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Manganese accumulation in nail clippings as a biomarker of welding fume exposure and neurotoxicity☆

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ABSTRACT

Occupational exposure to welding fumes (WF) is thought to cause Parkinson's disease (PD)-like neurological dysfunction. An apprehension that WF may accelerate the onset of PD also exists. Identifying reliable biomarkers of exposure and neurotoxicity are therefore critical for biomonitoring and neurological risk characterization of WF exposure. Manganese (Mn) in welding consumables is considered the causative factor for the neurological deficits seen in welders. Hence, we sought to determine if Mn accumulation in blood or nail clippings can be a marker for adverse exposure and neurotoxicity. To model this, rats were exposed by intratracheal instillation to dissolved or suspended fume components collected from gas metal arc-mild steel (GMA-MS) or manual metal arc-hard surfacing (MMA-HS) welding. Trace element analysis revealed selective Mn accumulation in dopaminergic brain areas, striatum (STR) and midbrain (MB), following exposure to the two fumes. This caused dopaminergic abnormality as evidenced by loss of striatal tyrosine hydroxylase (Th; 25–32% decrease) and Parkinson disease (autosomal recessive, early onset) 7 (Park7; 25–46% decrease) proteins. While blood Mn was not detectable, Mn levels in nails strongly correlated with the pattern of Mn accumulation in the striatum ($R^2 = 0.9386$) and midbrain ($R^2 = 0.9332$). Exposure to manganese chloride ($MnCl_2$) caused similar Mn accumulation in STR, MB and nail. Our findings suggest that nail Mn has the potential to be a sensitive and reliable biomarker for long-term Mn exposure and associated neurotoxicity. The non-invasive means by which nail clippings can be collected, stored, and transported with relative ease, make it an attractive surrogate for biomonitoring WF exposures in occupational settings.

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1. Introduction

An estimated 466,400 workers are employed full-time in welding operations in the United States (Bureau of Labor Statistics, U.S. Department of Labor, 2011), and globally this figure exceeds two million workers. Welding generates fumes that are a complex mixture of gases (carbon monoxide, carbon dioxide, nitrous oxide, ozone) and metal particulates (iron, manganese, chromium, nickel). These aerosols are comprised of high concentrations of fine and ultrafine metal particles, including manganese, chromium and nickel, which are known to be toxic. The aerodynamic diameter of WF aerosols in the welder's breathing zone is reported to range from 100 nm to 1 μm (Zimmer and Biswas, 2001; Jenkins et al., 2005; Antonini et al., 2006), which are respirable and can deposit in the olfactory and lower respiratory tracts. Hence, exposure to airborne WF particulates is of significant occupational concern. Welders are a heterogeneous workforce employed in a variety of workplace conditions that include open, well-ventilated (e.g., outdoors on a construction site) or confined, poorly ventilated (e.g., ship hull, building crawl space and pipeline) spaces. 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.

Exposure to WF has been linked to occupational respiratory diseases, cancer and ischemic heart diseases (Ozdemir et al., 1995; Hansen et al., 1996; Bradshaw et al., 1998; Sjögren et al., 2002,

Abbreviations: Actb, beta-actin; ANOVA, analysis of variance; BCA, bicinchoninic acid; GMA-MS, gas metal arc-mild steel; ICP-AES, inductively coupled plasma-atomic emission spectroscopy; MB, midbrain; MMA-HS, manual metal arc-hard surfacing; MRI, magnetic resonance imaging; Park7, Parkinson disease (autosomal recessive, early onset) 7; PBST, phosphate-buffered saline (pH 7.2) containing 0.5% (v/v) Tween-20; PD, Parkinson's disease; Snap25, synaptosomal-associated protein 25; STR, striatum; Th, tyrosine hydroxylase; WF, welding fume.

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2006). An emerging apprehension is that WF exposure may be associated with the development of a neurological dysfunction similar to PD (Racette et al., 2001, 2005; Josephs et al., 2005; Bowler et al., 2006, 2007a). Much of this concern has been attributed to the presence of Mn in welding electrodes/rods. Chronic overexposure to Mn in occupational settings like mining, smelting, ferroalloy and dry battery industries has been shown to impair motor function, consequently leading to Parkinsonism (Couper, 1837; Rodier, 1955; Emara et al., 1971; Wang et al., 1989; Wennberg et al., 1991; Lucchini et al., 1997, 1999). Our recent experimental studies have shown that repeated exposure to WF caused selective accumulation of Mn in the brain leading to dopaminergic abnormality (Sriram et al., 2010a, 2010b), as assessed by changes in mitochondrial function and expression of Th, Park5 and Park7 proteins. Further, our studies revealed that the neurotoxicity extended beyond globus pallidus, conventionally thought to be the site of damage in Mn intoxication, to striatum (STR) and midbrain (MB), brain areas typically associated with neurodegeneration in PD (Sriram et al., 2010a, 2010b).

The emerging findings of neurotoxicity and PD-like neurological manifestations linked to Mn-containing WF, calls for identification and validation of reliable biomarkers of exposure and adverse neurotoxicity. Such information is critical for workplace exposure monitoring, disease-risk assessment, and establishment of occupational exposure limits. Together, these measures can contribute to achieving the primary goal of occupational safety and health programs, which is prevention of adverse health effects resulting from workplace exposures. Occupational health risks are primarily dependent on three critical factors, (i) inherent hazard or toxicity of the agent, (ii) population of workforce employed in handling the agent, and (iii) nature of usage/application of agent that poses a potential risk for exposure (Sargent, 2006). All these features are pertinent to WF exposures; wherein, there is potential for exposure to toxic metal aerosols, a large number of workers are employed in welding operations, and welding in complex workplace settings like confined spaces, can potentially increase the risk of exposure.

Biological monitoring of Mn exposures in whole blood, urine and hair has been attempted in an effort to demonstrate toxicological outcomes following occupational exposures (Roels et al., 1992; Mergler et al., 1994; Lucchini et al., 1995; Myers et al., 2003; Ellingsen et al., 2003; Bowler et al., 2007b). However, accurate measurements of Mn concentrations in blood have often been hampered by technical and analytical challenges resulting in considerable variations in reported values (Järvisalo et al., 1992; Myers et al., 2003; Ellingsen et al., 2003; Bowler et al., 2007b; Jiang et al., 2007). It is thought that a poor correlation between blood and tissue Mn levels is a likely cause for the variability in reported values (Takeda et al., 1995; Apostoli et al., 2000; Lu et al., 2005). Further, blood levels of essential trace elements are tightly regulated; thus changes in blood metals may be transient in nature (Zheng et al., 2000), and therefore less likely to reflect long-term exposures. This warrants identification of reliable, sensitive, minimally invasive and cost-effective biological sample sources for effective biomonitoring of Mn exposures. To that end, utilizing an experimental animal model of WF neurotoxicity (Sriram et al., 2010b), we explored if nail clippings can serve as a reliable surrogate for assessment of adverse exposures to WF. Specifically, we examined if Mn accumulation in nail clippings can be a potential marker of WF exposure and neurotoxicity.

2. Materials and methods

2.1. Welding fume generation

Bulk samples of MMA-HS and GMA-MS fumes were provided as a gift by Lincoln Electric Company (Cleveland, OH). The fumes were generated in a cubical open front fume chamber (volume = 1 m^3) by a skilled welder using a manual or semi-automatic technique appropriate to the electrode and collected on 0.2 μm Nuclepore filters (Nuclepore, Pleasanton, CA). The fumes were generated using two different processes: (1) manual metal arc welding using a flux-covered stainless steel hard-surfacing electrode (MMA-HS; Wearshield 15CrMn, Lincoln Electric, Cleveland, OH) and (2) gas metal arc welding using a mild steel E70S-3 electrode (GMA-MS; L-50 carbon steel electrode, Lincoln Electric, Cleveland, OH).

