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INORGANIC COMPOUNDS

# Dopaminergic neurotoxicity following pulmonary exposure to manganese-containing welding fumes

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**Abstract** The potential for development of Parkinson's disease (PD)-like neurological dysfunction following occupational exposure to aerosolized welding fumes (WF) is an area of emerging concern. Welding consumables contain a complex mixture of metals, including iron (Fe) and manganese (Mn), which are known to be neurotoxic. To determine whether WF exposure poses a neurological risk particularly to the dopaminergic system, we treated Sprague–Dawley rats with WF particulates generated from two different welding processes, gas metal arc-mild steel (GMA-MS; low Mn, less water-soluble) and manual metal arc-hard surfacing (MMA-HS; high Mn, more water-soluble) welding. Following repeated intratracheal instillations (0.5 mg/rat, 1/week × 7 weeks) of GMA-MS or MMA-HS, elemental analysis and various molecular indices of neurotoxicity were measured at 1, 4, 35 or 105 days after last exposure. MMA-HS exposure, in particular, led to increased deposition of Mn in striatum and midbrain. Both fumes also caused loss of tyrosine hydroxylase (TH) protein in the striatum (~20%) and midbrain (~30%) by 1 day post-exposure. While the loss of TH following GMA-MS was transient, a sustained loss (34%) was observed in the midbrain 105 days after cessation of MMA-HS exposure. In addition, both fumes caused persistent down-regulation of dopamine D2 receptor (Drd2; 30–40%) and vesicular monoamine transporter 2 (Vmat2; 30–55%) mRNAs in the midbrain. WF exposure also modulated factors associated with synaptic transmission, oxidative stress, neuroinflammation and gliosis. Collectively, our findings demonstrate that repeated exposure to Mn-containing WF can cause persistent molecular alterations in dopaminergic targets. Whether such perturbations will lead to PD-like neuropathological manifestations remains to be elucidated.

**Keywords** Brain · Dopaminergic dysfunction · Manganese · Neurotoxicity · Neurodegeneration · Occupational exposure · Parkinson's disease · Parkinsonism · Welding · Welding fume

**Abbreviations**

| | |
|---|---|
| BALF | Bronchoalveolar lavage fluid |
| CCL2 | Chemokine–chemokine ligand 2 |
| CXCL2 | Chemokine-X-chemokine ligand 2 |
| CNS | Central nervous system |
| COX | Cycloxygenase |
| DMT1 | Divalent metal transporter 1 |
| DRD2 | Dopamine D2 receptor |
| EMR1 | Egf-like module containing mucin-like hormone receptor-like 1 |
| FLISA | Fluorescence-linked immunosorbent assay |
| GFAP | Glial fibrillary acidic protein |
| GMA-MS | Gas metal arc-mild steel |
| HO | Heme-oxygenase |

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| | |
|---|---|
| ICP-AES | Inductive coupled plasma atomic absorption spectroscopy |
| IL6 | Interleukin 6 |
| ITGAM | Integrin αM |
| LDH | Lactate dehydrogenase |
| MMA-HS | Manual metal arc-hard surfacing |
| NOS | Nitric oxide synthase |
| PCR | Polymerase chain reaction |
| PD | Parkinson's disease |
| PMN | Polymorphonuclear leukocyte |
| SDS | Sodium dodecyl sulfate |
| TH | Tyrosine hydroxylase |
| TNF | Tumor necrosis factor |
| VMAT2 | Vesicular monoamine transporter 2 |
| WF | Welding fume |

## Introduction

Welding generates a complex mixture of gaseous products (carbon monoxide, carbon dioxide, nitrous oxide, ozone), as well as high concentrations of aerosolized fine and ultrafine metal particulates (iron, manganese, chromium, nickel) that are potentially toxic. Thus, exposure to airborne welding fume (WF) particulates is of immense occupational concern. The unique metal composition and physical characteristics (shape, size, surface area, solubility) of WFs may influence their deposition within the respiratory tract and subsequent translocation to extrapulmonary organs. 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), which are respirable and can deposit in the olfactory and lower respiratory tracts. As ultrafine particles or the soluble metal fractions can translocate and accumulate in the brain (Hunter and Undem 1999; Oberdörster et al. 2002; Oberdorster et al. 2004; Elder et al. 2006), questions have been raised regarding a causal association between WF exposure and neurological effects seen in welders. Particularly, the presence of manganese (Mn) in welding consumables is thought to be associated with the appearance of a Parkinson's disease (PD)-like neurological manifestation.

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 makes it difficult to comprehensively evaluate the adverse health effects associated with WF exposure. Occupational exposure to WF has been associated with health effects primarily involving respiratory diseases, cancer and ischemic heart diseases (Sferlazza and Beckett 1991; Sjögren et al. 2002; Antonini et al. 2003; Meo and Al-Khlaiwi 2003; Sjogren et al. 2006). More recently, there is growing concern that exposure to WF may also be associated with the development of neurological and psychological/psychiatric disturbances, including PD-like symptoms (Josephs et al. 2005; Bowler et al. 2006, 2007a, b). Neuroepidemiological findings, albeit limited, suggest a potential relationship between welding and Parkinsonism (Racette et al. 2001, 2005). Welding is not only a high-risk occupation for the development of manganism (Nelson et al. 1993; Kim et al. 1999; Sadek et al. 2003) but also has been implicated as a risk factor for PD (Racette et al. 2001). Indeed, among the occupations with a high mortality odds ratio for neurodegenerative diseases, welding is thought to enhance the risk of PD, particularly with an early onset (Park et al. 2005; Racette et al. 2005), although other studies have argued against such an association (Fored et al. 2006; Marsh and Gula 2006; Park et al. 2006; Stampfer 2009; Tanner et al. 2009).

Occupational exposure to Mn, a component of WF, has been linked to neurotoxicity (Donaldson 1987; Mergler and Baldwin 1997), deficits in the ability to perform rapid hand movements, loss of coordination and balance and symptoms such as forgetfulness, anxiety or insomnia (Bowler et al. 2007a, b). Mn in occupational settings such as mining and dry battery industries has been shown to cause a parkinsonian syndrome referred to as manganism (Rodier 1955; Emara et al. 1971). Cumulative Mn exposure in workers at foundries and ferroalloy industries has been shown to be associated with preclinical signs of motor and cognitive function (Wennberg et al. 1991; Lucchini et al. 1999). High prevalence of parkinsonian disorders has also been reported in populations living in the vicinities of ferroalloy industries, which has been linked to high Mn dust levels (Lucchini et al. 2007). Environmental Mn pollution from fuel additives has also been linked to increased risk of PD-like disorders (Finkelstein and Jerrett 2007). Clinically, several features of Mn intoxication are akin to PD, particularly symptoms such as tremor, bradykinesia and rigidity (Calne et al. 1994; Pal et al. 1999). However, the two syndromes are thought to differ in their extent of dystonia, response to levodopa, dopaminergic brain areas affected and age of onset (Nelson et al. 1993; Calne et al. 1994; Lu et al. 1994; Shinotoh et al. 1995; Koller et al. 2004). In the light of these observations, it is critical to evaluate the effects of WF exposure on central nervous system (CNS) function. In the brain, WF particulates or its soluble metal components, including Mn, could accumulate in dopaminergic brain areas, causing neurotoxicological and neurobehavioral changes characteristic of Parkinsonism.

However, at present, there is a paucity of information on the neurological effects of inhaled WFs generated at the workplace. Controversy exists regarding the adverse neurological health effects seen in workers with WF exposure. The limited availability of epidemiological data and the lack of well-designed case control studies warrant the need for laboratory-based investigations that will aid in determining whether the above concerns are factual. These studies may help support or refute findings that suggest an association between WF exposure and the appearance of a PD-like neurological disorder. Further, understanding the mechanistic basis of WF-related neurotoxicity will help in development of appropriate bio-monitoring and prevention strategies.

Here, we evaluated the potential neurotoxicological effects of low (GMA-MS) and high (MMA-HS) Mn-containing WFs in a rodent model. Specifically, we investigated if pulmonary exposure to WFs results in accumulation of Mn in the striatum and midbrain causing dopaminergic dysfunction.

## Materials and methods

### Welding fumes

Bulk samples of GMA-MS and MMA-HS were collected in the laboratory of Kenneth Brown of Lincoln Electric Company (Cleveland, OH). The fumes were generated in a cubical open front fume chamber (volume = 1 $m^3$) using a manual or semi-automatic technique appropriate to the electrode and collected on 0.2-μm Nuclepore filters (Nuclepore, Pleasanton, CA). The fume samples were generated using two different processes: (1) gas metal arc welding using a mild steel E70S-3 electrode (GMA-MS; L-50 carbon steel electrode, Lincoln Electric, Cleveland, OH); and (2) manual metal arc welding using a flux-covered stainless steel hard-surfacing electrode (MMA-HS; Wearshield 15CrMn, Lincoln Electric, Cleveland, OH) that contains elevated amounts of Mn compared to standard welding electrodes/rods/wires.

### Inductively coupled plasma atomic emission spectroscopy (ICP-AES)

To determine the metal composition, as well as, the ratio of water-soluble and water-insoluble metal components in the WFs, elemental analysis was carried out by ICP-AES. Accurately weighed (1 mg) WF samples (GMA-MS and MMA-HS) were suspended in 1 ml of distilled water, pH 7.4, and sonicated for 1 min with a Sonifier 450 Cell Disruptor (Branson Ultrasonics, Danbury, CT) to disperse the particulates (designated as total suspension). To determine the soluble and insoluble metal components of the WFs, similar sets of total suspension samples were prepared, incubated at 37°C for 24 h and centrifuged at 16,000*g* for 30 min. The entire supernatant (soluble fraction) was carefully recovered and filtered through a 0.2-μm filter (Millipore Corp., Bedford, MA). The pellet (insoluble fraction) was air-dried and saved. ICP-AES analysis was performed by Bureau Veritas North America, Inc (Novi, MI) according to NIOSH method 7303 (NIOSH 2003). The total metal composition and solubility ratios of the two WFs were calculated.

