Inflammasome inhibition prevents α-synuclein pathology and dopaminergic neurodegeneration in mice

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Parkinson’s disease (PD) is characterized by a profound loss of dopaminergic neurons in the substantia nigra, accompanied by chronic neuroinflammation, mitochondrial dysfunction, and widespread accumulation of α-synuclein–rich protein aggregates in the form of Lewy bodies. However, the mechanisms linking α-synuclein pathology and dopaminergic neuronal death to chronic microglial neuroinflammation have not been completely elucidated. We show that activation of the microglial NLR family pyrin domain containing 3 (NLRP3) inflammasome is a common pathway triggered by both fibrillar α-synuclein and dopaminergic degeneration in the absence of α-synuclein aggregates. Cleaved caspase-1 and the inflammasome adaptor protein apoptosis-associated speck-like protein containing a C-terminal caspase recruitment domain (ASC) were elevated in the substantia nigra of the brains of patients with PD and in multiple preclinical PD models. NLRP3 activation by fibrillar α-synuclein in mouse microglia resulted in a delayed but robust activation of the NLRP3 inflammasome leading to extracellular interleukin-1β and ASC release in the absence of pyroptosis. Nanomolar doses of a small-molecule NLRP3 inhibitor, MCC950, abolished fibrillar α-synuclein–mediated inflammasome activation in mouse microglial cells and extracellular ASC release. Furthermore, oral administration of MCC950 in multiple rodent PD models inhibited inflammasome activation and effectively mitigated motor deficits, nigrostriatal dopaminergic degeneration, and accumulation of α-synuclein aggregates. These findings suggest that microglial NLRP3 may be a sustained source of neuroinflammation that could drive progressive dopaminergic neuropathology and highlight NLRP3 as a potential target for disease-modifying treatments for PD.

INTRODUCTION

Parkinson’s disease (PD) is the most prevalent synucleinopathy and the second most common neurodegenerative disorder worldwide, affecting about 2% of the population over the age of 60 (1). Although symptomatic treatments exist, no current therapies can effectively slow or halt disease progression (1). The pathological hallmark of PD is a profound loss of nigrostriatal dopaminergic neurons that is preceded by the accumulation and spread of characteristic Lewy body and neurite inclusions, consisting primarily of misfolded fibrillar α-synuclein (1). Accumulating evidence suggests that pathological fibrils are the major neurotoxic form of α-synuclein, which can self-propagate through interconnected brain regions and drive progressive dopaminergic degeneration (2–7). Chronic microglial neuroinflammation is evident early in the nigrostriatal system in living patients with PD and remains a prominent feature in postmortem PD brains (8–10). Compelling evidence from clinical, preclinical, and epidemiological studies supports a pathogenic role for chronic neuroinflammation during dopaminergic degeneration (10–15). However, a clear mechanism linking fibrillar α-synuclein pathology, progressive dopaminergic degeneration, and the underlying chronic neuroinflammation evident in clinical and experimental PD has not been completely defined.

Inflammasomes are multiprotein complexes that function as intracellular sensors of environmental and cellular stress (16–18). The NLR family pyrin domain containing 3 (NLRP3) inflammasome is composed of the NLRP3 sensor, the signaling adapter apoptosis-associated speck-like protein containing a caspase recruitment domain (ASC), and the caspase-1 protease. Assembly of the NLRP3 complex in immune cells upon cellular stress triggers caspase-1 activation and caspase-1–mediated release of interleukin-1β (IL-1β) and IL-18, thereby initiating inflammatory responses (18). In neurodegenerative conditions such as Alzheimer’s disease (AD), persistent accumulation of misfolded protein aggregates can trigger and sustain inflammasome activation and thereby drive central nervous system (CNS) inflammation and neuropathology (19). Recent findings in AD models have demonstrated that microglia-derived inflammasome components such as ASC specks can cross-seed pathogenic amyloid fibrils (20). In the brains of patients with PD, the inflammasome pathway can potentially be activated by oxidative stress and insoluble α-synuclein aggregates (18, 21, 22). However, the interplay between chronic NLRP3 activation, ASC release, and progressive α-synuclein pathology in PD is still unclear.

We provide herein direct evidence of NLRP3 inflammasome activation in postmortem PD brains and in multiple rodent models involving fibrillar α-synuclein pathology, as well as mitochondrial dysfunction and oxidative stress. We also confirm that reactive microglia are the source of NLRP3 inflammasomes in human PD brains and provide new insight into the mechanisms of α-synuclein–mediated NLRP3 activation and ASC release in microglia that have
implications for PD pathophysiology. The potent small-molecule NLRP3 inhibitor MCC950 is active in the CNS after oral delivery and can functionally inhibit NLRP3 activation in multiple mouse models of PD. Furthermore, daily oral dosing with MCC950 is neuroprotective and reduces the loss of striatal dopamine and nigrostriatal dopaminergic degeneration in PD mice. We show that chronic NLRP3 inhibition with MCC950 protects against motor deficits, and accumulation of hyperphosphorylated α-synuclein aggregates in multiple brain regions in the PD mice. Our results suggest that chronic NLRP3 activation might be a key pathological mechanism that drives PD pathology and that NLRP3 inhibition with orally active and CNS-penetrant drugs, such as MCC950, may represent a promising therapeutic strategy to mitigate progressive dopaminergic degeneration in PD.

