Manganese activates NLRP3 inflammasome signaling and propagates exosomal release of ASC in microglial cells

Souvarish Sarkar1*, Dharmin Rokad1, Emir Malovic1, Jie Luo1, Dilshan S. Harischandra1, Huajun Jin1, Vellareddy Anantharam1, Xuemei Huang2, Mechelle Lewis2, Arthi Kanthasamy1, Anumantha G. Kanthasamy1†

Chronic, sustained inflammation underlies many pathological conditions, including neurodegenerative diseases. Divalent manganese (Mn2+) exposure can stimulate neurotoxicity by increasing inflammation. In this study, we examined whether Mn2+ activates the multiprotein NLRP3 inflammasome complex to promote neuroinflammation. Exposing activated mouse microglial cells to Mn2+ substantially augmented NLRP3 abundance, caspase-1 cleavage, and maturation of the inflammatory cytokine interleukin-1β (IL-1β). Exposure of mice to Mn2+ had similar effects in brain microglial cells. Furthermore, Mn2+ impaired mitochondrial ATP generation, basal respiratory rate, and spare capacity in microglial cells. These data suggest that Mn-induced mitochondrial defects drove the inflammasome signal amplification. We found that Mn induced cell-to-cell transfer of the inflammasome adaptor protein ASC in exosomes. Furthermore, primed microglial cells exposed to exosomes from Mn-treated mice released more IL-1β than did cells exposed to exosomes from control-treated animals. We also observed that welders exposed to manganese-containing fumes had plasma exosomes that contained more ASC than did those from a matched control group. Together, these results suggest that the divalent metal manganese acts as a key amplifier of NLRP3 inflammasome signaling and exosomal ASC release.

INTRODUCTION

Hyperactivation of immune cells, which leads to the production of a plethora of proinflammatory factors, is a key pathophysiological hallmark of multiple disorders including neurodegenerative disorders, autoimmune diseases, and inflammatory bowel syndrome (1–3). Although inflammation exacerbates multiple pathological conditions, the signaling cascade underlying this hyperactivated state is unclear. The etiology of neurodegenerative disorders including Alzheimer’s disease (AD) and Parkinson’s disease (PD) is mult factorial. Microglial cells, as well as various inflammatory factors, including tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β), are increased in patients with PD (1, 4, 5). Blocking microglial activation is neuroprotective in animal models of PD (5). Furthermore, use of anti-inflammatory agents is protective in cell culture and animal models of PD (6–10). Use of anti-inflammatory drugs reduces the incidence of PD (11). Although the effects of chronic inflammation are well documented in neurodegenerative disorders such as PD, the mechanism underlying this sustained immune response remains unclear (12).

Inflammasome activation is a key determinant of chronic inflammation in a variety of inflammatory cells (13) and pathological conditions. A multimeric protein complex, inflammasomes that contain NLRP3 (nucleotide binding and oligomerization domain–like receptor family pyrin domain–containing 3), NLRC4 (NLR family CARD domain–containing protein 4), and AIM2 (absent in melanoma 2) among others, is involved in neurodegenerative disorders such as PD, AD, and traumatic brain injury (TBI) (14). The NLRP3 inflammasome oligomeric complex is composed of an adapter protein ASC (apoptosis-associated speck–like protein containing a CARD), caspase-1, and NLRP3. Multiple external signals, including misfolded β-amyloid (15) and αSynPF (16), activate the NLRP3 inflammasome complex. NLRP3 inflammasome signaling requires two signals for its activation. Lipopolysaccharide (LPS), TNF-α, or any signal capable of activating nuclear factor κB (NFκB) can act as signal 1 (or a priming signal) to increase the expression of NLRP3 and pro–IL-1β. A second activation signal, such as mitochondrial dysfunction and lysosomal damage, that is known as signal 2 triggers the assembly and activation of the NLRP3 inflammasome complex. The adapter molecule ASC links the NLRP3 protein to the recruited caspase-1, which promotes caspase-1 dimerization, autocalytic processing, and the generation of active caspase-1. Active caspase-1 processes pro–IL-1β into its mature IL-1β form that stimulates inflammation (17, 18). NLRP3 inflammasome propagation is modulated by a cell-to-cell transmission of the ASC adaptor protein by an unknown mechanism (19). However, the exact mechanisms of inflammasome activation and propagation remain elusive.

Chronic exposure to transition metals like manganese (Mn), iron (Fe), copper (Cu), and zinc (Zn) from occupational or environmental sources (e.g., various food products and well water) is linked to various neurodegenerative disorders such as AD and PD. Chronic exposure to Mn in occupational settings is observed in miners (20, 21) and welders (22). Occupational exposure to Mn increases the risk of developing PD in welder populations (23). Mn toxicity can also manifest as a disease called manganism, which shares similar symptoms as PD (24, 25). Mn accumulates mainly in the globus pallidus and in the striatum leading to Parkinsonian neurological deficits (26).

Mn toxicity is well documented in neurodegeneration and glial dysfunction (26–31). More specifically, Mn induces TNF-α and inducible nitrogen synthase (iNOS) in astrocytes (32, 33), which causes
mitochondrial dysfunction by accumulating in mitochondria (34). In addition, iNOS induces neurotoxicity in a rat model of Mn neurotoxicity by modulating microgliosis (35). Mn exposure induces classical activation (M1 activation) in microglial cells (36) and astrocytes (31) in the central nervous system (CNS), leading to the production of proinflammatory cytokines. A plethora of proinflammatory factors causes neuronal damage (30). Furthermore, Mn enhances LPS-induced production of proinflammatory factors in microglial cells (29). Mn-induced microglial activation also activates astrocytes, leading to enhanced production of proinflammatory factors (37). However, the molecular signaling mechanism of Mn-induced microglial activation is still not well understood. Microglia express multiple functional inflammasomes, including NLRP3, NLRC4, and AIM2 inflammasomes (13). However, the effect of Mn on microglial inflammasome activation and propagation remains ill-defined.

In this study, we characterized the role of Mn in inflammasome activation in microglial cells. We found that Mn acted as signal 2 to activate the NLRP3 inflammasome in microglial cells, which stimulated mitochondrial dysfunction in microglial cells. Mn exposure reduced the abundance of mitochondrial fusion protein 2 (also Mitofusin 2 or Mfn2) and vacuolar protein sorting-associated protein 35 (VPS35), a retromer complex protein that promotes Mfn2 ubiquitination. Uptake of ASC-containing exosomes played a role in Mn-induced inflammasome propagation. Lastly, welder serum exosomes had a higher ASC load compared to age-matched controls, and welder serum had increased amounts of serum proinflammatory cytokines.