To determine metal content in animal tissues (lung and discrete brain areas), 1 ml of 3 N hydrochloric acid/10% trichloroacetic acid solution was added to pre-weighed lung (dry weight) or brain (wet weight) tissues and heated at 70°C for 18 h to digest the tissue. After centrifugation at 600*g* for 10 min, concentrations of metals in the supernatant were quantified by ICP-AES (Model Optima 4300D, Perkin Elmer, Norwalk, CT) and expressed as μg/g tissue.

### Animals

Male Sprague–Dawley [Hla:(SD) CVF] rats (~2 months old; 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 and hardwood Beta-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.

### Animal exposures

GMA-MS or MMA-HS WFs 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 (0.5 mg/animal in 300 μl of sterile saline) were carried out once a week for 7 weeks. Control animals were intratracheally instilled with 300 μl of sterile saline. The 0.5 mg/animal dose was chosen based on results from a preliminary pulmonary dose–response study

(Antonini et al. 1996), which demonstrated pulmonary inflammation and cytotoxicity but not lung fibrosis. The relevance of this exposure paradigm to actual workplace exposure conditions that a welder may experience was estimated and is presented under results.

Immediately after euthanasia, the lungs were lavaged; the brains excised and specific brain areas (olfactory bulb, striatum, hippocampus, cerebellum and midbrain) from the left and right hemispheres were dissected free-hand. Brain tissues from the left hemisphere were placed in RNA-*Later*® (Ambion, Austin, TX) for mRNA expression analysis, and tissues from the right hemisphere were processed in 10 volumes of T-PER tissue protein extraction reagent (Pierce, Rockford, IL) for analysis of proteins. A separate set of animals were exposed similarly to obtain specific brain regions (as indicated above) for elemental analysis.

### Bronchoalveolar lavage (BAL)

At 1 day after the last weekly treatment, the rats were deeply anesthetized with an intraperitoneal injection of sodium pentobarbital (>100 mg/kg body weight; Sleep-away. Fort Dodge Animal Health, Wyeth, Madison, NJ) and then exsanguinated. The lungs were first lavaged with a 1 ml/100 g body wt aliquot of calcium- and magnesium-free phosphate-buffered saline (PBS, pH 7.4). The first fraction of the recovered lavage fluid was centrifuged at 500*g* for 10 min, and the resultant cell-free supernatant was analyzed for various biochemical parameters as described below. The lungs were further lavaged with 6 ml aliquots of PBS until 30 ml were collected. These samples also were centrifuged at 500*g* for 10 min and the cell-free lavage fluid discarded. The cell pellets from all washes for each rat were combined, washed and resuspended in 1 ml of PBS buffer, and BAL cell differentials were determined.

### Lactate dehydrogenase (LDH) activity and albumin content in BAL fluid

Using the acellular first fraction of BAL, albumin content, an index to quantify increased permeability of the bronchoalveolar-capillary barrier, and LDH activity, an indicator of general cytotoxicity, were measured. Albumin content was determined colorimetrically at 628 nm based on albumin binding to bromocresol green using an albumin BCG diagnostic kit (Sigma Chemical Co., St. Louis, MO). LDH activity was determined by measuring the oxidation of lactate to pyruvate coupled with the formation of NADH at 340 nm. Measurements were performed on a COBAS MIRA auto-analyzer (Roche Diagnostic Systems, Montclair, NJ).

### BAL cell differentials

Total cell numbers in the BAL were determined using Coulter Multisizer II and AccuComp software (Coulter Electronics, Hialeah, FL). Cells were differentiated using a Cytospin®3 centrifuge (Shandon Life Sciences International, Cheshire, UK). Cell suspensions ($5 \times 10^4$ cells) were spun for 5 min at 800 rpm and pelleted onto a slide. Recovered lung cells (200/rat) were identified as either neutrophils (PMNs) or lung macrophages after labeling with Leukostat stain (Fisher Scientific, Pittsburgh, PA).

### RNA isolation, cDNA synthesis and real-time PCR

The brain tissues (striatum and midbrain) were homogenized in Tri Reagent® (Molecular Research Center, Inc., Cincinnati, OH) and the aqueous phase separated with MaXtract High Density gel (Qiagen, Valencia, CA). Total RNA from the aqueous phase was then isolated using RNeasy mini spin columns (Qiagen, Valencia, CA), and on-column digestion of DNA was performed using DNAse I. RNA concentrations were determined using NanoDrop® ND-1000 UV–Vis Spectrophotometer (NanoDrop Technologies, Wilmington, DE). First strand cDNA synthesis was carried out using total RNA (2 μg), random hexamers and MultiScribe™ reverse transcriptase (High Capacity cDNA Reverse Transcription Kit; Applied Biosystems, Foster City, CA) in a 20 μl reaction. Real-time PCR amplification was performed using the 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA) in combination with TaqMan® chemistry. Specific primers and FAM™ dye-labeled TaqMan® MGB probe sets (TaqMan® Gene Expression Assays) were procured from Applied Biosystems (Foster City, CA) and 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® Universal master mix (Applied Biosystems, Foster City, CA), respectively. Sequence detection software (version 1.7; Applied Biosystems, Foster City, CA) results were exported as tab-delimited text files and imported into Microsoft Excel for further analysis. Following normalization to $\beta$-Actin, relative quantification of gene expression was performed using the comparative threshold ($C_T$) method as described by the manufacturer (Applied Biosystems, Foster City, CA; User Bulletin 2). The values are expressed as percent change from saline-treated controls.

### Preparation of brain tissues for protein analysis

Brain tissue (striatum and midbrain) homogenates were prepared in T-PER tissue protein extraction reagent (Pierce

Biotechnologies, Inc., Rockford, IL) containing protease inhibitors and EDTA. The samples were then centrifuged at 10,000*g* for 3 min, 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. The protein extracts were stored at −75°C until use.

### Fluorescence-linked immunosorbent assay (FLISA) of tyrosine hydroxylase (TH)

TH protein content was measured by a "sandwich" FLISA, using a near-infrared fluorescent dye for detection. Both the capture and the detection antibodies used in the assay were individually tested for specificity on a western blot before application in the FLISA. The total protein load for sample and standards were empirically determined. In brief, Fluorotrac 600 high-binding microtiter plates (Greiner Bio-One, Monroe, NC) were coated with a mouse monoclonal antibody to TH (Capture antibody; Sigma, St. Louis, MO), sealed and incubated overnight at 4°C. The wells were washed twice with Wash Buffer (phosphate buffered saline containing 0.1% Tween-20; PBST) and blocked using Odyssey Blocking Buffer (LI-COR Biosciences, Lincoln, NE) for 1 h at room temperature. Following two washes, 100 µl of appropriate dilutions of sample homogenate or standards (0–12 µg) prepared in PBST were loaded onto pre-designated wells. The plates were sealed and incubated for 2 h at 37°C. Following four washes, 100 µl of rabbit polyclonal antibody to TH (Detection antibody; Calbiochem, San Diego, CA) was added to "sandwich" the TH protein and the plates were incubated at room temperature for 1 h. The plates were again washed four times, and 100 µl of IRDye 800CW-conjugated anti-rabbit IgG (LI-COR Biosciences, Lincoln, NE) was added to each well and incubated for 30 min at room temperature. The plates were protected from light to minimize any photo-bleaching of the fluorescent dye. The plates were washed four times, rinsed with MilliQ water and dried briefly by centrifugation. Near-infrared fluorescence was measured using Odyssey Imaging System (LI-COR Biosciences, Lincoln, NE), and the signal intensities (*k* counts) were obtained. The relative signal intensities of the samples were derived from the standard curve and represented as percent of saline-treated control.

### Western immunoblotting

Aliquots of brain homogenates (10 µg total protein) were diluted in Laemmli sample buffer, boiled and loaded on 10% SDS–polyacrylamide gels (Laemmli, 1970). Proteins then were electrophoretically resolved and transferred to 0.45 µm Immobilon-FL PVDF Membranes (Millipore, Billerica, MA) according to a previously described method (Towbin et al. 1979). 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, washed (1 × 5 min; 2 × 10 min) with PBST and incubated overnight at 4°C with appropriate primary antibody. Following incubation with antibodies to either, phospho-Synapsin I (Ser553; rabbit monoclonal, Abcam, Cambridge, MA; detects Ser551 in rat), Synapsin I (rabbit polyclonal, Stressgen, Ann Arbor, MI) or α-Tubulin (mouse monoclonal, Santa Cruz Biotechnology, Inc., Santa Cruz, CA), 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 an endogenous control, α-Tubulin.

### Statistical analysis

Data were analyzed by one- or two-way ANOVA followed by Student's-Newman-Keuls (SNK) multiple comparison test, as appropriate, using SigmaStat 3.1 statistical software (Systat Software Inc., San Jose, CA). Where data failed equal variance or normality tests, they were analyzed by one-way ANOVA on ranks followed by Dunn's multiple comparison test. Results are presented as Mean ± SE, and the differences are considered significant at $P < 0.05$.

## Results

### Elemental analysis of GMA-MS and MMA-HS WFs

ICP-AES analysis of GMA-MS WF revealed that Fe was the major (90.4% of total metals) metal component, while Mn accounted for about 6.9% (Table 1). On the other hand, MMA-HS WF comprised mainly of Mn (43.7%), Fe (30.4%) and Cr (8.3%), as well as flux materials like K (13.4%) that are added to manufacture this type of welding rods/electrodes. It must be noted that the weight % calculated here are ratios of individual metal to total metal in the aerosolized fume samples collected, and not the actual % composition of the electrodes as provided by the manufacturers.

