

Award Number: W81XWH-05-1-0239

TITLE: Manganese Research Health Project (MHRP)

PRINCIPAL INVESTIGATOR: Michael Aschner, PhD

CONTRACTING ORGANIZATION: Vanderbilt University Medical Center
Nashville, TN 27203

REPORT DATE: February 2011

TYPE OF REPORT: Final Addendum

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT:

Approved for public release; distribution unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

REPORT DOCUMENTATION PAGE				Form Approved OMB No. 0704-0188	
Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Department of Defense, Washington Headquarters Services, Directorate for Information Operations and Reports (0704-0188), 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302. Respondents should be aware that notwithstanding any other provision of law, no person shall be subject to any penalty for failing to comply with a collection of information if it does not display a currently valid OMB control number. PLEASE DO NOT RETURN YOUR FORM TO THE ABOVE ADDRESS.					
1. REPORT DATE (DD-MM-YYYY) 01-02-2011		2. REPORT TYPE Final Addendum		3. DATES COVERED (From - To) 1 JAN 2010 - 31 JAN 2011	
4. TITLE AND SUBTITLE Manganese Research Health Project (MHRP)				5a. CONTRACT NUMBER	
				5b. GRANT NUMBER W81XWH-05-1-0239	
				5c. PROGRAM ELEMENT NUMBER	
6. AUTHOR(S) Michael Aschner, PhD E-Mail: michael.aschner@vanderbilt.edu				5d. PROJECT NUMBER	
				5e. TASK NUMBER	
				5f. WORK UNIT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Vanderbilt University Medical Center Nashville, TN 27203				8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012				10. SPONSOR/MONITOR'S ACRONYM(S)	
				11. SPONSOR/MONITOR'S REPORT NUMBER(S)	
12. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited					
13. SUPPLEMENTARY NOTES					
14. ABSTRACT Questions persists regarding a possible association between neurological effects in welders and the presence of manganese in welding fume. Researchers have suggested that welding is not only a high-risk occupation for the development of manganism, but it may also be a risk factor for or can accelerate the onset of idiopathic Parkinson's disease. However, toxicology studies investigating this issue are lacking. The objective was to examine the potential neurotoxic effect of manganese in rats after pulmonary exposure to different welding fumes. Manganese was found to translocate from the lungs via the circulation to dopaminergic brain areas. Consistent with the observed accumulation of manganese in the brain, welding fumes differentially elicited neuroinflammatory responses in the olfactory bulb, striatum, and midbrain and altered the expression of Parkin (Park2), Uchl1 (Park5) and Dj1 (Park7) proteins in dopaminergic brain areas. As mutations in PARK genes have been linked to early-onset PD in humans, and because welding is implicated as a risk factor for Parkinsonism, PARK genes may play a critical role in WF-mediated dopaminergic dysfunction. Whether these molecular alterations culminate in neurobehavioral and neuropathological deficits reminiscent of PD remains to be ascertained.					
15. SUBJECT TERMS Manganese, welding, neurotoxicity, inhalation					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT UU	18. NUMBER OF PAGES 91	19a. NAME OF RESPONSIBLE PERSON USAMRMC
a. REPORT U	b. ABSTRACT U	c. THIS PAGE U			19b. TELEPHONE NUMBER (include area code)

Table of Contents

Introduction.....	4
BODY.....	4
Key Research Accomplishments.....	8
Reportable Outcomes.....	9
Conclusions.....	11
References.....	12
Appendices (Prior Reports attached).....	13

Introduction

Epidemiology suggest that inhalation of welding fumes may cause adverse health effects in exposed workers. However, more information is required to determine causality, to evaluate temporal and dose-response relationships, and to elucidate mechanisms. To accomplish this, it is necessary to develop a welding fume generation and animal inhalation exposure system to perform long-term toxicology studies. The Health Effects Laboratory Division within CDC-NIOSH at Morgantown, WV has constructed a completely automated, robotic welding fume inhalation system that can expose laboratory animals to tightly-controlled, well-characterized welding fumes generated from different welding processes and materials.

Serious questions have been raised regarding a possible causal association between neurological effects in welders and the presence of manganese in welding consumables. Some researchers have suggested that welding is not only a high-risk occupation for the development of manganism, but that it may also be a risk factor for or can accelerate the onset of idiopathic Parkinson's disease. However, toxicology studies currently investigating this issue are greatly lacking.