RESULTS
Mn induces NLRP3 inflammasome activation in microglial cells in vitro and in vivo
The NLRP3 inflammasome can be activated by various external signals including adenosine triphosphate (ATP), pathogen-activated molecular patterns, and danger-associated molecular patterns (15). Because the role of environmental PD neurotoxicants, such as Mn, in inducing inflammasome activation has yet to be described, we investigated the ability of Mn to stimulate the processing and release of IL-1β and caspase-1 cleavage from LPS-primed microglial cells. Microglial cells were primed with LPS for 3 hours after which the cells were treated with Mn for a further 24 hours. We found no detectable mature IL-1β protein in unstimulated, only LPS-primed, or only Mn-treated cells (Fig. 1A). Mature IL-1β was only detected in LPS-primed, Mn-treated microglial cells (Fig. 1A). Similarly, a multiplexed Luminex assay confirmed that LPS-primed microglial cells released IL-1β after Mn exposure in a dose-dependent manner (Fig. 1B). However, Mn treatment alone had no effect on IL-1β processing and release. Furthermore, Western blot analysis revealed that Mn stimulated the cleavage of caspase-1 in LPS-primed cells when compared to either unstimulated or LPS-primed cells (Fig. 1A). These results confirmed that Mn exposure served as signal 2 to augment microglial inflammasome signaling (38).

ASC, a component of inflammasome activation, can form speck-like structures that propagate inflammasome activation from cell to cell (19). Using immunofluorescence analysis, we found that ASC was evenly distributed throughout unstimulated or LPS-primed cells. In contrast, the LPS-primed microglial cells exposed to Mn formed intense ASC specks, which are indicative of inflammasome activation (Fig. 1C). When we tested by Western blot which inflammasome was activated by Mn, we found that NLRP3 abundance significantly increased in LPS-primed and Mn-treated, LPS-primed microglial cells (Fig. 1D). Immunocytochemical (ICC) analysis further confirmed that NLRP3 abundance was increased (Fig. 1E) by LPS priming of microglial cells. Similarly, quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) analysis showed that LPS increased mRNA expression of pro–IL-1β in microglial cells and treatment with various doses of Mn did not further augment expression (Fig. 1F). Furthermore, using qRT-PCR analysis, we found that LPS priming and LPS + Mn increased NLRP3 mRNA expression but not expression of AIM2 and NLRC4 (Fig. 1G). Mn-treated, unprimed cells did not increase expression of pro–IL-1β or NLRP3, indicating that Mn exposure had no effect on the priming step of the NLRP3 inflammasome. To further verify the specificity of Mn in metal-induced inflammasome activation, we treated primary microglial cells with Mn, Cu, and Zn. Because Zn can be toxic to microglial cells, a nontoxic dose of Zn was used (fig. S1A). Cu was used at the same concentration as Mn. Our Luminex analysis revealed that Mn increased IL-1β release in LPS-primed cells, whereas Cu and Zn did not (fig. S1B). In contrast, LPS-treated cells stimulated with Mn, Cu, or Zn released comparable amounts of TNF-α (fig. S1C). These data validated that inflammasome activation was not a property of all divalent transition metals. Moreover, Mn exposure increased LPS-induced Nos2 mRNA expression (fig. S2A) and protein abundance (fig. S2, B and C), as well as nitrite release (fig. S2D). All these findings together showed that Mn activated the NLRP3 inflammasome in primed microglial cells.

The etiology of PD is complex and multifactorial, and gene-environment interaction is likely involved in PD pathogenesis (39–41). Aggregated αSyn is an integral component of PD-related Lewy bodies and Lewy neurites, which can induce NLRP3 inflammasome activation (16). We hypothesized that Mn may augment αSynPFF-induced microglial NLRP3 inflammasome activation. Co-treatment of Mn with αSynPFF increased the abundance of αSynPFF-induced NLRP3 and iNOS proteins (Fig. 2A). Luminex assay revealed that Mn further increased the αSynPFF-induced release of IL-1β (Fig. 2B) but not IL-6 (fig. S3A) or TNF-α (fig. S3B). These findings indicate that Mn increased αSynPFF-induced inflammasome activation in microglial cells.

We verified that Mn can promote NLRP3 inflammasome-mediated inflammation in an animal model of Mn neurotoxicity. Mn exposure induces a neurotoxic effect (42) and production of factors like TNF-α from neurons (43), which can potentially act as signal 1. These stress signals can activate microglial cells. Hence, in an in vivo scenario, Mn may act as both signal 1 and signal 2. When C57BL/6 mice were orally exposed to Mn for 30 days, we found that Mn specifically induced NLRP3 mRNA expression, but not AIM2 or NLRC4, as revealed by qRT-PCR (Fig. 2C). Western blot analysis showed that Mn significantly increased caspase-1 cleavage and mature IL-1β (Fig. 2D), although we did not observe increased abundance of pro–IL-1β. The Mn-induced increase in cleaved caspase-1 was strongly correlated with an increase in the amount of released IL-1β (fig. S4). Immunohistochemistry (IHC) studies further revealed that this NLRP3 increase occurred in ionized calcium-binding adapter molecule 1 (IBA1, a microglial marker)–positive cells (Fig. 2E). Collectively, all these findings showed that Mn activated the NLRP3 inflammasome in microglial cells both in vitro and in vivo. This NLRP3 signaling cascade could be the putative mechanism by which chronic inflammation is sustained in Mn-induced Parkinsonism.
Fig. 1. Manganese treatment induced NLRP3 inflammasome activation in microglial cells in vitro. (A) Western blot analysis of caspase-1 cleavage and IL-1β maturation in lysates from an LPS-primed microglial cell line treated with Mn, as indicated. Blots (left) are representative of four independent experiments. Normalized band intensity data (right) are means ± SEM from all experiments. Con, control. (B) Luminex analysis of IL-1β production by a microglial cell line primed with LPS and treated for 24 hours with Mn at the indicated dose. Data are means ± SEM pooled from eight independent experiments. (C) Immunofluorescence microscopy analysis of ASC specks formation in a microglial cell line primed with LPS and treated as indicated. Images (left) are representative of three independent experiments. Quantified data (right) are means ± SEM from all experiments. Scale bar, 20 μm. (D and E) Western blot (D) and immunofluorescence microscopy (E) analyses of NLRP3 abundance in a microglial cell line primed with LPS and treated as indicated. Blots and images are representative of three independent experiments. Normalized band intensity data (D, bottom) are means ± SEM from all experiments. Scale bar, 20 μm. (F and G) qRT-PCR analysis of IL-1β (F) and NLRP3, NLRC4, and AIM2 (G) mRNA expression after treatment of an LPS-primed microglial cell line. Data are means ± SEM pooled from three independent experiments. *P < 0.05, ***P < 0.001 by analysis of variance (ANOVA) with Tukey post hoc analysis.
Fig. 2. Manganese exposure induced NLRP3 inflammasome activation in microglial cells in vivo. (A) Western blot analysis of the NLRP3 and iNOS expression in wild-type microglial cells treated with Mn and αSyn Agg as indicated. Blots (left) are representative of three independent experiments. Normalized band intensity data (right) are means ± SEM from all experiments. (B) Luminex analysis of IL-1β production by WT microglial cells treated with Mn and αSyn Agg as indicated. Data are means ± SEM pooled from four independent experiments. (C) qRT-PCR analysis of NLRP3, NLRC4, and AIM2 mRNA expression in the striata of C57BL mice exposed to Mn for 30 days. Data are means ± SEM pooled from five mice from three experiments. (D) Western blot analysis of caspase-1 cleavage and IL-1β maturation in lysates from striatum samples from mice treated as indicated. Blots (top) are representative of six mice from three experiments. Normalized band intensity data (bottom) are means ± SEM from all experiments. (E) Immunofluorescence microscopy analysis of NLRP3 abundance in IBA1-positive microglial cells in the striatal region of mice treated as indicated. Images are representative of three mice from three experiments. Scale bar, 15 μm. *P < 0.05 and ***P < 0.001 by ANOVA with Tukey post hoc analysis.
Mn leads to mitochondrial dysfunction in primed microglial cells

