrain in WT and A53T mice. Even though neither method will cause a microglial-specific knockdown of the proteins, the results might prove useful in implicating the NLRP3 inflammasome as being instrumental in PD progression. Similar results were obtained with the APP/PS1 AD mouse model. When crossed to NLRP3-deficient or Caspase-1 deficient mice, AD pathology was almost completely attenuated (Heneka et al., 2013). We anticipate similar results with the A53T model. Finally, we intend

to cross the A53T line with our global Fyn knockout mouse line to generate A53T/Fyn$^{-/-}$ double transgenic mice. Inflammasome activation, microgliosis, and neuronal pathology will be assessed at various time points in these mice.

References

Introduction, Literature Review & Conclusions

Aarum, J., Sandberg, K., Haeberlein, S.L., and Persson, M.A. (2003). Migration and differentiation of neural precursor cells can be directed by microglia. Proceedings of the National Academy of Sciences of the United States of America *100*, 15983-15988.

Abulafia, D.P., de Rivero Vaccari, J.P., Lozano, J.D., Lotocki, G., Keane, R.W., and Dietrich, W.D. (2009). Inhibition of the inflammasome complex reduces the inflammatory response after thromboembolic stroke in mice. J Cereb Blood Flow Metab *29*, 534-544.

Adamczak, S., Dale, G., de Rivero Vaccari, J.P., Bullock, M.R., Dietrich, W.D., and Keane, R.W. (2012). Inflammasome proteins in cerebrospinal fluid of brain-injured patients as biomarkers of functional outcome: clinical article. J Neurosurg *117*, 1119-1125.

Anantharam, V., Kitazawa, M., Wagner, J., Kaul, S., and Kanthasamy, A.G. (2002). Caspase-3-dependent proteolytic cleavage of protein kinase Cdelta is essential for oxidative stress-mediated dopaminergic cell death after exposure to methylcyclopentadienyl manganese tricarbonyl. The Journal of neuroscience : the official journal of the Society for Neuroscience *22*, 1738-1751.

Angin, Y., Steinbusch, L.K., Simons, P.J., Greulich, S., Hoebers, N.T., Douma, K., van Zandvoort, M.A., Coumans, W.A., Wijnen, W., Diamant, M., *et al*. (2012). CD36 inhibition prevents lipid accumulation and contractile dysfunction in rat cardiomyocytes. Biochem J *448*, 43-53.

Appleby, M.W., Gross, J.A., Cooke, M.P., Levin, S.D., Qian, X., and Perlmutter, R.M. (1992). Defective T cell receptor signaling in mice lacking the thymic isoform of p59fyn. Cell *70*, 751-763.

Asaithambi, A., Kanthasamy, A., Saminathan, H., Anantharam, V., and Kanthasamy, A.G. (2011). Protein kinase D1 (PKD1) activation mediates a compensatory protective response during early stages of oxidative stress-induced neuronal degeneration. Molecular neurodegeneration *6*, 43.

Aschner, M., Allen, J.W., Kimelberg, H.K., LoPachin, R.M., and Streit, W.J. (1999). Glial cells in neurotoxicity development. Annu Rev Pharmacol Toxicol *39*, 151-173.

Banati, R.B., Daniel, S.E., and Blunt, S.B. (1998). Glial pathology but absence of apoptotic nigral neurons in long-standing Parkinson's disease. Mov Disord *13*, 221-227.

Baroja-Mazo, A., Martin-Sanchez, F., Gomez, A.I., Martinez, C.M., Amores-Iniesta, J., Compan, V., Barbera-Cremades, M., Yague, J., Ruiz-Ortiz, E., Anton, J., *et al*. (2014). The NLRP3 inflammasome is released as a particulate danger signal that amplifies the inflammatory response. Nat Immunol *15*, 738-748.

Bartels, A.L., and Leenders, K.L. (2007). Neuroinflammation in the pathophysiology of Parkinson's disease: evidence from animal models to human in vivo studies with [11C]-PK11195 PET. Mov Disord *22*, 1852-1856.

Basu, A., Krady, J.K., Enterline, J.R., and Levison, S.W. (2002). Transforming growth factor beta1 prevents IL-1beta-induced microglial activation, whereas TNFalpha- and IL-6-stimulated activation are not antagonized. Glia *40*, 109-120.

Bauernfeind, F.G., Horvath, G., Stutz, A., Alnemri, E.S., MacDonald, K., Speert, D., Fernandes-Alnemri, T., Wu, J., Monks, B.G., Fitzgerald, K.A., *et al*. (2009). Cutting edge: NF-kappaB activating pattern recognition and cytokine receptors license NLRP3 inflammasome activation by regulating NLRP3 expression. J Immunol *183*, 787-791.

Bhatt, K.H., Sodhi, A., and Chakraborty, R. (2011). Role of mitogen-activated protein kinases in peptidoglycan-induced expression of inducible nitric oxide synthase and nitric oxide in mouse peritoneal macrophages: extracellular signal-related kinase, a negative regulator. Clin Vaccine Immunol *18*, 994-1001.

Block, M.L., Zecca, L., and Hong, J.S. (2007). Microglia-mediated neurotoxicity: uncovering the molecular mechanisms. Nat Rev Neurosci *8*, 57-69.

Boggon, T.J., and Eck, M.J. (2004). Structure and regulation of Src family kinases. Oncogene *23*, 7918-7927.

Bonneh-Barkay, D., Reaney, S.H., Langston, W.J., and Di Monte, D.A. (2005). Redox cycling of the herbicide paraquat in microglial cultures. Brain Res Mol Brain Res *134*, 52-56.

Bower, J.H., Maraganore, D.M., Peterson, B.J., Ahlskog, J.E., and Rocca, W.A. (2006). Immunologic diseases, anti-inflammatory drugs, and Parkinson disease: a case-control study. Neurology *67*, 494-496.

Boyden, E.D., and Dietrich, W.F. (2006). Nalp1b controls mouse macrophage susceptibility to anthrax lethal toxin. Nat Genet *38*, 240-244.

Bruey, J.M., Bruey-Sedano, N., Luciano, F., Zhai, D., Balpai, R., Xu, C., Kress, C.L., Bailly-Maitre, B., Li, X., Osterman, A., *et al*. (2007). Bcl-2 and Bcl-XL regulate proinflammatory caspase-1 activation by interaction with NALP1. Cell *129*, 45-56.

Bruey, J.M., Bruey-Sedano, N., Newman, R., Chandler, S., Stehlik, C., and Reed, J.C. (2004). PAN1/NALP2/PYPAF2, an inducible inflammatory mediator that regulates NF-kappaB and caspase-1 activation in macrophages. The Journal of biological chemistry *279*, 51897-51907.

Burguillos, M.A., Deierborg, T., Kavanagh, E., Persson, A., Hajji, N., Garcia-Quintanilla, A., Cano, J., Brundin, P., Englund, E., Venero, J.L., *et al*. (2011). Caspase signalling controls microglia activation and neurotoxicity. Nature *472*, 319-324.

Butovsky, O., Ziv, Y., Schwartz, A., Landa, G., Talpalar, A.E., Pluchino, S., Martino, G., and Schwartz, M. (2006). Microglia activated by IL-4 or IFN-gamma differentially induce neurogenesis and oligodendrogenesis from adult stem/progenitor cells. Mol Cell Neurosci *31*, 149-160.

Caicedo, M.S., Desai, R., McAllister, K., Reddy, A., Jacobs, J.J., and Hallab, N.J. (2009). Soluble and particulate Co-Cr-Mo alloy implant metals activate the inflammasome danger signaling pathway in human macrophages: a novel mechanism for implant debris reactivity. J Orthop Res *27*, 847-854.

Cao, S., Standaert, D.G., and Harms, A.S. (2012). The gamma chain subunit of Fc receptors is required for alpha-synuclein-induced pro-inflammatory signaling in microglia. Journal of neuroinflammation *9*, 259.

Carson, M.J. (2002). Microglia as liaisons between the immune and central nervous systems: functional implications for multiple sclerosis. Glia *40*, 218-231.

Carvey, P.M., Punati, A., and Newman, M.B. (2006). Progressive dopamine neuron loss in Parkinson's disease: the multiple hit hypothesis. Cell Transplant *15*, 239-250.

Cassel, S.L., Eisenbarth, S.C., Iyer, S.S., Sadler, J.J., Colegio, O.R., Tephly, L.A., Carter, A.B., Rothman, P.B., Flavell, R.A., and Sutterwala, F.S. (2008). The Nalp3 inflammasome is essential for the development of silicosis. Proceedings of the National Academy of Sciences of the United States of America *105*, 9035-9040.

Castano, A., Herrera, A.J., Cano, J., and Machado, A. (1998). Lipopolysaccharide intranigral injection induces inflammatory reaction and damage in nigrostriatal dopaminergic system. J Neurochem *70*, 1584-1592.

Castano, A., Herrera, A.J., Cano, J., and Machado, A. (2002). The degenerative effect of a single intranigral injection of LPS on the dopaminergic system is prevented by dexamethasone, and not mimicked by rh-TNF-alpha, IL-1beta and IFN-gamma. J Neurochem *81*, 150-157.

Chen, G.Y., Liu, M., Wang, F., Bertin, J., and Nunez, G. (2011). A functional role for Nlrp6 in intestinal inflammation and tumorigenesis. J Immunol *186*, 7187-7194.