The objective of the study was to examine the potential neurotoxic effect of manganese in rats after pulmonary exposure to different welding fumes. Rats were exposed by inhalation or intratracheal instillation to welding fumes that contained differing levels of manganese. The translocation of deposited metals from the respiratory tract to other organs systems, including the central nervous system, was determined. In addition, molecular and biochemical markers of neuroinflammation, metal transport, and neuronal cell injury were examined.

Body

STUDY 1- Inhalation of gas metal arc-mild steel welding fume

To evaluate temporal and dose-response relationships and to elucidate the mechanisms associated with the potential adverse health effects of welding, a welding fume generation and animal inhalation exposure system is needed to perform long-term toxicology studies. A completely automated, robotic welding fume inhalation system that exposes laboratory animals to tightly-controlled, well-characterized welding fumes generated from different welding processes and materials has been developed. The physical and chemical composition of welding fumes and gases generated by the system have been characterized and found to be comparable to what is observed in the workplace.

Male Sprague-Dawley rats were exposed to 40 mg/m³ of gas metal arc-mild steel (GMA-MS) welding fume for 3 hours/day for 10 days. Longer-term exposures to GMA-MS fume for up to 90 days and to a fume with a greater manganese content also are planned as part of the study but have not been completed at this time. GMA-MS was initially chosen for study in the initial experiments because a large majority of welders in the U.S. (~90 %) are exposed to this particular fume. In the characterization of the generated fume, the majority of the collected particles was observed in the fine size range with cut-off diameters of 0.10-1.0 µm. Additional nanometer-sized particles in the range of 0.010-0.10 µm as well as larger, coarse particles with diameters >1.0 µm in size also

were collected. The mass median aerodynamic diameter was calculated to be approximately 0.31 μm . Electron microscopic analysis demonstrated that most of the aerosols generated were arranged in homogeneous, chain-like agglomerates of nanometer-sized primary particles. Metal analysis indicated that the particles were composed primarily of iron (80.6 %) and manganese (14.7 %).

Significant elevations in iron and manganese were observed in lungs after 10 days of exposure to GMA-MS welding fume compared to air control. Despite the relatively high GMA-MS welding fume concentration used, no evidence of lung inflammation, as determined by neutrophil influx, or injury, as determined by lactate dehydrogenase and albumin measurements in recovered lung lining fluid samples, was observed after the 10-day exposure. Light and electron microscopic analyses indicated that a significant number of inhaled GMA-MS welding particles were engulfed by lung macrophages after exposure. Intact primary MS welding particles were observed to reside in phagolysosomes after macrophage uptake. SEM-EDS analyses indicated that the particles residing in the macrophages were mostly intact with no change in metal profile and little evidence of particle dissolution over the 10 day treatment period. Several welding particles were analyzed, and iron and manganese were observed to be present in all phagocytized particles.

A slight, but not significant, increase in manganese was measured in whole blood of animals exposed to the GMA-MS welding fume. In nearly every case, there was a slight increase in iron and manganese measured in the liver, heart, kidney, and spleen after exposure to GMA-MS welding fume compared to air control. However, significant increases were observed only for liver iron and kidney manganese in the welding fume group compared to air control. In the assessment of metal deposition in specific brain regions after welding fume inhalation, a significant increase in manganese concentration was observed in the cerebellum, cortex, and olfactory bulb at 1 day after 10 days of exposure to GMA-MS fume compared to air control. Iron was not significantly elevated in any brain region after GMA-MS welding fume inhalation for 10 days.

Following 10 days of exposure to GMA-MS welding fume a significant increase (1.5 to 2.3-fold) in the expression of the divalent metal ion transporter 1 (Dmt1) was observed in the dopaminergic targets, striatum and midbrain. The expression of this transporter is suggestive of potential translocation of divalent metals, including Mn into neural cells in these dopaminergic brain areas.

Following 10 days of exposure to GMA-MS welding fume, a small but significant increase (~1.5-fold) in the expression of proinflammatory chemokines (Ccl2, Cxcl2) and cytokines (Il1 β , Tnf α) predominantly in the striatum is indicative of an early inflammatory response. This neuroinflammatory response in the striatum was associated with a subtle increase (~1.5-fold) in the mRNA expression of the astroglial marker, glial fibrillary acidic protein (GFAP). Similarly, GFAP protein levels increased in the striatum and globus pallidus by 27 % and 70 %, respectively. The induction of neuroinflammation and gliosis in the striatum and globus pallidus are suggestive of an early insult in these basal ganglia targets, which are predominantly involved in dopaminergic neurodegeneration characteristic of Parkinson-like neurological diseases.

