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Mitochondrial dysfunction and loss of Parkinson's disease-linked proteins contribute to neurotoxicity of manganese-containing welding fumes

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ABSTRACT **Welding generates complex metal aerosols, inhalation of which is linked to adverse health effects among welders. An important health concern of welding fume (WF) exposure is neurological dysfunction akin to Parkinson's disease (PD), thought to be mediated by manganese (Mn) in the fumes. Also, there is a proposition that welding might accelerate the onset of PD. Our recent findings link the presence of Mn in the WF with dopaminergic neurotoxicity seen in rats exposed to manual metal arc-hard surfacing (MMA-HS) or gas metal arc-mild steel (GMA-MS) fumes. To elucidate the molecular mechanisms further, we investigated the association of PD-linked (Park) genes and mitochondrial function in causing dopaminergic abnormality. Repeated instillations of the two fumes at doses that mimic ~1 to 5 yr of worker exposure resulted in selective brain accumulation of Mn. This accumulation caused impairment of mitochondrial function and loss of tyrosine hydroxylase (TH) protein, indicative of dopaminergic injury. A fascinating finding was the altered expression of Parkin (Park2), Uchl1 (Park5), and Dj1 (Park7) proteins in dopaminergic brain areas. A similar regimen of manganese chloride ($MnCl_2$) also caused extensive loss of striatal TH, mitochondrial electron transport components, and Park proteins. As mutations in PARK genes have been linked to early-onset PD in humans, and because welding is implicated as a risk factor for parkinsonism, PARK genes might play a critical role in WF-mediated dopaminergic dysfunction. Whether these molecular alterations culminate in neurobehavioral and neuropathological deficits reminiscent of PD remains to be ascertained.—Sriram, K., Lin, G. X., Jefferson, A. M., Roberts, J. R., Wirth, O., Hayashi, Y., Krajnak, K. M., Soukup, J. M., Ghio, A. J., Reynolds, S. H., Castranova, V., Munson, A. E., Antonini, J. M. Mitochondrial dysfunction and loss of Parkinson's disease-linked proteins contribute to neurotoxicity of manganese-containing welding fumes. *FASEB J.* 24, 000–000 (2010). www.fasebj.org**

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WELDING FUME (WF) AEROSOLS are complex mixtures of gases (carbon monoxide, carbon dioxide, nitrous oxide, ozone) and metal particulates (iron, manganese, chromium, nickel) that are potentially toxic. The aerodynamic diameter of WF aerosols in the breathing zone is reported to range from 100 nm to 1 μm (1–3). Therefore, welding particulates are respirable and can deposit in the olfactory and lower respiratory tracts. Exposure to airborne WF particulates is subsequently of immense occupational concern. The unique elemental composition and physicochemical characteristics of WF might influence deposition within the respiratory tract and subsequent translocation to extrapulmonary organs. Welders are a heterogeneous workforce employed in a variety of workplace conditions that include open, well-ventilated (*e.g.*, outdoors on construction sites) or confined, poorly ventilated spaces (*e.g.*, ship hulls, building crawl spaces, and pipelines). The complexity of the workplace settings combined with exposure to diverse aerosols generated from different welding processes can potentially increase the risk of exposure and associated adverse health effects.

One growing concern is that WF exposure might be associated with the development of neurological dysfunction similar to Parkinson's disease (PD). Much of this concern has been attributed to the presence of manganese (Mn) in WF consumables. Indeed, Mn intoxication following chronic human exposure in other occupational settings, such as mining, smelting, and ferroalloy and dry battery industries, has been documented to cause a PD-like syndrome called manganism (4–8). Although some studies describe a poten-

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tial link between welding and Parkinsonism and suggest the possibility of an early-onset parkinsonism among welders (9–14), these claims are disputed by other studies (15–18). Thus, conclusive epidemiological evidence for an association between WF exposure and parkinsonism is lacking, warranting further experimental investigations to help unravel the existing ambiguity regarding WF exposure and the appearance of a PD-like neurological dysfunction.

PD is characterized by progressive neurodegeneration of nigrostriatal dopaminergic neurons. While most forms of PD are sporadic, ~10% are linked to genetic defects. Although the etiology of the sporadic form of the disorder remains unknown, mitochondrial dysfunction and oxidative stress are considered to be critical contributors to the pathogenesis. The identification of single-gene mutations in familial forms of PD has garnered interest in the evaluation of such genes to elucidate and understand the molecular basis of PD. Deletions and loss-of-function mutations in the human *DJ-1* (*PARK7*) locus has been associated with autosomal recessive early-onset parkinsonism (19–21). Similarly, loss-of-function homozygous deletional mutations in *Parkin* (*PARK2*) have been associated with autosomal recessive juvenile parkinsonism (22) and autosomal recessive early (<45 yr) onset parkinsonism (23–25). The physiological functions of these proteins involve protection against oxidative stress resulting from mitochondrial dysfunction, and they are targeted to the mitochondria on mitochondrial damage. Both mitochondrial dysfunction and oxidative stress can modulate the ubiquitin-proteasome pathway and have been implicated as causative factors for abnormal accumulation of proteins in familial forms of PD. Whether the factors associated with early-onset PD are similarly linked to Mn- and/or WF-related dopaminergic neurotoxicity remains unknown. However, a recent discovery links PD susceptibility genes to environmental risk factors like Mn and suggests that interaction of PD-linked genes can modulate the neurotoxic outcome of environmental exposures (26). Therefore, evaluation of these PD-linked genes might enhance our understanding of the molecular basis of Mn- and WF-related neurotoxicity and provide adequate information to determine whether WF exposure can cause dopaminergic neurodegeneration similar to that seen in PD. Further, changes in PD-associated genes might serve as biological markers of Mn accumulation in dopaminergic brain areas and the progressive appearance of neurological dysfunction.

Manual metal arc–hard surfacing electrode (MMA-HS) welding is a welding process that finds application in specialized settings such as in ship building and railroad industries. Gas metal arc–mild steel electrode (GMA-MS) welding, however, is a more common process used in a variety of industries. The elemental compositions of the electrodes used in these two processes differ considerably, including their Mn content. Consequently, the fumes generated by these processes also exhibit diverse elemental composition (27) that can potentially contribute to distinct biological responses. We examined the potential neurotoxicological effects of repeated pulmonary exposure to MMA-HS or GMA-MS fumes in a rodent model. Specifically, we investigated whether pulmonary exposure to WF results in mitochondrial dysfunction, oxidative stress, and alterations in PD-linked proteins leading to dopaminergic neurotoxicity.

MATERIALS AND METHODS

WF generation

Bulk samples of MMA-HS and GMA-MS WFs were provided by the laboratory of Kenneth Brown (Lincoln Electric Company, Cleveland, OH, USA). The fumes were generated in a cubical open-front fume chamber (volume=1 m^3) by a skilled welder using a manual or semiautomatic technique appropriate to the electrode and collected on 0.2-μm Nucleoprotein filters (Nucleoprotein, Pleasanton, CA, USA). The fumes were generated using two different processes: MMA-HS welding using a flux-covered stainless steel hard-surfacing electrode (Wearshield 15CrMn; Lincoln Electric); and GMA-MS welding using a mild steel E70S-3 electrode (L-50 carbon steel; Lincoln Electric).

Elemental analysis of fumes and tissues

The elemental composition, as well as the ratios of the water-soluble and water-insoluble elemental fractions in the MMA-HS and GMA-MS fumes, were determined by inductively coupled plasma atomic emission spectroscopy (ICP-AES) and reported recently (27). In brief, MMA-HS fumes were comprised mainly of Mn (43.7% of total metals analyzed in the collected aerosol), Fe (30.4%), and Cr (8.3%), as well as flux materials like K (13.4%), while GMA-MS fumes were comprised mainly of Fe (90.4% of total metals) and smaller amounts of Mn (6.9%).

To determine elemental content in tissues and organs (blood, lung, lung-associated lymph nodes, or discrete brain areas), 1 ml of 3N hydrochloric acid/10% trichloroacetic acid solution was added to preweighed tissues and heated at 70°C for 18 h to digest the tissue. After centrifugation at 600 *g* for 10 min, concentrations of elements in the supernatant were quantified using ICP-AES (Optima 4300D; Perkin Elmer, Norwalk, CT, USA). Blood Mn levels were measured as micrograms per milliliter of whole blood. Mn content in lung or lung-associated lymph nodes were measured as micrograms per gram dry weight and brain Mn content as micrograms per gram wet weight.