Particle size distribution of the two fumes was not determined in the current study as bulk samples were obtained as a gift from Lincoln Electric Company (Cleveland, OH). However, fumes generated in a similar manner in our laboratory using a robotic welder were size characterized. Particle-size distribution of GMA-MS particulates was determined in an animal exposure chamber using a Micro-Orifice Uniform Deposit Impactor (MOUDI, MSP Model 110, MSP Corporation, Shoreview, MN) and a Nano-MOUDI (MSP Model 115, MSP Corporation, Shoreview, MN) that is specifically designed for sampling aerosols in size ranges down to 0.010 μm. Using the two MOUDI impactors in series or in tandem, WF aerosols were separated for size characterization and determination of mass median aerodynamic diameter (MMAD). GMA-MS fume particles generated this way exhibited a particle size distribution ranging from 0.10 to 1.0 μm in diameter (Antonini et al., 2011). The MMAD of GMA-MS fume particles were calculated to be 0.30 μm (Antonini et al., 2011).

2.2. 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 (Sriram et al., 2010a). Briefly, GMA-MS fumes comprise predominantly of Fe (90.4% of total metals) and small amounts of Mn (6.9%), Cr (0.04%), Cu (0.9%) and Zn (1.3%), while MMA-HS fumes are composed of Fe (30.4%), Mn (43.7%), Cr (8.2%), Cu (0.03%), and Zn (0.7%), respectively. Further, GMA-MS fumes are mostly water-insoluble; only a small fraction (1.7% of the total metal content) being water-soluble, while MMA-HS fumes contain a larger fraction (17.9% of total metal content) of water-soluble metals (Sriram et al., 2010a).

To determine elemental content in tissues/organs (striatum, midbrain, lung, liver, kidney or blood), 1 ml of 3 N hydrochloric acid/10% trichloroacetic acid solution was added to pre-weighed 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 by ICP-AES (kind help from Dr. Andrew Ghio and Ms. Joleen Sokup, US-EPA). Nail samples were analyzed by ICP-AES following NMAM 7300 method modified for bulk tissue samples (NIOSH, 2003). Briefly, nail samples were ashed with a concentrated mixture of nitric acid/perchloric acid (4:1) at 120 °C, using a hotplate. Samples were transferred to volumetric flasks and diluted to 10 ml prior to ICP-AES analysis. Recommended multi-element standards were run concurrently. Whole blood elemental levels were measured as μg/ml. Elemental content of lung, liver, heart or kidney were measured as μg/g dry weight. Elemental content of brain tissues and nails were measured as μg/g wet weight. Values are expressed as μg/g (Table 1) or as percent of saline-treated controls (in figures) for better comparison and clarity, due to large differences in the basal levels of metals among various organs.

2.3. Animals

Male Sprague-Dawley [Hla:(SD) CVF] rats (250–300 g) were procured from Hilltop Lab Animals (Scottdale, PA). The rats were acclimated for at least 6 days after arrival and were housed in ventilated polycarbonate cages with Alpha-Dri cellulose chips as bedding, with provision for HEPA-filtered air, irradiated Teklad 2918 diet 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 institution's Animal Care and Use Committee.

2.4. Animal exposures

Rats were exposed by intratracheal instillation to dissolved or suspended fume components collected from gas metal arc-mild steel (GMA-MS) or manual metal arc-hard surfacing (MMA-HS) welding. GMA-MS or MMA-HS fume particulates were prepared in sterile saline and sonicated for 1 min in a Sonifier 450 Cell Disruptor (Branson Ultrasonics, Danbury, CT) to disperse the particulates. Rats were lightly anesthetized by an intraperitoneal injection of 0.6 ml of a 1% solution of sodium methohexital (Brevital®, Eli Lilly, Indianapolis, IN). Intratracheal instillations (Reasor and Antonini, 2000) of GMA-MS or MMA-HS fume condensates (2 mg/animal in 300 μl of sterile saline; $n = 4$ per treatment group) were carried out once a week for 28 weeks. Based on our estimates of Mn content in GMA-MS (6.9%) and MMA-HS (43.7%), the amount of elemental Mn administered per dose of each WF, amounts to ~0.14 mg and ~0.87 mg, respectively. To relate the pulmonary (intra-tracheal instillation) dosing paradigm employed in this study to workplace exposures of welders, we utilized a mathematical calculation (Sriram et al., 2010a) to determine the daily lung burden of a welder on an 8 h work schedule exposed to a fume concentration of 5 mg/m^3. This concentration was the threshold limit value (TLV) previously established for WF by the American Conference of Governmental Industrial

Table 1
Elemental analysis of various organs and specific brain regions.

Target organ/region	Treatment group	Cr (μg/g)	Cu (μg/g)	Fe (μg/g)	Mn (μg/g)	Zn (μg/g)
Brain (striatum)	Saline	0.35 (0.02)	4.42 (0.17)	21.08 (1.88)	0.48 (0.01)	13.92 (0.17)
	GMA-MS	0.53 (0.04)	4.37 (0.25)	29.68 (5.16)	0.73* (0.03)	14.69 (0.34)
	MMA-HS	0.35 (0.06)	4.42 (0.09)	21.40 (1.13)	1.61* (0.05)	13.98 (0.31)
	$MnCl_2$	0.63* (0.04)	4.88 (0.10)	25.11 (0.76)	1.41* (0.06)	15.26* (0.20)
Brain (midbrain)	Saline	0.63 (0.01)	3.37 (0.17)	20.31 (1.01)	0.75 (0.01)	10.57 (0.34)
	GMA-MS	0.73 (0.05)	3.93 (0.31)	23.46 (0.53)	1.04* (0.04)	11.22 (0.36)
	MMA-HS	0.44* (0.03)	3.27 (0.16)	18.19 (0.48)	2.29* (0.09)	9.96 (0.19)
	$MnCl_2$	0.78 (0.09)	3.84 (0.25)	25.36 (1.58)	2.12* (0.06)	12.46 (1.24)
Blood	Saline	ND	0.31 (0.02)	74.68 (5.81)	ND	1.89 (0.07)
	GMA-MS	ND	0.28 (0.01)	83.06 (6.68)	ND	2.09 (0.09)
	MMA-HS	ND	0.31 (0.09)	66.92 (8.53)	ND	1.92 (0.28)
	$MnCl_2$	ND	0.28 (0.02)	80.21 (3.24)	ND	2.13 (0.11)
Nail	Saline	4.50 (0.30)	17.90 (1.40)	16.60 (1.90)	0.30 (0.04)	140.60 (9.90)
	GMA-MS	3.90 (0.10)	17.40 (1.60)	25.20 (6.90)	0.60* (0.01)	143.50 (2.30)
	MMA-HS	7.50 (1.40)	21.00 (2.60)	34.30 (4.30)	2.20* (0.50)	167.00 (30.80)
	$MnCl_2$	4.70 (0.70)	17.00 (0.90)	21.50 (0.70)	2.10* (0.20)	135.90 (8.00)
Lung	Saline	0.24 (0.12)	15.71 (1.40)	1043.91 (67.08)	2.14 (0.18)	216.37 (17.85)
	GMA-MS	41.19* (7.32)	551.79* (168.54)	61,725.01* (3763.77)	3897.45* (613.63)	242.43 (19.22)
	MMA-HS	3903.69* (175.60)	15.24 (1.08)	22,784.58* (418.72)	4292.62* (160.58)	155.49 (15.90)
	$MnCl_2$	0.68* (0.47)	18.17 (2.01)	1043.90 (148.00)	612.59* (166.04)	172.79 (16.28)
Liver	Saline	0.015 (0.00)	0.33 (0.01)	16.75 (2.70)	0.19 (0.01)	2.24 (0.12)
	GMA-MS	0.004 (0.00)	0.31 (0.02)	11.85 (1.63)	0.16 (0.01)	2.07 (0.18)
	MMA-HS	0.041* (0.02)	0.33 (0.06)	17.26 (2.47)	0.25 (0.05)	2.21 (0.50)
	$MnCl_2$	0.005 (0.00)	0.33 (0.03)	16.77 (1.33)	0.22 (0.02)	2.61 (0.55)
Heart	Saline	0.06 (0.00)	7.67 (0.15)	114.25 (4.46)	0.67 (0.02)	24.07 (0.77)
	GMA-MS	0.06 (0.01)	7.11 (0.29)	107.35 (3.24)	0.82* (0.04)	22.33 (1.15)
	MMA-HS	0.40* (0.03)	7.65 (0.52)	120.18 (4.45)	1.43* (0.11)	24.78 (1.81)
	$MnCl_2$	0.06 (0.01)	8.52 (0.57)	113.48 (11.01)	1.05* (0.07)	26.45 (1.73)
Kidney	Saline	ND	4.39 (0.24)	61.87 (12.49)	0.41 (0.02)	2.24 (0.42)
	GMA-MS	ND	4.01 (0.35)	45.58 (3.78)	0.58* (0.06)	2.07 (0.63)
	MMA-HS	ND	4.37 (0.61)	46.44 (6.55)	0.78* (0.16)	2.21 (1.09)
	$MnCl_2$	ND	4.31 (0.91)	41.46 (3.94)	0.50 (0.04)	2.61 (0.81)