Although 10 days of exposure to GMA-MS welding fume resulted in increases in inflammatory cytokines as well as GFAP in the striatum and globus pallidus, these indices of insult were not accompanied by any alterations in dopamine or its metabolites.

As manganese exposure has been shown to affect brain GABA levels, additional HPLC analyses of these areas for GABA content indicated this exposure regimen had no effect on this biochemical parameter.

Ten days of inhalation to GMA-MS welding fume did not cause neurodegeneration in any brain regions as determined by histopathological analysis. Specific regional targets, the striatum and globus pallidus, were examined with increased interest as they showed subtle differences between control and fume treated animals by other measures (i.e., RNA). The striatum of welding fume-treated rats appeared identical to control, air-treated animals. GFAP immunohistochemistry revealed no differences in astrocyte morphology between control and welding fume-treated rats, indicating astrogliosis in response to overt neuronal damage had not been initiated. Microglia stained by Iba-1 were observed in the ramified or resting state in both control and welding fume-treated rats suggesting insufficient cause for activation. In summary, our goal was to develop an animal model to measure the accumulation of manganese in specific brain areas and to examine the potential neurological responses associated with the inhalation of GMA-MS welding fume. Short-term exposure to high concentrations of GMA-MS fume led to an accumulation of manganese in the olfactory bulb, cerebellum, and cortex. Manganese most likely reached these brain regions via transport by olfactory neurons. However, because of anatomical and physiological differences between rats and humans, one must be cautious in the interpretation of these results because the relevance of these findings to human manganese inhalation exposure and the risks for neurotoxicity are unknown. There was no evidence of observable changes in neuronal cell injury as assessed by histopathology. However, subtle changes in cell markers of neuroinflammation and astrogliosis were observed. There is a need to extend the welding exposures for longer periods of time (e.g., subchronic exposures for 90 days) and to include inhalation exposure to other fumes that contain varying concentrations of manganese. The neurofunctional significance of these findings currently are being investigated in longer, more chronic welding fume exposure studies.

STUDY 2- Intratracheal Instillation of Welding Fumes Containing Different Levels of Manganese

The objective was to compare the neurotoxicity and translocation of metals from the respiratory tract to specific brain regions and other organ systems after intratracheal instillation of a welding fume that is high in manganese content compared to one that is lower in manganese content. Male Sprague-Dawley rats were treated with GMA-MS welding fume or manual metal arc-hardsurfacing (MMA-HS) welding fume. These welding fumes were chosen on the basis of their varying metal composition, as well as, differences in their solubility, factors that could influence translocation. The GMA-MS fume was composed of iron (~85 %) and manganese (~15 %) and was mostly insoluble in water with a soluble/insoluble ratio of 0.014. The MMA-HS fume was higher in manganese content (~51 %) with lower levels of iron (~20 %) and found to be more water soluble with a soluble/insoluble ratio of 0.218. The rats were treated by intratracheal instillation with 0.5 mg/rat of the GMA-MS or MMA-HS fumes once a week for 7 or 11 weeks. Control animals received intratracheal instillations of saline vehicle.

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. High levels of lung iron and copper were detected following 7 and 11 week intratracheal exposure to GMA-MS fumes as well as a significant increase in manganese compared to control, while substantially high levels of chromium and manganese were measured following exposure to MMA-HS fumes. Lung lining fluid was recovered 1 day following the last instillation of GMA-MS or MMA-HS fumes for both the 7 and 11 weeks treatment periods. Large increases in recovered neutrophils were observed following instillation of GMA-MS (~14-fold) and MMA-HS (~117-fold). MMA-HS exposure caused a significant increase (2.2-fold) in the number of lung macrophages. Similarly, GMA-MS did not alter the lung fluid levels of albumin, extravasation of which is an index of compromised alveolar-capillary barrier, or lactate dehydrogenase, an index of cellular integrity. On the other hand, both albumin (2.2-fold) and LDH (2.7-fold) were significantly higher in the MMA-HS exposed animals indicating that MMA-HS (i) caused pulmonary inflammation, leading to recruitment of inflammatory cells, (ii) disrupted the air-blood barrier, causing extravasation of albumin and (iii) caused cell damage leading to release of cytoplasmic lactate dehydrogenase.