Animals

Male Sprague-Dawley [Hla:(SD) CVF] rats (250–300 g) were procured from Hilltop Lab Animals (Scottdale, PA, USA). The rats were acclimated for ≥6 d after arrival and were housed in ventilated polycarbonate cages with Alpha-Dri cellulose chips as bedding (Shepherd Specialty Papers, Watertown, TN, USA), with provision for HEPA-filtered air, and irradiated Teklad 2918 diet (Harlan Teklad, Madison, WI, USA) and tap water *ad libitum*. The National Institute for Occupational Safety and Health (NIOSH) animal facility is

specific-pathogen free, environmentally controlled, and accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC). All animal procedures used during the study have been reviewed and approved by the NIOSH Animal Care and Use Committee.

Animal exposures

MMA-HS or GMA-MS fumes were prepared in sterile saline and sonicated for 1 min in a Sonifier 450 Cell Disruptor (Branson Ultrasonics, Danbury, CT, USA) to disperse the particulates. $MnCl_2$ solution was prepared similarly in sterile saline. Rats were lightly anesthetized by an intraperitoneal injection of 0.6 ml of a 1% solution of sodium methohexital (Brevital; Eli Lilly, Indianapolis, IN, USA). Intratracheal instillations (28) of MMA-HS, GMA-MS, or $MnCl_2$ (0.125 mg or 2 mg/animal in 300 μl of sterile saline) were performed 1/wk for 7 or 28 wk. Control animals were intratracheally instilled with 300 μl of sterile saline. Following each weekly exposure, animals were monitored daily for any signs of abnormal behavior, until 1 wk following the last exposure. No gross behavioral changes were observed. Animals were euthanized 1 d after the 7 weekly exposures or 1 wk after the 28 weekly exposures. Euthanasia was performed by administration of an intraperitoneal injection of sodium pentobarbital (Sleepaway; >100 mg/kg body weight; Fort Dodge Animal Health; Wyeth, Madison, NJ, USA), and the animals were exsanguinated prior to collection of tissues.

Immediately after euthanasia, the right lung was removed for elemental analysis. The brains were excised, and brain areas (OB, olfactory bulb; STR, striatum; FCT, frontal cortex; PCT, parietal cortex; HIP, hippocampus; THL, thalamus; MB, midbrain; PM, pons/medulla; CER; cerebellum) from the left and right hemispheres were dissected freehand. Brain tissues from left hemisphere were processed for RNA and protein; tissues from the right hemisphere of the 28-wk exposure group were utilized for elemental analysis by ICP-AES.

RNA isolation, cDNA synthesis, and real-time PCR

The brain tissues (STR and MB) were homogenized in Tri Reagent (Molecular Research Center, Inc., Cincinnati, OH, USA), and the aqueous phase was separated with MaXtract High Density gel (Qiagen, Valencia, CA, USA). Total RNA from the aqueous phase was then isolated using RNeasy mini-spin columns (Qiagen), and concentrations were determined with a NanoDrop ND-1000 UV-Vis spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). The isolated RNA was stored at −75°C until use.

First-strand cDNA synthesis was performed using total RNA (1 μg), random hexamers, and MultiScribe reverse transcriptase (High Capacity cDNA Reverse Transcription Kit; Applied Biosystems, Foster City, CA, USA) in a 20-μl reaction. Real-time PCR amplification was performed using the 7500 Real-Time PCR System (Applied Biosystems) in combination with TaqMan chemistry. Specific primers and FAM dye-labeled TaqMan MGB probe sets (TaqMan Gene Expression Assays; Applied Biosystems) were used according to the manufacturer's recommendation. All PCR amplifications (40 cycles) were performed in a total volume of 25 μl, containing 1 μl cDNA, 1.25 μl of the specific TaqMan gene expression assay, and 12.5 μl of TaqMan Gene Expression Master Mix (Applied Biosystems), respectively. Sequence detection software (version 1.7; Applied Biosystems) results were exported as tab-delimited text files and imported into Microsoft Excel (Microsoft, Redmond, WA, USA) for further analysis. Following normalization to β-actin, relative quantification of gene expression was performed using the comparative threshold (C_T) method as described by the manufacturer (Applied Biosystems; User Bulletin 2). The values are expressed as fold change over saline-treated controls.

Preparation of brain tissues for protein analysis

Brain tissues (STR and MB) were homogenized in T-PER tissue protein extraction reagent (Pierce Biotechnologies, Inc., Rockford, IL, USA) containing protease inhibitors and EDTA. The homogenates were centrifuged to pellet the cell/tissue debris, and the supernatant was collected carefully. Total protein was determined according to the microbicinchoninic acid (BCA) method (Pierce Biotechnologies) using bovine serum albumin as a standard. Protein extracts were stored at −75°C until use.

Western immunoblotting

Aliquots of brain homogenates (5–20 μg total protein) were diluted in Laemmli sample buffer, boiled, and loaded on to 10% SDS-polyacrylamide gels. Proteins then were resolved electrophoretically and transferred to 0.45-μm Immobilon-FL PVDF Membranes (Millipore, Billerica, MA, USA). Following transfer, immunoblot analysis was performed. Briefly, membranes were blocked using Odyssey Blocking Buffer (LI-COR Biosciences, Lincoln, NE, USA) for 1 h at room temperature, washed (1×5 min; 2×10 min) with PBST, and incubated overnight at 4°C with the appropriate primary antibody. Following incubation with an antibody (30–50 ng/ml primary antibody buffer) to either, tyrosine hydroxylase (TH; rabbit polyclonal; EMD Biosciences; Gibbstown, NJ, USA), Parkin (Park2; rabbit polyclonal; Abcam, Cambridge, MA, USA), PGP9.5 (Uchl1/Park5; rabbit polyclonal; Abcam), DJ-1 (Park7; rabbit polyclonal), mitochondrial complex I–V proteins (mouse monoclonal; MitoSciences, Eugene, OR, USA), α-tubulin (Tuba; mouse monoclonal; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) or cyclophilin A (rabbit polyclonal; EMD Biosciences, Gibbstown, NJ, USA), blots were washed with PBST (1×5 min; 3×10 min) and incubated for 1 h at room temperature with appropriate IRDye 680 or 800 secondary antibodies (LI-COR Biosciences). The membranes were protected from light to minimize any photobleaching of the fluorescent dyes. Membranes were washed (1×5 min; 4×10 min) in PBST, followed by washes (2×3 min) in PBS. Near-infrared fluorescence detection was performed on the Odyssey Imaging System (LI-COR Biosciences), and the fluorescent signal intensities (k counts) of the individual bands were determined and normalized to endogenous controls, α-tubulin (55 kDa) or cyclophilin A (15 kDa), as appropriate.

Statistical analysis

Data were analyzed by 1-way ANOVA followed by Student's-Newman-Keuls (SNK) multiple-comparison test, using SigmaStat 3.1 statistical software (Systat Software Inc., San Jose, CA, USA). Where data failed equal variance or normality tests, they were analyzed by 1-way ANOVA on ranks, followed by Dunn's or SNK multiple-comparison tests. Results were con-

sidered significant at $P < 0.05$. Graphical representations are means ± SE.