**Table 1** Metal composition of aerosolized WF samples

| Metal | GMA-MS (%) | MMA-HS (%) |
|---|---|---|
| Fe | 90.35 | 30.38 |
| Mn | 6.88 | 43.74 |
| Ca | 0.00 | 0.65 |
| Cr | 0.04 | 8.25 |
| Cu | 0.88 | 0.03 |
| K | 0.13 | 13.39 |
| Mg | 0.02 | 0.78 |
| Ni | 0.00 | 0.31 |
| Ti | 0.26 | 1.29 |
| Zn | 1.25 | 0.73 |

Metal composition of GMA-MS and MMA-HS fumes was determined by ICP-AES following the NIOSH Method 7303. A total of 31 elements were analyzed and the table depicts some of the major elements in the respective aerosol fractions. Values are expressed as weight % relative to all metals analyzed in the WF aerosol. WF samples were analyzed in triplicates. The Mean and S.E. weight % of Mn in GMA-MS fumes was 6.88 ± 0.17 and in MMA-HS fumes was 43.74 ± 1.33

Solubility studies to determine the water-insoluble and water-soluble metal constituents of the WFs revealed that the GMA-MS fume was predominantly water-insoluble. Only a small fraction (1.72% of the total metal content) was water-soluble, and the soluble to insoluble metal ratio of GMA-MS fume was determined to be 0.018. On the other hand, MMA-HS fumes contained more water-soluble metals (17.9% of total metal content) with a soluble to insoluble metal ratio of 0.218. Further, the MMA-HS fumes contain nearly 8 times more water-soluble metals than GMA-MS fumes (Table 2).

## Relevance of instillation dose to actual work-zone fume concentrations and exposure duration

To determine whether our instillation dose is comparable to total WF and Mn fume concentrations observed in a welder's work atmosphere, we calculated the approximate fume concentration necessary to achieve a daily lung burden (DLB) in a welder that would be equivalent of the DLB in rats exposed in our study. The calculations made here do not account for clearance or influence of any confounding factors, but provide an estimate of the plausible welder exposure concentrations that our exposure paradigm mimics.

We assumed our 0.5 mg/week intratracheal instillation exposure paradigm, on a divided daily dose basis (of a 5 day week), will give a DLB of 0.1 mg in the rat (average body weight of 0.4 kg in this study).

$$\mathrm{DLB}_{\mathrm{WF(rat)}} = \frac{\text{Dose per week}}{\text{Days per week}} = \frac{0.5\ \text{mg/week}}{5\ \text{days/week}} = 0.1\ \text{mg/day}$$

The corresponding DLB in a welder when body mass (average human body weight of 75 kg) is used as dose metric is,

$$\mathrm{DLB}_{\mathrm{WF(welder)}} = \frac{\mathrm{DLB}_{\mathrm{WF(rat)}} \times \text{Body mass}_{\mathrm{(human)}}}{\text{Body mass}_{\mathrm{(rat)}}} = \frac{0.1\ \text{mg} \times 75\ \text{kg}}{0.4\ \text{kg}} = 18.75\ \text{mg/day}$$

Incorporating this value, the fume concentration was calculated using the following formula,

$$\mathrm{FC}_{\mathrm{WF}} = \frac{\mathrm{DLB}_{\mathrm{WF(welder)}}}{\left[\mathrm{MRV}_{\mathrm{(human)}} \times \mathrm{ED} \times \mathrm{DE}\right]}$$

where $\mathrm{FC}_{\mathrm{WF}}$ is total WF concentration, $\mathrm{DLB}_{\mathrm{WF(welder)}}$ is the daily lung burden in a welder, $\mathrm{MRV}_{\mathrm{(human)}}$ is human respiratory minute volume (20 L/min × $10^{-3}$ $m^3$/L), ED is the exposure duration assuming a 8 h work schedule (8 h/days × 60 min/h) and DE is the deposition efficiency of particles in the pulmonary alveoli (predicted as 15% based on mass median aerodynamic diameter of 0.25 μm for WF; Antonini et al. 2006; also see ICRP 1994),

$$\mathrm{FC}_{\mathrm{WF}} = \frac{18.75\ \text{mg/day}}{[20\ \text{L/min} \times 10^{-3}\ \text{m}^3/\text{L} \times (8\ \text{h/day} \times 60\ \text{min/h}) \times 0.15]} = \frac{18.75\ \text{mg}}{1.44\ \text{m}^3} = 13\ \text{mg/m}^3$$

When calculated using surface area (SA) of alveolar epithelium (rat = 0.4 $m^2$; human = 102 $m^2$; Stone et al.

**Table 2** Solubility characteristics of WF samples

| Groups | Total metals (TM) mg/g | Soluble metals (SM) mg/g | Insoluble metals (IM) mg/g | SM/IM ratio | % SM/TM |
|---|---|---|---|---|---|
| GMA-MS | 551.4 ± 25.8 | 9.5 ± 0.7 | 541.9 ± 25.9 | 0.018 | 1.72 |
| MMA-HS | 430.2 ± 40.6 | 77.0 ± 13.7 | 353.2 ± 39.6 | 0.218 | 17.90 |

Metal composition of the soluble and insoluble fractions of GMA-MS and MMA-HS WFs was determined by ICP-AES and calculated as mg/g of aerosolized WF. The solubility ratio and weight % of soluble metal fraction were then derived from the corresponding mean values. *GMA-MS* gas metal arc-mild steel; *MMA-HS* manual metal arc-hard surfacing; *SM* soluble metals; *IM* insoluble metals; *TM* total metals. All measurements were made in triplicate

1992) as dose metric, the DLB in a welder and the FC necessary to achieve this are,

$$\mathrm{DLB_{WF(welder)}} = \frac{\mathrm{DLB_{WF(rat)}} \times \mathrm{SA_{(human)}}}{\mathrm{SA_{(rat)}}} = \frac{0.1\ \mathrm{mg} \times 102\ \mathrm{m^2}}{0.4\ \mathrm{m^2}} = 25.50\ \mathrm{mg/day}$$

$$\mathrm{FC_{WF}} = \frac{25.5\ \mathrm{mg/day}}{[20\ \mathrm{L/min} \times 10^{-3}\ \mathrm{m^3/L} \times (8\ \mathrm{h/day} \times 60\ \mathrm{min/h}) \times 0.15]} = \frac{25.50\ \mathrm{mg}}{1.44\ \mathrm{m^3}} = 17.7\ \mathrm{mg/m^3}.$$

Thus, our instillation doses would mimic a daily worker exposure to total WF concentrations of about 13–18 $\mathrm{mg/m^3}$ either based on body mass or alveolar epithelial surface area as dose metric.

Next, to determine how our instillation doses relate to Mn exposure, present as a component of the total WF aerosol, we calculated Mn fume concentrations after determining the percent composition of Mn in the total WFs. ICP-AES elemental analysis of GMA-MS and MMA-HS fumes indicated that the Mn composition of these aerosols were 6.9 and 43.7%, respectively. Using this Mn % composition and the DLB of a welder for total WF [$\mathrm{DLB_{WF(welder)}}$ = 18.75 mg; derived using body mass as dose metric], we determined the DLB for Mn in a welder [$\mathrm{DLB_{Mn(welder)}}$] and Mn fume concentration [$\mathrm{FC_{Mn}}$] as follows,

$$\mathrm{DLB_{Mn(welder)}} = \mathrm{DLB_{WF(welder)}} \times \%\ \mathrm{Mn\ content\ in\ WF}$$

For GMA-MS fumes,

$$\mathrm{DLB_{Mn(welder)}} = 18.75\ \mathrm{mg} \times 6.9\% = 1.29\ \mathrm{mg}, \text{ and}$$

$$\mathrm{FC_{Mn}} = \frac{\mathrm{DLB_{Mn(welder)}}}{[\mathrm{MRV_{(human)}} \times \mathrm{ED} \times \mathrm{DE}]} = \frac{1.29\ \mathrm{mg/day}}{[20\ \mathrm{L/min} \times 10^{-3}\ \mathrm{m^3/L} \times (8\ \mathrm{h/day} \times 60\ \mathrm{min/h}) \times 0.15]} = 0.9\ \mathrm{mg/m^3}$$

For MMA-HS,

$$\mathrm{DLB_{Mn(welder)}} = 18.75\ \mathrm{mg} \times 43.7\% = 8.19\ \mathrm{mg}, \text{ and}$$

$$\mathrm{FC_{Mn}} = \frac{\mathrm{DLB_{Mn(welder)}}}{[\mathrm{MRV_{(human)}} \times \mathrm{ED} \times \mathrm{DE}]} = \frac{8.19\ \mathrm{mg/day}}{[20\ \mathrm{L/min} \times 10^{-3}\ \mathrm{m^3/L} \times (8\ \mathrm{h/day} \times 60\ \mathrm{min/h}) \times 0.15]} = 5.7\ \mathrm{mg/m^3}$$

Thus, our instillation doses would mimic a daily worker exposure to Mn concentrations of 0.9 $\mathrm{mg/m^3}$ and 5.7 $\mathrm{mg/m^3}$, respectively, for the welding fume types examined in this study.

## MMA-HS causes pulmonary inflammation and cytotoxicity

Pulmonary toxicity was evaluated as it is the primary site of particle deposition. Pulmonary exposure to GMA-MS or MMA-HS resulted in deposition of large amounts of various metals in the lungs depending on the composition of the metals in the respective fumes (Fig. 1). Higher levels of Fe and Cu were detected following exposure to GMA-MS, while higher levels of Cr and Mn were measured following exposure to MMA-HS fumes (Fig. 1).

BAL was performed 1 day following the last instillation of GMA-MS or MMA-HS fumes. Large increases in BAL polymorphonuclear neutrophils (PMNs) were observed following instillation of GMA-MS ($\sim$14-fold; $P < 0.05$) and MMA-HS ($\sim$117-fold; $P < 0.001$; Table 3). While GMA-MS did not alter the levels of lavagable alveolar macrophages (AMs), MMA-HS exposure caused significant increase (2.2-fold; $P < 0.05$) in the number of AMs (Table 3). Similarly, GMA-MS did not alter the levels of BAL albumin, extravasation of which is an index of compromised alveolar-capillary barrier, or BAL lactate dehydrogenase (LDH), an index of cellular integrity. On the other hand, BAL levels of both albumin (2.2-fold; $P < 0.05$) and LDH (2.7-fold; $P < 0.05$) were significantly higher (Table 3) in the MMA-HS exposed animals indicating that MMA-HS (1) caused pulmonary inflammation leading to recruitment of inflammatory cells (2) disrupted the air–blood barrier causing extravasation of albumin and (3) caused cell damage leading to release of cytosolic LDH.