Although the mechanisms of inflammasome activation are currently unclear, mitochondrial dysfunction and mitochondrial superoxide generation may stimulate inflammasome activation (17, 18, 44–46). Because Mn severely impairs mitochondria under stress conditions (47–50), we tested the effects of Mn on mitochondrial bioenergetics of LPS-primed microglia. Primary microglial cells were primed with LPS for 3 hours, followed by 24 hours of treatment with Mn before analysis of oxygen consumption and extracellular acidification on a Seahorse Bioscience extracellular flux analyzer. Exposing LPS-primed microglia to Mn significantly impaired mitochondrial function (Fig. 3A), as indicated by the decreased mitochondrial ATP production (Fig. 3B), basal respiration rate (Fig. 3C), maximal respiration (Fig. 3D), and spare respiratory capacity (Fig. 3E) of the cells after exposure to Mn. Mn exposure in unprimed microglial cells showed no substantial changes in mitochondrial dynamics. This is consistent with no increase in oxidative stress in N9 microglia after a high-concentration Mn exposure (29). Exposure of LPS-primed primary microglia to Mn significantly decreased the stressed oxygen consumption rate (OCR) without changing the stressed extracellular acidification rate (ECAR), as measured by the cell energy phenotype test (fig. S5). These data suggest that Mn exposure reduced the mitochondrial metabolism rate without modulating nonmitochondrial function.

Mitochondrial dysfunction can lead to superoxide generation. When we assayed reactive oxygen species activity using MitoSoX staining, we found that LPS-primed microglial cells treated with Mn for 24 hours generated substantial amounts of superoxide (Fig. 3F), as expected. These data further confirmed the effects of Mn treatment on mitochondrial dysfunction in LPS-primed microglia. Because LPS-primed cells and unprimed microglial cells exposed to Mn both exhibited minimal or no changes in mitochondrial superoxide production (Fig. 3F), we hypothesized that LPS may increase Mn transporters in microglia. Our qRT-PCR analysis revealed that LPS activation significantly increased the Mn importer SLC11A2 (fig. S6A), whereas Mn exposure alone stimulated expression of the Mn exporter SLC30A10 (fig. S6B) without changing importer expression. Mn uptake was increased in LPS-primed, Mn-treated microglial cells when compared to unprimed Mn-treated cells, as determined by inductively coupled plasma mass spectroscopy (ICP-MS) analysis (fig. S6C). These data suggest that LPS priming increased the uptake of Mn and Mn transporter expression. Further studies in transporter knockdown (KD) cells are required to understand the mechanism of Mn uptake in LPS-treated microglia.

Mfn2 protein promotes mitochondrial quality control by eliminating damaged mitochondria using mitochondrial fusion (51–53). Although our qRT-PCR analysis of LPS-primed microglial cells showed no significant change in Mfn2 expression (Fig. 3G), our immunofluorescence (Fig. 3H) and Western blot (Fig. 3I) analyses showed that Mfn2 abundance was decreased in primed microglial cells treated with Mn. These data suggest that the Mn-induced mitochondrial dysfunction in primed microglia may be due to Mfn2 degradation. One candidate for ubiquitinating Mfn2 is mitochondrial ubiquitin ligase 1 (Mul1). The mRNA expression of Mul1 in Mn-treated, LPS-primed cells doubled when compared to other groups (Fig. 3J). Furthermore, the increased expression of Mul1 was Mn dose-dependent (fig. S7). ICC analysis also indicated an increase in the Mul1 protein abundance in Mn-treated, LPS-primed cells (Fig. 3K). Collectively, these data suggest that Mn-induced inflammasome activation in microglia was related to mitochondrial dysfunction and may be promoted by Mul1-mediated degradation of Mfn2.

Retromer complex proteins are involved in Mn-induced mitochondrial dysfunction in LPS-primed microglia

Mutating or reducing the abundance of the retromer complex protein VPS35 (Park 17) induces mitochondrial dysfunction by increasing Mul1 in dopaminergic neurons (54). Because we observed that Mn exposure increased the amount of Mul1 expressed in LPS-primed microglia, we further investigated whether this increased Mul1 might be due to decreased expression of VPS35. We treated an LPS-primed microglial cell line with Mn for 24 hours in preparation for qRT-PCR, ICC, and immunoblot analyses. Our qRT-PCR analysis revealed that Mn exposure reduced VPS35 mRNA expression in LPS-primed microglial cells when compared to other groups (Fig. 4A). Expression of another retromer complex protein, VPS29, was also decreased in primed microglial cells treated with Mn (Fig. 4B). Furthermore, ICC (Fig. 4C) and Western blot (Fig. 4D) analyses confirmed that VPS35 is diminished in Mn-exposed, LPS-primed microglial cells. We further validated our cell culture findings in an animal model of Mn toxicity. Mice were similarly treated with Mn as described above. Striatal VPS35 mRNA expression decreased in Mn-gavaged mice (Fig. 4E).

To further elucidate the role of VPS35 in maintaining microglial mitochondria, we developed a stable VPS35 KD microglial cell line using the CRISPR-Cas9 system (fig. S8A). Our Western blot analysis showed that VPS35 KD cells have less Mfn2 (Fig. 4F). Mn treatment enhanced the loss of Mfn2 in VPS35 KD cells (fig. S8B). Moreover, qRT-PCR revealed that Mul1 mRNA was significantly elevated in the VPS35 KD cells (Fig. 4G). These data collectively suggest that VPS35 is upstream of the Mfn2 and Mul1 pathway and played a role in maintaining mitochondrial integrity.

Exosomes mediate Mn-induced cell-to-cell transmission of the inflammasome component ASC in LPS-primed microglia

A major inflammasome adaptor component ASC propagates inflammasome activation through cell-to-cell transmission (19). We hypothesized that ASC may migrate from one cell to another through exosomes and that Mn may promote inflammasome propagation by increasing ASC-loading in exosomes. To determine whether exosomal uptake plays a role in inflammasome propagation, we used the CRISPR-Cas9 system to knock down the endocytosis mediators, caveolin and clathrin, that are responsible for distinct pathways of exosomal uptake in microglial cells. Control, Cav1-KD, and Cltc-KD microglial cells were treated with Mn for 24 hours after LPS priming. Reduced caveolin expression inhibited Mn-stimulated expression of pro–IL-1β (Fig. 5A) and NLRP3 (Fig. 5B) mRNA in LPS-primed cells. The expression of Nos2 mRNA was reduced when both caveolin and clathrin abundance were limited (fig. S9A). Furthermore, Luminex assay revealed that Mn-exposed, LPS-primed Cav1-KD and Cltc-KD cells produced significantly less mature IL-1β (Fig. 5C), without affecting TNF-α abundance (fig. S9B). These data suggest that endocytosis played a role in inflammasome activation and that limiting exosomal uptake reduced Mn-induced inflammasome propagation.