**RESULTS**

**Patients with PD and preclinical PD models show extensive inflammasome activation and ASC up-regulation**

To determine whether inflammasome activation occurs in clinical PD, we first assessed key inflammasome components in the substantia nigra of postmortem brains of patients with PD. We found substantially increased cleaved caspase-1 (p20) and adaptor protein ASC in the brains of patients with PD compared to age-matched controls (Fig. 1, A and B). Immunohistochemistry studies confirmed NLRP3 and ASC up-regulation in PD substantia nigra sections, which was almost exclusively localized to Iba-1+ microglia (Fig. 1, C and D, and fig. S1, A and B), consistent with previous reports linking chronic inflammasome activation to persistent reactive microglia (18, 19). A proportion of ASC was also identified outside of microglial cells (fig. S1B, blue arrows), supporting similar findings recently documented in patients with AD (20). These results clearly show that increased inflammasome activation is evident at the sites of dopaminergic cell loss in patients with end-stage PD. Downstream inflammasome activation markers including IL-1β have been shown to be elevated systemically in the blood of patients with PD and in cerebrospinal fluid (9, 23). Similarly, we also found significantly elevated circulating caspase-1 in a cohort of 21 patients with PD (P = 0.023; fig. S1C), suggesting that systemic inflammasome activation might also be involved in PD pathophysiology (18, 20, 22).

In addition to the central role for α-synuclein in PD progression, mitochondrial dysfunction is considered a major underlying pathological mechanism in PD (24). To further investigate the contribution of NLRP3 during dopaminergic degeneration, we examined the activation of the NLRP3 inflammasome in distinct animal models of PD involving dopaminergic degeneration that is driven by mitochondrial dysfunction, oxidative stress, or α-synuclein pathology. Consistent with our finding in human PD cases, immunohistochemistry studies revealed an almost exclusive localization of NLRP3 in hypertrophic reactive microglia (Fig. 1E), along with ASC expression (Fig. 1F) in the striatum of mice 3 days after 6-hydroxydopamine (6-OHDA) administration. Striatal NLRP3 expression after 6-OHDA treatment was also confirmed using NLRP3–green fluorescent protein gene knockin mice (fig. S2, A and B). Cleaved caspase-1 (p20) and ASC protein detected by Western blotting were also increased before motor dysfunction in 6-OHDA mice at day 3 (Fig. 1, G and H), as was Nlrp3, ASC, and Caspase-1 gene expression (fig. S2, C to E), suggesting that the up-regulation and subsequent activation of inflammasome components occur at the early stages of dopaminergic degeneration in this model, correlating with the onset of reactive microgliosis. We also confirmed the lack of cleaved caspase-1 generation in 6-OHDA–administered NLRP3 knockout mice (fig. S2F).

Next, we investigated MitoPark mice, a levodopa-responsive genetic model of PD, where targeted inactivation of the mitochondrial transcription factor A (Tfam) gene in dopaminergic neurons leads to progressive degeneration over 32 weeks, accompanied by parkinsonian motor deficits (25). Similar to 6-OHDA mice, we observed elevated Nlrp3 gene expression, NLRP3 and ASC immunostaining (fig. S3, A and B), and increased generation of cleaved caspase-1 in the substantia nigra of 12- and 20-week-old MitoPark mice (Fig. 1, I and J) before the onset of overt motor deficits. Together, these results suggest that robust microglial NLRP3 inflammasome activation occurs early in the disease process in mitochondrial dysfunction models of PD and can occur in the absence of α-synuclein aggregates. Given the central role of neurotoxic α-synuclein fibrils in PD, we used the α-synuclein preformed fibril (PPF) model of PD (3) to determine whether inflammasome activation occurs with α-synuclein pathology in vivo. At 30 days after fibrillar α-synuclein infusion, we detected microglial NLRP3 immunostaining (fig. S4), increases in ASC adaptor protein, and caspase-1 cleavage to the active caspase-1 (p20) fragment (Fig. 1, K and L) in the striatum of PFF mice compared to phosphate-buffered saline (PBS)–injected controls, demonstrating that neurotoxic α-synuclein fibrils can indeed drive early inflammasome activation in vivo. Collectively, these results demonstrate that robust inflammasome activation occurs in the brains of patients with PD and multiple disease models driven by mitochondrial dysfunction and α-synuclein pathology.

**Fibrillar α-synuclein drives delayed microglial NLRP3 activation and extracellular ASC release in the absence of detectable pyroptosis**