## Pulmonary exposure to MMA-HS results in deposition of Mn in various brain areas

To determine whether pulmonary exposure to WFs causes translocation of WF particulates or its soluble metal components to the brain, metal content in various brain areas was determined. Pulmonary exposure to GMA-MS did not alter the levels of any of major metals (Fe, Cu, Cr, Mn) in olfactory bulb (OB), striatum (STR), midbrain (MB), hippocampus (HIP) or cerebellum (CER) when examined 1 day post-exposure (Fig. 2). Similarly, exposure to MMA-HS did not significantly alter the levels of Fe, Cu or Cr in any of the above brain areas (Fig. 2). However, by 1 day post-exposure, MMA-HS caused significant deposition of Mn in OB (74%; $P < 0.001$), STR (70%; $P < 0.001$) and MB (45%; $P < 0.001$) (Fig. 2). In addition, Mn accumulation was also observed in HIP (48%; $P < 0.001$) and CER (26%; $P < 0.05$). By 105 days post-exposure, Mn levels in both STR and MB were similar to saline-treated controls. The time course of accumulation and clearance of Mn in the STR and MB were markedly different, highlighting the regional heterogeneity in the bio-distribution and transport of Mn,

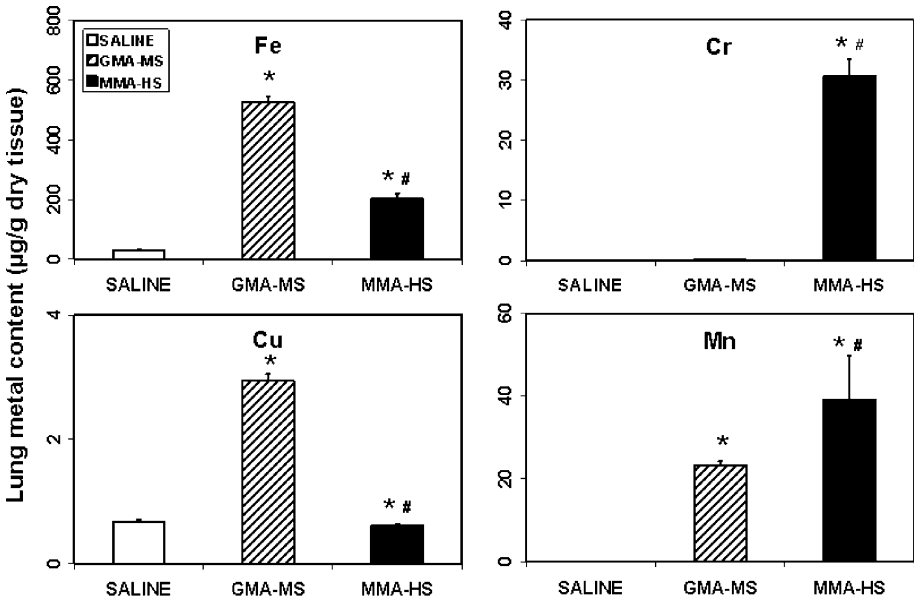


**Fig. 1** Elemental analysis of lung tissue following WF exposure. Lung concentrations of Fe, Cr, Cu and Mn were determined by ICP-OES, 1 day after repeated weekly instillations (0.5 mg/rat; 1/week × 7 weeks) of GMA-MS or MMA-HS fumes. The levels of each element were calculated as µg/g dry tissue. Graphical representations are Mean ± SE ($n = 5$/group). *Significantly different from saline-treated control ($P < 0.05$). # Significantly different from GMA-MS fume ($P < 0.05$)

**Table 3** Lung injury and inflammation parameters

| Groups | LDH (U/L) | Albumin (mg/ml) | PMNs ($10^6$ cells) | AMs ($10^6$ cells) |
|---|---|---|---|---|
| Saline | 103 ± 12.9 | 0.20 ± 0.01 | 0.12 ± 0.06 | 10.9 ± 1.07 |
| GMA-MS | 141 ± 8.7 | 0.28 ± 0.03 | 1.64 ± 0.17* | 15.0 ± 1.58 |
| MMA-HS | 282 ± 33.7* | 0.45 ± 0.04* | 14.10 ± 1.59* | 24.1 ± 1.90* |

Lung toxicity indices 1 day after intratracheal instillation (0.5 mg/rat; 1/week × 7 weeks) of low or high Mn-containing WFs. Control animals received sterile saline. Values are Mean ± SE ($n = 5$)

* Significantly higher than saline-treated control for a particular parameter ($P < 0.05$)

*LDH* lactate dehydrogenase; *PMN* polymorphonuclear neutrophils; *AM* alveolar macrophages

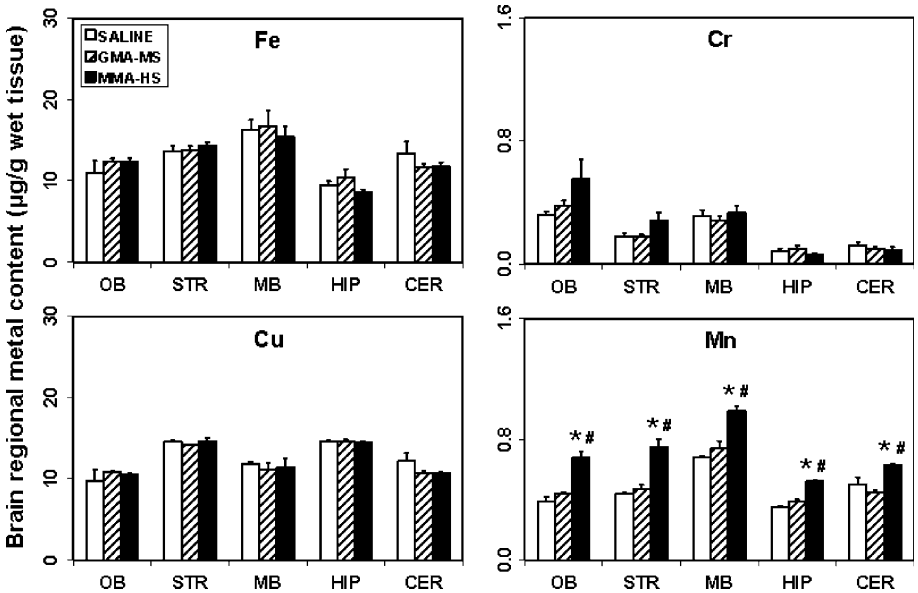


**Fig. 2** Selective accumulation of Mn in the brain following WF exposure. Concentrations of Fe, Cr, Cu and Mn were determined in discrete brain areas by ICP-OES, 1 day after repeated weekly instillations (0.5 mg/rat; 1/week × 7 weeks) of GMA-MS or MMA-HS fumes. The levels of each element were calculated as µg/g wet tissue. Graphical representations are Mean ± SE ($n = 4$–5/group). *Significantly different from saline-treated control ($P < 0.05$). # Significantly different from GMA-MS fume ($P < 0.05$). *OB* olfactory bulb, *STR* striatum, *MB* midbrain, *HIP* hippocampus, *CER* cerebellum

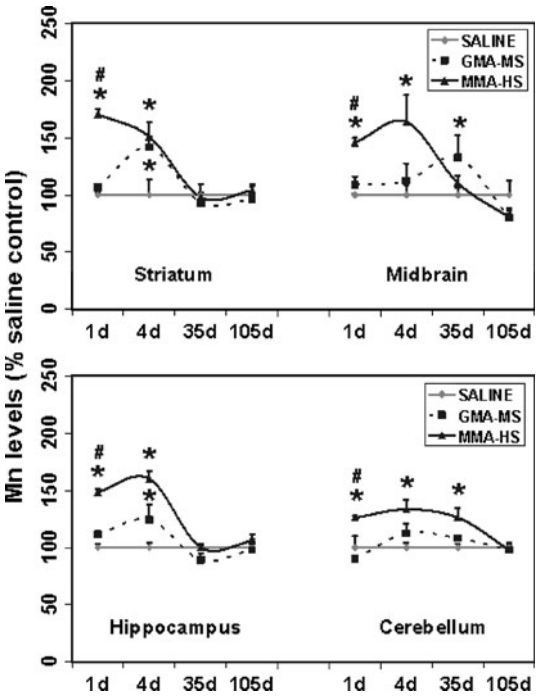


**Fig. 3** Mn accumulation and clearance in discrete brain areas following WF exposure. Concentrations of Mn were determined in discrete brain areas by ICP-OES at 1, 4, 35 and 105 days after repeated weekly instillations (0.5 mg/rat; 1/week × 7 weeks) of GMA-MS or MMA-HS fumes. The levels of each element were calculated as µg/g wet tissue and are expressed as percent of saline-treated control. Graphical representations are Mean ± SE ($n$ = 4-5/group). *Significantly different from saline control ($P < 0.05$). # Significantly different from GMA-MS fume ($P < 0.05$)

as well as, regional metal ion homeostasis (Fig. 3). A detailed evaluation of the pulmonary accumulation, clearance and extra-pulmonary translocation of various WF-related metals was recently reported by us (Antonini et al. 2010).