RESULTS

Relevance of rat dosing paradigm to workplace exposures

To relate the pulmonary (intratracheal instillation) dosing paradigm used in this study to workplace exposures of welders, we utilized a mathematical calculation (27, 29) to determine the daily lung burden of a welder on an 8-h work schedule. Incorporating factors such as fume concentration [5 mg/m^3, previous threshold limit value (TLV) for WFs], human (worker) minute ventilation volume (20,000 ml/min × 10^{-6} m^3/ml), exposure duration (8 h/d × 60 min/h) and a predicted deposition efficiency of 15% (3), it was determined that the daily lung burden of a welder is ~7.2 mg. Using surface area of alveolar epithelium (rat, 0.4 m^2; human, 102 m^2) as dose metric (30), the daily lung burden for a similar exposure in a rat amounts to 0.0282 mg. Factoring the cumulative dosing paradigms used in this study (2 mg × 7 instillations = 14 mg or 2 mg × 28 instillations = 56 mg) and the estimated daily lung burden for a rat (0.0282 mg), the number of welder exposure days was derived for the two exposure paradigms as 14 mg/0.0282 mg = 496.5 d, and 56 mg/0.0282 mg = 1985.8 d. Thus, our short- and long-term pulmonary exposures in rats mimic worker exposures of ~1.4 and 5.4 yr, respectively. While the estimates of worker exposure to total WFs or Mn presented here was derived using TLV, it is likely that in certain work environments, workers might be exposed to much higher concentrations of fumes, and consequently, toxicological effects might be greater in such cases. Indeed, total WF levels measured in various industries (31, 32), especially in confined spaces (33), have been observed to often exceed the previous TLV of 5 mg/m^3 for WFs.

Tissue elemental analysis following exposure to MMA-HS and GMA-MS WFs

Following repeated weekly instillations of WFs or $MnCl_2$ for 28 wk, elemental analysis of blood, lung, lung-associated lymph nodes, and brain regions were performed by ICP-AES to determine accumulation of specific metals and identify target brain areas. The distribution and accumulation of important elements (Al, Cr, Cu, Fe, Mn, Ni, Ti, Zn) was studied. Measurements of blood Mn were near or at limit of detection and as a consequence variable, rendering it unreliable to report. In the lung, the primary target of WF deposition following intratracheal instillation, significant increases in levels of all elements, except Zn were detected (**Table 1**). Lung Zn levels were lower than control in the MMA-HS and $MnCl_2$ groups. Lung Fe levels following MMA-HS or a GMA-MS treatment were 22- and 59-fold above saline-treated controls (**Fig. 1**), while in lung-associated lymph nodes, Fe levels increased by 16- and 25-fold, respectively. High amounts of Cr were observed in the lungs (~16,000-fold over controls) and lung-associated lymph nodes (690-fold over controls) after MMA-HS treatment, while GMA-MS treatment caused a 171-fold increase in the lung (Fig. 1). Mn levels in the lung and lung-associated lymph nodes increased by ~2000-fold and ~700-fold, respectively, following MMA-HS or GMA-MS treatment (Fig. 1). Not surprisingly, Mn was observed to persist in the lung and lung-associated lymph nodes following welding exposure but not following $MnCl_2$ treatment, possibly due to the highly soluble nature of the $MnCl_2$ (Fig. 1). Further, despite the difference in Mn content of MMA-HS and GMA-MS fumes (27), as determined by their elemental composition (Mn weight % relative to all metals analyzed in MMA-HS and GMA-MS fumes were 43.7 and 6.9%, respectively), lung Mn levels following 28 weekly exposures to either fumes were not significantly different (Fig. 1). Thus, over time, a steady-state concentration of Mn was achieved in the lung.

In the brain significant deposition of Mn (**Fig. 2**), but not other elements (data not shown), was observed in all

TABLE 1. *Elemental analysis of lung and lung-associated lymph node (LALN) tissue following WF exposure*

Tissue	Al	Cu	Ni	Ti	Zn
Lung					
Saline	2.5 ± 0.7	15.7 ± 1.4	0.2 ± 0.1	0.1 ± 0.0	216.4 ± 17.9
MMA-HS	92.7 ± 2.3*	15.2 ± 1.1	48.7 ± 1.3*	276.8 ± 28.3*	155.5 ± 15.9#
GMA-MS	14.7 ± 2.2*	551.8 ± 168.5*	11.2 ± 1.3*	23.2 ± 4.2*	242.4 ± 19.2
$MnCl_2$	1.3 ± 0.2	18.2 ± 2.0	0.2 ± 0.1	0.1 ± 0.0	172.8 ± 16.3#
LALN					
Saline	2.5 ± 0.3	5.1 ± 0.9	0.6 ± 0.1	0.2 ± 0.0	18.3 ± 1.4
MMA-HS	17.9 ± 3.2*	3.9 ± 0.7	7.1 ± 2.4*	59.3 ± 16.3*	24.6 ± 5.3
GMA-MS	1.2 ± 0.4	39.8 ± 6.9*	1.1 ± 0.2	1.0 ± 0.2	34.4 ± 5.0*
$MnCl_2$	17.7 ± 17.9	5.3 ± 0.9	0.4 ± 0.1	0.3 ± 0.2	20.5 ± 1.2

Lungs and LALNs were excised 1 wk after repeated weekly instillations (2 mg/rat; 1/wk × 28 wk) of MMA-HS, GMA-MS, or $MnCl_2$. Control animals were treated with sterile saline. Elemental analysis was performed by ICP-AES; values are mean ± SE tissue concentration (μg/g dry tissue; n=4/group). Levels of Fe, Cr and Mn were also determined and are depicted in Fig. 1. *Significantly higher than saline-treated controls. #Significantly lower than saline-treated controls.

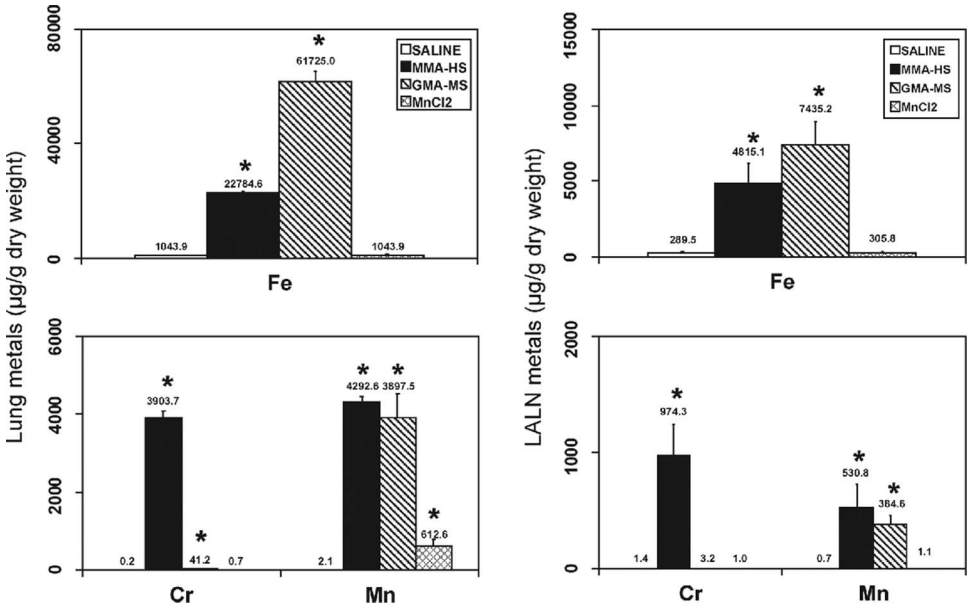

Figure 1. Elemental analysis of lung or lung-associated lymph node (LALN) tissue following Mn or WF exposure. Concentrations of Fe, Cr, and Mn were determined by ICP-AES, 1 wk after repeated weekly instillations (2 mg/rat; 1/wk × 28 wk) of GMA-MS, MMA-HS or $MnCl_2$. Levels of each element were calculated as micrograms per gram dry tissue. Bars represent means ± SE (n=4/group). *$P < 0.05$ *vs.* saline-treated controls.

brain areas (OB, STR, MB, PM, THL, CER, HIP, PCT, FCT) examined. Greater deposition of Mn occurred in dopaminergic brain areas following 28 weekly exposures to both types of fumes or $MnCl_2$. Large increases in Mn were observed with MMA-HS (115–230% over controls in various brain areas) and $MnCl_2$ (75–190% over controls in various brain areas) treatments. Smaller increases in Mn levels following GMA-MS (40–50% increase over controls) were seen in OB, STR, PM, THL, and MB, but not in other brain areas (Fig. 2). Based on our observation of Mn burden in the lung (Fig. 1), we hypothesize that Mn translocation to the brain following MMA-HS or GMA-MS is predominantly from the soluble or solubilized fractions of the fumes, consistent with that observed for the soluble $MnCl_2$. The kinetics of extrapulmonary translocation and accumulation is dependent on the slow dissolution of the poorly soluble components in the lungs, as reported recently by us (27, 29). Although GMA-MS caused only a small increase in brain Mn levels, it persisted for longer durations in the brain (27), and the speciation state of Mn in this fume can significantly influence its ability to cause molecular/cellular alterations. Thus, not only a large presence of Mn, but also biopersistence of smaller amounts of toxic Mn species, can potentially elicit neurotoxicity.