The pathological hallmark of PD is the presence of high–molecular weight, misfolded α-synuclein–rich Lewy body inclusions (2, 5). Although persistent insoluble protein aggregates are considered the major triggers for chronic sterile inflammasome activation in the CNS (18, 19), the contribution of microglial neuroinflammation and the NLRP3 inflammasome pathway to α-synuclein pathology and its cell-to-cell transmission is still unclear. Furthermore, an emerging analogous paradigm in inflammasome biology is that prion-like activities of extracellular polymeric ASC can propagate chronic inflammation by spreading inflammasome signaling from cell to cell (20, 26). Previous studies in monocytic and immortalized microglial cell lines have shown that α-synuclein can trigger inflammasome activation similar to amyloid-β (21, 22); nonetheless, the specific contribution of NLRP3 and ASC to inflammasome activation by α-synuclein is still not well defined. Hence, we evaluated the microglial response to fibrillar α-synuclein in primary microglia isolated from wild-type and NLRP3 knockout mice using highly characterized synthetic α-synuclein fibrils prepared by in vitro aggregation and sonication (fig. S5, A to C), which have been shown to drive α-synuclein pathology in vivo (3). In microglia primed with ultrapure lipopolysaccharide (LPS) and activated with fibrillar α-synuclein, we observed a time-dependent secretion of IL-1β that was detectable at 18 hours (Fig. 2A). The amount of IL-1β secreted 24 hours after α-synuclein treatment was equivalent to that obtained at 1 hour with activation using the powerful NLRP3 activator adenosine 5’-triphosphate (ATP), suggesting that fibrillar α-synuclein mediates a delayed but robust activation of the inflammasome pathway in microglia. We also confirmed the presence of caspase-1...
Fig. 1. Extensive inflammasome activation and microglial NLRP3 expression are observed in the brains of patients with PD and animal models. (A) Western blot and (B) densitometric analysis for caspase-1 (casp-1) and ASC from substantia nigra tissue lysates obtained from patients with PD and control subjects (n = 5 to 6 per group). GAPDH, glyceraldehyde-3-phosphate dehydrogenase. (C and D) Immunohistochemistry of key inflammasome components NLRP3 (C, green) and ASC (D, red), and Iba-1+ microglia in postmortem substantia nigra tissue sections of patients with PD and age-matched controls. Magnification, ×40. Scale bars, 20 μm. (E and F) Immunohistochemistry within the striatum of mice 3 days after 6-OHDA or PBS injection, showing NLRP3 (E, green) and ASC (F, red) localized to hypertrophic activated Iba-1+ microglia in 6-OHDA mice. Magnification, ×40. Scale bars, 20 μm. (G) Western blot and (H) densitometric analysis for cleaved caspase-1 and ASC in ipsilateral striatal tissue of 6-OHDA- and PBS-injected mice at 3 days after injection (n = 4 mice per group). (I) Western blot and (J) densitometric analysis for cleaved caspase-1 and ASC in substantia nigra tissue from MitoPark mice (MP) and littermate controls (Ctrl) at 12 and 24 weeks of age (n = 2 to 4 mice per group). (K) Western blot and (L) densitometric analysis for cleaved caspase-1 and ASC in ipsilateral striatal tissue from PBS- and α-synuclein PFF–injected mice at 30 days after injection (n = 9 mice per group). Data shown as means ± SEM. *P < 0.05, **P < 0.01, and ***P < 0.001 by Student’s t test (H and J) or Mann-Whitney test (B and L).

caspase-1 (p20) and cleaved IL-1β (p17) in supernatants starting at 18 hours, demonstrating delayed inflammasome activation (Fig. 2B). We found substantially increased extracellular ASC protein in the supernatants of patients with PD and age-matched controls (Fig. 2B), supporting our immunohistochemistry findings in patients with PD and suggesting that extracellular ASC might have a role in the propagation of inflammasome-driven pathology triggered by α-synuclein fibrils (16, 20, 26).

Inflammasome “priming” induces transcriptional up-regulation of inflammasome components, which facilitates the assembly and activation of the inflammasome complex (18). To determine whether priming was a requirement for α-synuclein–mediated inflammasome activation, we repeated the experiments in the absence of the LPS-priming step. We found that α-synuclein fibrils alone were able to induce IL-1β release at 24 hours, although only up to ~35% of the level of primed cells (Fig. 2C). Because IL-18 is also a key inflammatory mediator that is released during inflammasome activation (17, 27), we quantified
IL-18 concentrations in α-synuclein–activated microglia. Under the same conditions, no IL-18 was detectable with α-synuclein (Fig. 2D) despite ATP stimulation releasing substantial amounts of IL-18 from microglia. We also confirmed the formation of characteristic ASC specks in α-synuclein–treated microglia at 24 hours (Fig. 2E) and the presence of oligomeric ASC in cross-linking experiments (Fig. 2F). Because α-synuclein fibrils caused increased extracellular ASC together with the release of inflammasome components, we evaluated the potential mechanisms of cellular ASC release. In macrophages and other immune cells stimulated with NLRP3 activators such as nigericin or monosodium urate crystals, the inflammasome complex is released during caspase-1–dependent cell lysis by a process termed pyroptosis (16). Extracellular release of ASC by pyroptosis has been shown to be the primary mechanism by which inflammasome activation propagates inflammation in systemic inflammatory models (20, 26). Therefore, we assayed whether inflammasome activation by α-synuclein fibrils triggered pyroptosis in microglia. Even at 24 hours, when inflammasome activation was maximal, α-synuclein did not cause detectable pyroptosis, which was quantified using lactate dehydrogenase (LDH) release (Fig. 2G). In contrast, nigericin treatment readily triggered caspase-1–dependent pyroptosis, ns, not significant. Nigericin and VX-765 (20 μM) were used as positive controls. (H) Western blot (left) and densitometric analysis (right) for cleaved caspase-1 (p20), cleaved IL-1β, and ASC in the supernatants of primed microglia treated with α-synuclein for 24 hours. Expression of pro–caspase-1, NLRP3, and GAPDH was determined in cell lysates, and 1-hour ATP treatment (5 mM) was used as a positive control. (I) Comparison of α-synuclein–mediated IL-1β secretion in unprimed or LPS-primed primary microglia at 24 hours. (J) IL-18 secretion in microglia treated with ATP (0.5 mM, 1 hour) was used as a positive control. Data are means ± SEM from at least three independent experiments. *P < 0.05 and ***P < 0.001 by one-way analysis of variance (ANOVA) with Bonferroni’s post hoc test (B, C, F, and H) or Kruskal-Wallis test with Dunn’s post hoc test (A, D, and G).
Orally administered NLRP3 inhibitor MCC950 is active in the CNS and blocks inflammasome activation in multiple preclinical PD models