## Up-regulation of divalent metal transporter 1 (Dmt1) mRNA in striatum and midbrain following WF exposure

Dmt1, a metal-proton symporter for divalent metal ions, functions to transport specific metal ions across the plasma membrane and in/out of the endosomal compartment of the cell. To determine whether the accumulation of Mn in the dopaminergic brain areas is due to increased cellular trafficking, we measured the expression of Dmt1 mRNA in discrete brain areas (Fig. 4). Repeated pulmonary exposure of rats to GMA-MS resulted in selective up-regulation of Dmt1 mRNA in the STR and MB (1.6 to 2.0-fold; $P < 0.05$) by 1 day post-exposure (Fig. 4). Similarly, MMA-HS caused up-regulation of Dmt1 mRNA in the STR (1.6-fold; $P < 0.05$) and a small but non-significant increase in the MB (Fig. 4). On the contrary, Dmt1 expression was down-regulated in other non-dopaminergic

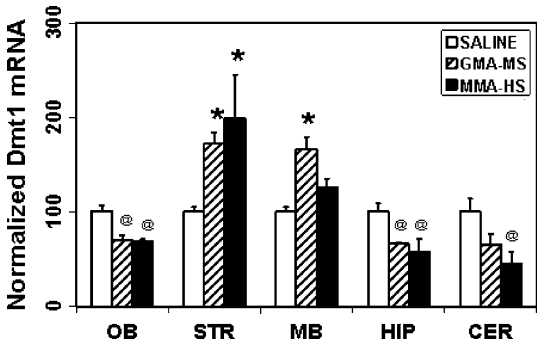


**Fig. 4** Divalent metal transporter 1 (Dmt1) mRNA is up-regulated in dopaminergic brain areas following WF exposure. Dmt1 mRNA expression in discrete brain areas was assayed by TaqMan® real-time PCR, 1 day after repeated weekly instillations (0.5 mg/rat; 1/week × 7 weeks) of GMA-MS or MMA-HS fumes. Following normalization to endogenous control ($\beta$-Actin), the mRNA levels are expressed as percent of saline control. Graphical representations are Mean ± SE ($n$ = 4/group). *Significant increase over saline-treated control ($P < 0.05$). @ Significant decrease from saline-treated control ($P < 0.05$). *OB* olfactory bulb, *STR* striatum, *MB* midbrain, *HIP* hippocampus, *CER* cerebellum

brain areas examined (Fig. 4). Dmt1 mRNA levels in either STR or MB were not significantly different from saline-treated controls at 105 days post-exposure.

## Loss of tyrosine hydroxylase (TH) protein in the striatum and midbrain following WF exposure

TH, a marker of dopaminergic neurons, is the rate-limiting enzyme in dopamine synthesis and loss of TH function or protein is an index of injury to the dopaminergic neurons. Exposure to either GMA-MS or MMA-HS decreased TH protein content in STR and MB. GMA-MS caused a small decrease in striatal TH protein (13%; did not reach statistical significance), while in the MB, a 30% ($P < 0.05$) loss of TH protein was observed (Fig. 5) at 1 day post-exposure. However, exposure to the more soluble MMA-HS fume decreased TH levels in the STR and MB by 24% ($P < 0.05$) and 34% ($P < 0.05$), respectively (Fig. 5). While the loss of striatal TH was transient, recovering to control levels by 105 days post-exposure, persistent loss of TH in the MB (34%; $P < 0.05$) was observed 105 days following cessation of MMA-HS exposure (Fig. 5).

## Down-regulation of dopamine D2 (Drd2) receptor mRNA in the midbrain following WF exposure

The actions of the neurotransmitter dopamine are mediated by interaction with G-protein coupled dopamine receptors that regulate dopaminergic signaling and function. To determine whether exposure to Mn-containing WFs modulate dopamine receptor expression and thereby dopaminergic signaling, we evaluated the mRNA expression of

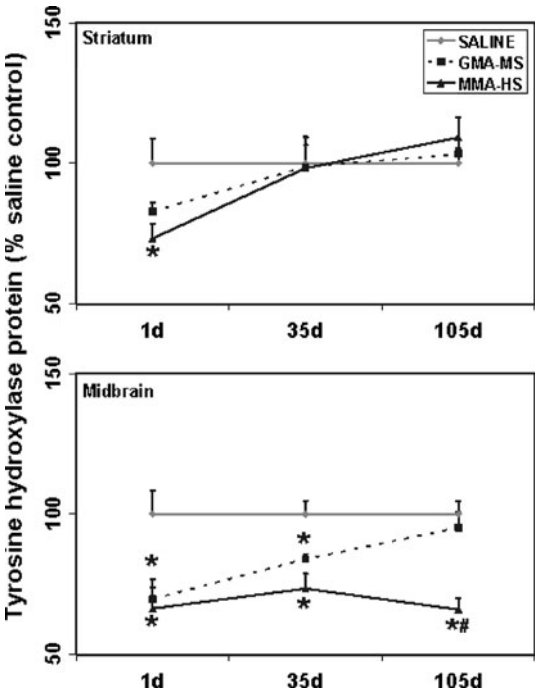


**Fig. 5** Loss of tyrosine hydroxylase (TH) protein in dopaminergic brain areas following WF exposure. TH protein content in STR and MB was assayed by ELISA at 1, 35 and 105 days after repeated weekly instillations (0.5 mg/rat; 1/week × 7 weeks) of GMA-MS or MMA-HS fumes. TH levels were calculated as μg/mg total protein and are expressed as percent of saline-treated control. Graphical representations are Mean ± SE ($n = 4$–5/group). *Significant decrease from saline-treated control ($P < 0.05$). # Significantly different from GMA-MS fume ($P < 0.05$)

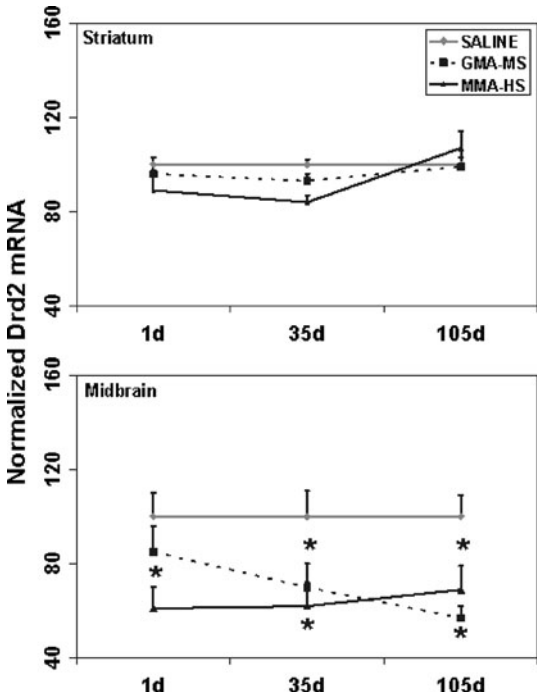


**Fig. 6** Decreased dopamine D2 receptor (Drd2) mRNA expression in midbrain following WF exposure. Drd2 mRNA expression in STR and MB was assayed by TaqMan® real-time PCR at 1, 35 and 105 days after repeated weekly instillations (0.5 mg/rat; 1/week × 7 weeks) of GMA-MS or MMA-HS fumes. Following normalization to endogenous control (β-Actin), the mRNA levels are expressed as percent of saline control. Graphical representations are Mean ± SE ($n = 4$–5/group). *Significant decrease from saline-treated control ($P < 0.05$)

several dopamine receptors (Drd1, Drd2, Drd3, Drd4 and Drd5) in the STR and MB. By 1 day post-exposure, MMA-HS caused down-regulation of Drd2 mRNA (39% decrease; $P < 0.05$) in the MB, but not STR (Fig. 6). On the other hand, GMA-MS-mediated loss of Drd2 (30%; $P < 0.05$) was observed only after 35 days post-exposure (Fig. 6). The loss of Drd2 mRNA in MB persisted at 105 days following cessation of exposure to either MMA-HS or GMA-MS (30–40% decrease; $P < 0.05$; Fig. 6). Neither fumes caused alterations in the expression of Drd1, Drd3 or Drd5 (data not shown). Drd4 expression was undetectable in both brain areas.

## Down-regulation of vesicular monoamine transporter 2 (Vmat2) in the midbrain following WF exposure

Vmat2 functions to sequester cytosolic monoamines into intracellular storage vesicles, a cellular process critical to avert oxidation of monomines like dopamine that can render them neurotoxic. Loss of Vmat2 expression can impair regulation of neurotransmitter release and increase sensitivity to neurotoxicity. To determine whether WF exposure causes alterations in the expression of Vmat2 and as a consequence plays a role in WF-related dopaminergic neurotoxicity, we examined the expression of Vmat2 mRNA expression in the striatum and midbrain. Exposure to MMA-HS resulted in a significant decrease in Vmat2 mRNA in MB by 1 day post-exposure (32% decrease; $P < 0.05$) and the loss persisted (54% decrease; $P < 0.05$) even at 105 days following exposure (Fig. 7). On the other hand, GMA-MS did not cause early loss of Vmat2 mRNA, but produced significant loss (52% decrease; $P < 0.05$) by 105 days post-exposure (Fig. 7). Neither of the fumes altered the expression of Vmat2 mRNA in the STR.

## Exposure to WFs elicits neuroinflammatory responses in striatum and midbrain

Proinflammatory cytokines and chemokines such as Tnfα, Il1α, Il6, Ccl2 and Cxcl2 have been implicated as etiological factors in several neurodegenerative diseases. In the brain, these factors are elaborated by activated microglia and play a key role in the glial response to neuronal injury (Sriram and O'Callaghan 2005). Concomitant with WF-mediated loss of TH immunoreactivity, subtle increases in the expression of proinflammatory cytokines and chemokines

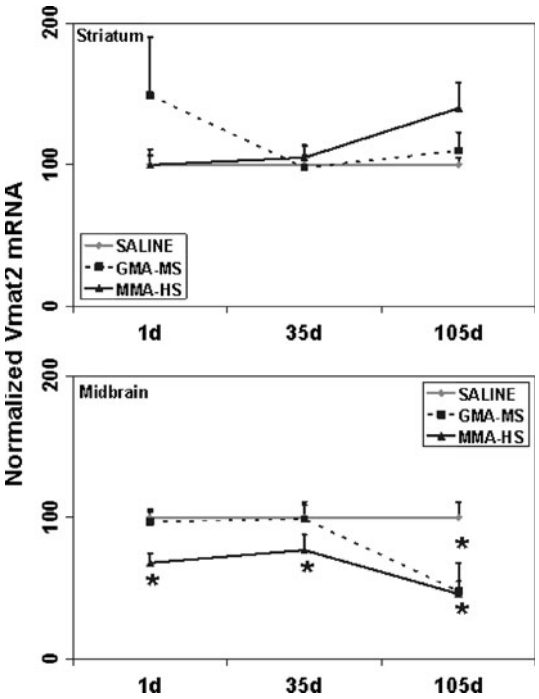


**Fig. 7** Decreased vesicular monoamine transporter 2 (Vmat2) mRNA expression in midbrain following WF exposure. Vmat2 mRNA expression in STR and MB was assayed by TaqMan® real-time PCR at 1, 35 and 105 days after repeated weekly instillations (0.5 mg/rat; 1/week × 7 weeks) of GMA-MS or MMA-HS fumes. Following normalization to endogenous control ($\beta$-Actin), the mRNA levels are expressed as percent of saline control. Graphical representations are Mean ± SE ($n = 4$–5/group). *Significant decrease from saline-treated control ($P < 0.05$)