To further identify the importance of exosome cargo for inflammasome propagation, we isolated exosomes from treatment medium of microglial cells treated with Mn for 24 hours. Immunoblot analysis
Fig. 3. Mn treatment induced mitochondrial damage by modulating Mfn2 and Mul1 expression in microglial cells. (A to E) Seahorse analysis of the OCR in Mn-exposed LPS-primed primary microglial cells. Data were analyzed to extrapolate mitochondrial ATP production (B), basal respiration rate (C), maximal respiration (D), and spare respiratory capacity (E). Data are means ± SEM pooled from five independent experiments. (F) Immunofluorescence microscopy analysis of mitochondrial superoxide generation from microglial cells primed with LPS and exposed to Mn. Images are representative of three independent experiments. Scale bar, 100 μm. (G) qRT-PCR analysis of Mfn2 mRNA expression in Mn-exposed microglial cells. Data are means ± SEM pooled from three independent experiments. (H and I) Immunofluorescence microscopy (H) and Western blot analysis (I) of Mfn2 abundance in microglial cells primed with LPS and exposed to Mn. Images and blots are representative of four experiments. Normalized band intensity data (I, bottom) are means ± SEM from all experiments. Scale bar, 100 μm. (J) qRT-PCR analysis of Mul1 mRNA expression in Mn-exposed microglial cells. Data are means ± SEM pooled from three independent experiments. (K) Immunofluorescence microscopy analysis measuring Mul1 protein on Mn exposure. Images are representative of three independent experiments. Scale bar, 20 μm. *P < 0.05 and ***P < 0.001 by ANOVA with Tukey post hoc analysis.
Fig. 4. VPS35, a retromer complex protein, can modulate Mfn2 degradation during Mn-induced inflammasome activation. (A and B) qRT-PCR analysis of the retromer complex components VPS35 and VPS29 mRNA expression. Data are means ± SEM pooled from three independent experiments. (C and D) Immunofluorescence microscopy (C) and Western blot (D) analysis of VPS35 abundance in microglial cells exposed to Mn. Images and blots are representative of three independent experiments. Normalized band intensity data (D, bottom) are means ± SEM from all experiments. Scale bar, 20 μm. (E) qRT-PCR analysis of VPS35 mRNA expression in the striatum of mice exposed to Mn. Data are means ± SEM pooled from six mice from three independent experiments. (F) Western blot analysis of Mfn2 abundance in lysates from wild-type and VPS35 CRISPR-Cas9 KD microglial cells. Blots are representative of four independent experiments. Normalized band intensity data (D, bottom) are means ± SEM from all experiments. (G) qRT-PCR analysis of Mul1 mRNA expression in VPS35 KD microglial cells compared to wild-type cells. Data are means ± SEM pooled from three independent experiments. *P < 0.05, **P < 0.01, ***P < 0.001 by ANOVA with Tukey post hoc analysis.
Fig. 5. Exosomal release of the inflammasome component ASC propagates Mn-induced inflammasome activation. (A and B) qRT-PCR analysis of pro–IL-1β (A) and NLRP3 (B) mRNA expression in wild-type (WT) and Cav1-KD microglial cells after Mn treatment. Data are means ± SEM pooled from three independent experiments. (C) Luminex analysis of IL-1β secreted by wild-type and Cav1-KD or Cltc-KD microglial cells after Mn treatment. Data are means ± SEM pooled from eight independent experiments. (D) Western blot analysis of ASC abundance in lysates of exosomes (exo) isolated from Mn-treated microglial cells treated as indicated. The blot (left) is representative of three independent experiments. Normalized band intensity data (right) are means ± SEM from all experiments. (E) NanoSight analysis of the number of exosomes released from wild-type and ASC KO microglial cells. Data are means ± SEM from three independent experiments. (F) Immunofluorescence microscopy analysis of CFP expression in wild-type microglial cells after treatment with exosomes isolated from cells overexpressing ASC-CFP. Images (left) are representative of three independent experiments. Quantified data (right) are means ± SEM from all experiments. Scale bar, 5 µm. (G and H) qRT-PCR analysis of NLRP3 (G) and pro–IL-1β (H) mRNA expression in microglial cells after treatment with exosomes isolated from wild-type and ASC KO microglial cells. Data are means ± SEM from three independent experiments. (I) Luminex analysis of IL-1β secreted by microglial cells treated with exosomes isolated from the serum of Mn-exposed animals. Data are means ± SEM pooled from eight independent experiments. *P < 0.05, **P < 0.01, ***P < 0.001 by ANOVA with Tukey post hoc analysis.
showed that Mn exposure enhanced exosomal release of ASC from LPS-primed microglial cells (Fig. 5D), but exposure to LPS or Mn alone had only minimal effects. Furthermore, Mn treatment significantly enhanced the number of exosomes released from primed microglial cells (Fig. 5E) without altering exosome size (Fig. S10). When we exposed ASC knockout (KO) cells to Mn, we found no decrease in exosome number (Fig. 5E). These data indicated that the increase in exosome number was independent of inflammasome activation. To test whether exosomes transfer ASC between cells, we collected exosomes from the medium of ASC–cyan fluorescent protein (CFP)–overexpressing microglial cells untreated or treated with Mn for 24 hours after LPS priming. When wild-type microglial cells were exposed to an equal number of exosomes for 24 hours, confocal microscopy revealed that exosomes from Mn-treated, LPS-primed cells transferred ASC-CFP, whereas exosomes from untreated cells did not (Fig. 5F). These data confirmed that ASC migrated from cell to cell through exosomes and that Mn increased the exosomal ASC content. When we exposed cells to exosomes from LPS-primed Mn-treated microglial cells, qRT-PCR analysis revealed significantly increased NLRP3 (Fig. 5G) and pro–IL-1β (Fig. 5H) mRNA expression relative to exposure to exosomes from untreated microglial cells. This increase in NLRP3 and pro–IL-1β mRNA was not seen when wild-type microglial cells were treated with exosomes isolated from Mn-treated, LPS-primed ASC KO cell line (Fig. 5, G and H), further validating that exosomal transfer of ASC is involved in propagation of inflammasome activation. In addition, exosomes isolated from serum of Mn-gavaged animals significantly elevated the release of IL-1β when administered to LPS-primed primary microglial cells for 24 and 48 hours (Fig. 5I); however, no significant change in IL-6 or TNF-α release was observed (Fig. S11, A and B). Collectively, these findings suggest that Mn induced cell-to-cell transmission of ASC through exosomes, further propagating inflammasome activation.