MMA-HS or GMA-MS WFs cause loss of striatal TH

Loss of TH function or protein is an index of injury to the dopaminergic neurons. Repeated exposure for 28 wk to either MMA-HS or GMA-MS decreased TH protein content in the STR as determined by immunoblot analysis (**Fig. 3**). MMA-HS and GMA-MS decreased TH protein by 45% (P<0.05) and 54% (P<0.05), respectively. A similar mass dose of $MnCl_2$ also caused significant loss of striatal TH (73%; P<0.05; Fig. 3). These findings indicate that Mn or Mn-containing WF exposure can alter TH, the rate-limiting enzyme in dopamine synthesis, and presumably dopaminergic function. Contrary to loss in the STR (Fig. 3), TH levels in

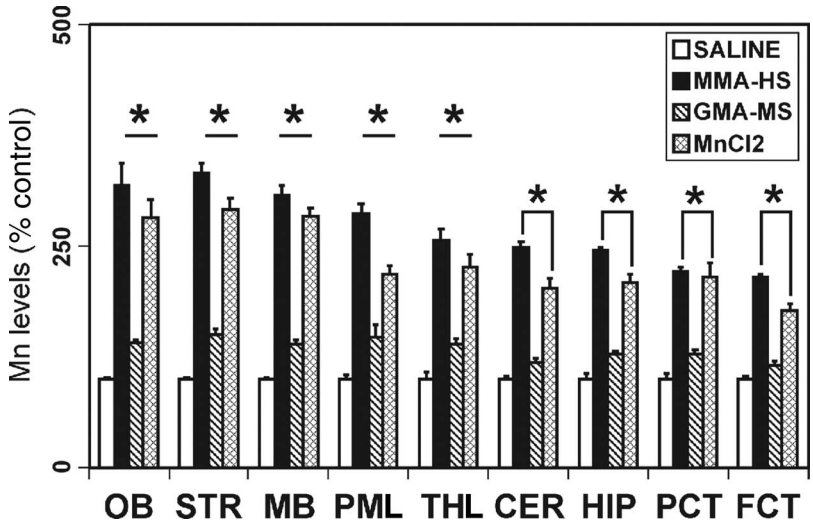

Figure 2. Deposition of Mn in the brain regions following Mn or WF exposure. Tissue concentrations of Mn were determined in discrete brain areas by ICP-AES, 1 wk after repeated weekly instillations (2 mg/rat; 1/wk × 28 wk) of GMA-MS, MMA-HS, or $MnCl_2$. Levels of Mn were calculated as micrograms per gram wet tissue and are represented as percentage of saline-treated control. Bars represent means ± SE (n=4/group). OB, olfactory bulb; STR, striatum; MB, midbrain; PM, pons/medulla; THL, thalamus; CER, cerebellum; HIP, hippocampus; PCT, parietal cortex; FCT, frontal cortex. *$P < 0.05$ *vs.* saline-treated controls.

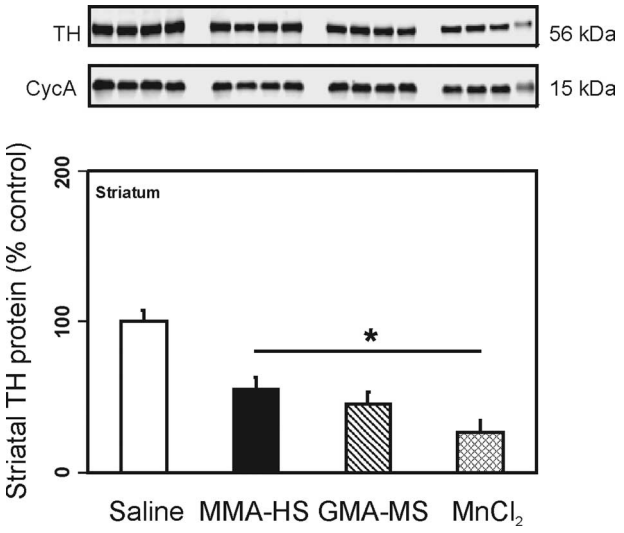

Figure 3. Loss of TH protein in dopaminergic brain areas following Mn or WF exposure. Striatal TH protein expression was determined by Western immunoblot analysis after repeated weekly instillations (2 mg/rat; 1/wk × 28 wk) of GMA-MS, MMA-HS, or $MnCl_2$. Following normalization to endogenous control (CycA), TH levels are expressed as percentage of saline-treated control. Bars represent means ± SE (n=4/group). *$P < 0.05$ *vs.* saline-treated controls.

MB, the brain area comprising dopaminergic cell bodies, were not altered significantly following exposure to either of the fumes or $MnCl_2$ (data not shown). Similarly, TH levels were not significantly different from controls in other brain regions innervated by dopaminergic nerve terminals, such as, FCT, PCT and OB (data not shown).

MMA-HS or GMA-MS WFs elicit neuroinflammation and oxidative stress

Increasing evidence indicates that inflammation plays a crucial role in the pathogenesis of neurodegenerative disorders, including PD. A number of proinflammatory cytokines such as interleukin (Il)1β, Il6, interferon-γ (Ifnγ) and tumor necrosis factor α (Tnfα) have been implicated as etiological factors in PD (34–37). Such factors are released by activated microglia and play a key role in the neuronal injury process (38). As neuroinflammation and microglial activation are known to precede dopaminergic neurotoxicity (27, 39, 40), we analyzed the expression of specific proinflammatory mediators after 7 weekly exposures to the two fumes or $MnCl_2$. Small increases in the expression of *Ifn*γ (2.3-fold; $P<0.05$) and *Tnf*α (1.3- to 1.5-fold; $P<0.05$) were observed in the STR (**Fig. 4**). However, in the MB, only $MnCl_2$ induced *Tnf*α (1.8-fold; $P<0.05$), indicating region-specific influences of these exposures on the induction of proinflammatory cytokines (data not shown). The induction of Tnfα in the dopaminergic targets is consistent with our earlier reports on the obligatory role of this cytokine in dopaminergic neurotoxicity (39–41).

Generation of reactive oxygen and nitrogen species has been suggested to be a key mediator in the pathophysiology of experimental and human PD (42, 43). Increases in nitric oxide synthase (Nos) activity or expression can result in the production of nitric oxide and peroxynitrite radicals that are thought to play a major role in neurodegenerative processes (44). To determine whether WF exposure might result in oxidative stress, we examined the expression of *Nos2* as an index of oxidative stress. Both fumes and $MnCl_2$ caused selective up-regulation of *Nos2* (1.6- to 3.7-fold; $P<0.05$) in the STR, 1 d following 7 weekly exposures (Fig. 4). No significant changes in the expression of *Nos1* or *Nos3* were observed in this brain region (Fig. 4). The subtle but significant neuroinflammatory and nitrosative stress responses are perhaps initiators of a more persistent and progressive loss of factors associated with dopaminergic signaling and function, events that precede overt neuropathology.