Having confirmed that the NLRP3 inflammasome is activated in both clinical and experimental PD, we evaluated the therapeutic potential of blocking NLRP3 activation using the potent small-molecule NLRP3 inhibitor MCC950 (30). We confirmed that MCC950 blocked ATP- and nigericin-dependent NLRP3 activation in primary microglia, with a median inhibitory concentration (IC50) of 7.7 nM (Fig. 3A and fig. S6, A and B), similar to the potency reported for macrophages (30). Furthermore, pretreatment with 100 nM MCC950 completely blocked the release of active IL-1β (p17), caspase-1 (p20), and ASC by ATP and nigericin treatment in microglia (fig. S6 C to F). Nanomolar doses of MCC950 abrogated α-synuclein–mediated IL-1β release into the supernatant (Fig. 3B) and the release of cleaved caspase-1, cleaved IL-1β, and ASC in Western blots of supernatants (Fig. 3, C and D). MCC950 also effectively blocked ASC oligomerization (Fig. 3, E and F) induced by α-synuclein fibrils in microglia. Together, these results show that nanomolar concentrations of the small-molecule NLRP3 inhibitor MCC950 are sufficient to block several pathogenic mediators of microglial inflammasome activation by α-synuclein aggregates. We next determined whether MCC950 could be effective in vivo to block CNS NLRP3 inflammasome activation in PD models. First, CNS pharmacokinetic studies were performed, which confirmed that oral dosing with 20 mg/kg was well tolerated and resulted in brain concentrations substantially above the IC50 for NLRP3 activation over a 24-hour period (Fig. 3G). Daily oral dosing with MCC950 at 20 mg/kg blocked the generation of cleaved caspase-1 in the striatum of α-synuclein PFF–injected mice at 30 days (Fig. 3H). Oral dosing with MCC950 also blocked cleaved caspase-1 generation in MitoPark mice (Fig. 3I) and in the 6-OHDA model of PD (Fig. 3J). These results demonstrate that MCC950 is orally active in the CNS and can effectively block nigrostriatal inflammasome activation in distinct PD models driven by both mitochondrial dysfunction and α-synuclein pathology.

Daily oral dosing with MCC950 protects against nigrostriatal dopaminergic degeneration in experimental PD

Because MCC950 was orally bioavailable and crossed the blood–brain barrier at therapeutically relevant concentrations, we next examined whether daily MCC950 dosing protects against neuropathology and dopaminergic degeneration. The unilateral 6-OHDA model induces a partial striatal lesion with progressive retrograde nigrostriatal pathology and allows assessment based on behavioral and neurochemical parameters relevant to PD (31). At 21 days after 6-OHDA lesioning, MCC950-treated mice displayed a marked reduction in amphetamine-induced ipsilateral rotations (Fig. 4A and movie S1). Mice that received MCC950 also displayed improved balance beam performance compared to untreated 6-OHDA mice (Fig. 4B). Quantification of striatal dopamine and metabolites 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) confirmed that MCC950–treated mice were protected against the loss of dopamine that results from nigrostriatal degeneration and dopaminergic neuron loss in this model (Fig. 4, C to E). We also found higher striatal tyrosine hydroxylase (TH) expression in MCC950–treated mice (Fig. 4, F and G). The neuroprotective effect of oral MCC950 treatment was further confirmed by unbiased stereological counts of TH+ dopaminergic neurons in the substantia nigra (Fig. 4, H and I), which demonstrated partial preservation of the nigrostriatal system in drug-treated mice. We also verified these findings using NLRP3 knockout mice, which showed substantially improved amphetamine-induced rotational behavior and prevention from the loss of dopamine and its metabolites DOPAC and HVA after 6-OHDA administration (Fig. 4, J to M). These results demonstrate that pharmacological inhibition of NLRP3 with an orally active, CNS-permeable inhibitor can protect against dopaminergic degeneration in vivo in rodent models of PD, confirming a pathologic role for NLRP3 inflammasome activation in driving disease progression in mice.

Chronic dosing with MCC950 protects against dopaminergic degeneration induced by pathological α-synuclein aggregates

Having confirmed the neuroprotective efficacy of MCC950 in an acute neurotoxicant model in which pathology is driven by oxidative stress and mitochondrial dysfunction, we next evaluated the efficacy of MCC950 in a chronic progressive model of α-synuclein pathology. For these studies, we used the PFF model in which a single striatal injection of synthetic α-synuclein fibrils drives transmission of pathological misfolded α-synuclein resulting in Lewy body–like pathology in the interconnected nigrostriatal system (3). PFF mice develop progressive motor deficits, loss of striatal dopamine, and degeneration of TH+ dopaminergic neurons in the substantia nigra. For our studies, we used synthetic human α-synuclein fibrils prepared by in vitro aggregation and sonication as previously described (3). We first confirmed that drinking water administration of MCC950 could enter the brain, by treating mice (0.3 mg/ml) for 5 days and confirming presence of drug in perfused brain tissue (table S1). Separate mice were then inoculated with 8 μg of α-synuclein PFFs in the right dorsal striatum and were administered MCC950 in their drinking water. Control mice received an equivalent intrastral injection of PBS, the vehicle used for preparing α-synuclein fibrils. At 6 months, we performed a battery of standard motor function tests to evaluate parkinsonian motor deficits in PFF mice. Mice undergoing chronic MCC950 treatment showed improved performance on the rotarod (Fig. 5A). MCC950–treated PFF mice also displayed improved performance on the balance beam test, requiring less time to cross the beam compared to untreated PFF mice (Fig. 5B). Similar results were obtained with the wire-hang test (Fig. 5C) in which MCC950–treated PFF mice had latency times comparable to those of PBS–injected controls. We also used an automated open-field activity monitor to measure motor behavior parameters and subsequently quantified striatal dopamine and its metabolites at 8 months. In open-field assessments, we found increased rotational behavior in PFF mice compared to PBS–injected controls, which was reduced in MCC950–treated PFF mice (Fig. 5D). Similarly, stereotypic behavior was prevented in MCC950–treated PFF mice (Fig. 5D).
increased in PFF mice compared to PBS controls, which was reduced in the MCC950-treated PFF group (Fig. 5E).