Elemental analysis was performed by ICP-AES. Absolute levels of major elements present in various organs or dopaminergic brain areas (striatum and midbrain) following GMA-MS, MMA-HS or $MnCl_2$ are presented. Levels of metals in striatum, midbrain and nail are expressed as μg/g wet tissue; levels in lung, liver, heart and kidney as μg/g dry tissue; levels in blood as μg/ml whole blood. Values are mean ± SE (n = 4/group) and are rounded up to the second decimal.
ND = not detected.

* Significantly different from saline controls ($P < 0.05$), demonstrating a treatment-related effect. Significance of all other pairwise comparisons are appropriately depicted in the figures.

Hygienists (ACGIH), calculated as a TWA for a normal 8-h workday and a 40-h workweek (ACGIH, 1994). Currently, however, neither ACGIH nor the Occupational Safety and Health Administration (OSHA) have a recommended TLV for WF. Incorporating factors such as fume concentration (5 mg/m^3, previous TLV for WF), human (worker) minute ventilation volume (20,000 ml/min × 10^{-6} m^3/ml), exposure duration (8 h/day × 60 min/h) and a predicted deposition efficiency of 15% (Antonini et al., 2006), it was determined that the daily lung burden of a welder is about 7.2 mg. Using surface area of alveolar epithelium (rat = 0.4 m^2; human = 102 m^2) as dose metric (Stone et al., 1992), the daily lung burden for a similar exposure in the rat amounts to 0.0282 mg. Factoring the cumulative dosing paradigm used in this study (2 mg × 28 instillations = 56 mg) and the estimated daily lung burden for rat (0.0282 mg), the number of welder exposure days necessary to achieve this lung burden at a fume concentration equivalent to the previous TLV for WF (5 mg/m^3) is estimated to be 56 mg/0.0282 mg = 1985.8 days or ~5.4 years (Sriram et al., 2010b). While the estimates of worker exposure to total WF presented here was derived using TLV, it is likely that in certain work environments, workers may be exposed to much higher concentrations of fumes and consequently toxicological effects may be greater in such cases. Indeed, total WF levels measured in various industries (Korczynski, 2000; Susi et al., 2000), especially in confined spaces (Harris et al., 2005), have been observed to often exceed the previous TLV of 5 mg/m^3 for WF.

For comparison, animals were similarly exposed to soluble manganese chloride ($MnCl_2$), prepared in sterile saline. The mass percent composition of elemental Mn in $MnCl_2$ is 43.7%, which is identical to the content of elemental Mn in MMA-HS fume condensates. Thus, the amount of elemental Mn administered per dose of $MnCl_2$ is ~0.87 mg. Control animals were instilled with 300 μl of sterile saline. Animals were periodically monitored for any signs of abnormal behavior. No gross behavioral changes were observed, as previously reported (Sriram et al., 2010b).

Animals were euthanized 1 week after the last exposure. 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), and the animals were exsanguinated prior to collection of tissues. Immediately after euthanasia, the right lobe of the lung, liver, heart and kidneys were removed for elemental analysis. The brains were excised and brain areas (STR and MB) from the left and right hemispheres were dissected free-hand and processed for protein studies or elemental analysis. Claws (nails) present at the tip of the digits of each limb were gently trimmed with a stainless steel surgical scissor. The nail clippings from each animal were pooled and collected in pre-weighed screw-top tubes to obtain accurate sample weights. As the animals were exposed to WF via intratracheal instillation, no further cleaning of the nail samples were necessary (as would be required following a whole-body inhalation exposure), since external contamination is less likely to occur through this mode of exposure.

2.5. Western immunoblotting

Brain tissues (STR and MB) were homogenized in a tissue protein extraction reagent (T-PER; Pierce Biotechnologies, Inc., Rockford, IL) containing protease inhibitors and EDTA. The homogenates were centrifuged to pellet the cell/tissue debris and the supernatant was carefully collected. Total protein was determined according to the micro-bicinchoninic acid (BCA) method (Pierce Biotechnologies, Inc., Rockford, IL) using bovine serum albumin as a standard. Protein extracts were stored at −75 °C until use. Aliquots of brain homogenates (10 μg total protein) were diluted in Laemmli sample buffer, boiled and loaded on to 10% SDS-polyacrylamide gels. Proteins then were electrophoretically resolved and transferred to 0.45 μm Immobilon-FL PVDF Membranes (Millipore, Billerica, MA). Following transfer, immunoblot analysis was performed. Briefly, membranes were blocked using

Odyssey Blocking Buffer (LI-COR Biosciences, Lincoln, NE) for 1 h at room temperature and washed (1 × 5 min; 2 × 10 min) with phosphate-buffered saline (pH 7.2) containing 0.5% (v/v) Tween-20 (PBST). Following incubation with primary antibody (30–50 ng/ml of primary antibody buffer) to tyrosine hydroxylase [Th; rabbit polyclonal, cat #657012, EMD Chemicals, Gibbstown, NJ], Parkinson disease (autosomal recessive, early onset) 7 [Park7 or Dj1; rabbit polyclonal, cat #Ab18257, Abcam, Cambridge, MA], synaptosomal-associated protein 25 [Snap25; rabbit polyclonal, cat #487912, EMD Chemicals, Gibbstown, NJ] or β-actin [Actb; rabbit polyclonal, cat #Ab8227, Abcam, Cambridge, MA], 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, Lincoln, NE). The membranes were protected from light to minimize any photo-bleaching 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, Lincoln, NE), and the fluorescent signal intensities (*k* counts) of the individual bands were determined and normalized to the endogenous control, β-actin (Actb; 47 kDa).

2.6. Statistical analysis

Data were analyzed by one-way ANOVA followed by Tukey's multiple-comparison test, using SAS for Windows statistical software version 9.2 (SAS, Cary, NC). Pairwise Pearson's product-moment correlation coefficients (*r*) were calculated to estimate linear associations between various measurements. Results were considered significant at $P < 0.05$. Graphical representations are mean ± SE.

3. Results

3.1. Accumulation of Mn in dopaminergic brain areas following WF exposure

Repeated pulmonary (intratracheal instillation) exposure to GMA-MS or MMA-HS resulted in significant deposition of Mn in the STR and MB. Exposure to GMA-MS fumes caused a small increase in Mn in the STR (51% over control, $P < 0.05$) and MB (39% over control, $P < 0.05$), while exposure to MMA-HS fumes caused larger increases in the levels of Mn in the STR (232% over control, $P < 0.05$) and MB (207% over control, $P < 0.05$; Fig. 1; also see Table 1 for absolute values of metals quantified as μg/g). In comparison, repeated pulmonary exposure to $MnCl_2$ increased Mn levels in the STR (192% over control, $P < 0.05$) and MB (184% over control, $P < 0.05$), similar to that caused by the more soluble MMA-HS fumes (Fig. 1). A small increase in Cr was seen in STR following $MnCl_2$ treatment, but not following exposure to the two fumes (Fig. 1). Cu, Fe or Zn levels were not altered in these brain regions, following exposure to the fumes or $MnCl_2$ (Fig. 1).