To determine if intratracheal instillation exposure to welding fume results in translocation of the particulates or soluble metal components to the brain, metal content in discrete brain areas was determined. Pulmonary exposure to GMA-MS did not alter the levels of any of major metals (iron, copper, chromium, manganese) in olfactory bulb (OB), striatum (STR), midbrain (MB), hippocampus (HIP) or cerebellum (CER) after 7 weeks of treatment. Similarly, exposure to MMA-HS did not significantly alter the levels of iron, copper or chromium in any of the above brain areas. However, accumulation of manganese was observed in OB (74 %), STR (70 %) and MB (45 %), dopaminergic areas known to be associated with Parkinsonian-type of neurological disorders. In addition, Mn accumulation was also observed in HIP (48 %) and CER (26 %).

To determine if the accumulation of manganese in the dopaminergic brain areas is due to increased cellular trafficking, we measured the mRNA expression of the divalent metal transporter 1 (Dmt1) that functions as a metal-proton symporter for divalent metals at 1 day after 7 weeks of intratracheal treatments. Following exposure to either GMA-MS or MMA-HS, Dmt 1 was selectively up-regulated in the STR (1.7 to 2.0-fold) and MB (1.3 to 1.6-fold), but not in other brain regions. Exposure to either GMA-MS or MMA-HS decreased tyrosine hydroxylase protein content in STR and MB after 7 weeks of treatment. GMA-MS exposure caused a small decrease in striatal TH protein (13 %), while in the MB a 30 % loss of TH protein was observed. However, exposure to the more soluble MMA-HS fume, decreased striatal and MB TH levels by 24 % and 34 %, respectively.

Proinflammatory cytokines and chemokines like, $Tnf\alpha$, $Il1\alpha$, $Il6$, $Ccl2$ 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. Concomitant with welding fume-mediated loss of TH immuno-reactivity, increased expression of proinflammatory cytokines were observed in the STR and MB. Exposure to GMA-MS, induced the mRNA expression of $Tnf\alpha$ (1.5-fold), $Il6$ (1.8-fold) and $Cxcl2$ (Mip2; 2.1-fold) in STR. Similarly, exposure to MMA-HS also induced $Tnf\alpha$ (1.5-fold), $Il6$ (2.2-fold) and $Cxcl2$ (2.1-fold) in STR. GMA-MS, but not MMA-HS, also induced the expression of $Tnf\alpha$ (1.8-fold) in the midbrain.

Collectively, these observations suggest that exposure to manganese-containing welding fumes could potentially cause dopaminergic neurotoxicity.

To further elucidate the molecular mechanisms, we investigated the association of PD-linked (Park) genes and mitochondrial function in causing dopaminergic abnormality. Repeated instillations of the two fumes at doses that mimic ~1 to 5 years of worker exposure resulted in selective brain accumulation of Mn. This caused impairment of mitochondrial function and loss of tyrosine hydroxylase (TH) protein, indicative of dopaminergic injury. A fascinating finding was the altered expression of Parkin (Park2), Uchl1 (Park5) and Dj1 (Park7) proteins in dopaminergic brain areas. A similar regimen of manganese chloride (MnCl₂) also caused extensive loss of striatal TH, mitochondrial electron transport components and Park proteins. As mutations in PARK genes have been linked to early-onset PD in humans, and because welding is implicated as a risk factor for Parkinsonism, PARK genes may play a critical role in WF-mediated dopaminergic dysfunction. Whether these molecular alterations culminate in neurobehavioral and neuropathological deficits reminiscent of PD remains to be ascertained.

Key Research Accomplishments

- A welding fume generation and inhalation exposure system was developed to expose laboratory animals.
- The generated welding fume was comparable to fume generated in the workplace in terms of particle size, morphology, and metal composition.
- Investigated the neurotoxicological potential following pulmonary exposure to diverse welding fumes. Specifically, investigated the potential of welding fumes to cause dopaminergic neurotoxicity.
- Determined the regional metal distribution in the brain following pulmonary exposure to welding fumes of varying manganese composition.
- Demonstrated the accumulation of manganese from welding fumes in target dopaminergic brain areas.
- Demonstrated that pulmonary exposure to manganese-containing welding fumes caused loss of tyrosine hydroxylase protein, a marker of dopaminergic neurons.
- Demonstrated that short-term inhalation exposure to manganese-containing welding fume elicited neuroinflammation and gliosis in specific brain areas, including dopaminergic targets.
- Demonstrated that acute inhalation exposure to manganese-containing welding fume alters the expression of divalent metal transporters in distinct brain areas.