MMA-HS or GMA-MS WFs cause mitochondrial dysfunction

Mitochondrial impairment is emerging as a common theme in the pathogenesis of sporadic and some familial forms of PD (45). Experimental models of PD have also recapitulated similar dysfunction, suggesting that a loss of mitochondria plays a critical role in PD. Neurotoxicants like 1-methyl-4-phenyl-1,2,3,6,-tetrahydropyridine (MPTP) and rotenone have been shown to inhibit the mitochondrial electron transport chain (42, 46–48). Similarly, Mn neurotoxicity has also been linked to mitochondrial dysfunction (49–51). To determine whether Mn-containing WF also affected mitochondrial function, we measured the protein expression of various mitochondrial electron transport complex proteins in the STR and

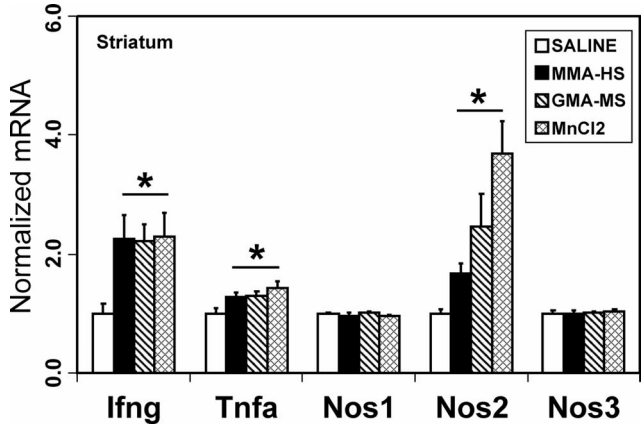

Figure 4. Neuroinflammatory and oxidative stress responses in STR following Mn or WF exposure. Striatal *Ifn*γ, *Tnf*α, *Nos1*, *Nos2*, and *Nos3* expression was assayed by TaqMan real-time PCR analysis following repeated weekly instillations (2 mg/rat; 1/wk × 7 wk) of GMA-MS, MMA-HS, or $MnCl_2$. Following normalization to endogenous control (β-actin; *Actb*), mRNA levels are expressed as fold-change over saline-treated controls. Bars represent means ± SE (n=8–10/group). *$P < 0.05$ *vs.* saline-treated controls.

MB of WF-treated animals. Following repeated exposure to MMA-HS, GMA-MS or $MnCl_2$ for 7 wk, region-specific decreases in various mitochondrial complex proteins were observed. In the STR, both GMA-MS and $MnCl_2$ treatment caused significant loss of complex III (CIII; 22 to 27%; $P<0.05$), complex IV (CIV; 28 to 50%; $P<0.05$), and complex V (CV; 20 to 30%; $P<0.05$) proteins (**Fig. 5**). In the MB, both GMA-MS and $MnCl_2$ decreased the levels of complex I (CI; 23 to 40%; $P<0.05$), CIV (45 to 50%; $P<0.05$), and CV (33%; $P<0.05$) proteins. However, MMA-HS treatment caused only a small decrease in CV (14 to 18%; $P<0.05$) protein in both, STR and MB (Fig. 5). Collectively, these findings suggest that exposure to Mn-containing WFs can alter mitochondrial function.

MMA-HS or GMA-MS WFs alter the expression of Park2, Park5, and Park7 proteins in the STR and MB

In humans, loss-of-function mutations in PARK genes are associated with early-onset parkinsonism (19, 20, 23, 24). In the brain, these genes are normally involved in affording neuroprotection against oxidative stress resulting from mitochondrial dysfunction. Because Mn or Mn-containing WFs caused mitochondrial dysfunction, we explored whether this is a consequence of alterations in certain PD-linked genes that are associated with mitochondrial function. Specifically, we examined the expression of Park2, Park5, and Park7. In the STR, GMA-MS and $MnCl_2$ treatments caused significant increases (46–65%; $P<0.05$) in Park2 protein following 7 weekly exposures (**Fig. 6**). However, following 28 weekly exposures, Park2 protein levels did not differ significantly from saline-treated controls. While a small (15%) but nonsignificant decrease in Park5 was seen after 7 weekly exposures to either of the fumes or $MnCl_2$, significant loss (39–73%; $P<0.05$) was observed after 28 weekly exposures (Fig. 6). However, loss (25–35%; $P<0.05$) of Park7 protein was seen by 7 wk of exposure to GMA-MS or $MnCl_2$, respectively. These levels decreased further (40–80%; $P<0.05$) after 28 weekly exposures to MMA-HS, GMA-MS, or $MnCl_2$ (Fig. 6). In the MB, only $MnCl_2$ treatment altered the expression of Park2 (230% increase over controls; $P<0.05$), Park5 (62% decrease; $P<0.05$), and Park7 (62% decrease; $P<0.05$) proteins (**Fig. 7**). Neither the WF nor $MnCl_2$ treatment altered the expression of these proteins in the HIP, suggesting that the dopaminergic brain areas might be more susceptible to the neurotoxic effects of Mn- or Mn-containing fumes, despite Mn deposition in other brain regions (as seen in Fig. 2). Administration of a lower dose of the WF or $MnCl_2$ also caused subtle alterations in the expression of these Park proteins (**Fig. 8**). Particularly, low doses of GMA-MS and $MnCl_2$ caused significant increases (19–

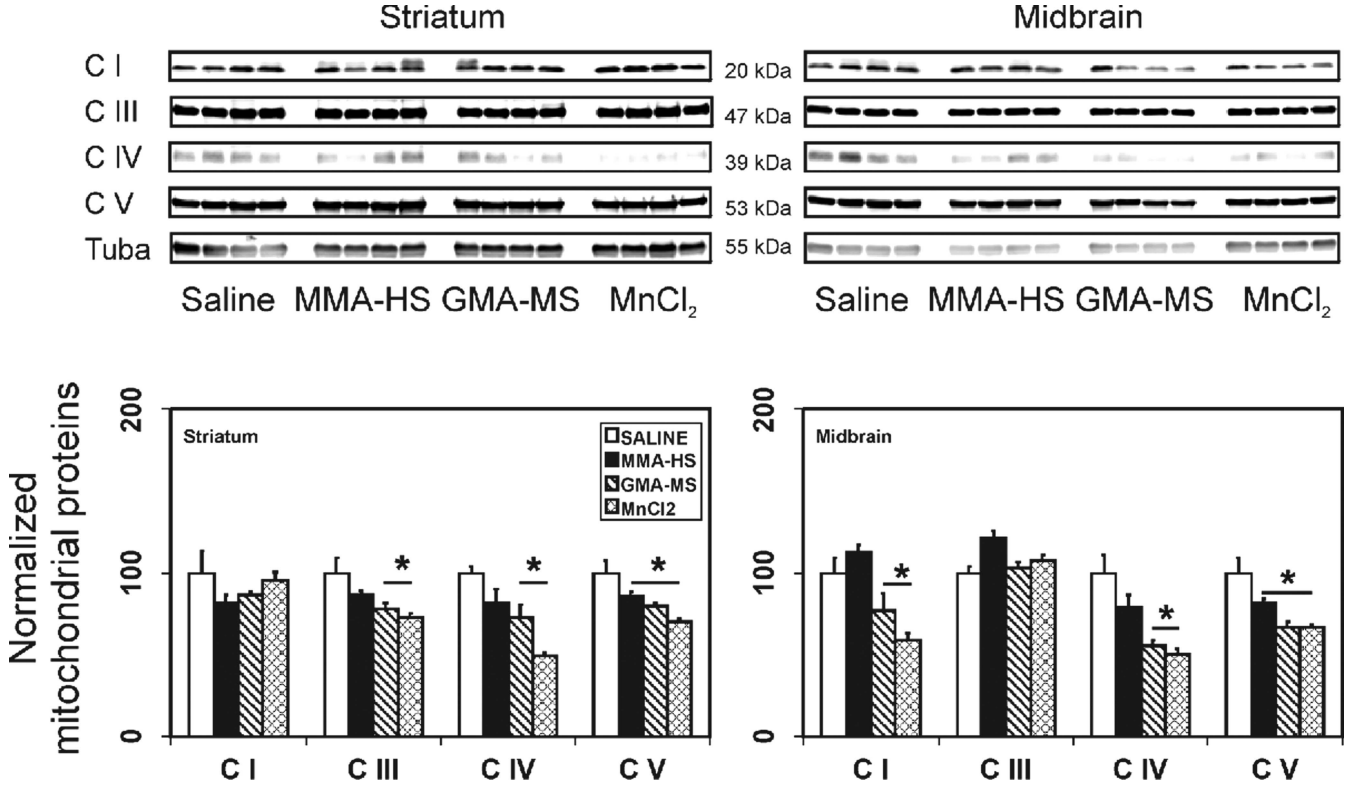

Figure 5. Loss of mitochondrial electron transport components in dopaminergic brain areas following Mn or WF exposure. Changes in expression of mitochondrial complex proteins (CI, CIII, CIV, and CV) in STR and MB were evaluated by Western immunoblot analysis following repeated weekly instillations (2 mg/rat; 1/wk × 7 wk) of GMA-MS, MMA-HS, or $MnCl_2$. Following normalization to endogenous control (α-tubulin; *Tuba*), protein levels are expressed as percentage of saline-treated control. Signal intensity of CII was too weak to reliably quantify and is therefore not presented. Bars represent means ± SE (n=4/group). *$P < 0.05$ *vs.* saline-treated controls.