Chen, H., Zhang, S.M., Hernan, M.A., Schwarzschild, M.A., Willett, W.C., Colditz, G.A., Speizer, F.E., and Ascherio, A. (2003). Nonsteroidal anti-inflammatory drugs and the risk of Parkinson disease. Arch Neurol *60*, 1059-1064.

Cheng, N., He, R., Tian, J., Dinauer, M.C., and Ye, R.D. (2007). A critical role of protein kinase C delta activation loop phosphorylation in formyl-methionyl-leucyl-phenylalanine-induced phosphorylation of p47(phox) and rapid activation of nicotinamide adenine dinucleotide phosphate oxidase. J Immunol *179*, 7720-7728.

Chong, Y.P., Mulhern, T.D., and Cheng, H.C. (2005). C-terminal Src kinase (CSK) and CSK-homologous kinase (CHK)--endogenous negative regulators of Src-family protein kinases. Growth Factors *23*, 233-244.

Chu, J., Thomas, L.M., Watkins, S.C., Franchi, L., Nunez, G., and Salter, R.D. (2009). Cholesterol-dependent cytolysins induce rapid release of mature IL-1beta from murine macrophages in a NLRP3 inflammasome and cathepsin B-dependent manner. J Leukoc Biol *86*, 1227-1238.

Chung, C.Y., Koprich, J.B., Siddiqi, H., and Isacson, O. (2009). Dynamic changes in presynaptic and axonal transport proteins combined with striatal neuroinflammation precede dopaminergic neuronal loss in a rat model of AAV alpha-synucleinopathy. The Journal of neuroscience : the official journal of the Society for Neuroscience *29*, 3365-3373.

Codolo, G., Plotegher, N., Pozzobon, T., Brucale, M., Tessari, I., Bubacco, L., and de Bernard, M. (2013). Triggering of inflammasome by aggregated alpha-synuclein, an inflammatory response in synucleinopathies. PLOS One *8*, e55375.

Cole, P.A., Shen, K., Qiao, Y., and Wang, D. (2003). Protein tyrosine kinases Src and Csk: a tail's tale. Curr Opin Chem Biol *7*, 580-585.

Coll, R.C., Robertson, A.A., Chae, J.J., Higgins, S.C., Munoz-Planillo, R., Inserra, M.C., Vetter, I., Dungan, L.S., Monks, B.G., Stutz, A., *et al*. (2015). A small-molecule inhibitor of the NLRP3 inflammasome for the treatment of inflammatory diseases. Nat Med *21*, 248-255.

Conti, B.J., Davis, B.K., Zhang, J., O'Connor, W., Jr., Williams, K.L., and Ting, J.P. (2005). CATERPILLER 16.2 (CLR16.2), a novel NBD/LRR family member that negatively regulates T cell function. The Journal of biological chemistry *280*, 18375-18385.

Cooke, M.P., Abraham, K.M., Forbush, K.A., and Perlmutter, R.M. (1991). Regulation of T cell receptor signaling by a src family protein-tyrosine kinase (p59fyn). Cell *65*, 281-291.

Cookson, M.R. (2009). alpha-Synuclein and neuronal cell death. Molecular neurodegeneration *4*, 9.

Corbalan-Garcia, S., and Gomez-Fernandez, J.C. (2006). Protein kinase C regulatory domains: the art of decoding many different signals in membranes. Biochimica et biophysica acta *1761*, 633-654.

Craven, R.R., Gao, X., Allen, I.C., Gris, D., Bubeck Wardenburg, J., McElvania-Tekippe, E., Ting, J.P., and Duncan, J.A. (2009). Staphylococcus aureus alpha-hemolysin activates the NLRP3-inflammasome in human and mouse monocytic cells. PLOS One *4*, e7446.

Cummings, R., Zhao, Y., Jacoby, D., Spannhake, E.W., Ohba, M., Garcia, J.G., Watkins, T., He, D., Saatian, B., and Natarajan, V. (2004). Protein kinase Cdelta mediates lysophosphatidic acid-induced NF-kappaB activation and interleukin-8 secretion in human bronchial epithelial cells. The Journal of biological chemistry *279*, 41085-41094.

D'Osualdo, A., Weichenberger, C.X., Wagner, R.N., Godzik, A., Wooley, J., and Reed, J.C. (2011). CARD8 and NLRP1 undergo autoproteolytic processing through a ZU5-like domain. PLOS One *6*, e27396.

Daniele, S.G., Beraud, D., Davenport, C., Cheng, K., Yin, H., and Maguire-Zeiss, K.A. (2015). Activation of MyD88-dependent TLR1/2 signaling by misfolded alpha-synuclein, a protein linked to neurodegenerative disorders. Sci Signal *8*, ra45.

Dauer, W., and Przedborski, S. (2003). Parkinson's disease: mechanisms and models. Neuron *39*, 889-909.

de Rivero Vaccari, J.P., Lotocki, G., Alonso, O.F., Bramlett, H.M., Dietrich, W.D., and Keane, R.W. (2009). Therapeutic neutralization of the NLRP1 inflammasome reduces the innate immune response and improves histopathology after traumatic brain injury. J Cereb Blood Flow Metab *29*, 1251-1261.

de Rivero Vaccari, J.P., Lotocki, G., Marcillo, A.E., Dietrich, W.D., and Keane, R.W. (2008). A molecular platform in neurons regulates inflammation after spinal cord injury. The Journal of neuroscience : the official journal of the Society for Neuroscience *28*, 3404-3414.

Delaloye, J., Roger, T., Steiner-Tardivel, Q.G., Le Roy, D., Knaup Reymond, M., Akira, S., Petrilli, V., Gomez, C.E., Perdiguero, B., Tschopp, J., *et al*. (2009). Innate immune sensing of modified vaccinia virus Ankara (MVA) is mediated by TLR2-TLR6, MDA-5 and the NALP3 inflammasome. PLoS Pathog *5*, e1000480.

Devary, Y., Gottlieb, R.A., Smeal, T., and Karin, M. (1992). The mammalian ultraviolet response is triggered by activation of Src tyrosine kinases. Cell *71*, 1081-1091.

DeYoung, K.L., Ray, M.E., Su, Y.A., Anzick, S.L., Johnstone, R.W., Trapani, J.A., Meltzer, P.S., and Trent, J.M. (1997). Cloning a novel member of the human interferon-inducible gene family associated with control of tumorigenicity in a model of human melanoma. Oncogene *15*, 453-457.

Doppler, H., and Storz, P. (2007). A novel tyrosine phosphorylation site in protein kinase D contributes to oxidative stress-mediated activation. The Journal of biological chemistry *282*, 31873-31881.

Dostert, C., Petrilli, V., Van Bruggen, R., Steele, C., Mossman, B.T., and Tschopp, J. (2008). Innate immune activation through Nalp3 inflammasome sensing of asbestos and silica. Science *320*, 674-677.

Du, Y., Ma, Z., Lin, S., Dodel, R.C., Gao, F., Bales, K.R., Triarhou, L.C., Chernet, E., Perry, K.W., Nelson, D.L., *et al*. (2001). Minocycline prevents nigrostriatal dopaminergic neurodegeneration in the MPTP model of Parkinson's disease. Proceedings of the National Academy of Sciences of the United States of America *98*, 14669-14674.

Duewell, P., Kono, H., Rayner, K.J., Sirois, C.M., Vladimer, G., Bauernfeind, F.G., Abela, G.S., Franchi, L., Nunez, G., Schnurr, M., *et al*. (2010). NLRP3 inflammasomes are required for atherogenesis and activated by cholesterol crystals. Nature *464*, 1357-1361.

Elinav, E., Strowig, T., Kau, A.L., Henao-Mejia, J., Thaiss, C.A., Booth, C.J., Peaper, D.R., Bertin, J., Eisenbarth, S.C., Gordon, J.I., *et al*. (2011). NLRP6 inflammasome regulates colonic microbial ecology and risk for colitis. Cell *145*, 745-757.

Emoto, Y., Manome, Y., Meinhardt, G., Kisaki, H., Kharbanda, S., Robertson, M., Ghayur, T., Wong, W.W., Kamen, R., Weichselbaum, R., *et al*. (1995). Proteolytic activation of protein kinase C delta by an ICE-like protease in apoptotic cells. EMBO J *14*, 6148-6156.

Faustin, B., Lartigue, L., Bruey, J.M., Luciano, F., Sergienko, E., Bailly-Maitre, B., Volkmann, N., Hanein, D., Rouiller, I., and Reed, J.C. (2007). Reconstituted NALP1 inflammasome reveals two-step mechanism of caspase-1 activation. Mol Cell *25*, 713-724.

Fellner, L., Irschick, R., Schanda, K., Reindl, M., Klimaschewski, L., Poewe, W., Wenning, G.K., and Stefanova, N. (2013). Toll-like receptor 4 is required for alpha-synuclein dependent activation of microglia and astroglia. Glia *61*, 349-360.

Fernandes-Alnemri, T., Wu, J., Yu, J.W., Datta, P., Miller, B., Jankowski, W., Rosenberg, S., Zhang, J., and Alnemri, E.S. (2007). The pyroptosome: a supramolecular assembly of ASC dimers mediating inflammatory cell death via caspase-1 activation. Cell Death Differ *14*, 1590-1604.

Fernandes-Alnemri, T., Yu, J.W., Juliana, C., Solorzano, L., Kang, S., Wu, J., Datta, P., McCormick, M., Huang, L., McDermott, E., *et al*. (2010). The AIM2 inflammasome is critical for innate immunity to Francisella tularensis. Nat Immunol *11*, 385-393.