3.2. Accumulation of Mn in lung and other organs following WF exposure

Repeated pulmonary exposure to GMA-MS or MMA-HS resulted in accumulation ($(180–200) \times 10^3$% over control, $P < 0.05$) of Mn in the lung (Fig. 2), the primary target of deposition following inhalation. Similarly, high amounts of Cr accumulated in the lung following GMA-MS (16.3×10^3% over control, $P < 0.05$) or MMA-HS (1567×10^3% over control, $P < 0.05$) treatment (Fig. 2). High levels of Fe were also observed in the lung following GMA-MS (5.8×10^3% over control, $P < 0.05$) or MMA-HS (2.1×10^3% over control, $P < 0.05$) exposure (Fig. 2). In the liver, a large increase in Cr, but not other metals, was seen following exposure to MMA-HS (720% over control, $P < 0.05$) fumes (Fig. 2). GMA-MS or $MnCl_2$ did not affect the levels of the metals examined in the liver (Fig. 2).

In the heart, increases in Mn was seen following MMA-HS (113% over control, $P < 0.05$) or $MnCl_2$ (57% over control; $P < 0.05$) treatment, but not GMA-MS (Fig. 3). A large increase in Cr, but not other metals, was also observed following exposure to MMA-HS (666% over control, $P < 0.05$) fumes (Fig. 3). Neither of the fumes nor $MnCl_2$ altered the levels of Cu, Fe or Zn in the heart (Fig. 3). In the kidney, a small increase in Mn, but not other metals was seen following MMA-HS (90% over control, $P < 0.05$) treatment (Fig. 3). GMA-MS or $MnCl_2$ did not alter elemental content in the kidney (Fig. 3).

3.3. Mn levels in blood and nail clippings following WF exposure

Blood Mn and Cr levels were undetectable following repeated pulmonary exposure to GMA-MS, MMA-HS or $MnCl_2$ (Fig. 4). The levels of Cu, Fe and Zn remained unaltered following exposure to either the fumes or $MnCl_2$ (Fig. 4). On the other hand, increase in Mn was seen in nail clippings, following GMA-MS (100% over control, $P < 0.05$), MMA-HS (633% over control, $P < 0.05$) or $MnCl_2$ (600% over control, $P < 0.05$) exposure (Fig. 4). Neither of the fumes or $MnCl_2$ altered the levels of other metals examined (Fig. 4). The pattern of Mn accumulation in the nail clippings appeared to reflect that seen in the brain but not lung (Fig. 5). A strong correlation was found between the levels of Mn in nails and the Mn content in STR ($R^2 = 0.9386$; $P < 0.0001$) or MB ($R^2 = 0.9332$; $P < 0.0001$; Fig. 6). Significant correlations were also observed between levels of Mn in nails and the Mn content in liver ($R^2 = 0.5112$; $P = 0.043$) or heart ($R^2 = 0.7815$; $P = 0.0004$; Table 2). No significant correlation was observed when a similar comparison was made with lung or kidney Mn (data not shown), suggesting that nail Mn, rather than blood Mn, may better predict Mn accumulation in target organs.

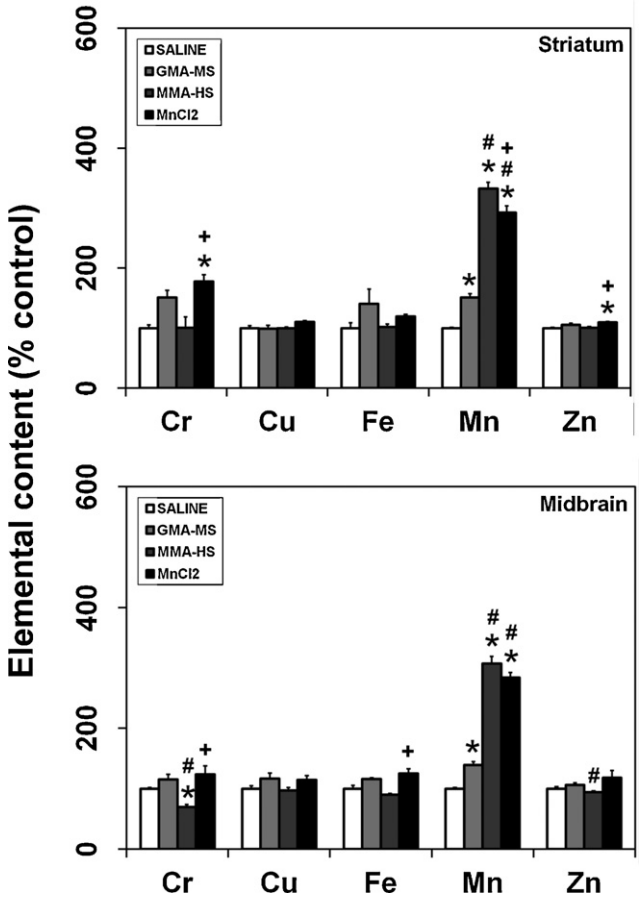

Fig. 1. Elemental analysis of brain tissues following WF exposure. Concentrations of Cr, Cu, Fe, Mn and Zn in striatum and midbrain were determined by ICP-AES, 1 week after repeated weekly instillations (2 mg/rat; 1/week × 28 weeks) of GMA-MS or MMA-HS fumes. Graphical representations are mean ± SE ($n = 4$/group) of values expressed as percent of saline-treated controls. Table 1 provides actual elemental concentrations calculated as μg/g. *Significantly different from saline-treated control ($P < 0.05$). #Significantly different from GMA-MS treatment ($P < 0.05$). +Significantly different from MMA-HS treatment ($P < 0.05$).

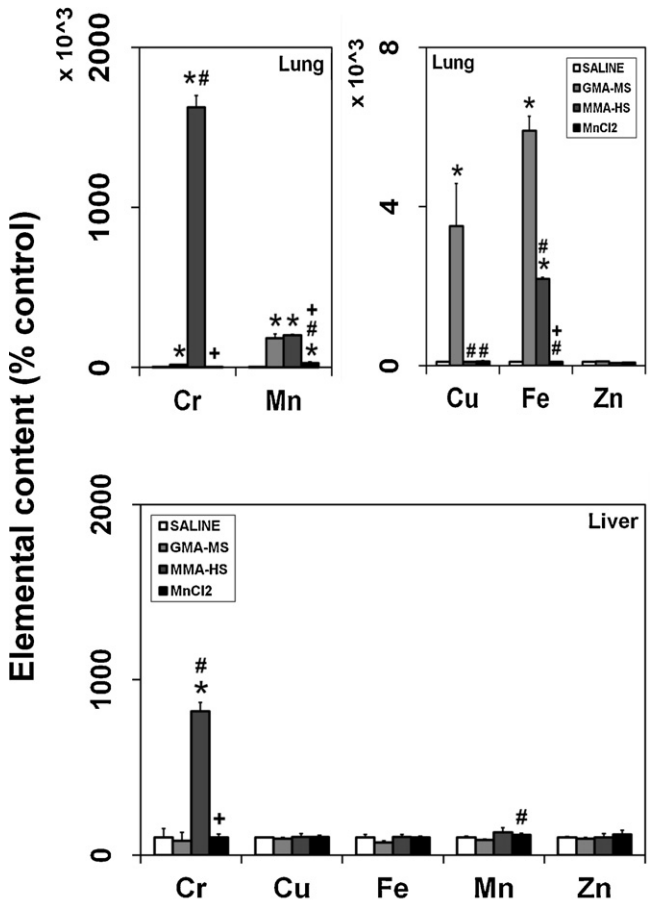

Fig. 2. Elemental analysis of lung and liver tissues following WF exposure. Concentrations of Cr, Mn, Cu, Fe, and Zn in lung and liver were determined by ICP-AES, 1 week after repeated weekly instillations (2 mg/rat; 1/week × 28 weeks) of GMA-MS or MMA-HS fumes. Graphical representations are mean ± SE (n = 4/group) of values expressed as percent of saline-treated controls. Table 1 provides actual elemental concentrations calculated as μg/g. *Significantly different from saline-treated control (P < 0.05). #Significantly different from GMA-MS treatment (P < 0.05). +Significantly different from MMA-HS treatment (P < 0.05).