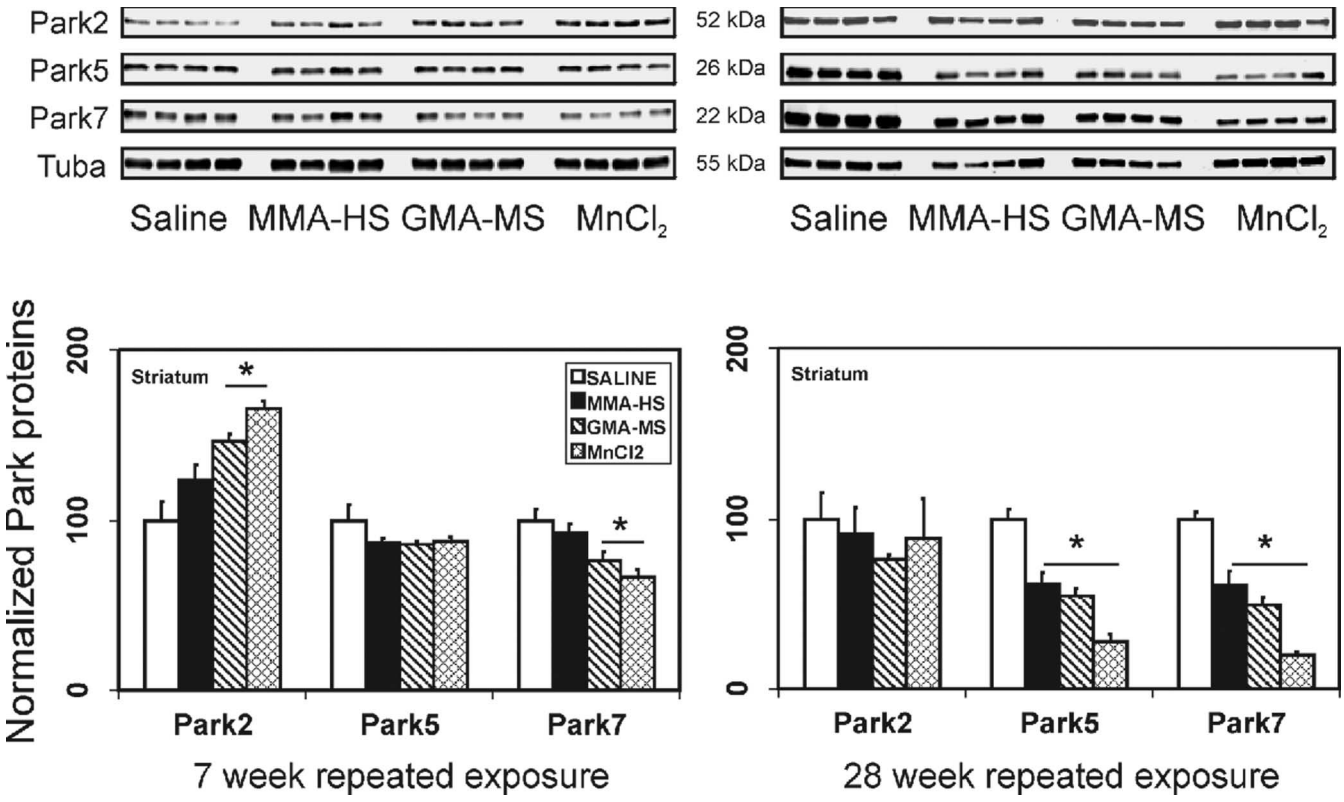

Figure 6. Expression of PD-linked proteins in STR following Mn or WF exposure. Changes in protein expression of Park2, Park5, or Park7 were investigated by Western immunoblot analysis following repeated weekly instillations (2 mg/rat) for 7 or 28 wk to GMA-MS, MMA-HS, or $MnCl_2$. Following normalization to endogenous control (α-tubulin; *Tuba*), protein levels are expressed as percentage of saline-treated control. Bars represent means ± SE (n=4/group). *$P < 0.05$ *vs.* saline-treated controls.

34%; $P<0.05$) in Park2 protein levels in the STR (Fig. 8). The low dose of GMA-MS also appeared to decrease (22%) Park7 protein in the STR but did not reach statistical significance (Fig. 8).

DISCUSSION

Neuroepidemiological findings, albeit limited, suggest a potential relationship between welding and parkinsonism (9, 10). While welding is considered a high-risk occupation for the development of manganism (52–54), it is also implicated as a potential risk factor for PD (9, 14). Moreover, concerns remain that welding might enhance the risk of PD, particularly with early-onset forms (10, 55). However, epidemiological findings to date remain controversial and inconclusive, primarily due to lack of well-defined case control studies. This finding is complicated further by the heterogeneity of the welding processes and diverse workplace environments where exposure might occur, as well as, other confounding factors. Well-controlled laboratory-based studies might provide ample health risk information to support or refute such relationships, if any. Our findings show that repeated exposure to Mn or Mn-containing WF causes mitochondrial dysfunction and alterations in

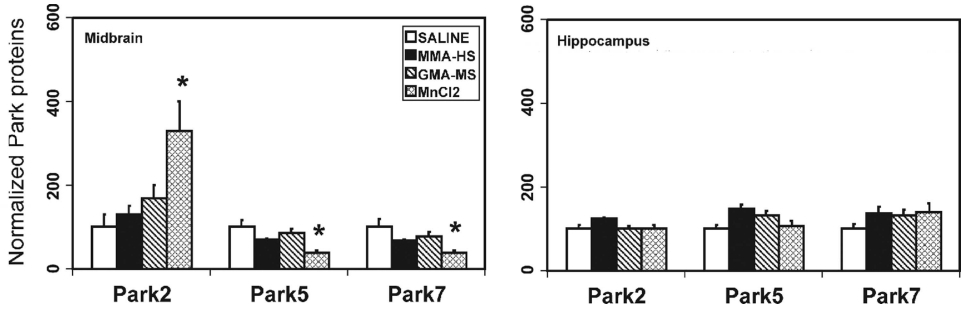

Figure 7. Expression of PD-linked proteins in MB and HIP following Mn or WF exposure. Changes in protein expression of Park2, Park5, or Park7 were analyzed by Western immunoblot analysis following repeated weekly instillations (2 mg/rat; 1/wk × 28 wk) of GMA-MS, MMA-HS or $MnCl_2$. Following normalization to endogenous control (α-tubulin), protein levels are expressed as percentage of saline-treated control. Bars represent means ± SE (n=4/group). *$P < 0.05$ *vs.* saline-treated controls.

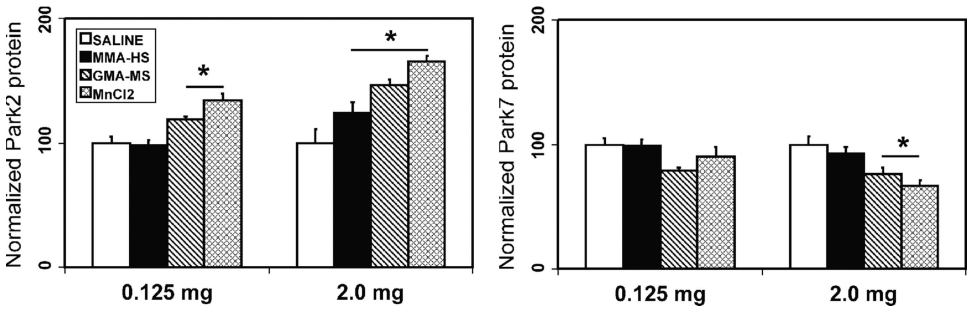

Figure 8. Expression of PD-linked proteins in STR following exposure to low and high doses of Mn or WF. Changes in protein expression of Park2 or Park7 were evaluated by Western immunoblot analysis following repeated weekly instillations (0.125 or 2 mg/rat; 1/wk × 7 wk) of GMA-MS, MMA-HS or $MnCl_2$. Following normalization to endogenous control (α-tubulin), protein levels are expressed as percentage of saline-treated control. Bars represent means ± SE (n=4/group). *$P < 0.05$ *vs.* saline-treated controls.

the expression of Park proteins in dopaminergic brain areas, events that contribute to dopaminergic neurotoxicity. A schematic illustration linking Mn, mitochondria, proteasome and Park genes is depicted in **Fig. 9**.