Ferrari, C.C., Pott Godoy, M.C., Tarelli, R., Chertoff, M., Depino, A.M., and Pitossi, F.J. (2006). Progressive neurodegeneration and motor disabilities induced by chronic expression of IL-1beta in the substantia nigra. Neurobiology of disease *24*, 183-193.

Finger, J.N., Lich, J.D., Dare, L.C., Cook, M.N., Brown, K.K., Duraiswami, C., Bertin, J., and Gough, P.J. (2012). Autolytic proteolysis within the function to find domain (FIIND) is required for NLRP1 inflammasome activity. The Journal of biological chemistry *287*, 25030-25037.

Fontalba, A., Gutierrez, O., and Fernandez-Luna, J.L. (2007). NLRP2, an inhibitor of the NF-kappaB pathway, is transcriptionally activated by NF-kappaB and exhibits a nonfunctional allelic variant. J Immunol *179*, 8519-8524.

Franchi, L., Amer, A., Body-Malapel, M., Kanneganti, T.D., Ozoren, N., Jagirdar, R., Inohara, N., Vandenabeele, P., Bertin, J., Coyle, A., *et al*. (2006). Cytosolic flagellin requires Ipaf for activation of caspase-1 and interleukin 1beta in salmonella-infected macrophages. Nat Immunol *7*, 576-582.

Franklin, B.S., Bossaller, L., De Nardo, D., Ratter, J.M., Stutz, A., Engels, G., Brenker, C., Nordhoff, M., Mirandola, S.R., Al-Amoudi, A., *et al*. (2014). The adaptor ASC has extracellular and 'prionoid' activities that propagate inflammation. Nat Immunol *15*, 727-737.

Frederick Lo, C., Ning, X., Gonzales, C., and Ozenberger, B.A. (2008). Induced expression of death domain genes NALP1 and NALP5 following neuronal injury. Biochem Biophys Res Commun *366*, 664-669.

Furumoto, Y., Gomez, G., Gonzalez-Espinosa, C., Kovarova, M., Odom, S., Ryan, J.J., and Rivera, J. (2005). The role of Src family kinases in mast cell effector function. Novartis Found Symp *271*, 39-47; discussion 47-53, 95-39.

Gao, H.M., Hong, J.S., Zhang, W., and Liu, B. (2002). Distinct role for microglia in rotenone-induced degeneration of dopaminergic neurons. The Journal of neuroscience : the official journal of the Society for Neuroscience *22*, 782-790.

Gao, H.M., Liu, B., Zhang, W., and Hong, J.S. (2003a). Critical role of microglial NADPH oxidase-derived free radicals in the in vitro MPTP model of Parkinson's disease. Faseb J *17*, 1954-1956.

Gao, H.M., Liu, B., Zhang, W., and Hong, J.S. (2003b). Synergistic dopaminergic neurotoxicity of MPTP and inflammogen lipopolysaccharide: relevance to the etiology of Parkinson's disease. Faseb J *17*, 1957-1959.

Gasse, P., Riteau, N., Charron, S., Girre, S., Fick, L., Petrilli, V., Tschopp, J., Lagente, V., Quesniaux, V.F., Ryffel, B., *et al*. (2009). Uric acid is a danger signal activating NALP3 inflammasome in lung injury inflammation and fibrosis. Am J Respir Crit Care Med *179*, 903-913.

Ghayur, T., Hugunin, M., Talanian, R.V., Ratnofsky, S., Quinlan, C., Emoto, Y., Pandey, P., Datta, R., Huang, Y., Kharbanda, S., *et al*. (1996). Proteolytic activation of protein kinase C delta by an ICE/CED 3-like protease induces characteristics of apoptosis. J Exp Med *184*, 2399-2404.

Ghosh, A., Kanthasamy, A., Joseph, J., Anantharam, V., Srivastava, P., Dranka, B.P., Kalyanaraman, B., and Kanthasamy, A.G. (2002). Anti-inflammatory and neuroprotective effects of an orally active apocynin derivative in pre-clinical models of Parkinson's disease. Journal of neuroinflammation *9*, 241.

Ghosh, A., Roy, A., Liu, X., Kordower, J.H., Mufson, E.J., Hartley, D.M., Ghosh, S., Mosley, R.L., Gendelman, H.E., and Pahan, K. (2007). Selective inhibition of NF-kappaB activation prevents dopaminergic neuronal loss in a mouse model of Parkinson's disease. Proceedings of the National Academy of Sciences of the United States of America *104*, 18754-18759.

Gilfillan, A.M., and Rivera, J. (2009). The tyrosine kinase network regulating mast cell activation. Immunol Rev *228*, 149-169.

Ginhoux, F., Lim, S., Hoeffel, G., Low, D., and Huber, T. (2013). Origin and differentiation of microglia. Front Cell Neurosci *7*, 45.

Glass, C.K., Saijo, K., Winner, B., Marchetto, M.C., and Gage, F.H. (2010). Mechanisms underlying inflammation in neurodegeneration. Cell *140*, 918-934.

Glezer, I., Simard, A.R., and Rivest, S. (2007). Neuroprotective role of the innate immune system by microglia. Neuroscience *147*, 867-883.

Goldmann, T., Wieghofer, P., Muller, P.F., Wolf, Y., Varol, D., Yona, S., Brendecke, S.M., Kierdorf, K., Staszewski, O., Datta, M., *et al*. (2013). A new type of microglia gene targeting shows TAK1 to be pivotal in CNS autoimmune inflammation. Nature neuroscience *16*, 1618-1626.

Gomez, G., Gonzalez-Espinosa, C., Odom, S., Baez, G., Cid, M.E., Ryan, J.J., and Rivera, J. (2005). Impaired FcepsilonRI-dependent gene expression and defective eicosanoid and cytokine production as a consequence of Fyn deficiency in mast cells. J Immunol *175*, 7602-7610.

Grenier, J.M., Wang, L., Manji, G.A., Huang, W.J., Al-Garawi, A., Kelly, R., Carlson, A., Merriam, S., Lora, J.M., Briskin, M., *et al*. (2002). Functional screening of five PYPAF family members identifies PYPAF5 as a novel regulator of NF-kappaB and caspase-1. FEBS Lett *530*, 73-78.

Gris, D., Ye, Z., Iocca, H.A., Wen, H., Craven, R.R., Gris, P., Huang, M., Schneider, M., Miller, S.D., and Ting, J.P. (2010). NLRP3 plays a critical role in the development of experimental autoimmune encephalomyelitis by mediating Th1 and Th17 responses. J Immunol *185*, 974-981.

Gschwendt, M., Kittstein, W., and Marks, F. (1986). A novel type of phorbol ester-dependent protein phosphorylation in the particulate fraction of mouse epidermis. Biochem Biophys Res Commun *137*, 766-774.

Gu, Y., Kuida, K., Tsutsui, H., Ku, G., Hsiao, K., Fleming, M.A., Hayashi, N., Higashino, K., Okamura, H., Nakanishi, K., *et al*. (1997). Activation of interferon-gamma inducing factor mediated by interleukin-1beta converting enzyme. Science *275*, 206-209.

Guler, R., Afshar, M., Arendse, B., Parihar, S.P., Revaz-Breton, M., Leitges, M., Schwegmann, A., and Brombacher, F. (2011). PKCdelta regulates IL-12p40/p70 production by macrophages and dendritic cells, driving a type 1 healer phenotype in cutaneous leishmaniasis. Eur J Immunol *41*, 706-715.

Hafner-Bratkovic, I., Bencina, M., Fitzgerald, K.A., Golenbock, D., and Jerala, R. (2012). NLRP3 inflammasome activation in macrophage cell lines by prion protein fibrils as the source of IL-1beta and neuronal toxicity. Cellular and molecular life sciences : CMLS *69*, 4215-4228.

Hakansson, A., Westberg, L., Nilsson, S., Buervenich, S., Carmine, A., Holmberg, B., Sydow, O., Olson, L., Johnels, B., Eriksson, E., *et al*. (2005). Interaction of polymorphisms in the genes encoding interleukin-6 and estrogen receptor beta on the susceptibility to Parkinson's disease. Am J Med Genet B Neuropsychiatr Genet *133B*, 88-92.

Halle, A., Hornung, V., Petzold, G.C., Stewart, C.R., Monks, B.G., Reinheckel, T., Fitzgerald, K.A., Latz, E., Moore, K.J., and Golenbock, D.T. (2008). The NALP3 inflammasome is involved in the innate immune response to amyloid-beta. Nat Immunol *9*, 857-865.

Heneka, M.T., Kummer, M.P., Stutz, A., Delekate, A., Schwartz, S., Vieira-Saecker, A., Griep, A., Axt, D., Remus, A., Tzeng, T.C., *et al*. (2013). NLRP3 is activated in Alzheimer's disease and contributes to pathology in APP/PS1 mice. Nature *493*, 674-678.

Hernandez-Hansen, V., Bard, J.D., Tarleton, C.A., Wilder, J.A., Lowell, C.A., Wilson, B.S., and Oliver, J.M. (2005). Increased expression of genes linked to FcepsilonRI Signaling and to cytokine and chemokine production in Lyn-deficient mast cells. J Immunol *175*, 7880-7888.

Herrera, A.J., Castano, A., Venero, J.L., Cano, J., and Machado, A. (2000). The single intranigral injection of LPS as a new model for studying the selective effects of inflammatory reactions on dopaminergic system. Neurobiol Dis *7*, 429-447.

Hickey, W.F., and Kimura, H. (1988). Perivascular microglial cells of the CNS are bone marrow-derived and present antigen in vivo. Science *239*, 290-292.