**Serum exosomes and serum from welder population have higher load of ASC and higher proinflammatory cytokines compared to age-matched controls**

Welders exposed to Mn exhibit a dose-dependent progression in Parkinsonian syndrome (55). To demonstrate the human relevance of our findings from cell culture and animal models of Mn exposure, we used serum exosomes collected from welders. When we counted serum exosomes and normalized our Western blot analysis to the number of exosomes isolated from an equal volume of serum, we found that welders’ serum exosomes had significantly more ASC than age-matched controls (Fig. 6B). These data further corroborate our in vitro and in vivo data, suggesting that Mn exposure increases exosomal release of ASC. We analyzed the amount of Mn in whole blood from control and welder samples, but we did not observe any significant differences (Fig. S12). Most of the welders used in the study were exposed to low amounts of Mn, and their blood Mn concentration measured at a single time point may not be an accurate measure of toxicity for low-grade exposures. A better measure of chronic weak Mn exposure can be achieved by taking magnetic resonance imaging T1-weighted brain scans (56).

We also analyzed welder serum for cytokine and chemokine profiling (Fig. 5, C to F and Fig. S12). We found that the serum proinflammatory cytokines TNF-α (Fig. 6C) and IL-17 (Fig. 6D) and the chemokines RANTES (Fig. 6E) and MIP1β (macrophage inflammasome protein 1b) (Fig. 6F) significantly increased in welders when compared to age-matched controls. However, the small amount of IL-1β (Fig. S13A) detected in the serum of welders and control individuals may be attributed to the short half-life of IL-1β (57). To verify whether the exosomes isolated from welder serum can induce NLRP3 expression, we treated microglial cells with serum exosomes from four welders and five age-matched controls for 24 hours. Our qRT-PCR analysis revealed that the cells treated with welder exosomes significantly increased expression of pro–IL-1β (Fig. 6G) and NLRP3 (Fig. 6H) when compared to exposure to serum exosomes from healthy donors. Together, these data suggested that exosomal ASC was increased in human populations occupationally exposed to Mn and that environmental exposure to Mn induced an inflammatory response. Moreover, exosomes from welders stimulated NLRP3 inflammasome signaling.

**DISCUSSION**

A diverse variety of signals including ATP (58), various crystals (59, 60), mitochondrial inhibitors (17, 61), and misfolded proteins (15, 16) stimulate NLRP3 inflammasome activation. Inflammasome activation is linked to various neurodegenerative disorders including PD, AD, and TBI (14). In the present study, we showed that the divalent metal Mn promoted NLRP3 inflammasome activation in microglial cells in vitro and in vivo. Furthermore, this inflammasome activation in microglial cells was associated with mitochondrial dysfunction and correlated with Mfn2 degradation. We also showed that Mn-stimulated exosomes that contained the inflammasome component ASC propagated NLRP3 inflammasome activation. Lastly, we demonstrated that occupational exposure to Mn in welders increased serum exosomal ASC load and circulating proinflammatory cytokine abundance when compared to age-matched controls.

The link between Mn and neurodegenerative and neuroimmune processes is becoming clearer. Chronic Mn exposure damages the nigrostriatal region (37, 42, 62–66) and induces mitochondrial activation in the substantia nigra pars compacta region in a primate model (67) and astroglial inflammation (37). Mn exposure also promotes the proinflammatory cytokine produced by glial cells (68–70) and increases the NFκB and p38 mitogen-activated protein kinase pathway signaling in glial cells (30, 69). Although the mechanism underlying sustained and chronic inflammation in Mn-induced neurotoxicity is not well understood, inflammasome activation promotes inflammatory responses in various disease models (13). Exposure to silica and asbestos also stimulates NLRP3 inflammasome activation in various cell types including lymphocytes and macrophages, among others (60). Furthermore, NLRP3 inflammasome activation is linked to Parkinsonian syndrome (16, 71). Here, using cell culture and animal models of Mn toxicity, we showed that Mn exposure activated the NLRP3 inflammasome specifically in microglial cells.

Although a variety of stimuli has been shown to act as signal 2 in activating the NLRP3 inflammasome cascade, the exact mechanism underlying its assembly is not well understood. Various factors, including mitochondrial dysfunction (17), mitochondrial superoxide generation (18), and lysosomal dysfunction (72), can promote NLRP3 inflammasome assembly and activation (43). Mn exposure causes mitochondrial dysfunction by accumulating in mitochondria (26, 34, 50, 73) in neurons and astrocytes. In this study, we showed that Mn exposure leads to mitochondrial dysfunction and mitochondrial superoxide generation in microglial cells. Mfn2 plays an important role in mitochondrial fusion and is one of the targets of
Fig. 6. Serum exosomes and serum from a welder population have a higher load of ASC and higher proinflammatory cytokines, respectively, when compared to age-matched controls. (A and B) Western blot analysis of exosomal ASC load in welders and age-matched controls. Blot (A) is representative of 17 controls and 20 welder samples. Normalized band intensity data (B) are means ± SEM from all experiments. (C to F) Luminex analysis of TNF-α (C), IL-17 (D), RANTES (E), and MIP1b (F) abundance in control and welder serum. Data are means ± SEM from 17 controls and 20 welder samples. (G and H) qRT-PCR analysis of pro–IL-1β (G) and NLRP3 (H) mRNA expression in microglial cells treated with exosomes isolated from welders or age-matched controls. Data are means ± SEM from five welders and four age-matched controls. *P < 0.05 by Student’s t test.
the PD-associated gene PARKIN (74, 75). Manipulating Mfn2 expression alters mitochondrial dynamics (53, 54, 76), and Mfn2 plays a role in Mn-induced neurotoxicity in neuronal cells (77). In this study, we demonstrated that Mn exposure leads to decreased expression of Mfn2 in microglial cells, which was accompanied by mitochondrial dysfunction and inflammasome activation.

Most studies with VPS35 focus on neurons but largely ignore the role of this retromer complex protein in microglia. VPS35 mutation or reduced expression can increase mitochondrial dysfunction either by promoting Mfn2 degradation in dopaminergic neurons (54) or by recycling the dynamin-like protein 1 complex (78). Here, we showed that Mn exposure reduced VPS35 in LPS-primed microglial cells and that VPS35 increased Mfn2 degradation that correlated with Mul1 expression. Although exactly how Mn treatment decreases VPS35 abundance is not clear, another retromer complex protein VPS29 contains Mn binding sites, and VPS35 wraps around these metal-binding sites (79, 80). Thus, Mn-induced reduction in retromer complex proteins may be due to excessive binding of Mn to these metal-binding sites, but more studies are required to confirm the exact mechanism. In addition, VPS35 modulates the cellular biogenesis and release of exosomes (81, 82). Retromer pathway dysfunction also promotes amyloid precursor protein processing (83).