Other open-field parameters such as average velocity, total distance traveled, or time spent in the center were not significantly different between any of the groups tested ($P > 0.05$; fig. S7, A to C), which is consistent with previous reports using this model (3). A separate cohort of PFF mice was also treated with MCC950, and motor deficits were examined at 8 months before neuro- stereology. Similar to the first cohort of mice, balance beam and wire-hang performances were found to be improved in MCC950-treated PFF mice at this 8-month termination point (Fig. 5, F and G, and movies S2 and S3).

Previous studies using the PFF model have shown that there is a progressive loss of dopaminergic neurons in the substantia nigra, accompanied by a reduction in striatal dopamine on the injected side (3). Consistent with these reports, in untreated PFF mice, we found a substantial reduction in striatal dopamine and dopamine metabolites at 8 months (Fig. 5H). In contrast, MCC950-treated PFF mice had significantly higher striatal dopamine concentrations ($P < 0.01$; Fig. 5H), indicating that they were protected against dopaminergic degeneration induced by $\alpha$-synuclein pathology. MCC950-treated mice also had higher dopamine metabolites DOPAC (Fig 5I) and HVA (Fig. 5I) compared to the untreated PFF mice. Next, unbiased stereological counts of TH$^+$ dopaminergic neurons in the substantia nigra were performed.
About 45% loss in TH⁺ neurons was observed in PFF mice compared to PBS-injected control mice (Fig. 5, K and L), which is comparable to previous reports in this model (3). MCC950-treated PFF mice had significantly more surviving TH⁺ neurons than untreated PFF mice (P < 0.01; Fig. 5, K and L). Together, these results indicate that chronic MCC950 administration can protect mice against progressive motor deficits and dopaminergic degeneration induced by pathological fibrillar α-synuclein aggregates in vivo.

**Pharmacological inhibition with MCC950 prevents α-synuclein pathology in the CNS**

The propagation of α-synuclein pathology in the CNS is considered to be crucial to the disease process in clinical PD (2–5, 32). The transmission of Lewy body pathology seen in PD is recapitulated in the PFF model after the inoculation of synthetic α-synuclein fibrils in wild-type mice and can be followed by quantifying the deposition of hyperphosphorylated serine 129 (pS129) α-synuclein (3, 5, 32).

We examined the transmission of pathological α-synuclein to regions outside of the striatum in our PFF mouse model by evaluating the presence of hyperphosphorylated α-synuclein in the substantia nigra and cortex at 8 months after PFF injection. We detected pS129 α-synuclein both in the nigral tract and in the extra-nigral regions of PFF mice (Fig. 8A and Fig. 8B, and fig. S9, A and B). Most of pS129⁺ α-synuclein inclusions in the substantia nigra not only were localized to TH⁺ dopaminergic neurons (Fig. 6A and fig. S10, A to F) but also were found to be localized to Iba-1⁺–stained microglia (Fig. 6B).
Moreover, pS129 α-synuclein colocalized with ubiquitin-positive aggregates (fig. S10, G to I), in line with previous findings in this model and in human PD (3). To evaluate whether inhibition of NLRP3 inflammasome activation could mitigate pathological hyperphosphorylated α-synuclein accumulation, we examined the brains of MCC950-treated PFF mice. PFF mice that were chronically dosed with MCC950 had markedly fewer pS129+ α-synuclein inclu-
sions both within the nigrostriatal system (Fig. 6, A, C, and D, and fig. S8) and in cortical brain regions (fig. S9) compared to untreated PFF mice. Total α-synuclein expression was not altered by MCC950 treatment (fig. S11). These findings therefore indicate that chronic NLRP3 activation contributes to the propagation of pS129 α-synuclein aggregates in the PFF mouse model in vivo and that pharmacological NLRP3 inhibition with MCC950 can effectively mitigate this central pathological process.

Because we previously found NLRP3 and ASC up-regulation at the site of α-synuclein PFF infusion at early stages of this model, we also examined the substantia nigra from 8-month-old PFF mice and found increased NLRP3 and ASC expression (Fig. 6, E to G), suggesting that there is a concomitant inflammasome pathology in line with the propagation of hyperphosphorylated α-synuclein. In addition, mice treated with MCC950 had reduced NLRP3 and ASC expression (Fig. 6, E to G). We also examined other key markers associated with neuroinflammation and α-synuclein pathology in PD and found reduced amounts of substantia nigra gp91phox (NOX2) and nitrated α-synuclein in MCC950-treated mice (Fig. 6, H and I). These results indicate that chronic NLRP3 inflammasome activation can contribute to downstream mechanisms of α-synuclein pathology.