Hickman, S.E., Kingery, N.D., Ohsumi, T.K., Borowsky, M.L., Wang, L.C., Means, T.K., and El Khoury, J. (2013). The microglial sensome revealed by direct RNA sequencing. Nature neuroscience *16*, 1896-1905.

Hirsch, E.C., and Hunot, S. (2009). Neuroinflammation in Parkinson's disease: a target for neuroprotection? Lancet Neurol *8*, 382-397.

Hirsch, E.C., Hunot, S., and Hartmann, A. (2005). Neuroinflammatory processes in Parkinson's disease. Parkinsonism & related disorders *11 Suppl 1*, S9-S15.

Hoegen, T., Tremel, N., Klein, M., Angele, B., Wagner, H., Kirschning, C., Pfister, H.W., Fontana, A., Hammerschmidt, S., and Koedel, U. (2011). The NLRP3 inflammasome contributes to brain injury in pneumococcal meningitis and is activated through ATP-dependent lysosomal cathepsin B release. J Immunol *187*, 5440-5451.

Hornung, V., Bauernfeind, F., Halle, A., Samstad, E.O., Kono, H., Rock, K.L., Fitzgerald, K.A., and Latz, E. (2008). Silica crystals and aluminum salts activate the NALP3 inflammasome through phagosomal destabilization. Nat Immunol *9*, 847-856.

Hornung, V., and Latz, E. (2010). Intracellular DNA recognition. Nat Rev Immunol *10*, 123-130.

Hsieh, P.F., Chia, L.G., Ni, D.R., Cheng, L.J., Ho, Y.P., Tzeng, S.F., Chang, M.H., and Hong, J.S. (2002). Behavior, neurochemistry and histology after intranigral lipopolysaccharide injection. Neuroreport *13*, 277-280.

Hunot, S., Brugg, B., Ricard, D., Michel, P.P., Muriel, M.P., Ruberg, M., Faucheux, B.A., Agid, Y., and Hirsch, E.C. (1997). Nuclear translocation of NF-kappaB is increased in

dopaminergic neurons of patients with parkinson disease. Proceedings of the National Academy of Sciences of the United States of America *94*, 7531-7536.

Hwang, M.K., Kang, N.J., Heo, Y.S., Lee, K.W., and Lee, H.J. (2009). Fyn kinase is a direct molecular target of delphinidin for the inhibition of cyclooxygenase-2 expression induced by tumor necrosis factor-alpha. Biochem Pharmacol *77*, 1213-1222.

Ichinohe, T., Lee, H.K., Ogura, Y., Flavell, R., and Iwasaki, A. (2009). Inflammasome recognition of influenza virus is essential for adaptive immune responses. J Exp Med *206*, 79-87.

Idzko, M., Hammad, H., van Nimwegen, M., Kool, M., Willart, M.A., Muskens, F., Hoogsteden, H.C., Luttmann, W., Ferrari, D., Di Virgilio, F., *et al*. (2007). Extracellular ATP triggers and maintains asthmatic airway inflammation by activating dendritic cells. Nat Med *13*, 913-919.

Imamura, K., Hishikawa, N., Sawada, M., Nagatsu, T., Yoshida, M., and Hashizume, Y. (2003). Distribution of major histocompatibility complex class II-positive microglia and cytokine profile of Parkinson's disease brains. Acta neuropathologica *106*, 518-526.

Inoue, M., Williams, K.L., Gunn, M.D., and Shinohara, M.L. (2012). NLRP3 inflammasome induces chemotactic immune cell migration to the CNS in experimental autoimmune encephalomyelitis. Proceedings of the National Academy of Sciences of the United States of America *109*, 10480-10485.

Iyer, S.S., Pulskens, W.P., Sadler, J.J., Butter, L.M., Teske, G.J., Ulland, T.K., Eisenbarth, S.C., Florquin, S., Flavell, R.A., Leemans, J.C., *et al*. (2009). Necrotic cells trigger a sterile inflammatory response through the Nlrp3 inflammasome. Proceedings of the National Academy of Sciences of the United States of America *106*, 20388-20393.

Jamilloux, Y., Pierini, R., Querenet, M., Juruj, C., Fauchais, A.L., Jauberteau, M.O., Jarraud, S., Lina, G., Etienne, J., Roy, C.R., *et al*. (2013). Inflammasome activation restricts Legionella pneumophila replication in primary microglial cells through flagellin detection. Glia *61*, 539-549.

Jayaraman, P., Sada-Ovalle, I., Nishimura, T., Anderson, A.C., Kuchroo, V.K., Remold, H.G., and Behar, S.M. (2013). IL-1beta promotes antimicrobial immunity in macrophages by regulating TNFR signaling and caspase-3 activation. J Immunol *190*, 4196-4204.

Jelic, D., Mildner, B., Kostrun, S., Nujic, K., Verbanac, D., Culic, O., Antolovic, R., and Brandt, W. (2007). Homology modeling of human Fyn kinase structure: discovery of rosmarinic acid as a new Fyn kinase inhibitor and in silico study of its possible binding modes. J Med Chem *50*, 1090-1100.

Jenner, P., and Olanow, C.W. (2006). The pathogenesis of cell death in Parkinson's disease. Neurology *66*, S24-36.

John, G.R., Chen, L., Rivieccio, M.A., Melendez-Vasquez, C.V., Hartley, A., and Brosnan, C.F. (2004). Interleukin-1beta induces a reactive astroglial phenotype via deactivation of the

Rho GTPase-Rock axis. The Journal of neuroscience : the official journal of the Society for Neuroscience *24*, 2837-2845.

Joly, S., Ma, N., Sadler, J.J., Soll, D.R., Cassel, S.L., and Sutterwala, F.S. (2009). Cutting edge: Candida albicans hyphae formation triggers activation of the Nlrp3 inflammasome. J Immunol *183*, 3578-3581.

Kang, S.J., Wang, S., Hara, H., Peterson, E.P., Namura, S., Amin-Hanjani, S., Huang, Z., Srinivasan, A., Tomaselli, K.J., Thornberry, N.A., *et al*. (2000). Dual role of caspase-11 in mediating activation of caspase-1 and caspase-3 under pathological conditions. J Cell Biol *149*, 613-622.

Kanthasamy, A.G., Kitazawa, M., Kanthasamy, A., and Anantharam, V. (2003). Role of Proteolytic Activation of Protein Kinase Cd in Oxidative
Stress-Induced Apoptosis. Antioxidants & Redox Signaling *5*.

Kato, H., Takahasi, K., and Fujita, T. (2011). RIG-I-like receptors: cytoplasmic sensors for non-self RNA. Immunol Rev *243*, 91-98.

Kaufman, A.C., Salazar, S.V., Haas, L.T., Yang, J., Kostylev, M.A., Jeng, A.T., Robinson, S.A., Gunther, E.C., van Dyck, C.H., Nygaard, H.B., *et al*. (2015). Fyn inhibition rescues established memory and synapse loss in Alzheimer mice. Ann Neurol *77*, 953-971.

Kaul, S., Anantharam, V., Yang, Y., Choi, C.J., Kanthasamy, A., and Kanthasamy, A.G. (2005). Tyrosine phosphorylation regulates the proteolytic activation of protein kinase Cdelta in dopaminergic neuronal cells. The Journal of biological chemistry *280*, 28721-28730.

Kaushal, V., Dye, R., Pakavathkumar, P., Foveau, B., Flores, J., Hyman, B., Ghetti, B., Koller, B.H., and LeBlanc, A.C. (2015). Neuronal NLRP1 inflammasome activation of Caspase-1 coordinately regulates inflammatory interleukin-1-beta production and axonal degeneration-associated Caspase-6 activation. Cell Death Differ *22*, 1676-1686.

Kaushik, D.K., Gupta, M., Kumawat, K.L., and Basu, A. (2012). NLRP3 inflammasome: key mediator of neuroinflammation in murine Japanese encephalitis. PLOS One *7*, e32270.

Kawai, T., and Akira, S. (2011). Toll-like receptors and their crosstalk with other innate receptors in infection and immunity. Immunity *34*, 637-650.

Kayagaki, N., Warming, S., Lamkanfi, M., Vande Walle, L., Louie, S., Dong, J., Newton, K., Qu, Y., Liu, J., Heldens, S., *et al*. (2011). Non-canonical inflammasome activation targets caspase-11. Nature *479*, 117-121.

Kayagaki, N., Wong, M.T., Stowe, I.B., Ramani, S.R., Gonzalez, L.C., Akashi-Takamura, S., Miyake, K., Zhang, J., Lee, W.P., Muszynski, A., *et al*. (2013). Noncanonical inflammasome activation by intracellular LPS independent of TLR4. Science *341*, 1246-1249.

Kharbanda, S., Yuan, Z.M., Rubin, E., Weichselbaum, R., and Kufe, D. (1994). Activation of Src-like p56/p53lyn tyrosine kinase by ionizing radiation. J Biol Chem *269*, 20739-20743.

Kilpatrick, L.E., Lee, J.Y., Haines, K.M., Campbell, D.E., Sullivan, K.E., and Korchak, H.M. (2002). A role for PKC-delta and PI 3-kinase in TNF-alpha-mediated antiapoptotic signaling in the human neutrophil. Am J Physiol Cell Physiol *283*, C48-57.

Kilpatrick, L.E., Standage, S.W., Li, H., Raj, N.R., Korchak, H.M., Wolfson, M.R., and Deutschman, C.S. (2011). Protection against sepsis-induced lung injury by selective inhibition of protein kinase C-delta (delta-PKC). J Leukoc Biol *89*, 3-10.