- Demonstrated altered expression of Parkin (Park2), Uchl1 (Park5) and Dj1 (Park7) proteins in dopaminergic brain areas after pulmonary exposure to manganese-containing welding fumes.

Reportable Outcomes

1. Manuscripts

-Antonini JM, Afshari AA, Stone S, Chen B, Schwegler-Berry D, Fletcher WG, Goldsmith WT, Vandestouwe KH, McKinney W, Castranova V, and Frazer DG. Design, Construction, and Characterization of a Novel Robotic Welding Fume Generation and Inhalation Exposure System for Laboratory Animals. *J Occup Environ Hyg* 3:194-203, 2006.

-Antonini JM, Santamaria A, Jenkins NT, Albin E, and Lucchini R. Fate of manganese associated with the inhalation of welding fumes: Potential neurological effects. *Neurotoxicol* 27:304-310, 2006.

-Antonini JM, O'Callaghan JP, Miller DB. Development of an animal model to study the potential neurotoxic effects associated with welding fume inhalation. *Neurotoxicol* 27:745-751, 2006.

-Antonini JM, Sriram K, Benkovic SA, Roberts JR, Stone S, Chen BT, Schwegler-Berry D, Jefferson AM, Billig BK, Felton CM, Hammer MA, Ma F, Frazer DG, O'Callaghan JP, and Miller DB. Mild steel welding fume causes manganese accumulation and subtle neuroinflammatory changes but not overt neuronal damage in discrete brain regions of rats after short-term inhalation exposure; *Neurotoxicol* 30:915-925, 2009.

-Sriram K, Lin, GX, Jefferson AM, Roberts JR, Chapman RS, Soukup JM, Ghio AJ, Chen BT, and Antonini JM. Dopaminergic neurotoxicity following pulmonary exposure to manganese-containing welding fumes. *Arch Toxicol* 84:521-540, 2010.

-Antonini JM, Roberts JR, Chapman R, Soukup JM, Ghio AJ, and Sriram K. Pulmonary toxicity and extrapulmonary tissue distribution of metals after repeated exposure to different welding fumes. *Inhal Toxicol* 22:805-816, 2010.

-Sriram K, Lin GX, Jefferson AM, Roberts JR, Wirth O, Hayashi Y, Krajnak KM, Soukup JM, Ghio AJ, Reynolds SH, Castranova V, Munson AE, and Antonini JM. Mitochondrial dysfunction and loss of Parkinson's disease-linked proteins contribute to neurotoxicity of manganese-containing welding fumes. *FASEB J* 24:4989-5002, 2010.

2. Abstracts

-Antonini JM, Miller DB, and O'Callaghan JP. Characterization of welding fumes and their neurotoxic effects. 22nd International Neurotoxicology Conference: Manganese Symposium, Research Triangle Park, NC, September 2005.

-Antonini JM, O'Callaghan JP, and Miller DB. Characterization of welding fumes and their potential neurotoxic effects. International Workshop: Neurotoxic Metals- Lead, Mercury, and Manganese, From Research to Prevention. Brescia, Italy, June 2006.

-Antonini JM, Roberts JR, Benkovic SA, Sriram K, O'Callaghan JP, and Miller DB. Potential neurotoxic responses in rats after pulmonary administration of welding fume with varying concentrations of manganese. 23rd International Neurotoxicology Conference: Health Effects of Manganese Exposure- Human and Animals Models, Little Rock, AR, September 2006.

-Antonini JM, Roberts JR, Sriram K, Benkovic SA, O'Callaghan JP, and Miller DB. Extrapulmonary tissue distribution of metals following repeated lung exposures to welding fumes with different elemental profiles. Society of Toxicology Annual Meeting, Seattle, WA, March 2008.

-Antonini JM, Stone S, Roberts JR, Schwegler-Berry D, Moseley A, Donlin M, Cumpston J, Afshari A, and Frazer DG. Pulmonary effects and tissue distribution of metals after inhalation of mild steel welding fume. American Thoracic Society International Conference, Toronto, Ontario, May 2008.

-Antonini JM, Schwegler-Berry D, Stone S, Chen TB, Zeidler-Erdely PC, Frazer DG, and Roberts JR. Comparison of the persistence of deposited particles and the inflammatory potential of stainless steel versus mild steel welding fume in rat lungs after inhalation. Society of Toxicology Annual Meeting, Baltimore, MD, March 2009.