**DISCUSSION**

The pathological hallmarks of progressive neurodegenerative diseases are the persistent accumulation and spread of disease-specific misfolded protein aggregates such as α-synuclein–rich Lewy bodies in PD. Although pathological involvement of misfolded protein aggregates in driving neurodegeneration and the accompanying
motor and/or cognitive deficits are now well recognized, the associated mechanisms remain unclear, making it difficult to design therapeutic approaches that are effective in vivo. Chronic activation of the NLRP3 inflammasome, which occurs in response to persistent misfolded proteins in the CNS, has emerged as a central neuroinflammatory mechanism that can drive neurodegeneration, making it an important therapeutic target (18–20). In PD, α-synuclein aggregates have been implicated as potential activators of the NLRP3 inflammasome (21), although direct in vivo evidence has been lacking. The clinical and experimental results outlined here suggest that the NLRP3 inflammasome might play a role in PD pathophysiology and progression. They also provide insight into the mechanisms of α-synuclein–mediated NLRP3 activation in mouse microglia that might be relevant to PD pathophysiology. In addition, our results demonstrate that, in multiple rodent models of PD, pharmacological inhibition of the NLRP3 inflammasome using an orally active CNS-permeable drug can protect against fibrillar α-synuclein pathology and prevent...
nigrostriatal dopaminergic degeneration driven by Lewy body–like pathology, as well as independently by mitochondrial dysfunction and oxidative stress.

Our results provide compelling clinical evidence for inflammasome activation in postmortem PD brains, confirming that key inflammasome markers are elevated in the substantia nigra of patients with PD. We have also confirmed that both NLRP3 and ASC localize to hypertrophic reactive microglia in patients with PD. Our mechanistic studies with α-synuclein fibrils demonstrate that the kinetics and NLRP3 activation profile induced by α-synuclein fibrils in mouse microglia are markedly different from conventional activators such as ATP and nigericin, indicating that α-synuclein fibrils trigger a strong but delayed activation of the NLRP3 inflammasome. The delayed activation observed with α-synuclein is likely due to the requirement for phagocytosis of α-synuclein to trigger the intracellular NLRP3 sensor (19, 33). Furthermore, α-synuclein–mediated NLRP3 activation failed to elicit IL-1β production from primary mouse microglia even at 24 hours, whereas NLRP3 activation with ATP resulted in IL-1β secretion within 1 hour. Conventional NLRP3 activation typically results in cell lysis via caspase-1–dependent pyroptosis, causing the release of stable polymeric ASC specks into the extracellular space. This, in turn, can drive further inflammasome activation and thereby propagate the inflammatory response (16, 26).

We observed substantial extracellular ASC release after NLRP3 activation with α-synuclein fibrils. However, α-synuclein treatment did not induce detectable microglial pyroptosis even at 24 hours, when the highest amounts of cleaved caspase-1, IL-1β, and ASC are detectible in the supernatant. In contrast, activation with nigericin readily induced caspase-1–dependent pyroptosis in microglia within 1 hour, similar to the macrophage response (16, 30). This suggests that the release of active IL-1β (p17), caspase-1 (p20), and ASC in the absence of pyroptosis is unique to NLRP3 activation with α-synuclein fibrils and that microglia are not intrinsically resistant to pyroptosis. Collectively, these findings provide insight into the neuroinflammatory pathology of PD, with potential therapeutic relevance given the emerging paradigm whereby extracellular ASC propagates inflammatory responses in vivo and can increase the formation of amyloid-β aggregates by acting as a cross-seed for misfolded proteins in the CNS (16, 20, 26). Our in vitro results using the NLRP3 inhibitor MCC950 demonstrate that nanomolar doses of drug can prevent ATP- and nigericin–mediated NLRP3 activation in microglia. Pretreatment with 100 nM of MCC950 almost completely blocked fibrillar α-synuclein–mediated NLRP3 activation and release of active IL-1β and caspase-1 (p20). In addition, MCC950 also blocked ASC release from microglia after NLRP3 activation by α-synuclein fibrils. Given that extracellular ASC release by microglia has been shown to cross-seed amyloid protein aggregates and propagate neuropathology (20), our mechanistic findings suggest that inhibition with MCC950 might be effective in blocking this key downstream pathological process.

Although α-synuclein pathology is now considered central to the spread of disease pathophysiology in clinical PD, there is also a strong clinical and experimental evidence for mitochondrial dysfunction, oxidative stress, and proteasomal impairment as key mechanisms that can initiate and drive dopaminergic neuron loss (2, 4, 34). For example, large-scale genome-wide association studies have shown that peroxisome proliferator–activated receptor γ coactivator 1α–responsive genes, which regulate mitochondrial biogenesis and cellular bioenergetics, are specifically down-regulated in patients with PD (24). Similarly, the loss of functional mitochondrial TFAM signaling results in progressive dopaminergic degeneration (25). Our findings in the 6-OHDA and MitoPark models confirm that NLRP3 inflammasome activation in the nigrostriatal system can also occur in the absence of α-synuclein pathology. Oral MCC950 treatment effectively blocked NLRP3 activation in all PD models that we tested, irrespective of the distinct upstream triggers in these models. Furthermore, functional inhibition of NLRP3 with MCC950 resulted in substantial neuroprotection in the 6-OHDA model, demonstrating that NLRP3 is also a key driver of neuropathology triggered by mitochondrial dysfunction and reactive oxygen species.