Kim, C., Ho, D.H., Suk, J.E., You, S., Michael, S., Kang, J., Joong Lee, S., Masliah, E., Hwang, D., Lee, H.J., *et al*. (2013a). Neuron-released oligomeric alpha-synuclein is an endogenous agonist of TLR2 for paracrine activation of microglia. Nature communications *4*, 1562.

Kim, D.C., Kim, S.H., Jeong, M.W., Baek, N.I., and Kim, K.T. (2005). Effect of rottlerin, a PKC-delta inhibitor, on TLR-4-dependent activation of murine microglia. Biochem Biophys Res Commun *337*, 110-115.

Kim, J., Byun, J.W., Choi, I., Kim, B., Jeong, H.K., Jou, I., and Joe, E. (2013b). PINK1 Deficiency Enhances Inflammatory Cytokine Release from Acutely Prepared Brain Slices. Experimental neurobiology *22*, 38-44.

Kim, J.H., Choi, D.J., Jeong, H.K., Kim, J., Kim, D.W., Choi, S.Y., Park, S.M., Suh, Y.H., Jou, I., and Joe, E.H. (2013c). DJ-1 facilitates the interaction between STAT1 and its phosphatase, SHP-1, in brain microglia and astrocytes: A novel anti-inflammatory function of DJ-1. Neurobiology of disease *60*, 1-10.

Kim, Y.J., Sano, T., Nabetani, T., Asano, Y., and Hirabayashi, Y. (2012). GPRC5B activates obesity-associated inflammatory signaling in adipocytes. Sci Signal *5*, ra85.

Kim, Y.S., Choi, D.H., Block, M.L., Lorenzl, S., Yang, L., Kim, Y.J., Sugama, S., Cho, B.P., Hwang, O., Browne, S.E., *et al*. (2007). A pivotal role of matrix metalloproteinase-3 activity in dopaminergic neuronal degeneration via microglial activation. Faseb J *21*, 179-187.

Kitazawa, M., Anantharam, V., and Kanthasamy, A.G. (2003). Dieldrin induces apoptosis by promoting caspase-3-dependent proteolytic cleavage of protein kinase Cdelta in dopaminergic cells: relevance to oxidative stress and dopaminergic degeneration. Neuroscience *119*, 945-964.

Kitazawa, M., Anantharam, V., Yang, Y., Hirata, Y., Kanthasamy, A., and Kanthasamy, A.G. (2005). Activation of protein kinase C delta by proteolytic cleavage contributes to manganese-induced apoptosis in dopaminergic cells: protective role of Bcl-2. Biochem Pharmacol *69*, 133-146.

Kitazawa, M., Wagner, J.R., Kirby, M.L., Anantharam, V., and Kanthasamy, A.G. (2002). Oxidative stress and mitochondrial-mediated apoptosis in dopaminergic cells exposed to methylcyclopentadienyl manganese tricarbonyl. J Pharmacol Exp Ther *302*, 26-35.

Klein, C., and Westenberger, A. (2012). Genetics of Parkinson's disease. Cold Spring Harb Perspect Med *2*, a008888.

Kofoed, E.M., and Vance, R.E. (2011). Innate immune recognition of bacterial ligands by NAIPs determines inflammasome specificity. Nature *477*, 592-595.

Kontny, E., Kurowska, M., Szczepanska, K., and Maslinski, W. (2000). Rottlerin, a PKC isozyme-selective inhibitor, affects signaling events and cytokine production in human monocytes. J Leukoc Biol *67*, 249-258.

Koriyama, H., Kouchi, Z., Umeda, T., Saido, T.C., Momoi, T., Ishiura, S., and Suzuki, K. (1999). Proteolytic activation of protein kinase C delta and epsilon by caspase-3 in U937 cells during chemotherapeutic agent-induced apoptosis. Cell Signal *11*, 831-838.

Kouadir, M., Yang, L., Tan, R., Shi, F., Lu, Y., Zhang, S., Yin, X., Zhou, X., and Zhao, D. (2012). CD36 participates in PrP(106-126)-induced activation of microglia. PLOS One *7*, e30756.

Kreutzberg, G.W. (1996). Microglia: a sensor for pathological events in the CNS. Trends Neurosci *19*, 312-318.

Kruger, R., Hardt, C., Tschentscher, F., Jackel, S., Kuhn, W., Muller, T., Werner, J., Woitalla, D., Berg, D., Kuhnl, N., *et al*. (2000). Genetic analysis of immunomodulating factors in sporadic Parkinson's disease. J Neural Transm (Vienna) *107*, 553-562.

Kubo-Murai, M., Hazeki, K., Sukenobu, N., Yoshikawa, K., Nigorikawa, K., Inoue, K., Yamamoto, T., Matsumoto, M., Seya, T., Inoue, N., *et al*. (2007). Protein kinase Cdelta binds TIRAP/Mal to participate in TLR signaling. Mol Immunol *44*, 2257-2264.

Kuda, O., Pietka, T.A., Demianova, Z., Kudova, E., Cvacka, J., Kopecky, J., and Abumrad, N.A. (2013). Sulfo-N-succinimidyl oleate (SSO) inhibits fatty acid uptake and signaling for intracellular calcium via binding CD36 lysine 164: SSO also inhibits oxidized low density lipoprotein uptake by macrophages. The Journal of biological chemistry *288*, 15547-15555.

Kumar, M., Roe, K., Orillo, B., Muruve, D.A., Nerurkar, V.R., Gale, M., Jr., and Verma, S. (2013). Inflammasome adaptor protein Apoptosis-associated speck-like protein containing CARD (ASC) is critical for the immune response and survival in west Nile virus encephalitis. J Virol *87*, 3655-3667.

Lamkanfi, M., and Dixit, V.M. (2014). Mechanisms and functions of inflammasomes. Cell *157*, 1013-1022.

Langlois, A., Chouinard, F., Flamand, N., Ferland, C., Rola-Pleszczynski, M., and Laviolette, M. (2009). Crucial implication of protein kinase C (PKC)-delta, PKC-zeta, ERK-1/2, and p38 MAPK in migration of human asthmatic eosinophils. J Leukoc Biol *85*, 656-663.

Langston, J.W., Forno, L.S., Tetrud, J., Reeves, A.G., Kaplan, J.A., and Karluk, D. (1999). Evidence of active nerve cell degeneration in the substantia nigra of humans years after 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine exposure. Ann Neurol *46*, 598-605.

Latchoumycandane, C., Anantharam, V., Kitazawa, M., Yang, Y., Kanthasamy, A., and Kanthasamy, A.G. (2005). Protein kinase Cdelta is a key downstream mediator of manganese-induced apoptosis in dopaminergic neuronal cells. J Pharmacol Exp Ther *313*, 46-55.

Lee, G., Thangavel, R., Sharma, V.M., Litersky, J.M., Bhaskar, K., Fang, S.M., Do, L.H., Andreadis, A., Van Hoesen, G., and Ksiezak-Reding, H. (2004). Phosphorylation of tau by fyn: implications for Alzheimer's disease. The Journal of neuroscience: the official journal of the Society for Neuroscience *24*, 2304-2312.

Lee, E.J., Woo, M.S., Moon, P.G., Baek, M.C., Choi, I.Y., Kim, W.K., Junn, E., and Kim, H.S. (2010). Alpha-synuclein activates microglia by inducing the expressions of matrix metalloproteinases and the subsequent activation of protease-activated receptor-1. J Immunol *185*, 615-623.

Lee, M.C., Wei, S.C., Tsai-Wu, J.J., Wu, C.H., and Tsao, P.N. (2008). Novel PKC signaling is required for LPS-induced soluble Flt-1 expression in macrophages. J Leukoc Biol *84*, 835-841.

Leverrier, S., Vallentin, A., and Joubert, D. (2002). Positive feedback of protein kinase C proteolytic activation during apoptosis. Biochem J *368*, 905-913.

Levy, O.A., Malagelada, C., and Greene, L.A. (2009). Cell death pathways in Parkinson's disease: proximal triggers, distal effectors, and final steps. Apoptosis *14*, 478-500.

Lin, C.S., Lin, F.Y., Ho, L.J., Tsai, C.S., Cheng, S.M., Wu, W.L., Huang, C.Y., Lian, C.H., Yang, S.P., and Lai, J.H. (2012). PKCdelta signalling regulates SR-A and CD36 expression and foam cell formation. Cardiovasc Res *95*, 346-355.

Luk, K.C., Kehm, V., Carroll, J., Zhang, B., O'Brien, P., Trojanowski, J.Q., and Lee, V.M. (2012a). Pathological alpha-synuclein transmission initiates Parkinson-like neurodegeneration in nontransgenic mice. Science *338*, 949-953.

Luk, K.C., Kehm, V.M., Zhang, B., O'Brien, P., Trojanowski, J.Q., and Lee, V.M. (2012b). Intracerebral inoculation of pathological alpha-synuclein initiates a rapidly progressive neurodegenerative alpha-synucleinopathy in mice. J Exp Med *209*, 975-986.

Majewski, M., Bose, T.O., Sille, F.C., Pollington, A.M., Fiebiger, E., and Boes, M. (2007). Protein kinase C delta stimulates antigen presentation by Class II MHC in murine dendritic cells. Int Immunol *19*, 719-732.