-Sriram K, Lin GX, Jefferson AM, Roberts JR, Stone S, Chen TB, Frazer DG, Soukup JM, Ghio AJ, and Antonini JM. Dopaminergic neurotoxicity following exposure to manganese-containing welding fumes. Society for Neuroscience Annual Meeting, Chicago, IL, October 2009. Program No. 154.26.

-Sriram K, Lin GX, Jefferson AM, Wirth O, Hayashi Y, Roberts JR, Chapman RS, Krajnak KM, and Antonini JM. Welding fume-related dopaminergic neurotoxicity. XVIII WFN World Congress on Parkinson's Disease and Related Disorders, Miami, FL, December 2009. Parkinsonism & Related Disorders 15 (2):S162, 2009.

-Antonini JM and Chen LC. Neurological responses after exposure to inhaled metal particles. Society of Toxicology Annual Meeting, Salt Lake City, UT, March 2010. Toxicol Sci: The Toxicologist 114:4, 2010.

Conclusions

An animal model was developed that assessed the potential neurological responses associated with welding fumes that contained differing levels of manganese. Two methods of treatment were used to expose the laboratory animals to welding fumes: intratracheal instillation and inhalation. Intratracheal instillation is a method by which welding particles are collected onto filters during generation and directly instilled into the lungs of animals via the trachea after suspension in aqueous solution. It is simple, cheap, and large number of animals and treatment groups can be treated at one time. Also, the welding particles are directly administered to the distal alveolar regions of the lungs bypassing upper airway deposition (e.g., nasal/olfactory). Thus, translocation of metals after exposure from the respiratory system would be known to originate from the alveolar regions to the circulation and would not result from olfactory uptake. The advantages of inhalation exposure are the procedure is more physiological, deposition of the particles is more evenly distributed in the lungs, and the upper airways are involved, allowing assessment of possible olfactory transport of metal particles to brain areas. Unfortunately, inhalation exposure can be technically challenging and be quite expensive.

Our research group at NIOSH has developed an automated robotic welder to expose laboratory animals. The fume generated by our generator has been observed to be comparable to welding fume collected in the workplace. For this study, short-term inhalation exposures to gas metal arc-mild steel welding fume, the most common in U.S. industries, were performed. Important findings from the short-term exposures indicate that manganese can translocate from the respiratory tract to other organ systems. Importantly, manganese was observed to deposit in the olfactory bulb. Due to the significant number of nanometer-sized particles ($<0.1\ \mu\text{m}$), it is possible that intact particles are being transported along olfactory nerve processes to the brain regions, bypassing the blood brain barrier. There was no evidence of observable changes in neuronal cell injury as assessed by histopathology. However, subtle changes in cell markers of neuroinflammation and gliosis were observed. The neurofunctional significance of these findings currently are being investigated in longer welding fume inhalation exposure studies.

Similar observations were made after exposing animals by the intratracheal instillation method with fumes containing differing levels of manganese. Manganese was found to translocate from the lungs via the circulation to other organs, in particular, dopaminergic brain areas. Consistent with the observed accumulation of manganese in specific brain regions, intratracheal instillation of welding fumes with varying levels of manganese were observed to induce subtle increases in metal transporter expression and neuroinflammatory responses in the olfactory bulb, striatum, and midbrain. These observations suggest that exposure to manganese-containing welding fumes could potentially cause dopaminergic neurotoxicity. In addition, altered expression of Parkin (Park2), Uchl1 (Park5) and Dj1 (Park7) proteins in dopaminergic brain areas was observed. As mutations in PARK genes have been linked to early-onset PD in humans, and because welding is implicated as a risk factor for Parkinsonism, PARK genes may play a critical role in WF-mediated dopaminergic dysfunction. Whether these molecular alterations culminate in neurobehavioral and neuropathological deficits reminiscent of PD remains to be ascertained.

Current faculty receiving support from the grant:

No investigator on study received salary support.