Although neurotoxicant models are useful for preclinical validation of new therapeutic agents, they do not recapitulate the relentless spread of α-synuclein–mediated Lewy body pathology that occurs in human PD and that is considered central to disease progression (35). Most of disease-modifying PD therapeutics that have been developed and validated using neurotoxicant models have failed to show efficacy in the clinic, underscoring the importance of targeting α-synuclein–driven pathology in PD (35). Until recently, the lack of robust preclinical models that reproduced the clinical features of α-synuclein pathology and progressive dopaminergic degeneration has limited the development of effective therapeutic strategies (35). The PFF model recapitulates many of the cardinal features of Lewy body pathology in PD and has been used to model the mechanisms of α-synuclein pathology that drive dopaminergic degeneration (3, 32).

Our results demonstrate that NLRP3 inflammasome activation occurs in the PFF model at early stages of neuropathology and support a crucial role for chronic microglial NLRP3–driven mechanisms in the propagation of fibrillar α-synuclein pathology (8). These findings are consistent with previous studies in transgenic AD mice lacking NLRP3 and ASC, which reported reduced amyloid-β deposition and enhanced microglial clearance of amyloid-β plaques (19, 20). Chronic NLRP3 inhibition with orally active MCC950 protected against motor deficits and nigrostriatal dopaminergic degeneration and markedly reduced the amount of hyperphosphorylated α-synuclein aggregates both within and outside of the nigrostriatal system in PFF mice, further underscoring the importance of chronic NLRP3 activation in propagating α-synuclein pathology in this preclinical model.

The NLRP3 inhibitor used in our studies, MCC950 (originally referred to as CP-465773), comes from a class of sulfonfonylurea-containing compounds initially reported as inhibitors of IL-1β posttranslational processing (36). Compared to other chemical classes reported to inhibit NLRP3 activation with micromolar potency, MCC950 and its analogs (37, 38) have nanomolar potency, with very high target selectivity. Data obtained from a U.S. Environmental Protection Agency ToxCast/Tox21 screen (39) indicate that MCC950/CP-465773 and a close chemical analog have extremely limited and very weak (>10 μM) off-target activity (table S2). Our data clearly demonstrate that the compound can effectively cross the blood-brain barrier, rendering it a good lead for medicinal chemistry optimization toward an optimized lead candidate suitable for CNS diseases. Although MCC950 itself cannot be commercialized because of previous lapsed patents, we have developed numerous analogs (37, 38) with improved chemical properties (table S3). These could lay the groundwork for future clinical translation of a therapy to treat neurodegenerative diseases.

There are important limitations and caveats with our study and findings. For example, our results do not establish the precise mechanisms by which sustained NLRP3 activation can drive α-synuclein...
pathology and dopaminergic degeneration. Recent reports have shown that activated inflammasomes components such as prionoid ASC can directly interact with and seed misfolded proteins (20). Similarly, studies in NLRP3 knockout mice indicate that sustained inflammasome activation can impair the clearance of pathological protein aggregates, causing their accumulation (19). It is also plausible that secreted inflammasome mediators could be directly neurotoxic to dopaminergic neurons. Additional studies would be required to clarify these mechanisms. Another important consideration with our study is that MCC950 was administered before the induction of neuropathology in rodents; therefore, treatment studies initiated after disease onset would be required to conclusively establish whether MCC950 can rescue or halt ongoing neuropathology, which would be more clinically relevant in late-stage PD.

Current therapies for PD, including levodopa treatment and deep brain stimulation, can manage symptoms but have little to no impact on the underlying disease pathology. Therefore, there is an urgent need to develop therapeutic strategies that halt or impede disease progression (1). Our results demonstrate that the NLRP3 inflammasome plays a key role in PD-like pathophysiology in rodents and might represent a feasible therapeutic target to mitigate neurotoxic α-synuclein pathology and the resulting nigrostriatal dopaminergic neuron loss in PD. The potency and specificity of MCC950 for NLRP3 inhibition in the CNS, combined with its neuroprotective efficacy and safety profile after long-term oral dosing, make it an excellent lead for optimization of drug candidates and potential clinical translation in PD.

MATERIALS AND METHODS

Study design
Studies were primarily designed (i) to determine whether increased NLRP3 inflammasome activation occurs in patients with PD and in distinct experimental PD models and (ii) to test the efficacy and CNS penetration of the small-molecule NLRP3 inhibitor, MCC950, in experimental models of α-synuclein pathology and dopaminergic degeneration relevant to PD. For in vivo studies, age-matched littermates were randomly assigned to drug or vehicle treatment groups. An additional study was performed with at least three replicates per study in addition to pilot optimization studies for dose and time point determination. Mice were humanely euthanized at defined study end points, and all experimental procedures were carried out in accordance with local institutional animal ethics approvals.

Statistical analysis
Statistical analyses were performed using GraphPad Prism 7.0 (GraphPad). Normally distributed data were analyzed using a two-tailed Student’s t test when comparing two groups or a one-way or two-way ANOVA with Bonferroni’s multiple comparison post hoc test when comparing more than two groups. For nonnormally distributed data (Shapiro-Wilk test, P < 0.05), a nonparametric two-tailed Mann-Whitney U test was used when comparing two groups or a Kruskal-Wallis test with Dunn’s multiple comparison post hoc test when assessing more than two groups. Differences with P < 0.05 were considered statistically significant. All data are presented as the mean ± SEM if not mentioned otherwise.