Mariathasan, S., Newton, K., Monack, D.M., Vucic, D., French, D.M., Lee, W.P., Roose-Girma, M., Erickson, S., and Dixit, V.M. (2004). Differential activation of the inflammasome by caspase-1 adaptors ASC and Ipaf. Nature *430*, 213-218.

Martinon, F. (2010). Update on biology: uric acid and the activation of immune and inflammatory cells. Curr Rheumatol Rep *12*, 135-141.

Martinon, F., Burns, K., and Tschopp, J. (2002). The inflammasome: a molecular platform triggering activation of inflammatory caspases and processing of proIL-beta. Mol Cell *10*, 417-426.

Maschberger, P., Bauer, M., Baumann-Siemons, J., Zangl, K.J., Negrescu, E.V., Reininger, A.J., and Siess, W. (2000). Mildly oxidized low density lipoprotein rapidly stimulates via activation of the lysophosphatidic acid receptor Src family and Syk tyrosine kinases and Ca2+ influx in human platelets. J Biol Chem *275*, 19159-19166.

McGeer, P.L., Itagaki, S., Boyes, B.E., and McGeer, E.G. (1988). Reactive microglia are positive for HLA-DR in the substantia nigra of Parkinson's and Alzheimer's disease brains. Neurology *38*, 1285-1291.

McNeela, E.A., Burke, A., Neill, D.R., Baxter, C., Fernandes, V.E., Ferreira, D., Smeaton, S., El-Rachkidy, R., McLoughlin, R.M., Mori, A., *et al*. (2010). Pneumolysin activates the NLRP3 inflammasome and promotes proinflammatory cytokines independently of TLR4. PLoS Pathog *6*, e1001191.

Meixenberger, K., Pache, F., Eitel, J., Schmeck, B., Hippenstiel, S., Slevogt, H., N'Guessan, P., Witzenrath, M., Netea, M.G., Chakraborty, T., *et al*. (2010). Listeria monocytogenes-infected human peripheral blood mononuclear cells produce IL-1beta, depending on listeriolysin O and NLRP3. J Immunol *184*, 922-930.

Meurer, S., Pioch, S., Gross, S., and Muller-Esterl, W. (2005). Reactive oxygen species induce tyrosine phosphorylation of and Src kinase recruitment to NO-sensitive guanylyl cyclase. J Biol Chem *280*, 33149-33156.

Miao, E.A., Alpuche-Aranda, C.M., Dors, M., Clark, A.E., Bader, M.W., Miller, S.I., and Aderem, A. (2006). Cytoplasmic flagellin activates caspase-1 and secretion of interleukin 1beta via Ipaf. Nat Immunol *7*, 569-575.

Miao, E.A., Mao, D.P., Yudkovsky, N., Bonneau, R., Lorang, C.G., Warren, S.E., Leaf, I.A., and Aderem, A. (2010). Innate immune detection of the type III secretion apparatus through the NLRC4 inflammasome. Proceedings of the National Academy of Sciences of the United States of America *107*, 3076-3080.

Miao, E.A., Rajan, J.V., and Aderem, A. (2011). Caspase-1-induced pyroptotic cell death. Immunol Rev *243*, 206-214.

Minghetti, L. (2005). Role of inflammation in neurodegenerative diseases. Curr Opin Neurol *18*, 315-321.

Minkiewicz, J., de Rivero Vaccari, J.P., and Keane, R.W. (2013). Human astrocytes express a novel NLRP2 inflammasome. Glia *61*, 1113-1121.

Mitchell, A.J., Yau, B., McQuillan, J.A., Ball, H.J., Too, L.K., Abtin, A., Hertzog, P., Leib, S.L., Jones, C.A., Gerega, S.K., *et al*. (2012). Inflammasome-dependent IFN-gamma drives pathogenesis in Streptococcus pneumoniae meningitis. J Immunol *189*, 4970-4980.

Moehle, M.S., Webber, P.J., Tse, T., Sukar, N., Standaert, D.G., DeSilva, T.M., Cowell, R.M., and West, A.B. (2012). LRRK2 inhibition attenuates microglial inflammatory responses. The Journal of neuroscience : the official journal of the Society for Neuroscience *32*, 1602-1611.

Mogi, M., Harada, M., Narabayashi, H., Inagaki, H., Minami, M., and Nagatsu, T. (1996). Interleukin (IL)-1 beta, IL-2, IL-4, IL-6 and transforming growth factor-alpha levels are elevated in ventricular cerebrospinal fluid in juvenile parkinsonism and Parkinson's disease. Neurosci Lett *211*, 13-16.

Mogi, M., Togari, A., Kondo, T., Mizuno, Y., Komure, O., Kuno, S., Ichinose, H., and Nagatsu, T. (2000). Caspase activities and tumor necrosis factor receptor R1 (p55) level are elevated in the substantia nigra from parkinsonian brain. J Neural Transm (Vienna) *107*, 335-341.

Moore, K.J., El Khoury, J., Medeiros, L.A., Terada, K., Geula, C., Luster, A.D., and Freeman, M.W. (2002). A CD36-initiated signaling cascade mediates inflammatory effects of beta-amyloid. The Journal of biological chemistry *277*, 47373-47379.

Mosley, R.L., Benner, E.J., Kadiu, I., Thomas, M., Boska, M.D., Hasan, K., Laurie, C., and Gendelman, H.E. (2006). Neuroinflammation, Oxidative Stress and the Pathogenesis of Parkinson's Disease. Clin Neurosci Res *6*, 261-281.

Muller, T., Vieira, R.P., Grimm, M., Durk, T., Cicko, S., Zeiser, R., Jakob, T., Martin, S.F., Blumenthal, B., Sorichter, S., *et al*. (2011). A potential role for P2X7R in allergic airway inflammation in mice and humans. Am J Respir Cell Mol Biol *44*, 456-464.

Munoz-Planillo, R., Franchi, L., Miller, L.S., and Nunez, G. (2009). A critical role for hemolysins and bacterial lipoproteins in Staphylococcus aureus-induced activation of the Nlrp3 inflammasome. J Immunol *183*, 3942-3948.

Muruve, D.A., Petrilli, V., Zaiss, A.K., White, L.R., Clark, S.A., Ross, P.J., Parks, R.J., and Tschopp, J. (2008). The inflammasome recognizes cytosolic microbial and host DNA and triggers an innate immune response. Nature *452*, 103-107.

Nakae, S., Komiyama, Y., Yokoyama, H., Nambu, A., Umeda, M., Iwase, M., Homma, I., Sudo, K., Horai, R., Asano, M., *et al*. (2003). IL-1 is required for allergen-specific Th2 cell activation and the development of airway hypersensitivity response. Int Immunol *15*, 483-490.

Nakamura, K., Hori, T., Sato, N., Sugie, K., Kawakami, T., and Yodoi, J. (1993). Redox regulation of a src family protein tyrosine kinase p56lck in T cells. Oncogene *8*, 3133-3139.

Nakamura, T., Yamashita, H., Takahashi, T., and Nakamura, S. (2001). Activated Fyn phosphorylates alpha-synuclein at tyrosine residue 125. Biochem Biophys Res Commun *280*, 1085-1092.

Napoli, I., and Neumann, H. (2010). Protective effects of microglia in multiple sclerosis. Exp Neurol *225*, 24-28.

Netea, M.G., Nold-Petry, C.A., Nold, M.F., Joosten, L.A., Opitz, B., van der Meer, J.H., van de Veerdonk, F.L., Ferwerda, G., Heinhuis, B., Devesa, I., *et al*. (2009). Differential requirement for the activation of the inflammasome for processing and release of IL-1beta in monocytes and macrophages. Blood *113*, 2324-2335.

Nigam, A., and Narula, A.A. (1990). Intramuscular papaveretum in the management of food bolus impaction. Ear Nose Throat J *69*, 737-738.

Nimmerjahn, A., Kirchhoff, F., and Helmchen, F. (2005). Resting microglial cells are highly dynamic surveillants of brain parenchyma in vivo. Science *308*, 1314-1318.

Nishimura, M., Mizuta, I., Mizuta, E., Yamasaki, S., Ohta, M., Kaji, R., and Kuno, S. (2001). Tumor necrosis factor gene polymorphisms in patients with sporadic Parkinson's disease. Neurosci Lett *311*, 1-4.

Nishimura, M., Mizuta, I., Mizuta, E., Yamasaki, S., Ohta, M., and Kuno, S. (2000). Influence of interleukin-1beta gene polymorphisms on age-at-onset of sporadic Parkinson's disease. Neurosci Lett *284*, 73-76.

Normand, S., Delanoye-Crespin, A., Bressenot, A., Huot, L., Grandjean, T., Peyrin-Biroulet, L., Lemoine, Y., Hot, D., and Chamaillard, M. (2011). Nod-like receptor pyrin domain-containing protein 6 (NLRP6) controls epithelial self-renewal and colorectal carcinogenesis upon injury. Proceedings of the National Academy of Sciences of the United States of America *108*, 9601-9606.

Nour, A.M., Yeung, Y.G., Santambrogio, L., Boyden, E.D., Stanley, E.R., and Brojatsch, J. (2009). Anthrax lethal toxin triggers the formation of a membrane-associated inflammasome complex in murine macrophages. Infect Immun *77*, 1262-1271.

Olanow, C.W. (2007). The pathogenesis of cell death in Parkinson's disease--2007. Mov Disord *22 Suppl 17*, S335-342.