Release of exosomes, the so-called “garbage bag” of cells, can promote the progression of neurodegenerative disorders including AD and PD. Exosomal export of aggregated αSyn to other cells (84, 85) acts as a key signal between cells. Intercellular communication through exosome cargo can also modulate neuroinflammation (86). NLRP3 inflammasome propagation has become an active area of research. A novel pyroptotic-phagocytic cascade has been shown to spread ASC and NLRP3 inflammasome assembly between cells, in a “prionoid” fashion, thus propagating the inflammasome (19). In this study, we demonstrated that ASC loaded in exosomes can stimulate inflammasome activation in neighboring cells. Mn exposure induced the exosomal release of ASC from LPS-primed microglial cells, whereas blocking exosomal uptake reduced IL-1β release (Fig. 5). This finding may have broad implications because exosomes can cross the blood-brain barrier and can communicate to various organs.

Mn can induce Parkinsonism in humans (20, 55, 62, 63, 87). Because of their exposure to welding-derived metal nanoparticles, Swedish welders have an increased inflammatory profile (88). Our data suggest a probable mechanism for inflammation stemming from occupational exposure to Mn. Although we were not able to detect substantial amounts of IL-1β in welder serum, we did show an increased ASC load in welder serum exosomes. The lack of elevated IL-1β may be attributed to its short half-life (Fig. 6) (57, 89). Moreover, we also showed that exosomes isolated from welder serum stimulated the expression of NLRP3 and pro–IL-1β, further validating the role of exosomes in modulating inflammasome propagation. Our findings may provide some insight into the mechanism of communication between peripheral inflammation and CNS inflammation in neurodegenerative disorders, including AD and Parkinsonian syndrome. Future studies should explore the role of exosomal biogenesis during inflammation in different disease models including PD. Overall, our study describes how Mn dysregulates VPS35-Mul1-Mfn signaling to activate and propagate the inflammasome (Fig. 7).
MATERIALS AND METHODS

Cell culture, primary culture, and treatments

Primary microglial cells were isolated using a magnetic bead separation technique described in our previous publications (90, 91). Primary microglial cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM)–F12, 10% fetal bovine serum (FBS), 1% sodium pyruvate, 1% glutamine, 1% penicillin-streptavidin, and 1% nonessential amino acids. The wild-type and ASC-CFP microglial cell lines were donations from D. T. Golenbock (University of Massachusetts Medical School, Worcester, MA). The ASC KO macrophage cell line was donated by K. Fitzgerald (University of Massachusetts). The wild-type microglial cell line was characterized by Halle et al. (15) and our group (92) and cultured in DMEM, 10% FBS, 1% glutamine, and 1% penicillin-streptavidin. Treatments were performed in 2% FBS-containing medium. For LPS-priming treatments, cells were treated with LPS (100 ng/ml) for 3 hours. Next, the cells were triple-washed with full serum medium to remove any excess LPS and then treated with 100 μM Mn for 6 to 24 hours, unless otherwise stated. For exosome treatment, cells were treated with an equal number of exosomes from welders and age-matched controls for 24 hours, after which qRT-PCR analysis was performed.

CRISPR-Cas9 KD cell generation

CRISPR guide RNA (gRNA) was obtained for VPS35, Cav1, and Cltc from Sigma-Aldrich and transfected using protocols from our previous publications (9, 93). After transfection, cells were incubated for 48 hours before treatment. The lentivirus-based CRISPR-Cas9 KO plasmid against gene Vps35, with target site 5'-CAATCCAGG-3' in a U6gRNA-Cas9-2A-RFP vector, was purchased from Sigma-Aldrich. The lentivirus-based CRISPR-Cas9 plasmids, pLV-U6g-EP CG-Cav1 and pLV-U6g-EP CG-Cltc with the Cav1 and Cltc gRNA target sequences 5'-CAATCCAGG-3' and 5'-CAATCCAGG-3', respectively, were purchased from Sigma-Aldrich. To make the lentivirus, the lenti–CRISPR-Cas9 Vps35 KO plasmid and nontarget control plasmid were transfected into 293FT cells using the Mission Lentiviral Packaging Mix from Sigma-Aldrich according to the manufacturer’s instructions. The lentivirus was harvested 48 hours after transfection and added to the microglial cell line at a multiplicity of infection of 100 to KD VPS35, caveolin-1, and clathrin expression.

Animal study

Eight-week-old male C57BL/6Ncr1 mice, obtained from Charles River Laboratories, were housed under standard conditions of constant temperature (22 ± 1°C), humidity (relative, 30%), and a 12-hour light/dark cycle. After acclimating for 3 days, mice were gavaged with Mn (15 mg/kg) for 30 days and then euthanized. This Mn dose regimen was chosen on the basis of previous studies in humans and animals (94–96). Use of the animals and protocol procedures were approved by the Institutional Animal Care and Use Committee at Iowa State University (Ames, IA, USA).

Western blot

Western blot analyses were performed following our previous publications (93, 97). Briefly, cells or tissues were lysed using modified radioimmunoprecipitation assay buffer. Proteins were normalized using a Bradford assay before loading on SDS–acylamide gels. For separation of proteins, 20 to 40 μg of protein was loaded in each well of 10 to 15% acrylamide gels. Acrylamide gels were run at 110 V for 2 hours at 4°C. After separation, proteins were transferred to a nitrocellulose membrane at 27 V for 18 hours at 4°C. After transfer, the membranes were blocked with a LI-COR blocking buffer for 45 min and incubated in primary antibodies following the manufacturer’s protocol. After primary antibody incubation, membranes were washed with phosphate-buffered saline (PBS–TWEEN 20 (0.05%) for 1 hour, incubated in LI-COR infrared (IR) secondary antibodies for 1 hour at room temperature, washed with PBS–TWEEN 20 for 1 hour, and imaged using an Odyssey scanner. The primary antibodies used are as follows: anti–caspase-1 (1:1000; AB_2490248, AdipoGen Life Sciences), anti-NLRP3 (1:1000; AdipoGen Life Sciences), anti-iNOS (1:1000; Santa Cruz Biotechnology), anti–IL-1β (1:500; R&D Systems), anti-Mfn2 (1:1000; Cell Signaling Technology), anti-VPS35 (1:500; Santa Cruz Biotechnology), and anti-ASC (1:1000; AB_2490440, AdipoGen Life Sciences). The secondary antibodies used are as follows: IR-800 conjugated goat anti-mouse immunoglobulin G (IgG) (1:20,000; LI-COR) and IR-700 conjugated goat anti-rabbit IgG (1:20,000; LI-COR). For loading control, β-actin (1:10,000; Sigma-Aldrich) antibody was used.

Human individuals

In central Pennsylvania, USA, and local communities, 81 individuals were recruited. Controls have no history of welding, whereas welders were defined as persons who have welded at any point in their lifetime. All individuals were male. Written informed consent was obtained in accordance with guidelines approved by the Penn State Health Milton S. Hershey Institutional Review Board. Controls were age-matched volunteers from the same regional community with various occupations. Before sending the serum samples to Iowa state, samples were blinded. This serum was used to isolate exosomes as described. Of the exosome samples, 39 samples were chosen randomly for further Western blot, qRT-PCR, and Luminex analysis.