SUPPLEMENTARY MATERIALS

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MATERIALS AND METHODS

Fig. 5. Inflammasome components are up-regulated in patients with PD.
Fig. 52. Inflammasome components are up-regulated, and activation occurs via NLRP3 in the 6-OHDA mouse model of PD.
Fig. 53. NLRP3 and ASC are up-regulated in the MitoPark mouse model of PD.
Fig. 54. NLRP3 is expressed by microglia in the α-synuclein PFF mouse model.
Fig. 55. Validation of α-synuclein PFFs.
Fig. 56. Validation of MCC950 as a potent inhibitor of NLRP3 inflammasome activation in microglia.
Fig. 57. Additional open-field activity measurements in PFF mice.
Fig. 58. Representative images of hyperphosphorylated α-synuclein staining in the substantia nigra of PFF mice.
Fig. 59. Representative images and quantitation of hyperphosphorylated α-synuclein staining in the cortex of PFF mice.
Fig. 60. Hyperphosphorylated α-synuclein is present within dopaminergic neurons and is associated with ubiquitin in the PFF mouse model.
Fig. 61. Total α-synuclein is not altered in PFF mice treated with MCC950.
Table S1. Brain and plasma concentrations of MCC950 after 5-day drinking water administration (0.3 mg/ml) to mice.
Table S2. Off-target activity of MCC950 and a structural analog from ToxCast/Tox21 datasets.
Table S3. Comparison of chemical and pharmacological properties of novel MCC950 analogs.
Table S4. Primary data (Excel file).
Movie S1. Representative amphetamine-induced rotation videos of 6-OHDA mice with and without MCC950 treatment.
Movie S2. Representative balance beam videos of PFF mice with and without MCC950 treatment.
Movie S3. Representative wire-hang videos of PFF mice with and without MCC950 treatment.

REFERENCES AND NOTES

MPTP-treated mice is not restricted to the nigrostriatal system: Microgliosis and B. M. A. targets Nod-like receptor protein 3 inflammasome to modulate neuroinflammation in D. J. 84–97 (2014). Triggers of inflammasome by aggregated D. H. 213–215 (2015). NLRP3 inhibitors that propagate inflammation. V. K. performed animal studies and provided guidance to M.R.L. who performed the MitoPark experiments. D.B.R. collected the human plasma samples and patient information. L.A.O. and K.S. provided guidance on NLRP3 mechanistic studies. R.G. and T.M.W. wrote the manuscript with input from K.S. and M.A.C., and all authors edited the final manuscript. Competing interests: A.A.B.R., L.A.O., K.S., and M.A.C. are co-inventors on patent applications for NLRP3 inhibitors from K.S. and M.A.C., and all authors edited the final manuscript. Author contributions: R.G., M.A.C., and T.M.W. conceived the work was supported by the National Health and Medical Research Council of Australia (NHMRC: project grant 1086786 to T.M.W., M.A.C., L.A.O., K.S., and A.A.B.R.), The Michael J. Fox Foundation for Parkinson’s Research and the Shake It Up Australia Foundation (grants 9916 and 12626 to T.M.W., M.A.C., K.S., R.G. and A.A.B.R.), and the NIH (R01 grants NINDS NS001090, NEIHS ES026892, and NEIHS NS088206 to A.A.G.K.). T.M.W. was supported by an NHMRC career development fellowship (1105420). R.G. was supported by an Advance Queensland mid-career fellowship. K.S. was supported by the Australian Research Council and by NHMRC fellowships (FT13010061 and 114113). Author contributions: R.G., M.A.C., and T.M.W. conceived the project, with K.S. assisting in the study design and the provision of NLRP3 reagents. R.G. and T.M.W. designed studies, and R.G. performed most of the experiments and data analysis together with E.A.A. and with help from D.C.C. and S.M. A.A.B.R. synthesized the MCC950, M.S.B. performed the MCC950 liquid chromatography–mass spectrometry, and both assisted on pharmacokinetic studies. V.K. performed dopamine studies and analysis. A.G.K. assisted on animal studies and provided guidance to M.R.L. who performed the MitoPark experiments. D.B.R. collected the human plasma samples and patient information. L.A.O. and K.S. provided guidance on NLRP3 mechanistic studies. R.G. and T.M.W. wrote the manuscript with input from K.S. and M.A.C., and all authors edited the final manuscript. Competing interests: A.A.B.R., L.A.O., K.S., and M.A.C. are co-inventors on patent applications for NLRP3 inhibitors that have been licensed to inflaZone Ltd, a biotechnology company that develops drugs to address unmet clinical needs in inflammatory disease by targeting inflammasomes (WO/2016/131098 and WO/2017/140778). Sulforaphene and related compounds and use of same). M.A.C. and L.A.O. are co-founders and shareholders of InflaZone and are acting as CEO and CSO, respectively. M.A.C. also holds a fractional Professorial Research Fellow appointment at The University of Queensland. K.S. was a member of the Scientific Advisory Board for InflaZone Ltd. from 2016 to 2017. All other authors declare that they have no competing interests. Data and materials availability: All the data are included in the main text or in the Supplementary Materials.
Inflammasome inhibition prevents α-synuclein pathology and dopaminergic neurodegeneration in mice


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Systemic inflammasome inhibitor for Parkinson’s disease

Brain accumulation of misfolded α-synuclein, progressive loss of dopaminergic neurons, and neuroinflammation are major hallmarks of Parkinson’s disease (PD). Although neuroinflammation has been shown to contribute to the pathophysiology of the disease, the mechanisms mediating the activation of inflammatory signals remain unclear. Gordon and colleagues now show that, in mouse models of PD, α-synuclein aggregates promote inflammasome activation in brain microglia. Oral treatment with an inflammasome inhibitor improved motor performance and reduced neuroinflammation, neurodegeneration, and α-synuclein accumulation in mouse models of α-synuclein-mediated toxicity. The results suggest that systemic delivery of inflammasome inhibitors might have therapeutic effects in PD.

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