Panicker, N., Saminathan, H., Jin, H., Neal, M., Harischandra, D.S., Gordon, R., Kanthasamy, K., Lawana, V., Sarkar, S., Luo, J., *et al*. (2015). Fyn Kinase Regulates Microglial Neuroinflammatory Responses in Cell Culture and Animal Models of Parkinson's Disease. The Journal of neuroscience : the official journal of the Society for Neuroscience *35*, 10058-10077.

Pappa, H., Murray-Rust, J., Dekker, L.V., Parker, P.J., and McDonald, N.Q. (1998). Crystal structure of the C2 domain from protein kinase C-delta. Structure *6*, 885-894.

Park, J.E., Kim, Y.I., and Yi, A.K. (2009). Protein kinase D1 is essential for MyD88-dependent TLR signaling pathway. J Immunol *182*, 6316-6327.

Parravicini, V., Gadina, M., Kovarova, M., Odom, S., Gonzalez-Espinosa, C., Furumoto, Y., Saitoh, S., Samelson, L.E., O'Shea, J.J., and Rivera, J. (2002). Fyn kinase initiates complementary signals required for IgE-dependent mast cell degranulation. Nat Immunol *3*, 741-748.

Persson, M., Brantefjord, M., Hansson, E., and Ronnback, L. (2005). Lipopolysaccharide increases microglial GLT-1 expression and glutamate uptake capacity in vitro by a mechanism dependent on TNF-alpha. Glia *51*, 111-120.

Pontillo, A., Catamo, E., Arosio, B., Mari, D., and Crovella, S. (2012). NALP1/NLRP1 genetic variants are associated with Alzheimer disease. Alzheimer Dis Assoc Disord *26*, 277-281.

Poyet, J.L., Srinivasula, S.M., Tnani, M., Razmara, M., Fernandes-Alnemri, T., and Alnemri, E.S. (2001). Identification of Ipaf, a human caspase-1-activating protein related to Apaf-1. The Journal of biological chemistry *276*, 28309-28313.

Proell, M., Gerlic, M., Mace, P.D., Reed, J.C., and Riedl, S.J. (2013). The CARD plays a critical role in ASC foci formation and inflammasome signalling. Biochem J *449*, 613-621.

Przedborski, S. (2005). Pathogenesis of nigral cell death in Parkinson's disease. Parkinsonism & related disorders *11 Suppl 1*, S3-7.

Qin, L., Wu, X., Block, M.L., Liu, Y., Breese, G.R., Hong, J.S., Knapp, D.J., and Crews, F.T. (2007). Systemic LPS causes chronic neuroinflammation and progressive neurodegeneration. Glia *55*, 453-462.

Qu, Y., Misaghi, S., Izrael-Tomasevic, A., Newton, K., Gilmour, L.L., Lamkanfi, M., Louie, S., Kayagaki, N., Liu, J., Komuves, L., *et al*. (2012). Phosphorylation of NLRC4 is critical for inflammasome activation. Nature *490*, 539-542.

Rajasekaran, K., Kumar, P., Schuldt, K.M., Peterson, E.J., Vanhaesebroeck, B., Dixit, V., Thakar, M.S., and Malarkannan, S. (2013). Signaling by Fyn-ADAP via the Carma1-Bcl-10-MAP3K7 signalosome exclusively regulates inflammatory cytokine production in NK cells. Nat Immunol *14*, 1127-1136.

Ramos, H.J., Lanteri, M.C., Blahnik, G., Negash, A., Suthar, M.S., Brassil, M.M., Sodhi, K., Treuting, P.M., Busch, M.P., Norris, P.J., *et al*. (2012). IL-1beta signaling promotes CNS-intrinsic immune control of West Nile virus infection. PLoS Pathog *8*, e1003039.

Rathinam, V.A., Jiang, Z., Waggoner, S.N., Sharma, S., Cole, L.E., Waggoner, L., Vanaja, S.K., Monks, B.G., Ganesan, S., Latz, E., *et al*. (2010). The AIM2 inflammasome is essential for host defense against cytosolic bacteria and DNA viruses. Nat Immunol *11*, 395-402.

Ren, J., Wang, Q., Morgan, S., Si, Y., Ravichander, A., Dou, C., Kent, K.C., and Liu, B. (2014). Protein kinase C-delta (PKCdelta) regulates proinflammatory chemokine expression through cytosolic interaction with the NF-kappaB subunit p65 in vascular smooth muscle cells. The Journal of biological chemistry *289*, 9013-9026.

Reynolds, A.D., Glanzer, J.G., Kadiu, I., Ricardo-Dukelow, M., Chaudhuri, A., Ciborowski, P., Cerny, R., Gelman, B., Thomas, M.P., Mosley, R.L., *et al*. (2008). Nitrated alpha-synuclein-activated microglial profiling for Parkinson's disease. J Neurochem *104*, 1504-1525.

Riteau, N., Gasse, P., Fauconnier, L., Gombault, A., Couegnat, M., Fick, L., Kanellopoulos, J., Quesniaux, V.F., Marchand-Adam, S., Crestani, B., *et al*. (2010). Extracellular ATP is a danger signal activating P2X7 receptor in lung inflammation and fibrosis. Am J Respir Crit Care Med *182*, 774-783.

Roskoski, R., Jr. (2004). Src protein-tyrosine kinase structure and regulation. Biochem Biophys Res Commun *324*, 1155-1164.

Salmond, R.J., Filby, A., Qureshi, I., Caserta, S., and Zamoyska, R. (2009). T-cell receptor proximal signaling via the Src-family kinases, Lck and Fyn, influences T-cell activation, differentiation, and tolerance. Immunol Rev *228*, 9-22.

Saminathan, H., Asaithambi, A., Anantharam, V., Kanthasamy, A.G., and Kanthasamy, A. (2011). Environmental neurotoxic pesticide dieldrin activates a non receptor tyrosine kinase to promote PKCdelta-mediated dopaminergic apoptosis in a dopaminergic neuronal cell model. Neurotoxicology *32*, 567-577.

Shaked, I., Tchoresh, D., Gersner, R., Meiri, G., Mordechai, S., Xiao, X., Hart, R.P., and Schwartz, M. (2005). Protective autoimmunity: interferon-gamma enables microglia to remove glutamate without evoking inflammatory mediators. J Neurochem *92*, 997-1009.

Shen, S., Yu, S., Binek, J., Chalimoniuk, M., Zhang, X., Lo, S.C., Hannink, M., Wu, J., Fritsche, K., Donato, R., *et al*. (2005). Distinct signaling pathways for induction of type II NOS by IFNgamma and LPS in BV-2 microglial cells. Neurochemistry international *47*, 298-307.

Shio, M.T., Eisenbarth, S.C., Savaria, M., Vinet, A.F., Bellemare, M.J., Harder, K.W., Sutterwala, F.S., Bohle, D.S., Descoteaux, A., Flavell, R.A., *et al*. (2009). Malarial hemozoin activates the NLRP3 inflammasome through Lyn and Syk kinases. PLoS Pathog *5*, e1000559.

Shukla, A., Lounsbury, K.M., Barrett, T.F., Gell, J., Rincon, M., Butnor, K.J., Taatjes, D.J., Davis, G.S., Vacek, P., Nakayama, K.I., *et al*. (2007). Asbestos-induced peribronchiolar cell proliferation and cytokine production are attenuated in lungs of protein kinase C-delta knockout mice. Am J Pathol *170*, 140-151.

Silverman, W.R., de Rivero Vaccari, J.P., Locovei, S., Qiu, F., Carlsson, S.K., Scemes, E., Keane, R.W., and Dahl, G. (2009). The pannexin 1 channel activates the inflammasome in neurons and astrocytes. The Journal of biological chemistry *284*, 18143-18151.

Simard, A.R., and Rivest, S. (2007). Neuroprotective effects of resident microglia following acute brain injury. J Comp Neurol *504*, 716-729.

Sitailo, L.A., Tibudan, S.S., and Denning, M.F. (2006). The protein kinase C delta catalytic fragment targets Mcl-1 for degradation to trigger apoptosis. The Journal of biological chemistry *281*, 29703-29710.

Smith-Garvin, J.E., Koretzky, G.A., and Jordan, M.S. (2009). T cell activation. Annu Rev Immunol *27*, 591-619.

Soto-Pena, G.A., and Vega, L. (2008). Arsenic interferes with the signaling transduction pathway of T cell receptor activation by increasing basal and induced phosphorylation of Lck and Fyn in spleen cells. Toxicol Appl Pharmacol *230*, 216-226.

Sriram, K., Matheson, J.M., Benkovic, S.A., Miller, D.B., Luster, M.I., and O'Callaghan, J.P. (2006). Deficiency of TNF receptors suppresses microglial activation and alters the susceptibility of brain regions to MPTP-induced neurotoxicity: role of TNF-alpha. FASEB journal : official publication of the Federation of American Societies for Experimental Biology *20*, 670-682.

Stott, S.R., and Barker, R.A. (2014). Time course of dopamine neuron loss and glial response in the 6-OHDA striatal mouse model of Parkinson's disease. Eur J Neurosci *39*, 1042-1056.

Stuart, L.M., Bell, S.A., Stewart, C.R., Silver, J.M., Richard, J., Goss, J.L., Tseng, A.A., Zhang, A., El Khoury, J.B., and Moore, K.J. (2007). CD36 signals to the actin cytoskeleton and regulates microglial migration via a p130Cas complex. The Journal of biological chemistry *282*, 27392-27401.

Su, X., Maguire-Zeiss, K.A., Giuliano, R., Prifti, L., Venkatesh, K., and Federoff, H.J. (2008). Synuclein activates microglia in a model of Parkinson's disease. Neurobiology of aging *29*, 1690-1701.