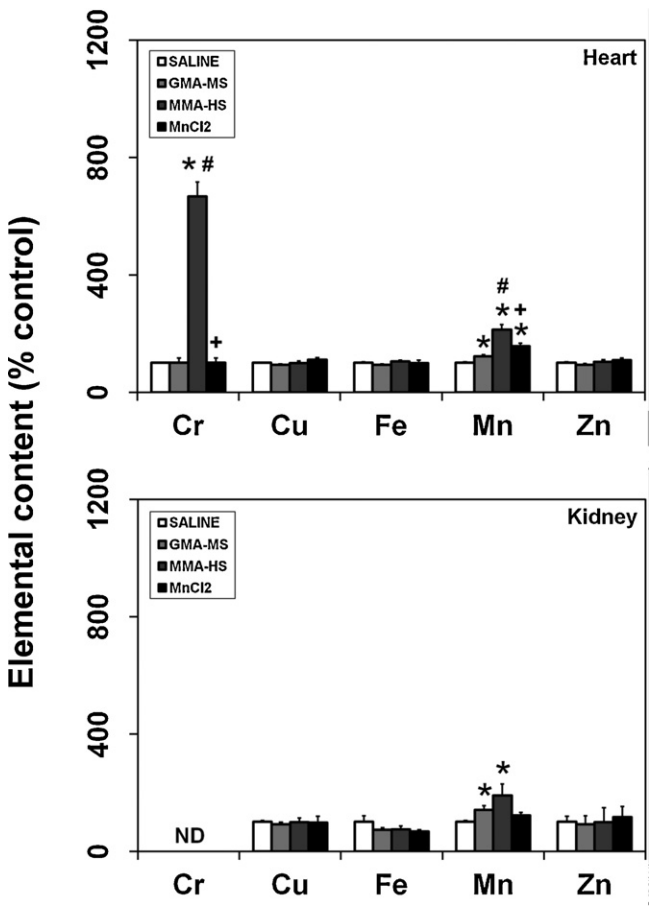

Fig. 3. Elemental analysis of heart and kidney tissues following WF exposure. Concentrations of Cr, Cu, Fe, Mn and Zn in heart and kidney were determined by ICP-AES, 1 week after repeated weekly instillations (2 mg/rat; 1/week × 28 weeks) of GMA-MS or MMA-HS fumes. Graphical representations are mean ± SE (n = 4/group) of values expressed as percent of saline-treated controls. Table 1 provides actual elemental concentrations calculated as μg/g. *Significantly different from saline-treated control (P < 0.05). #Significantly different from GMA-MS treatment (P < 0.05). +Significantly different from MMA-HS treatment (P < 0.05). ND = not detected.

3.4. Dopaminergic neurotoxicity following pulmonary exposure to GMA-MS or MMA-HS welding fumes

Chronic exposure to Mn has been linked to the development of a neurodegenerative condition resembling Parkinson's disease. We have recently shown that exposure to Mn-containing WFs similarly alters the expression of various indices of dopaminergic function, including Th, Park5 and Park7 proteins (Sriram et al., 2010a, 2010b), findings that suggest WF exposure may cause PD-like dysfunction. Accumulation of Mn in dopaminergic brain areas (as seen in Fig. 1) caused loss of Th protein, a marker of dopaminergic neurons and an index of dopaminergic injury (Fig. 7). GMA-MS and MMA-HS decreased Th protein in the STR by 32% (P < 0.05) and 26% (P < 0.05), respectively. Both GMA-MS and MMA-HS also caused loss (25–46% decrease, P < 0.05) of Park7 protein in the STR (Fig. 7). Exposure to GMA-MS or MMA-HS also resulted in a significant decrease in the levels of Snap25 in STR. Snap25 protein levels decreased by 24% (P < 0.05) following repeated exposure to GMA-MS, while MMA-HS decreased striatal Snap25 content by 47% (P < 0.05; Fig. 7). Collectively, these findings indicate that Mn-containing WFs cause dopaminergic abnormality. To determine if nail Mn can be a marker of dopaminergic injury, we examined the correlation between nail Mn content and various indices of striatal dopaminergic injury. A significant inverse correlation was seen between nail Mn and striatal Park7 protein (R^2 = 0.6782; P < 0.05; Fig. 8). Although striatal Th protein levels decreased following WF exposure, a significant correlation between Th levels in striatum and nail Mn was not reflected (Fig. 8), perhaps due to similar injury responses elicited by GMA-MS and MMA-HS fumes (Fig. 7), even though their Mn composition were different (Sriram et al., 2010a). Nevertheless, our findings reveal a potential association between Mn accumulation in nails and striatal dopaminergic injury.

4. Discussion

WF aerosols are comprised of toxic gases and metal particulates that are easily respirable (Zimmer and Biswas, 2001), thus inhalation exposure to airborne WF particulates is of immense occupational concern. Emerging evidence linking Mn-containing WF to the development of PD-like neurological dysfunction (Racette et al., 2001, 2005; Josephs et al., 2005; Bowler et al., 2007a) dictate the need for efficient biomonitoring of workplace exposures, in an effort to prevent adverse neurological health effects. Identifying reliable biomarkers of exposure and neurotoxicity are critical for neurological risk assessment of WFs. The limited utility of biological fluids, particularly blood, in effectively and reliably predicting Mn body burden (Roels et al., 1987; Jiang et al., 2007; Smith et al., 2007), motivated us to examine the efficacy of nail

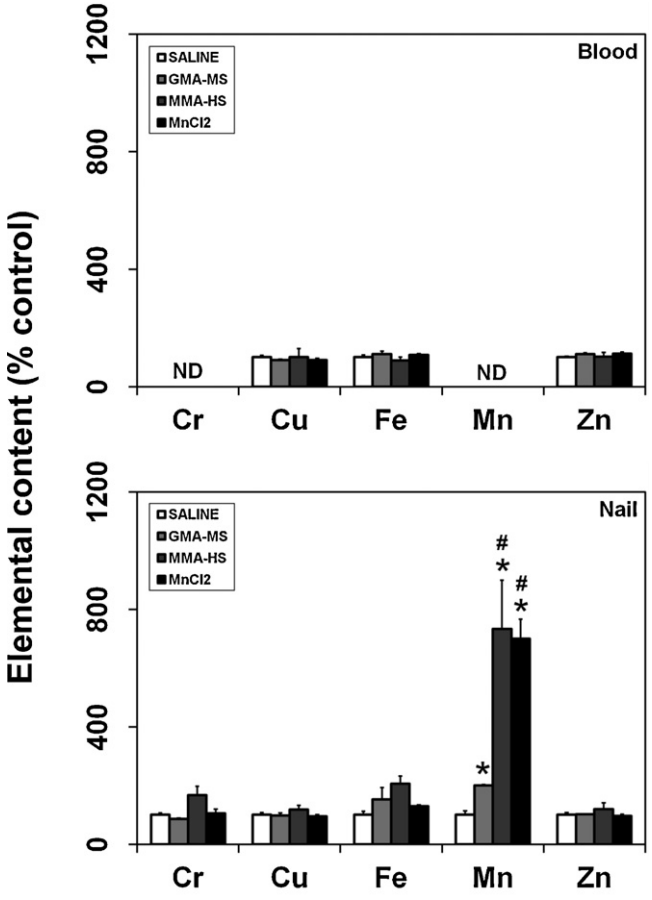

Fig. 4. Elemental analysis of blood and nail clippings following WF exposure. Concentrations of Cr, Cu, Fe, Mn and Zn in blood and nail clippings were determined by ICP-AES, 1 week after repeated weekly instillations (2 mg/rat; 1/week × 28 weeks) of GMA-MS or MMA-HS fumes. Graphical representations are mean ± SE (n = 4/group) of values expressed as percent of saline-treated controls. Table 1 provides actual elemental concentrations calculated as μg/ml (blood) or μg/g (nail). *Significantly different from saline-treated control (P < 0.05). #Significantly different from GMA-MS treatment (P < 0.05). ND = not detected.