Quantitative reverse transcription polymerase chain reaction

RNA was isolated from tissues and cells according to a previous publication (98, 99). Briefly, cells or tissues were lysed in 1 ml of TRIzol reagent and incubated for 5 min. After incubation, 0.2 ml of chloroform was added to each tube, incubated for 2 min, and centrifuged at 12,000 g for 15 min at 4°C. After centrifugation, the top clear layer containing RNA was transferred to a fresh tube containing 0.7 ml of isopropanol, washed with 75% ethanol, air-dried, and dissolved in water. A NanoDrop was used to quantify the RNA, and 1 μg of RNA was used for converting into complementary DNA (cDNA). For cDNA synthesis, the high-capacity cDNA synthesis kit from Applied Biosystems (catalog number 4368814) was used according to the manufacturer’s protocol. Quantitative SYBR green PCR assay was performed using qRT–PCR SYBR Green Mastermix (Agilent) and prevalidated primers. The validated primers used from QIAGEN were the following: pro–IL-1β (QT01048355), NLRP3 (QT00122458), NLRCA (QT00264670), AIM2 (QT00266819), Nos2 (QT00100275), Mfn2 (QT00134295), Mul1 (QT00132734), SLC30A10 (QT01199009), SLC11A2 (QT00147368), VPS35 (QT00160258), VPS29 (QT00137228), and 18S (QT02448075). The fold change in gene expression was determined by the ΔΔCt method, where Ct is the threshold value. 18S was used as the housekeeping gene.
IHC and immunocytochemistry
IHC was performed on striatal sections as described in our previous publications (6, 8, 100). Striatal sections (30 μm) were used for IHC. Citrate buffer was used to perform antigen retrieval [10 mM sodium citrate (pH 8.5)]. After antigen retrieval, sections were washed with PBS, blocked with blocking buffer (2% bovine serum albumin, 0.5% Triton X-100, and 0.05% Tween 20), and incubated in primary antibodies overnight at 4°C. Next, sections were washed with PBS, incubated in secondary antibodies for 1 hour, and stained with the nuclear dye Hoechst. Lastly, sections were mounted on precoated slides and dried overnight before visualizing them under a microscope. Confocal imaging was performed on these sections at the Iowa State University Microscopy Facility using a Leica DMI8 confocal microscope with 63× oil objective. For z-stacking, each section consisted of 10 to 15, 0.5-μm slices.

For ICC studies on microglial cells and microglial primary culture, 4% paraformaldehyde was used to fix the cells. Next, fixed cells were washed with PBS, blocked using blocking buffer, and incubated in primary antibodies following the manufacturer’s protocol. After primary antibody incubation, cells were washed with PBS, incubated in secondary antibody, and mounted on slides using Fluoromount aqueous mounting medium (Sigma-Aldrich). Samples were visualized using an inverted fluorescence microscope (Nikon TE2000-U). The primary antibodies used were the following: IBA1 (1:1000; AB_2314667, Wako), IBA1 (1:500; AB_870576, Abcam), M1 (1:500; AB_1868063, Abcam), NLRP3 (1:500; AB_2490202, AdipoGen Life Sciences), ASC (1:500; AdipoGen Life Sciences), Nos2 (1:500; AB_2152867, Santa Cruz Biotechnology), VPS35 (1:500; AB_2215220, Santa Cruz Biotechnology), and Mfn2 (1:500; Cell Signaling Technology). Alexa Fluor dye-conjugated secondary antibodies were used for ICC and IHC experiments.

Multiplex cytokine assay
Multiplex cytokine assay was performed according to our previous publication (93). Briefly, 40 μl of treatment medium was incubated overnight with 40 μl of primary antibodies conjugated with magnetic beads. After incubation with primary antibodies, samples were washed, incubated with a biotinylated antibody and biotin-streptavidin, and then read in a Bio-Plex plate reader. Standards were obtained from PeproTech. For welder serum cytokine analysis, the Bio-Plex beads. After incubation with primary antibodies, samples were washed with PBS, blocked using blocking buffer, and incubated in secondary antibodies for 1 hour, and stained with the nuclear dye Hoechst. Lastly, sections were mounted on precoated slides and dried overnight before visualizing them under a microscope. Confocal imaging was performed on these sections at the Iowa State University Microscopy Facility using a Leica DMI8 confocal microscope with 63× oil objective. For z-stacking, each section consisted of 10 to 15, 0.5-μm slices.

For ICC studies on microglial cells and microglial primary culture, 4% paraformaldehyde was used to fix the cells. Next, fixed cells were washed with PBS, blocked using blocking buffer, and incubated in primary antibodies following the manufacturer’s protocol. After primary antibody incubation, cells were washed with PBS, incubated in secondary antibody, and mounted on slides using Fluoromount aqueous mounting medium (Sigma-Aldrich). Samples were visualized using an inverted fluorescence microscope (Nikon TE2000-U). The primary antibodies used were the following: IBA1 (1:1000; AB_2314667, Wako), IBA1 (1:500; AB_870576, Abcam), M1 (1:500; AB_1868063, Abcam), NLRP3 (1:500; AB_2490202, AdipoGen Life Sciences), ASC (1:500; AdipoGen Life Sciences), Nos2 (1:500; AB_2152867, Santa Cruz Biotechnology), VPS35 (1:500; AB_2215220, Santa Cruz Biotechnology), and Mfn2 (1:500; Cell Signaling Technology). Alexa Fluor dye-conjugated secondary antibodies were used for ICC and IHC experiments.

MTS cell viability assay
Cells were plated in 96-well tissue plates. After treatment, 10 μl of 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2(4-sulphophenyl)-2H-tetrazolium (MTS) reagent (Promega) was added and incubated at 37°C for 1.5 hours. After incubation, a plate reader was used to read the absorbance at 490 nm.

Griess assay
Griess assay was performed as previously described in our publication (93). Microglial cells (50,000 to 100,000) were plated in 96-well plates and treated in 2% FBS-containing medium. After treatment, 50 μl of medium was collected and incubated with 50 μl of Griess reagent for 10 min. A plate reader was used to read the absorbance at 540 nm. Sodium nitrite solution was used for making standard curve.

Exosome isolation
Wild-type microglial cell line–produced exosomes were isolated by filtering the cell culture supernatant through 0.22-μm filters, followed by high-speed ultracentrifugation. Briefly, cells were grown in T-175 cell culture flasks and treated with 100 μM Mn. For each exosome sample, conditioned medium from two T-175 flasks were pooled with an original seeding density of 8 × 10^6 cells per flask. After treatment, the medium was collected and centrifuged at 300g for 10 min and 10,000g for 15 min successively to get rid of any cell debris before passing it through a 0.22-μm syringe filter. The filtrate was then centrifuged at 100,000g for 90 min using a Beckman Optima L-100 XP ultracentrifuge. The pellet containing exosomes was washed once with cold PBS and centrifuged again at 100,000g for 90 min using a Beckman Optima MAX ultracentrifuge.