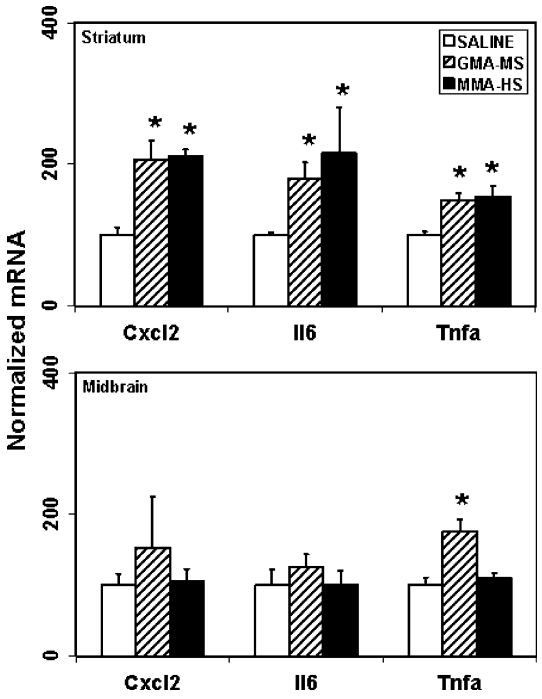


**Fig. 8** Subtle neuroinflammatory responses in dopaminergic brain areas following WF exposure. Cxcl2, Il6 and Tnfα mRNA expression in STR and MB was assayed by TaqMan® real-time PCR at 1 day after repeated weekly instillations (0.5 mg/rat; 1/week × 7 weeks) of GMA-MS or MMA-HS fumes. Following normalization to endogenous control ($\beta$-Actin), the mRNA levels are expressed as percent of saline control. Graphical representations are Mean ± SE ($n = 4$–5/group). *Significant increase over saline-treated control ($P < 0.05$)

were observed in the STR and MB (Fig. 8). At 1 day post-exposure, GMA-MS induced the mRNA expression of Cxcl2 (Mip2; 2.1-fold, $P < 0.05$), Il6 (1.8-fold, $P < 0.05$) and Tnfα (1.5-fold, $P < 0.05$), and in the STR (Fig. 8). Similarly, MMA-HS also induced Cxcl2 (2.1-fold, $P < 0.05$), Il6 (2.2-fold, $P < 0.05$) and Tnfα (1.6-fold, $P < 0.05$) in STR (Fig. 8). However, GMA-MS, but not MMA-HS, induced the expression of Tnfα (1.8-fold, $P < 0.05$) in the MB (Fig. 8). By 105 days post-exposure, the inflammatory response had resolved, both in STR and MB (data not shown). The induction of Tnfα in the dopaminergic targets is consistent with that observed with MPTP, corroborating the potential obligatory role of this proinflammatory cytokine in dopaminergic neurotoxicity (Sriram et al. 2006a, b).

## WFs increase the expression of inducible nitric oxide synthase (Nos2) in striatum and midbrain

In the brain, nitric oxide serves as an intracellular messenger at low physiological concentrations. However, excessive production of nitric oxide is detrimental and is thought to play a major role in the neurodegenerative process seen in PD, Alzheimer's disease and multiple sclerosis (Heales et al. 1999). To determine whether WF exposure causes oxidative stress, we examined the mRNA expression of various markers of oxidative stress. Pulmonary exposure to either GMA-MS or MMA-HS induced Nos2 (iNos) mRNA in the STR (2.6-fold, $P < 0.05$) by 1 day post-exposure (Fig. 9). Similarly, in the MB, both fumes induced Nos2 mRNA (1.9-fold, $P < 0.05$) and a small but non-significant raise in Nos1 mRNA (Fig. 9). Neither of the fumes induced the expression of Cox2 or Hmox1 (Ho1) in STR or MB, suggesting that WF neurotoxicity may be mediated by nitrosative stress-related mechanisms.

## Exposure to WFs causes glial activation in striatum and midbrain

Glial cells, particularly microglia and astrocytes, play a key role in the brain immune response and act as microsensors to detect subtle changes in neuronal milieu upon neuronal injury (Kreutzberg 1996; Streit 1999). Assessment of glial activation may therefore serve as an indicator of an

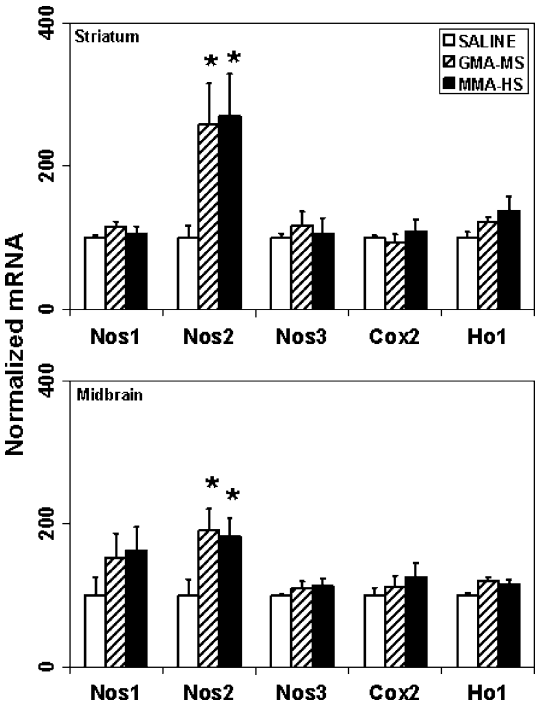


**Fig. 9** Nitrosative stress is associated with dopaminergic neurotoxicity following WF exposure. Nos1, Nos2, Nos3, Cox2, Hmox1 (Ho1) mRNA expression in STR and MB was assayed by TaqMan® real-time PCR at 1 day after repeated weekly instillations (0.5 mg/rat; 1/week × 7 weeks) of GMA-MS or MMA-HS fumes. Following normalization to endogenous control ($\beta$-Actin), the mRNA levels are expressed as percent of saline control. Graphical representations are Mean ± SE ($n$ = 4–5/group). *Significant increase over saline-treated control ($P < 0.05$)

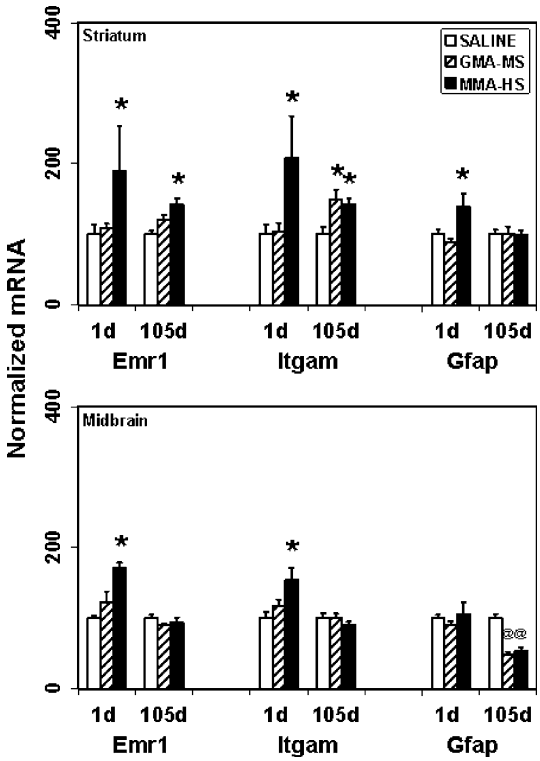


**Fig. 10** Micro- and astroglial responses following WF exposure. Emr1, Itgam (Ox42, Cd11b) and Gfap mRNA expression in STR and MB was assayed by TaqMan® real-time PCR at 1 day after repeated weekly instillations (0.5 mg/rat; 1/week × 7 weeks) of GMA-MS or MMA-HS fumes. Following normalization to endogenous control ($\beta$-Actin), the mRNA levels are expressed as percent of saline control. Graphical representations are Mean ± SE ($n$ = 4–5/group). *Significant increase over saline-treated control ($P < 0.05$). @ Significant decrease from saline-treated control ($P < 0.05$)

underlying neuronal injury. Pulmonary exposure to MMA-HS, in particular, induced the expression of specific microglial and astroglial markers in the STR and MB. In the STR, MMA-HS caused a small but significant increase in the mRNA expression of microglial markers, Emr1 (F4/80; 1.9-fold, $P < 0.05$) and Itgam (Ox42; 2.1-fold, $P < 0.05$; Fig. 10) by 1 day post-exposure, which persisted until 105 days post-exposure (1.4-fold, $P < 0.05$; Fig. 10). At 105 days post-exposure, GMA-MS also caused a small increase in Itgam mRNA (1.5-fold; $P < 0.05$; Fig. 10) expression. Similarly, in the MB, MMA-HS caused a small increase in the mRNA expression of Emr1 (1.7-fold, $P < 0.05$) and Itgam (1.5-fold, $P < 0.05$) by 1 day post-exposure, which unlike in STR, did not persist until 105 days after exposure (Fig. 10). A small but significant increase in the mRNA expression of the astroglial marker, Gfap (1.4-fold, $P < 0.05$), was also observed in the STR, but not MB, 1 day following MMA-HS exposure (Fig. 10). While the striatal Gfap mRNA expression was not significantly different from controls at 105 days post-exposure, both MMA-HS and GMA-MS fumes caused a significant down-regulation of Gfap mRNA (~50% decrease; $P < 0.05$) in the MB (Fig. 10).