References

Not applicable

AWARD NUMBER: W81XWH-05-1-0239

TITLE: Effects of manganese on glial-neuronal interactions

PRINCIPAL INVESTIGATOR: Lucio G. Costa, PhD

CONTRACTING ORGANIZATION: University of Washington, DEOHS, Seattle, WA 98105

REPORT DATE: January 2011

TYPE OF REPORT: Final report

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: (Check one)

- ☒ Approved for public release; distribution unlimited
- ☐ Distribution limited to U.S. Government agencies only;
report contains proprietary information

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

REPORT DOCUMENTATION PAGE				Form Approved OMB No. 0704-0188	
Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Department of Defense, Washington Headquarters Services, Directorate for Information Operations and Reports (0704-0188), 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302. Respondents should be aware that notwithstanding any other provision of law, no person shall be subject to any penalty for failing to comply with a collection of information if it does not display a currently valid OMB control number. PLEASE DO NOT RETURN YOUR FORM TO THE ABOVE ADDRESS.					
1. REPORT DATE (DD-MM-YYYY) 01-02-2011		2. REPORT TYPE Final Report		3. DATES COVERED (From - To) 4/15/2008-1/31/2011	
4. TITLE AND SUBTITLE Effects of manganese on glial-neuronal interactions				5a. CONTRACT NUMBER W81XWH-05-1-0239	
				5b. GRANT NUMBER	
				5c. PROGRAM ELEMENT NUMBER	
6. AUTHOR(S) Costa, Lucio G., PhD				5d. PROJECT NUMBER	
				5e. TASK NUMBER	
				5f. WORK UNIT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) University of Washington, DEOHS, Seattle, WA 98105				8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) University of Washington, DEOHS, Seattle, WA 98105				10. SPONSOR/MONITOR'S ACRONYM(S) UW	
				11. SPONSOR/MONITOR'S REPORT NUMBER(S)	
12. DISTRIBUTION / AVAILABILITY STATEMENT					
13. SUPPLEMENTARY NOTES Not applicable					
14. ABSTRACT Manganese (Mn) is a known neurotoxicant and developmental neurotoxicant. As Mn has been shown to accumulate in astrocytes, we sought to investigate whether Mn would alter astrocyte-neuronal interactions, specifically the ability of astrocytes to promote differentiation of neurons. We found that exposure of rat cortical astrocytes to Mn (50-500 uM) impairs their ability to promote axonal and neurite outgrowth in hippocampal neurons. This effect of Mn appears to be mediated by oxidative stress, as it is reversed by antioxidants and potentiated by glutathione depletion in astrocytes. As the extracellular matrix protein fibronectin plays an important role in astrocyte-mediated neuronal neurite outgrowth, we also investigated the effect of Mn on fibronectin. Mn caused a concentration-dependent decrease of fibronectin protein and mRNA in astrocytes, and these effects were also antagonized by antioxidants. Other oxidative stress-induced agents caused similar effects. These results indicate that Mn affects the ability of astrocytes to promote neuronal differentiation by a mechanism which is likely to involve oxidative stress.					
15. SUBJECT TERMS Manganese, neurotoxicity, glia-neuron interaction, oxidative stress, neurite outgrowth					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON Lucio G. Costa, PhD
a. REPORT	b. ABSTRACT	c. THIS PAGE			19b. TELEPHONE NUMBER (include area code) (206) 543-2831

Table of Contents

Cover.....	1
SF 298.....	2
Table of Contents.....	3
Introduction.....	4
Body.....	4
Key Research Accomplishments.....	9
Reportable Outcomes.....	9
References.....	10

Introduction

Manganese (Mn) is an essential metal, necessary for normal functioning of a variety of physiological processes in several tissues and organ systems, and an important cofactor for a number of enzymes such as glutamine synthetase or superoxide dismutase. Elevated exposure to Mn can lead to its accumulation in the brain and cause significant neurotoxicity. Concentrations of Mn as high as 200-300 μ M can be found in brain. Among brain cells, astrocytes, which have high capacity transporter for Mn, accumulate this metal; concentrations of Mn 50-60-fold higher than in neurons can be indeed found in astrocytes. The exact mechanism(s) of Mn neurotoxicity are not known, but there is evidence that Mn can elicit oxidative stress, cause mitochondrial dysfunction, alter the homeostasis of glutamate, cause astrocytic swelling and alter the expression of a number of genes involved in cell cycle regulation, signal transduction and inflammation.

There is emerging and convincing evidence that astrocytes play an essential role in fostering the development and survival of neurons. Indeed, astrocytes express and release a variety of factors, including neurotrophins, cytokines, growth factors, extracellular matrix proteins, proteoglycans and cholesterol, that have profound effects on neuronal proliferation, differentiation and survival of neurons, on neurite outgrowth and on synaptogenesis. By targeting astrocytes, neurotoxic compounds may thus indirectly affect neurons, by inhibiting several aspects of astrocyte-neuron interactions that are vital for the “well-being” of neuronal cells.