Inductively coupled plasma mass spectroscopy
Microglial cell line samples were carefully scraped and counted before sample preparation. Samples were analyzed for different metals including, Cd, Ca, Cr, Co, Cu, Fe, Mg, Mn, Mo, P, K, Se, Na, and Zn using ICP-MS (Analytik Jena Inc., Woburn, MA, USA) in CRI mode with hydrogen as the skimmer gas. Standards for elemental analyses were obtained from Inorganic Ventures (Christiansburg, VA), and digestion vessels, trace mineral grade nitric acid, and hydrochloric acid were obtained from Thermo Fisher Scientific (Pittsburgh, PA). Briefly, samples were digested in 70% nitric acid at 60°C for ≥12 hours. Preweighed samples were transferred to 15-ml tubes, and 0.25 ml of 70% nitric acid was added. All samples were digested overnight at 60°C. After digestion, all samples were diluted to 5 ml using 1% nitric acid with 0.5% hydrochloric acid and then analyzed using ICP-MS. For quality control, Bi, Sc, In, Li, Y, and Tb were used as internal standards for the ICP-MS.

NanoSight
Ultracentrifuged exosome samples were used for nanoparticle tracking analysis (NTA), as previously described (101). Briefly, isolated exosomes were resuspended in 500 to 1000 μl of PBS from which about 300 μl was loaded into the sample chamber of an LM10 unit (NanoSight, Amesbury, UK) using a disposable syringe. Sample durations of 30 to 60 s per sample were analyzed with NTA 2.3 software (NanoSight). Samples containing higher numbers of exosomes were diluted before the analysis, and their relative concentrations were then calculated according to the dilution factor. The size distribution of exosomes (fig. S10) was also determined using this method.

Mitochondrial dynamics analysis
A Seahorse XFe24 analyzer was used to measure mitochondrial OCR and ECAR using the Mito Stress test following a previously published protocol (43). Primary mouse microglia were plated 90,000 cells per well of a 24-well Seahorse plate. All treatments were performed in serum-free medium. For the Mito Stress test, 0.75 μM oligomycin, 1 μM carbonyl cyanide p-trifluoromethoxyphenylhydrazone, and 0.5 μM rotenone-antimycin were used. The Seahorse’s Mito Stress and cell phenotype report generators were used for analysis.

Statistical analysis
GraphPad 5.0 was used for statistical analysis. One-way ANOVA was used for comparing multiple groups with Tukey post hoc analysis, unless otherwise mentioned. For comparing two groups, Student’s t test was used.
SUPPLEMENTARY MATERIALS

**Fig. S3.** Mn exposure did not inhibit sTNαp6-induced proinflammatory cytokine production:

**Fig. S4.** Caspase-1-p20–induced cleavage of pro–IL-1β.

**Fig. S5.** Mn exposure inhibited microglial mitochondrial respiration.

**Fig. S6.** Mn increased Mn transporters and Mn uptake.

**Fig. S7.** Mn increased M1 profile expression in a dose-dependent manner.

**Fig. S8.** CRISPR-Cas9 VPS35 KD results in Mn2 degradation.

**Fig. S9.** Caveolin and clathrin KD reduced Mn-induced Nox2 expression but not TNF-α.

**Fig. S10.** Exposure did not change the size distribution of the exosomes secreted by microglial cells.

**Fig. S11.** Exposure to exosomes isolated from Mn-gavaged animals did not affect release of IL-6 and TNF-α.

**Fig. S12.** Mn concentration in whole blood is unaltered in welders.

**Fig. S13.** Pfrainflammatory cytokine profile of welder and age-matched control populations.

REFERENCES AND NOTES


35. The pro-inflammatory gene expression in activated glia.

17. Differences between interleukin 1


Acknowledgments: We acknowledge C. Wang (Department of Statistics, Iowa State University) for assistance with the statistical analysis. We also acknowledge G. Zenitsky for assistance in editing this manuscript and J. R. Kumar for technical assistance. Funding: This work was supported by NIH grants (#ES026892 to A.G.K. and #NS088206 to A.K.). Author contributions: S.S., A.K., and A.G.K. designed the research. S.S., D.R., E.M., J.L., and D.S.H. performed the research. S.S. and A.G.K. analyzed data. S.S., H.J., V.A., and A.G.K. wrote the paper. X.H. and M.L. collected the welder and controls serum samples. Competing interests: A.G.K. and V.A. have an equity interest in PK Biosciences Corporation located in Ames, IA. The terms of this arrangement have been reviewed and approved by Iowa State University in accordance with its conflict of interest policies. The other authors declare that they have no competing interests. Data and materials availability: All data needed to evaluate the conclusions in the paper are present in the paper and/or the Supplementary Materials.

Submitted 25 April 2018
Accepted 12 December 2018
Published 8 January 2019
10.1126/scisignal.aeat9900

Manganese activates NLRP3 inflammasome signaling and propagates exosomal release of ASC in microglial cells
Souvarish Sarkar, Dharmin Rokad, Emir Malovic, Jie Luo, Dilshan S. Harischandra, Huajun Jin, Vellareddy Anantharam, Xuemei Huang, Mechelle Lewis, Arthi Kanthasamy and Anumantha G. Kanthasamy

Sci. Signal. 12 (563), eaat9900.
DOI: 10.1126/scisignal.aat9900

Exosomes transfer inflammasome activation
Chronic occupational exposure to manganese is associated with the development of Parkinson's disease. Sarkar et al. found that exposure of primed microglial cells or mice to manganese increased NLRP3 inflammasome expression and activation. Manganese caused mitochondrial dysfunction in treated microglial cells and stimulated their release of exosomes containing the inflammasome adaptor protein ASC. The effects of manganese on inflammasome activation were sensitive to reduced endocytosis and transferable by exposure of cells to purified exosomes from ASC-sufficient cells. Similarly, serum exosomes from welders contained more ASC and were more inflammatory than those from normal donors, suggesting that occupational manganese exposure may increase systemic inflammasome activation due to exosome-mediated transfer of ASC.

ARTICLE TOOLS
http://stke.sciencemag.org/content/12/563/eaat9900

SUPPLEMENTARY MATERIALS
http://stke.sciencemag.org/content/suppl/2019/01/04/12.563.eaat9900.DC1

RELATED CONTENT
http://stm.sciencemag.org/content/scitransmed/10/465/eaah4066.full
http://stke.sciencemag.org/content/sigtrans/12/569/eaar2124.full
http://stke.sciencemag.org/content/sigtrans/12/572/eaau4543.full
http://stke.sciencemag.org/content/sigtrans/12/582/eaax4917.full
http://stke.sciencemag.org/content/sigtrans/12/589/eaay6126.full
http://stke.sciencemag.org/content/sigtrans/12/602/eaaz7370.full
http://stke.sciencemag.org/content/sigtrans/13/647/eabe5296.full
http://stke.sciencemag.org/content/sigtrans/12/663/eaax7119.full

REFERENCES
This article cites 98 articles, 14 of which you can access for free
http://stke.sciencemag.org/content/12/563/eaat9900#BIBL

PERMISSIONS
http://www.sciencemag.org/help/reprints-and-permissions

Use of this article is subject to the Terms of Service