## Exposure to WFs results in increased phosphorylation of Synapsin I

As Mn is known to influence the levels of neurotransmitters in the synapses (Takeda et al. 2002), we determined whether exposure to Mn-containing WFs alters the expression of proteins involved in synaptic neurotransmission. Specifically, we evaluated changes in Synapsin I phosphorylation, a major phosphoprotein in the nerve terminals that is involved in regulating neurotransmitter release. Exposure to either GMA-MS or MMA-HS led to an increase in the striatal phosphorylation of Synapsin I (Ser 551) by nearly 200% ($P < 0.05$; Fig. 11) at 1 day post-exposure. Similarly, MMA-HS, but not GMA-MS, also caused increased phosphorylation of Synapsin I in the MB (~200%, $P < 0.05$; data not shown) at 1 day post-exposure.

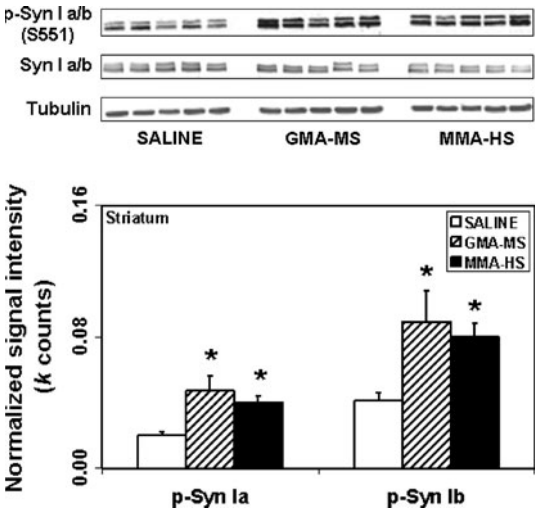


**Fig. 11** Increased phosphorylation of striatal Synapsin I (Syn I) following WF exposure. Phosphorylation of striatal Syn I at Ser551 (site 6), the primary site for Cdk5-mediated phosphorylation, was investigated by western immunoblot analysis 1 day after repeated weekly instillations (0.5 mg/rat; 1/week × 7 weeks) of GMA-MS or MMA-HS fumes. Following normalization to endogenous control (Tubulin), the relative signal intensities (*k* counts) of the two isoforms (Ia and Ib) were determined. Graphical representations are Mean ± SE ($n = 5$/group). *Significant increase over saline-treated control ($P < 0.05$)

## Discussion

Occupational exposure to aerosolized WF particulates is thought to be a risk factor for the development of a Parkinsonian-like pathology. Mn in welding consumables is considered the causative agent, although epidemiological evidence is not conclusive. To determine whether WF exposure is indeed linked to dopaminergic dysfunction, we evaluated the potential neurotoxic effects of low and high Mn-containing WFs. We report that repeated pulmonary exposure to WFs caused deposition of Mn in dopaminergic brain areas, decreased tyrosine hydroxylase protein content, down-regulated dopamine receptor expression, altered the expression of proteins involved in neurotransmitter release and elicited microglial activation.

Labor Statistics (Occupational Outlook Handbook 2008–2009, United States Department of Labor, Bureau of Labor Statistics) and projections data derived from the National Employment Matrix estimate that about 462,000 workers are employed full-time in welding operations in the United States, and this number is likely to increase by 5% over the next 5–7 years. Globally, this figure exceeds two million workers. Thus, there is potential for a large number of workers to be exposed to WF particulates at the workplace. Unfortunately, evaluating the adverse health effects of welders has proven difficult due to variations in workplace settings and exposure to diverse aerosols generated from a multitude of welding processes. Neuroepidemiological findings, in particular, are limited and sometimes controversial due to lack of well-designed case control studies (Racette et al. 2001; Racette et al. 2005). Therefore, extensive laboratory investigations of various WFs are warranted to fully understand their neurotoxic potential.

Based on our calculations to determine the relevance of the rat dosing paradigm to welder exposure, a daily lung burden in the rat of 0.1 mg is reflective of a worker's daily exposure to WF at a concentration of 13–18 mg/m$^3$. This value is about three times the prior threshold limit value (TLV) for WF (5 mg/m$^3$; ACGIH 1994). Currently, however, neither ACGIH nor OSHA has a recommended TLV for WF. The determined fume concentrations are not abnormally high, since excessive breathing zone fume concentrations have been reported to occur especially in locations where there is poor ventilation or in confined work environments. Time-weighted average concentrations of WF at the worker's breathing zone have been reported to range from 0.2 to 24.3 mg/m$^3$ (Matczak and Chmielnicka 1993; Matczak and Przybylska-Stanislawska 2004). Similarly, our predicted Mn concentrations of 0.9 and 5.7 mg/m$^3$ in GMA-MS or MMA-HS aerosols are comparable to the reported Mn concentrations of 0.01–4.93 mg/m$^3$ in the atmosphere around the welder's work zone (Karlsen et al. 1992; Korczynski 2000). Thus, our repeated intratracheal instillation exposure paradigm appears to reasonably mimic welder exposure at the workplace.

We recently reported that short-term inhalation exposure to GMA-MS fume caused deposition of Mn in olfactory bulb and striatum and elicited a subtle neuroinflammatory response (Antonini et al. 2009). However, we did not observe any changes in dopaminergic markers perhaps due to the short exposure regimen. Although a long-term inhalation exposure was desirable, operating and technical limitations of the welding system, based on the number of processes and types of consumables that can be handled, compelled us to adapt an alternate exposure method. Hence, we treated animals by intratracheal instillation, a procedure known to effectively deliver defined amounts of material to the lung (Driscoll et al. 2000). This method has been previously used for assessment of WF-related pulmonary toxicity (Antonini et al. 1996; Taylor et al. 2003). We acknowledge that this mode of exposure circumvents olfactory uptake, a prospective route of delivery to the brain during an inhalation exposure. However, it has been reported that the olfactory pathway may be ineffective in transporting several metals to the brain (Tjälve and Henriksson 1999). While olfactory transport could rapidly deliver Mn to the olfactory bulb (Gianutsos et al. 1997; Henriksson et al. 1999; Tjälve and Henriksson 1999), it was not efficient in facilitating translocation of Mn to

deeper brain structures, including striatum (Tjälve et al. 1996; Brenneman et al. 2000; Dorman et al. 2002). Moreover, interspecies differences in the nasal anatomy among rodents and humans (Dorman et al. 1997) can influence the way in which inhaled particles are deposited in the olfactory regions and their subsequent translocation. In rodents, the olfactory region constitutes a significantly larger portion of the central nervous system, and nearly half their nasal epithelium is ensheathed by the olfactory mucosa, compared to about 5% in humans (Aschner et al. 2005). This provides a larger nasal surface area for deposition and potential translocation of particles. Further, rodents are obligatory nasal breathers while humans are oronasal breathers. Computational modeling of nasal airflow estimate that about 3–9% of the inhaled air stream reaches the olfactory region in lower primates and humans (Kepler et al. 1998; Subramaniam et al. 1998), while in rodents, it can be as high as 20% (Kimbell et al. 1997). These differences in anatomic and respiratory patterns may predispose rodents to a greater olfactory deposition and/or translocation of inhaled particles compared to humans. On the other hand, deposition of inhaled particles in the lung can result in permeation of the particles across the lung–blood barrier by endocytosis, transcytosis or stochastic transport and enter systemic circulation. Ultrafine particles deposited in the lungs have been reported to translocate to the circulatory system and eventually to organs like liver, kidney and brain (Nemmar et al. 2001; Shimada et al. 2006). Thus, metal translocation to the brain, including Mn, may occur predominantly via systemic circulation after initial deposition in the lung, following an inhalation exposure. Dissolution of Mn from WF particles in the lung can account for extra-pulmonary distribution. Indeed, we have observed that particle solubility is a key determinant in the pulmonary translocation of toxic metals, such as, Fe, Mn, Cr, present in GMA-MS or MMA-HS fumes (Antonini et al. 2010). As the MMA-HS fumes are more water-soluble, Mn was rapidly translocated from the lungs following repeated intratracheal exposure. Concomitant increases of Mn in discrete brain regions were seen. Notably, elevated levels of Mn in the dopaminergic brain areas, STR and MB were observed. Other metal constituents of the WFs, such as, Fe, Cr, Ni and Cu, accumulated in the lungs and other extra-pulmonary organs (e.g., heart, spleen and kidney), but translocation of these metals to the brain was not observed. While the large presence of Fe in the fumes would be a prime candidate for eliciting neurotoxicity given its role in oxidative stress, the lack of accumulation of this element in the brain is suggestive of its existence as a water-insoluble component in the WFs. Thus, selective translocation of Mn to brain structures occurs following WF exposure. This selectivity may be cause for concern because chronic Mn intoxication associated with other occupations, such as mining and smelting, causes irreversible neurological dysfunction resembling PD, a syndrome known as 'manganism' (Huang et al. 1993; Mergler and Baldwin 1997; Pal et al. 1999).

It is postulated that translocation of Mn from systemic circulation to the brain involves transport across the blood–brain barrier (Murphy et al. 1991, Rabin et al. 1993) and is thought to be carrier-mediated (Aschner et al. 1999; Crossgrove et al. 2003). There is also a suggestion that the divalent metal transporter 1 (Dmt-1), a metal–proton symporter for divalent metal ions, is involved in the transport of Mn to the brain (Gunshin et al. 1997; Conrad et al. 2000) although others argue against its involvement (Crossgrove and Yokel 2004). Thus, while the exact transport mechanisms for Mn to the brain remain to be fully evaluated, it is nevertheless evident that Mn accumulates in the brain following WF exposure. In this study, we observed that exposure to either of the WFs induced the expression of Dmt1 mRNA selectively in the STR and MB, but not other brain regions. Up-regulation of Dmt1 in dopaminergic targets suggests preferential sequestration and increased cellular/intracellular trafficking of Mn in these regions. Dmt1 is known to transport specific metal ions across the plasma membrane and in/out of the endosomal compartment of the cell (Gunshin et al. 1997; Gruenheid et al. 1999) and has been immunolocalized to brain vasculature, neurons and astrocytes in the striatum (Burdo et al. 2001). In non-human primates, Dmt1 expression has been demonstrated in astrocytic processes and end-feet around cerebral blood vessels (Wang et al. 2001), suggesting a potential role in transport of divalent ions across the blood–brain barrier.