Recently, we reported that short-term repeated pulmonary exposure to MMA-HS or GMA-MS fumes resulted in selective deposition of Mn in the brain, particularly in dopaminergic brain areas (27). Other elemental constituents of the fumes like Fe, Cr, Ni or Cu did not appear to translocate to the brain despite their large accumulation in the lungs and lung-associated lymph nodes. In the same study, we showed that exposure to Mn-containing WF altered several molecular markers of dopaminergic neurotoxicity and that the injury response extended beyond the globus pallidus, considered the primary site of damage in manganism, to broader dopaminergic areas, including the STR and MB. The involvement of these additional brain regions bear resemblance to PD and suggests that the neurotoxic outcome following WF exposure might involve common/similar molecular mechanisms. Consistent with our earlier observations of Mn accumulation in the brain (27), a similar pattern of accumulation was observed in this study following long-term repeated pulmonary exposure, indicating that selective translocation of Mn to brain structures occurs following WF exposure. Specifically, greater deposition of Mn occurred in dopaminergic brain areas and in brain areas associated with dopaminergic signaling. MMA-HS or

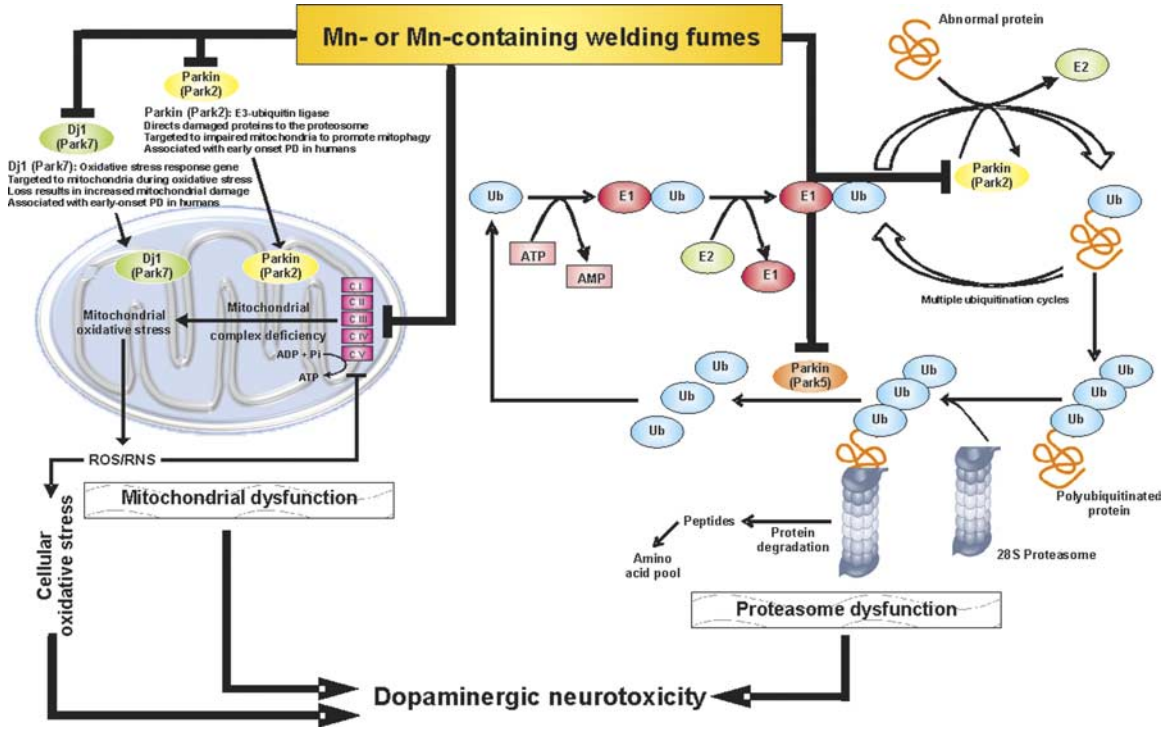

Figure 9. Schematic illustration of an association between mitochondria, proteasome, oxidative stress, and Park proteins in eliciting dopaminergic neurotoxicity of Mn-containing WFs. Inhibition of mitochondrial electron transport by Mn can induce mitochondrial oxidant stress and release of free radicals, which in turn can exacerbate mitochondrial and cellular stress. Impairment of mitochondria can lead to mitochondrial targeting of Park proteins to afford neuroprotection and/or to recruit damaged mitochondria toward autophagy/mitophagy. However, Mn-mediated loss of Park proteins can result in impairment of mitochondrial and proteasome function, contributing to accumulation of damaged proteins and/or organelles, events that underlie dopaminergic dysfunction.

$MnCl_2$ treatment resulted in a larger deposition of Mn as compared to GMA-MS, consistent with the elemental composition and solubility of the two fumes (27, 29). However, the neurotoxicological responses elicited by the two fumes were markedly different, with GMA-MS appearing to cause more toxicity than MMA-HS, based on the endpoints examined. Although GMA-MS exposure resulted in only a small increase in brain Mn levels, its persistence for longer durations in the brain than Mn from MMA-HS fumes (27), might likely have contributed to the greater neurotoxic effects that were observed in the current study.

Besides concentration and solubility, Mn speciation could also play a critical role in the neurotoxic response. Indeed Mn speciation, involving reduction-oxidation states are critical for evaluating the functional and toxicological properties of metals (56–58). As Mn exhibits complex redox states, its toxic potency might vary with its speciation (59). While the levels of Mn in blood rapidly peaked and cleared within a few hours following intratracheal instillation exposure to soluble Mn salts, Mn accumulation following exposure to insoluble Mn compounds was remarkably delayed (59). Our findings of Mn kinetics in the brain following MMA-HS or GMA-MS exposures (27) concur with the observations of Roels *et al.* (59). Thus, altered kinetics and speciation can contribute to differences in the biological responses. Based on our observations, we hypothesize that Mn speciation might be a more critical determinant of the neurotoxic response than solubility and that the Mn species in the two fumes are likely different.

It is reported that Mn (II) and Mn (III) forms of Mn can predominantly translocate to the brain and produce very different toxicological effects on cell function (57). Moreover, Mn (III) forms are known to accumulate to a greater extent and are retained for a longer duration in the blood and brain compared to Mn (II) forms (58). Perhaps our observations with the two types of WF are a consequence of such differences in the redox state of Mn in the WF. Indeed, X-ray diffraction (XRD), and extended X-ray absorption fine structure (EXAFS) analyses show that Mn phases in the WF produced by arc welding with covered electrodes are predominantly $MnFe_2O_4$ and $KMnO_4$ (60) and suggest that the valence of Mn ions in the fumes are primarily Mn (II) and Mn (III). Further, the oxidation states of Mn in WF appear to be very different depending on the welding techniques and type of welded steels (61), making it difficult to discern the exact Mn species that initiates and/or associates with the neurotoxicity. Comprehensive evaluation of the chemical characteristics of the two fumes used in this study are ongoing and will likely shed light on the influence of Mn speciation in eliciting neurotoxicity. Because much of the exposure at the workplace is to insoluble forms of Mn, primarily *via* inhalation, gradual dissolution and translocation of Mn from the lung to the brain might contribute to progressive neurotoxicity. Our observation of the persistence of Mn in the lungs and lung-associated lymph nodes of animals exposed to the two WF augments the probability of such an occurrence.