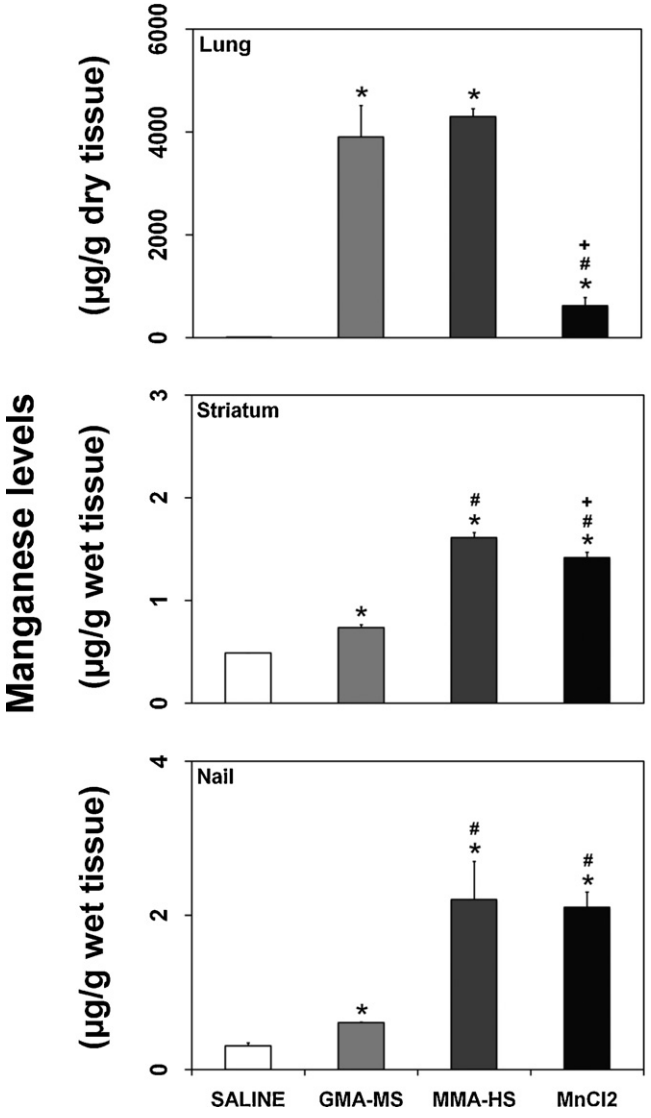

Fig. 5. Accumulation of Mn in nail clippings reflects the pattern of Mn accumulation in the brain following WF exposure. Mn concentrations in lung, striatum and nail clippings as determined in Figs. 1, 2 and 4, are compared here to illustrate the similarity in the pattern of Mn accumulation in brain and nail clippings. Concentrations of Mn in lung, striatum and nail clippings were determined by ICP-AES, 1 week after repeated weekly instillations (2 mg/rat; 1/week × 28 weeks) of GMA-MS or MMA-HS fumes. The levels of Mn were calculated either as μg/g dry tissue (lung) or as μg/g wet tissue (striatum, nail). Graphical representations are mean ± SE (n = 4/group). *Significantly different from saline-treated control (P < 0.05). #Significantly different from GMA-MS treatment (P < 0.05). +Significantly different from MMA-HS treatment (P < 0.05).

Table 2
Pairwise correlations between Mn levels in various organs.

Target organ	Target organ	Correlation (r)	Significance (P)
Midbrain	Striatum	0.979	<0.0001*
Lung	Striatum	0.343	0.194
Lung	Midbrain	0.285	0.285
Liver	Striatum	0.543	0.030*
Liver	Midbrain	0.410	0.114
Liver	Lung	0.035	0.898
Kidney	Striatum	0.518	0.040*
Kidney	Midbrain	0.470	0.066
Kidney	Lung	0.619	0.011*
Kidney	Liver	0.302	0.256
Heart	Striatum	0.865	<0.0001*
Heart	Midbrain	0.819	0.0001*
Heart	Lung	0.493	0.053
Heart	Liver	0.504	0.047*
Heart	Kidney	0.366	0.163
Nail	Striatum	0.899	<0.0001*
Nail	Midbrain	0.891	<0.0001*
Nail	Lung	0.215	0.425
Nail	Liver	0.511	0.043*
Nail	Kidney	0.454	0.078
Nail	Heart	0.782	0.0004*

Correlation between Mn levels in various organs following repeated exposure to WF (GMA-MS or MMA-HS) or $MnCl_2$. Pearson's correlation coefficients (r) were obtained to determine linear relationship.

* Data were considered significant at P < 0.05.

clippings as a potential surrogate for biomonitoring long-term WF exposures. Utilizing an experimental animal model of WF exposure, we show that Mn content in nail clippings following exposure to specific WFs were comparable to Mn levels in the brain but not other organs. Further, we show that brain Mn at concentrations similar to that seen in nail clippings was able to elicit dopaminergic abnormality. Thus, nail clippings may be a reasonable surrogate for monitoring Mn neurotoxicity following WF exposure.

Biological monitoring is critical for occupational health assessment of internal dose following exposures to hazardous materials (Schuhmacher et al., 2002; Nunes et al., 2010). Biomonitoring involves assessment of the presence and concentration of chemicals or toxicants in whole blood, serum, plasma, urine, saliva, breath, hair, nail, milk or tissues, following environmental or occupational exposures (Pirkle et al., 1995; DeCaprio, 1997; Sexton

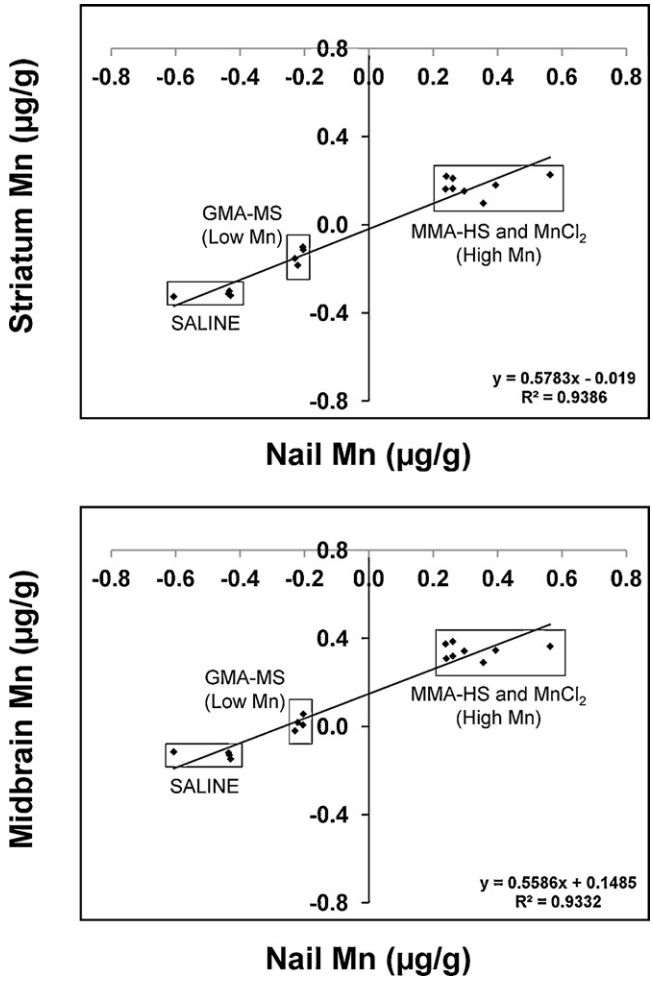

Fig. 6. Association between nail Mn and brain Mn levels following exposure to WF. Variables were transformed to log scale and Pearson's correlation coefficients (R^2) were obtained to determine linear relationship. A significant correlation was observed between Mn accumulation in nail clippings and Mn content in striatum ($R^2 = 0.9386$; $P < 0.0001$) or midbrain ($R^2 = 0.9332$; $P < 0.0001$), indicating that Mn in nail clippings was similar to Mn accumulation in the brain for each treatment group. Samples from the low (GMA-MS) and high (MMA-HS and $MnCl_2$) Mn treatment are grouped (box) for better clarity of data.