Exposure to WFs caused loss of TH protein in the STR and MB. The high Mn-containing MMA-HS fume caused more extensive loss of striatal TH compared to the low Mn-containing GMA-MS. While the loss of striatal TH mediated by either of the fumes was transient, exposure to MMA-HS resulted in a persistent loss of TH in the MB even after cessation of exposure. As the rate-limiting enzyme in the synthesis of dopamine, sustained loss of TH is suggestive of alterations in the levels of dopamine and dopaminergic signaling, although direct measurements of dopamine and its metabolites were not performed in this study. Several discrepancies exist on the effects of Mn on nigrostriatal dopamine concentrations. While some studies report a decrease in dopamine content (Autissier et al. 1982; Erikson et al. 1987; Sloot et al. 1994; Zhang et al. 2003; Díaz-Véliz et al. 2004), others find an increase (Bonilla 1980; Tomas-Camardiel et al. 2002) or even lack of an effect (Gwiazda et al. 2002; Normandin et al. 2002) following treatment with Mn or Mn-containing compounds. Thus, measurement of dopamine alone may not fully reflect the neurotoxic potential of Mn. Further, these

studies exemplify the complexity of Mn-mediated neurotoxicity and indicate that gaps exist in the understanding of various contributing factors. Despite such differences, our findings of the loss of TH protein suggest that WF exposure can modulate dopaminergic function. More comprehensive dose and time course evaluation of WF-related dopamine changes are currently being conducted, along with behavioral and motor assessments. These forthcoming studies will help identify whether WF-mediated dopamine loss associates with changes in dopaminergic function.

Concomitant with the sustained loss of TH in the MB, WF exposure also caused loss of dopamine D2 receptor (Drd2) and vesicular monoamine transporter 2 (Vmat2) mRNAs in this region. Marked decreases in Drd2 density following chronic Mn intoxication have been revealed by positron emission tomography, and such changes may be associated with neurodegeneration (Kessler et al. 2003). High levels of dopamine receptor mRNAs are present in all dopaminoceptive regions of the rat brain (Nagy et al. 1978; Mengod et al. 1989; Huntley et al. 1992). Specifically, Drd2 mRNA is widely distributed in the MB and hindbrain and its expression is prominent in dopaminergic neurons of the substantia nigra pars compacta and ventral tegmental area, while relatively low in substantia nigra pars reticulata (Meador-Woodruff et al. 1989). High levels of Drd2 mRNAs are also expressed in caudate-putamen, nucleus accumbens and olfactory tubercule (Meador-Woodruff et al. 1989). The large presence of Drd2 in the MB dopaminergic areas suggest that these receptors may play a role in regulation of dopamine release (Carlsson 1975), functioning as "autoreceptors" to regulate dopaminergic activity in the nigrostriatal and mesolimbic dopamine systems (Skirboll et al. 1979). It has also been shown that Drd2 mRNA co-localizes with TH in dopaminergic neurons of the substantia nigra and ventral tegmental area (Meador-Woodruff and Mansour 1991). Further, injury to the dopaminergic pathway caused concurrent loss of TH and Drd2 mRNA in the substantia nigra and the ventral tegmental area (Mansour et al. 1990). Our current findings of loss of TH protein and Drd2 mRNA in the MB are consistent with these observations and suggest the involvement of MB dopaminergic neurons in the neurotoxicity of Mn-containing WFs. These observations are in agreement with several studies demonstrating the involvement of MB dopaminergic neurons in Mn neurotoxicity (Gupta et al. 1980; Calne et al. 1994; Guilarte et al. 2006, 2008; Zhao et al. 2009; Stanwood et al. 2009), while others suggest that Mn preferentially accumulates in the globus pallidus and STR, while sparing the nigrostriatal dopaminergic system (Olanow et al. 1996; Pal et al. 1999; Olanow 2004; Spadoni et al. 2000; Perl and Olanow 2007). However, Olanow et al. (1996) do report the involvement of the substantia nigra pars reticularis in Mn neurotoxicity, in addition to the involvement of globus pallidus. Neuroanatomical and functional studies reveal that dopaminergic projections can significantly influence basal ganglia-thalamocortical circuits (motor-loops), areas that are associated with complex motor and behavioral activities (Alexander and Crutcher 1990). The STR serves as the input structure while the globus pallidus and substantia nigra form the output structures. These structures are connected by the direct and indirect pathways that are controlled by dopamine through modulation of its D1 and D2 receptors. A balance between these two pathways is critical for regulation of movement. Any abnormality of one or more components of the motor-loop may alter motor function. Thus, evaluation of dopaminergic pathway in its entirety is critical to understanding its role in welding fume/manganese-related neurotoxicity. Further, such an approach may perhaps highlight the differences in the pathology between PD and manganism that may be attributed to the specific dopaminergic nuclei affected in the midbrain, manifesting certain PD-related neurological deficits that are astonishingly similar. Given the differences in the time course of deposition and clearance pattern of Mn from the STR and MB observed by us (Antonini et al. 2010 and this study), the striatal effects appear early, while the MB responses are delayed and more persistent. It is likely that the differential regional susceptibility observed among various studies is at least in part, influenced by dissolution and translocation rate of Mn-containing compounds, animal species, exposure paradigms and/or stages of the disorder. Indeed, exposure to higher levels of manganese has been shown to cause loss of dopamine and neuromelanin in the nigra, suggesting that targets other than globus pallidus can become involved (Neff et al. 1969; Gupta et al. 1980). Thus, dose and duration of manganese exposure may also be key determinants for pre- and post-synaptic lesions to the dopaminergic system. Thus, consistent with the discrepancies in measures of dopamine, the target brain areas affected by Mn also appear to be debatable. Despite these intricacies, we have demonstrated that WFs have the ability to accumulate Mn in dopaminergic regions and cause alterations in the expression of several dopaminergic markers, persistent loss of which could prove detrimental.

The loss of Vmat2 in the MB further documents evidence that WF exposure can adversely affect dopamine neuron survival and function. Vmat2 is known to sequester cytosolic dopamine into storage vesicles for subsequent exocytosis and is a key regulator of dopamine homeostasis (Liu and Edwards 1997). Gain of function VMAT2 haplotype in humans was recently shown to be protective against PD (Glatt et al. 2006) confirming that perturbation of VMAT2 could lead to dopaminergic damage. A loss of Vmat2 seen following WF exposure in this study suggests that this factor may be a critical player in WF-related

neurotoxicity, in addition to other indices of dopaminergic signaling. Further, aberrant sequestration of dopamine into synaptic vesicles due to deficiency of Vmat2 may result in accumulation of cytosolic dopamine, which can undergo oxidation forming neurotoxic dopamine quinones and induce oxidative stress (Hastings et al. 1996; Hastings and Zigmond 1997; Rabinovic et al. 2000; Fuentes et al. 2007). The presence of metals, including Mn, can further enhance the oxidation of dopamine (Sistrunk et al. 2007). It remains to be investigated whether Mn-containing WFs cause similar oxidation of dopamine.

Consistent with the loss of Vmat2-mediated sequestration of dopamine in the MB, exposure to either of the WFs also appears to be involved in modulation of dopamine release from the striatal presynaptic terminals. Both GMA-MS and MMA-HS increased the phosphorylation of Synapsin I, a neuron-specific phosphoprotein that tethers synaptic vesicles to actin filaments to regulate neurotransmitter release. The phosphorylation of Synapsin I at S551 (site 6; in the rat) suggests the involvement of cyclin-dependent kinase 5 (Cdk5; Matsubara et al. 1996) in the regulation of striatal neurotransmitter release. Indeed, Cdk5 is thought to influence neurotransmitter release by regulating exocytosis and endocytosis through phosphorylation of several proteins associated with these two processes, although the exact mechanisms are yet to be identified (Chung 2008). The modulation of striatal synapsin I phosphorylation and Vmat2 expression in MB suggests that WF exposure may alter mechanisms associated with regulation of neurotransmitter release and function.

Furthermore, we demonstrate that WF exposure selectively up-regulates the expression of Nos2, but not Cox2 or Ho1 in the STR and MB, suggestive of nitrosative stress. Generation of reactive oxygen and nitrogen species has been suggested to be a key mediator in the pathophysiology of experimental and human PD (Sriram et al. 1997; Jenner 2003). In addition, up-regulation of mRNAs for microglial markers, Emr1 (F4/80) and Itgam (Cd11d, Ox42), was observed in the STR and MB after MMA-HS exposure; whereas, Gfap mRNA, an astroglial marker, was significantly down-regulated in the MB 105 days post-exposure, suggestive of impaired astroglial response that is consistent with previous reports (Aschner et al. 1999; Milatovic et al. 2007). Concurrent with the microglial activation, WF exposure also caused subtle increases in several cytokines and chemokines in the STR and MB, suggestive of a neuroinflammatory response. Particularly, increases in Cxcl2, Il6 and Tnfα mRNAs were observed in the STR following MMA-HS or GMA-MS exposure. These observations are consistent with the involvement of microglia-derived inflammatory and oxidative stress mediators in experimental and human PD (Boka et al. 1994; Mogi et al. 1996; Sriram et al. 2002; Sriram and O'Callaghan 2005, 2007), as well as, Mn-mediated neurotoxicity (Filipov et al. 2005; Zhang et al. 2009). The subtle 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.

In conclusion, we report that pulmonary exposure of rats to Mn-containing WFs results in accumulation of Mn in the brain, causes persistent loss of dopaminergic markers, affects factors involved in regulation of neurotransmission, induces oxidative stress and elicits neuroinflammation predominantly in dopaminergic brain areas. Whether these sustained events following WF exposure can elicit neurobehavioral and neurodegenerative manifestations reminiscent of PD is the topic of our ongoing investigations.

**Conflict of interest statement** 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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