Although the etiology of PD remains elusive, several molecular and biochemical abnormalities including neuroinflammation, oxidative stress, mitochondrial dysfunction, and ubiquitin-proteasome dysfunction are thought to contribute to the neurodegenerative process in sporadic forms of the disease. In addition, several gene mutations linked to familial forms of the disease have also been identified. Specifically, mutations in *PARK2* and *PARK7* are known to cause autosomal recessive early-onset parkinsonism (19–21, 23–25). Although welding has been implicated as a possible risk factor for development of PD and has been proposed to accelerate its onset (9, 10), no mechanistic evidence for such an association exists. To assess whether PD-linked genes contribute to Mn- or WF-related neurotoxicity, we examined the expression of Park2, Park5, and Park7 genes in discrete brain areas. Further, we evaluated the involvement of inflammation, oxidative stress, and mitochondrial dysfunction as potential pathogenic mechanisms that might contribute to the dopaminergic neurotoxicity following WF exposure. Repeated exposure to GMA-MS or $MnCl_2$, in particular, increased the expression of Park2 protein in the STR and MB. Park2 functions as an E3-ubiquitin ligase, catalyzing the addition of ubiquitin to proteins and targeting them for degradation by the 26S proteasome (62, 63). The increased expression of Park2 seen following Mn or Mn-containing WF exposure perhaps reflects a protective mechanism to clear over-expressed or defective/abnormal proteins induced by such treatments. Indeed, transient transfection of the Park2 gene in a dopaminergic cell line resulted in inhibition of Mn-induced cell death (64), suggesting that Park2 overexpression might afford neuroprotection. Such protection is likely a consequence of Park2-mediated regulation of divalent metal transporter 1 (Dmt1) expression through proteasomal mechanisms, resulting in decreased Mn uptake and toxicity (65). Our recent demonstration that WF or Mn exposure induces Dmt1 expression (27) lends support to the findings of Roth *et al.* (65), suggestive of an interaction between Park2 and Dmt1. Loss of Park2 on the other hand can result in a failure to target Park2 substrates to the proteasome due to lack of ubiquitination. Ineffective regulation of Dmt1 expression and/or toxic accumulation of Park2 substrates might subsequently contribute to enhanced Mn uptake and dopaminergic neurotoxicity.

Park2 also appears to play a role in maintaining the integrity and fidelity of mitochondria, as well as removal of damaged mitochondria. Park2 is selectively recruited to impaired mitochondria to promote their autophagy (66). Abnormality of mitochondrial permeability transition or failure of mitochondria to maintain ATP levels are also known to cause mitochondrial autophagy or mitophagy (67, 68) and might similarly be linked to Park2. Thus, Park2 plays a critical role in targeting and moving abnormal proteins and damaged organelles to degradation pathways. Mitochondrial dysfunction is impli-

cated strongly in the neurodegeneration seen in idiopathic and experimental PD (42, 69–73). A similar impairment of mitochondrial function following Mn or Mn-containing WF treatment, as evidenced by the loss of one or more electron transport complex proteins in dopaminergic brain regions, suggests that the underlying molecular mechanisms might be analogous to that seen in PD. Mn is thought to accumulate specifically in the mitochondria and disrupt its function through inhibition of electron transfer (74–76) or by enhancing free radical formation (77–79). Inhibition of mitochondrial electron transport itself can elicit free radicals, and likewise increased free radicals can impair mitochondrial respiration, thus triggering a vicious cycle of events that can result in exacerbation of mitochondrial damage. Both mitochondrial dysfunction and oxidative stress have been linked to manganese-induced neurotoxicity (51, 80). Our observations of selective brain accumulation of Mn, nitrosative stress, and mitochondrial dysfunction following WF exposure suggest that Mn from WF is a likely candidate for causing dopaminergic neurotoxicity.

Like Park2, Park5 is another key enzyme involved in the ubiquitin-proteasome pathway. Park5 is an ubiquitin carboxyl-terminal esterase/hydrolase L1 (Uchl1) that is expressed predominantly in neurons and neuroendocrine cells (81). Park5 functions to tag overexpressed or damaged proteins with ubiquitin for subsequent degradation by the proteasome. In addition, Park5 also is thought to have hydrolase activity whereby it deubiquitinates proteins and recycles the ubiquitin to sustain the protein degradation pathway. Although *PARK5* mutations are rare and their involvement in PD pathogenesis is debatable (82–84), compromised function or reduced expression of this gene has been associated with idiopathic PD (85). Further, mice mutant for *Park5* showed axonal loss and cell death (86). Thus, a deficiency or loss of Park5 can cause disruption of the ubiquitin-proteasome pathway and contribute to dopaminergic pathology. A similar loss of Park5 seen in dopaminergic brain areas following exposure to Mn or Mn-containing WF might likewise result in excessive accumulation of damaged, misshapen, or over-expressed proteins, and thereby progressively alter neuronal function.

Exposure to Mn or Mn-containing WF also caused loss of another PD-related protein, Park7, in the STR and MB. *PARK7* mutations account for ~1–2% of early-onset cases of PD (87, 88). Park7 has been shown to be expressed in the brain, including neurons within the substantia nigra pars compacta and STR, areas primarily affected in PD (89). Mice deficient in *Park7* exhibit exacerbation of MPTP-induced neurodegeneration (90). Disruption of *Park7* also results in nigrostriatal dopaminergic deficits, hypokinesia, and alterations in dopamine D2 receptor (Drd2)-related functions in the substantia nigra (91). Our recent findings of a persistent loss of *Drd2* following WF exposure (27) and the loss of Park7 in this study appear to concur with the observations of Goldberg *et al.* (91) on the involvement of Park proteins in dopaminergic dysfunction.

Park7 expression has been localized to the matrix and intermembrane space of mitochondria (92, 93) and is thought to function as an antioxidant protein (94). *Park7*-knockout mice exhibit an increase in mitochondrial free radical formation and inactivation of mitochondrial enzymes (95). Down-regulation of Park7 has been shown to enhance cell death through exacerbation of oxidative stress and inhibition of the proteasome (96). The early and persistent loss of Park7 seen following Mn or WF exposure concomitant with mitochondrial dysfunction suggests that Park7, like Park2, plays a critical role in maintaining mitochondrial integrity and function through protection from oxidant damage. Consequently, its loss might be indicative of progressive neural injury.

Modulation of PD-linked proteins by Mn or Mn-containing WF can impede the ubiquitin-proteasome and/or mitochondrial system, thereby disrupting normal dopaminergic neuron function. Corroborating evidence comes from the significant loss of striatal TH seen following such exposures. TH is a key enzyme involved in the synthesis of the neurotransmitter dopamine, and its loss or decreased activity is indicative of impaired dopaminergic function. Loss of TH immunoreactivity is also characteristic of PD. Collectively, our observations of mitochondrial dysfunction, modulation of Park proteins, and loss of TH in dopaminergic brain areas suggest that many of the early pathogenic mechanisms associated with Mn- or WF-related dopaminergic neurotoxicity bear similarity with the mechanistic features associated with PD. The involvement of such common biochemical and molecular associations in regulating the disease state in manganism or PD has recently been proposed (97). Whether such effects persist and cause neuropathological and neurobehavioral deficits reminiscent of PD remain unknown. Our ongoing investigations aimed at addressing the long term effects of such exposures, perhaps will help unravel the uncertainty surrounding WF exposure and the appearance of PD-like neurological dysfunction. FJ

The findings and conclusions of this article have not been formally disseminated by NIOSH and should not be construed to represent any agency determination or policy. K.S. conceived the study. K.S., J.M.A., O.W., and K.K. designed the study. K.S. headed the neurotoxicology studies, performed brain dissections, analyzed data, and wrote the paper. G.X.L. and A.M.J. conducted all neurotoxicity-related assays. J.M.A. and J.R.R. performed animal treatments and collected lung samples for elemental analysis. J.M.S. and A.J.G. performed tissue elemental analysis and acquired data. S.H.R., V.C., and A.E.M. were involved in experimental planning, design, and preparation of the manuscript. All authors reviewed and approved the final manuscript. The authors declare they have no proprietary, financial or personal interest of any kind or nature in any samples, products, supplies, service, or company that could be construed as being a conflict of interest.

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