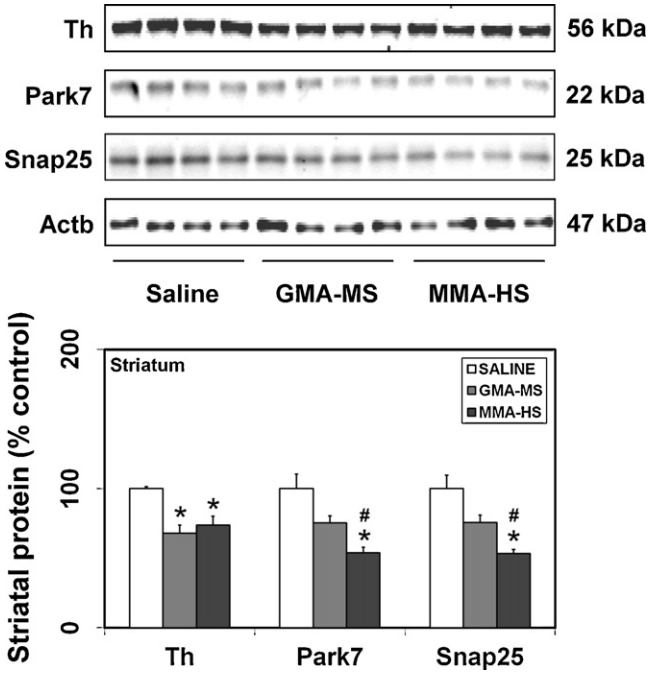

Fig. 7. Loss of dopaminergic and synaptic markers in striatum following WF exposure. Striatal TH, Park7 and Snap25 protein expression was determined by western immunoblot analysis after repeated weekly instillations (2 mg/rat; 1/week × 28 weeks) of GMA-MS, MMA-HS or $MnCl_2$. The fluorescent signal intensities (k counts) of the individual bands were determined. Following normalization to endogenous control β-actin (Actb), the protein levels are expressed as percent of saline-treated controls. Graphical representations are mean ± SE ($n = 4$/group). *Significant decrease from saline-treated controls ($P < 0.05$). #Significantly different from GMA-MS treatment ($P < 0.05$).

et al., 2004; Pausentbach and Galbraith, 2006). Biomonitoring WF-related Mn exposure is critical for unequivocally establishing adverse Mn exposure, determining body burden, and assessing toxicological impact. Development and validation of biomarkers that reflect Mn exposure and predict neurological disease risk among welders can contribute to reducing and preventing adverse exposures, and aid in better pre-job planning protocols, thus ensuring a safer workplace.

While brain magnetic resonance imaging (MRI) shows promise as a biomarker of Mn exposure, its application in bio-monitoring under clinical settings is limited by its operational costs and to some extent risks related to the static and oscillating magnetic fields used in MRI. Further, there is a possibility for the T_1 hyperintensity signals to fade upon withdrawal of exposure, perhaps due to gradual clearance of Mn from the brain. Indeed, a periodic follow-up MRI study among welders shows that there is a tendency for the T_1 hyperintensity signals to fade with time after withdrawal or cessation of exposure (Josephs et al., 2005; Han et al., 2008). However, it must be noted that the severity of the neurologic outcome does not appear to be related to the intensity of the MRI signal (Josephs et al., 2005) since abnormal T_1 hyperintensity has been observed in asymptomatic individuals with manganese exposure (Kim et al., 1999). The fact that MRI signals fade with time suggest that the solubility of Mn in WF appears to be a critical determinant of brain Mn concentrations. This is substantiated by studies that demonstrate inhalation exposure to soluble forms of Mn results in higher brain Mn concentrations than those achieved by exposure to insoluble form of Mn (Vitarella et al., 2000; Dorman et al., 2001). Further, it must be noted that the concentration of Mn required to cause abnormal T_1 hyperintensity on MRI is lower than the threshold necessary to elicit overt clinical symptoms (Kim, 2004).

Blood, serum and urine have been used for biological monitoring of Mn exposures in occupational settings, including welding (Roels et al., 1987; Mergler et al., 1994; Lucchini et al., 1995, 1997; Myers et al., 2003; Ellingsen et al., 2003; Wongwit et al., 2004; Bowler et al., 2007b). However, blood or plasma Mn concentrations have frequently exhibited poor correlation with workplace Mn exposures (Ellingsen et al., 2003; Lu et al., 2005) as it is dependent to a large extent on its solubility (Roels et al., 1997), as well as, the magnitude and duration of exposure (Dorman et al., 2008). Blood Mn levels peak rapidly, reaching maximum concentrations within 30 min following exposure to soluble Mn^{2+} salts (Roels et al., 1997). Due to their soluble nature they are likely to be cleared rapidly from blood and may not reliably reflect tissue Mn concentrations (Newland et al., 1987; Takeda et al., 1995; Zheng et al., 2000, 2011; Li et al., 2004). Further, as blood levels of trace elements are tightly regulated, changes in blood metal concentrations may be transient (Zheng et al., 2000), and therefore less likely to reflect long-term exposures. Existing evidence also suggest that serum Mn measurements primarily reflect levels that are protein bound. Upon saturation of circulating serum proteins, free Mn is rapidly redistributed to other tissues or is efficiently excreted from the body (Cotzias et al., 1968; Josephs et al., 2005). These observations imply that free Mn may not accumulate in blood or serum. In agreement,