Sun, F., Anantharam, V., Latchoumycandane, C., Kanthasamy, A., and Kanthasamy, A.G. (2005). Dieldrin induces ubiquitin-proteasome dysfunction in alpha-synuclein overexpressing dopaminergic neuronal cells and enhances susceptibility to apoptotic cell death. J Pharmacol Exp Ther *315*, 69-79.

Suzuki, T., Franchi, L., Toma, C., Ashida, H., Ogawa, M., Yoshikawa, Y., Mimuro, H., Inohara, N., Sasakawa, C., and Nunez, G. (2007). Differential regulation of caspase-1 activation, pyroptosis, and autophagy via Ipaf and ASC in Shigella-infected macrophages. PLoS Pathog *3*, e111.

Takeuchi, O., and Akira, S. (2010). Pattern recognition receptors and inflammation. Cell *140*, 805-820.

Tan, M.S., Tan, L., Jiang, T., Zhu, X.C., Wang, H.F., Jia, C.D., and Yu, J.T. (2014). Amyloid-beta induces NLRP1-dependent neuronal pyroptosis in models of Alzheimer's disease. Cell Death Dis *5*, e1382.

Theodore, S., Cao, S., McLean, P.J., and Standaert, D.G. (2008). Targeted overexpression of human alpha-synuclein triggers microglial activation and an adaptive immune response in a mouse model of Parkinson disease. Journal of neuropathology and experimental neurology *67*, 1149-1158.

Thornberry, N.A., Bull, H.G., Calaycay, J.R., Chapman, K.T., Howard, A.D., Kostura, M.J., Miller, D.K., Molineaux, S.M., Weidner, J.R., Aunins, J., *et al.* (1992). A novel heterodimeric cysteine protease is required for interleukin-1 beta processing in monocytes. Nature *356*, 768-774.

Um, J.W., Nygaard, H.B., Heiss, J.K., Kostylev, M.A., Stagi, M., Vortmeyer, A., Wisniewski, T., Gunther, E.C., and Strittmatter, S.M. (2012). Alzheimer amyloid-beta

oligomer bound to postsynaptic prion protein activates Fyn to impair neurons. Nature neuroscience *15*, 1227-1235.

Vawter, M.P., Dillon-Carter, O., Tourtellotte, W.W., Carvey, P., and Freed, W.J. (1996). TGFbeta1 and TGFbeta2 concentrations are elevated in Parkinson's disease in ventricular cerebrospinal fluid. Exp Neurol *142*, 313-322.

Virgone-Carlotta, A., Uhlrich, J., Akram, M.N., Ressnikoff, D., Chretien, F., Domenget, C., Gherardi, R., Despars, G., Jurdic, P., Honnorat, J., *et al*. (2013). Mapping and kinetics of microglia/neuron cell-to-cell contacts in the 6-OHDA murine model of Parkinson's disease. Glia *61*, 1645-1658.

Volpicelli-Daley, L.A., Luk, K.C., Patel, T.P., Tanik, S.A., Riddle, D.M., Stieber, A., Meaney, D.F., Trojanowski, J.Q., and Lee, V.M. (2011). Exogenous alpha-synuclein fibrils induce Lewy body pathology leading to synaptic dysfunction and neuron death. Neuron *72*, 57-71.

von Bohlen und Halbach, O., Schober, A., and Krieglstein, K. (2004). Genes, proteins, and neurotoxins involved in Parkinson's disease. Prog Neurobiol *73*, 151-177.

Walsh, J.G., Muruve, D.A., and Power, C. (2014). Inflammasomes in the CNS. Nat Rev Neurosci *15*, 84-97.

Walton, N.M., Sutter, B.M., Laywell, E.D., Levkoff, L.H., Kearns, S.M., Marshall, G.P., 2nd, Scheffler, B., and Steindler, D.A. (2006). Microglia instruct subventricular zone neurogenesis. Glia *54*, 815-825.

Wang, X.J., Kong, K.M., Qi, W.L., Ye, W.L., and Song, P.S. (2005). Interleukin-1 beta induction of neuron apoptosis depends on p38 mitogen-activated protein kinase activity after spinal cord injury. Acta Pharmacol Sin *26*, 934-942.

Whitton, P.S. (2007). Inflammation as a causative factor in the aetiology of Parkinson's disease. British journal of pharmacology *150*, 963-976.

Williamson, R., Usardi, A., Hanger, D.P., and Anderton, B.H. (2008). Membrane-bound beta-amyloid oligomers are recruited into lipid rafts by a fyn-dependent mechanism. FASEB J *22*, 1552-1559.

Willingham, S.B., Bergstralh, D.T., O'Connor, W., Morrison, A.C., Taxman, D.J., Duncan, J.A., Barnoy, S., Venkatesan, M.M., Flavell, R.A., Deshmukh, M., *et al*. (2007). Microbial pathogen-induced necrotic cell death mediated by the inflammasome components CIAS1/cryopyrin/NLRP3 and ASC. Cell Host Microbe *2*, 147-159.

Wilms, H., Rosenstiel, P., Sievers, J., Deuschl, G., Zecca, L., and Lucius, R. (2003). Activation of microglia by human neuromelanin is NF-kappaB dependent and involves p38 mitogen-activated protein kinase: implications for Parkinson's disease. FASEB journal :

official publication of the Federation of American Societies for Experimental Biology *17*, 500-502.

Wu, D.C., Jackson-Lewis, V., Vila, M., Tieu, K., Teismann, P., Vadseth, C., Choi, D.K., Ischiropoulos, H., and Przedborski, S. (2002). Blockade of microglial activation is neuroprotective in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine mouse model of Parkinson disease. The Journal of neuroscience : the official journal of the Society for Neuroscience *22*, 1763-1771.

Wu, D.C., Teismann, P., Tieu, K., Vila, M., Jackson-Lewis, V., Ischiropoulos, H., and Przedborski, S. (2003). NADPH oxidase mediates oxidative stress in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine model of Parkinson's disease. Proceedings of the National Academy of Sciences of the United States of America *100*, 6145-6150.

Wu, J., Fernandes-Alnemri, T., and Alnemri, E.S. (2010). Involvement of the AIM2, NLRC4, and NLRP3 inflammasomes in caspase-1 activation by Listeria monocytogenes. J Clin Immunol *30*, 693-702.

Yamasaki, K., Muto, J., Taylor, K.R., Cogen, A.L., Audish, D., Bertin, J., Grant, E.P., Coyle, A.J., Misaghi, A., Hoffman, H.M., *et al*. (2009). NLRP3/cryopyrin is necessary for interleukin-1beta (IL-1beta) release in response to hyaluronan, an endogenous trigger of inflammation in response to injury. The Journal of biological chemistry *284*, 12762-12771.

Yan, S.R., and Berton, G. (1996). Regulation of Src family tyrosine kinase activities in adherent human neutrophils. Evidence that reactive oxygen intermediates produced by adherent neutrophils increase the activity of the p58c-fgr and p53/56lyn tyrosine kinases. J Biol Chem *271*, 23464-23471.

Yan, Y., Jiang, W., Liu, L., Wang, X., Ding, C., Tian, Z., and Zhou, R. (2015). Dopamine controls systemic inflammation through inhibition of NLRP3 inflammasome. Cell *160*, 62-73.

Yang, Y., Kaul, S., Zhang, D., Anantharam, V., and Kanthasamy, A.G. (2004). Suppression of caspase-3-dependent proteolytic activation of protein kinase C delta by small interfering RNA prevents MPP+-induced dopaminergic degeneration. Mol Cell Neurosci *25*, 406-421.

Yu, J.R., and Leslie, K.S. (2011). Cryopyrin-associated periodic syndrome: an update on diagnosis and treatment response. Curr Allergy Asthma Rep *11*, 12-20.

Zecca, L., Wilms, H., Geick, S., Claasen, J.H., Brandenburg, L.O., Holzknecht, C., Panizza, M.L., Zucca, F.A., Deuschl, G., Sievers, J., *et al*. (2008). Human neuromelanin induces neuroinflammation and neurodegeneration in the rat substantia nigra: implications for Parkinson's disease. Acta neuropathologica *116*, 47-55.

Zhang, D., Anantharam, V., Kanthasamy, A., and Kanthasamy, A.G. (2007). Neuroprotective effect of protein kinase C delta inhibitor rottlerin in cell culture and animal models of Parkinson's disease. J Pharmacol Exp Ther *322*, 913-922.

Zhang, P., Lokuta, K.M., Turner, D.E., and Liu, B. (2010). Synergistic dopaminergic neurotoxicity of manganese and lipopolysaccharide: differential involvement of microglia and astroglia. J Neurochem *112*, 434-443.

Zhang, W., Wang, T., Pei, Z., Miller, D.S., Wu, X., Block, M.L., Wilson, B., Zhou, Y., Hong, J.S., and Zhang, J. (2005). Aggregated alpha-synuclein activates microglia: a process leading to disease progression in Parkinson's disease. FASEB journal : official publication of the Federation of American Societies for Experimental Biology *19*, 533-542.

Zhao, Y., Yang, J., Shi, J., Gong, Y.N., Lu, Q., Xu, H., Liu, L., and Shao, F. (2011). The NLRC4 inflammasome receptors for bacterial flagellin and type III secretion apparatus. Nature *477*, 596-600.

Zhou, R., Yazdi, A.S., Menu, P., and Tschopp, J. (2011). A role for mitochondria in NLRP3 inflammasome activation. Nature *469*, 221-225.

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