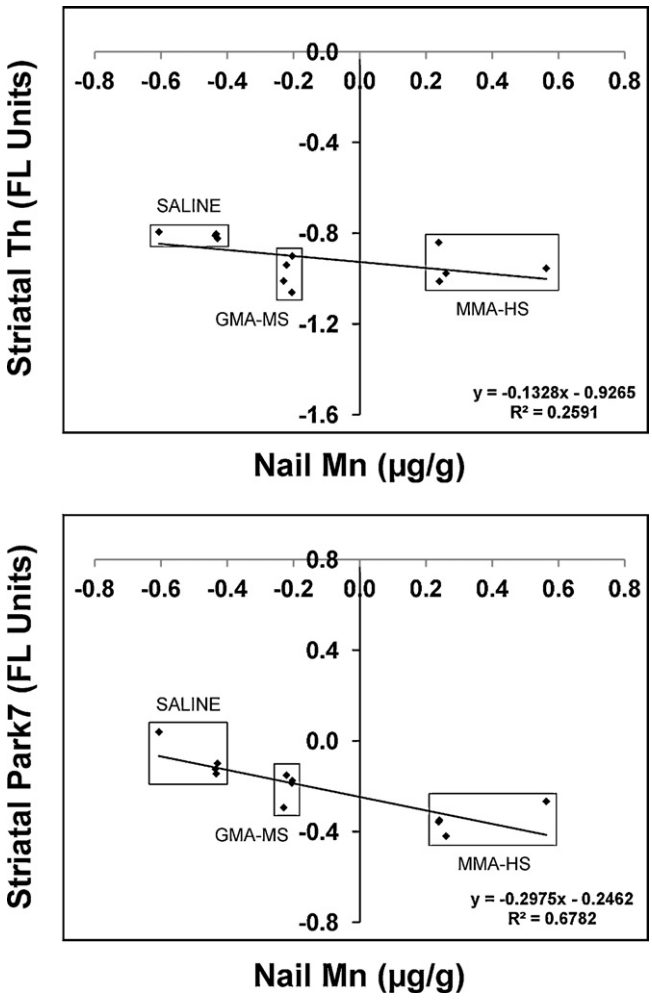

Fig. 8. Association between nail Mn and striatal neurotoxicity following WF exposure. Variables were transformed to log scale and Pearson's correlation coefficients (R^2) were obtained to determine linear relationship. A significant inverse-relationship was observed between Mn accumulation in nail clippings and striatal levels of Park7. With increasing Mn content in nail clippings, caused by exposure to a specific type of WF, a greater loss of striatal Park7 ($R^2 = 0.6782$; $P = 0.05$) was observed. Samples from the low (GMA-MS) and high (MMA-HS and $MnCl_2$) Mn treatment are grouped (box) for better clarity of data. FL units = normalized fluorescent signal intensities (k counts) of Th and Park7 proteins following immunoblot analysis. Near-infrared fluorescence detection was performed on the Odyssey Imaging System (LI-COR Biosciences, Lincoln, NE).

our findings in this study show that Cr and Mn were undetectable in the blood 1 week post-exposure, perhaps due to their rapid clearance or redistribution from blood. Consequently, blood or serum Mn levels may not correlate well with body or tissue burden.

The slow growth rate of nails can potentially reflect long-term retention of trace elements (Hopps, 1977), and perhaps may emulate metal burden in the body more efficiently than biological fluids. In rats, nail growth occurs at a rate of 1.5–3 mm/month (Godwin, 1959). In comparison, human fingernails grow at a rate of 2–4 mm/month (Weller et al., 2008) and toenails grow at the rate of ~1.7 mm/month (Yaemsiri et al., 2010) and can take up to 12–18 months to be completely replaced. The slow growth rate of nail can allow for continuous and chronological bio-monitoring of adverse exposures. This has advantage over biological fluids, such as, blood or serum, which appear to predominantly reflect recent or short-term exposure. Thus, nail may prove beneficial in monitoring long-term exposures and may better reflect body and/or target organ burden. Nails are a modified form of skin consisting of hard keratin that is rich in sulfur and glycine–tyrosine matrix proteins (Marshall, 1980, 1983; Lynch et al., 1986; Perrin et al., 2004; Alibardi et al., 2007). The sulfur-rich keratin present in hair and nail has high affinity for metal cations (Masri and Friedman, 1974; Fukuyama et al., 1978; Kokot et al., 1994; Bencko, 1995; Moreda-Piñeiro et al., 2007), thus nail can be a reliable matrix for evaluation of metal toxicity. Indeed, a few studies have examined the utility of nail as a biomarker of toxic metal exposure. Accumulation of arsenic or selenium in fingernails and toenails has been shown to be a useful indicator of intoxication of these elements following environmental exposures (Olguín et al., 1983; Agahian et al., 1990; Karagas et al., 1996; Slotnick and Nriagu, 2006). Increased accumulation of trace elements in nail samples has also been shown to occur in individuals exposed to such elements at the workplace. High levels of lead, cadmium, copper, manganese and nickel have been observed in fingernails of subjects working in railroad workshops and battery manufacturing industries (Mehra and Juneja, 2005). Our findings of increased Mn in nail clippings of rats exposed to WF suggest that nail can be a useful surrogate for biomonitoring WF exposure in the workplace. Further, the significant correlation that we observed between nail Mn accumulation, brain Mn content and striatal Park7 loss, indicates that nail Mn can potentially reveal WF-related neurotoxic Mn exposure. In a recent study of a small welder cohort from boiler-making plant, it was demonstrated that toenail Mn significantly correlated with cumulative Mn exposure that occurred for 7–12 months prior to toenail analysis, but not earlier (Laohaudomchok et al., 2011). The fact that toenail Mn did not correlate well with exposures 1–6 months prior to toenail analysis suggest that toenail may not adequately reflect acute or short-term exposures (Laohaudomchok et al., 2011). Whether similar outcomes are to be expected in the experimental studies remain unknown and warrant further investigation. We speculate that the lack of correlation with acute or short-term exposures may be due to slow dissolution of WF particulates deposited in the lung (Dorman et al., 2001; Antonini et al., 2011) that may contribute to a delayed accumulation of Mn in extra-pulmonary targets, including nails. This could further be influenced by type of WF, Mn content in the WF and the solubility of Mn (Antonini et al., 1999; Vitarella et al., 2000; Dorman et al., 2001; Taylor et al., 2003). On the other hand, we hypothesize that progressive disruption of Mn efflux transport mechanisms (Gavin et al., 1990; Yin et al., 2010) may lead to poor clearance and increased Mn accumulation, likely explaining the correlations observed with long-term exposures. Regardless, toenail Mn appears to be a valid measure of repeated long-term exposure to Mn. Further, Mn exposure over a typical work shift did not correlate well with Mn levels in blood nor urine (Laohaudomchok et al., 2011). Our experimental findings are in strong agreement with the observations of Laohaudomchok et al. (2011). The fact that our animal studies could mimic such workplace exposure conditions is promising, as the model can be utilized to delineate dose and time-dependent toxicological effects of various WF, factors that are difficult to discern from human exposures. Moreover, exposure and toxicity assessments can be performed under well-controlled conditions wherein process parameters can be uniquely regulated. These efforts will subsequently contribute towards establishing biomonitoring procedures, safe workplace practices, job planning protocols and occupational exposure limits. Additionally, our experimental model may facilitate rapid and economical screening of commonly used welding electrodes/rods to determine their toxicological profile, which is difficult to assess from human exposures.

It must be borne in mind that nail samples, particularly fingernails from workers employed in welding operations, have the potential for superficial contamination as a consequence of frequent handling of welding materials. Such contamination can be reasonably high if suitable protective equipment, such as gloves, is not worn during performance of work. Efforts to overcome such

contamination issues can be through establishment of appropriate hand-washing procedures prior to sample collection or elemental analysis (Chen et al., 1999). On the other hand, toenail samples may be more reliable as there is less likelihood of external contamination. Furthermore, the slower growth rate of toenails, compared to fingernails, will likely reflect long-term patterns of Mn accumulation related to adverse exposures. Mn concentrations in the nail clippings may typically reflect the average Mn exposure during the period when the nail was formed. Considering the slow growth rate of toenails, this could mean a reflection of 12–18 months of exposure, which can be a significant index of exposure and neurotoxicity. Our current findings call for a comprehensive study of the dose and time-dependent effects of WF exposure to determine the retention and/or clearance profile of Mn from nails. From a risk assessment perspective, such studies will also help understand the relationship between Mn accumulation in target organs to nail growth/length, dose, duration of exposure, etc.

In conclusion, nail Mn has the potential to be a sensitive and reliable biomarker for WF-related manganese exposure and neurotoxicity. The non-invasive manner by which nail clippings can be collected, stored, and transported with relative ease, make it an attractive surrogate for biomonitoring WF exposures in occupational settings.

Authors' contributions

KS conceived the study. KS and JMA designed the study. KS headed the neurotoxicology studies, performed brain dissections, analyzed data and wrote the paper. GXL and KS collected toenail samples. GXL and AMJ conducted all neurotoxicity-related assays. JMA and JRR performed animal treatments and collected lung, liver, heart and kidney samples. RNA performed elemental analysis of nail clippings. MLK is a biostatistician on the welding fume neurotoxicity project and performed the statistical analysis. All authors reviewed and approved the final manuscript.

Conflict of interest statement

The authors declare they have no proprietary, financial or personal interest of any kind or nature that could be construed as being a conflict of interest.

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