The general hypothesis of this proposal was that Mn which, as said, preferentially accumulates in astrocytes, would impair the ability of these cells to promote differentiation of neurons.

Body

Mn inhibits the ability of astrocytes to promote neuritogenesis in hippocampal neurons. When rat cortical astrocytes and rat hippocampal neurons were co-incubated for 48 h, astrocytes promoted the differentiation of neurons, which elongate axon and neurites (see Fig. 1A and quantification in Table 1). When astrocytes were incubated for 24 h with different concentrations of MnCl_2 (50, 100, 200, 500 μ M), followed by treatment wash-out before astrocytes and neurons were placed in co-culture, the ability of astrocytes to promote neurite outgrowth was significantly impaired. Fig. 1B-E shows the effect of MnCl_2 -treated astrocytes on neurons, and the results of quantitative morphometric analysis are shown in Table 1. At the concentration of 100 μ M and above, MnCl_2 decreased the average axon length and the average neurite length, and the number of neurites per cell. These concentrations of MnCl_2 did not affect the viability of astrocytes, as cytotoxicity (assessed by the MTT assay), was evident only at a MnCl_2 concentration of 500-1000 μ M (not shown). Furthermore, viability of neurons (also assessed by the MTT assay) following a 48 h co-incubation with MnCl_2 -treated astrocytes, was also not affected (not shown).

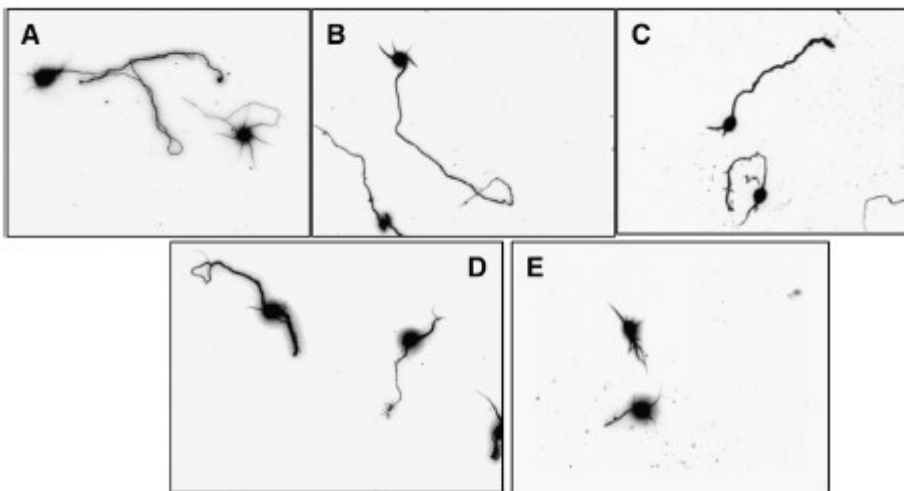


Fig. 1. Morphometric analysis of hippocampal neurons co-cultured with cortical astrocytes. Hippocampal neurons were plated in glass coverslips and inverted on top of treated or untreated cortical astrocytes for 48 h. Neurons were then immunostained with a neuronspecific-tubulin antibody. Pictures were taken with a fluorescence microscope, and neuronal extensions were measured by the MetaMorph software. The figure shows representative fields of neurons incubated in

the presence of cortical astrocytes previously treated with different concentrations of manganese: 0 uM (control) (A), 50 uM (B), 100 uM (C), 200 uM (D) or 500 uM (E).

Table 1. Quantitative morphometric analysis of the effect of manganese-treated astrocytes on rat hippocampal neurons

Treatment	Average Axon Length	Average Neurite Length	No. of Neurites/Cell
Control	154.7 ± 8.1	18.5 ± 2.1	8.3 ± 0.7
MnCl ₂ 50 uM	153.0 ± 10.0	18.0 ± 4.6	7.7 ± 0.6
MnCl ₂ 100 uM	115.2 ± 11.6**	15.3 ± 2.0**	7.3 ± 0.9
MnCl ₂ 200 uM	91.2 ± 6.2***	11.8 ± 2.0***	6.4 ± 1.1*
MnCl ₂ 500 uM	59.3 ± 3.8***	6.9 ± 1.5***	4.6 ± 0.5**

Rat astrocytes were incubated in the presence or absence of different concentrations of MnCl₂ for 24 hr. Cells were washed out and incubated with freshly prepared rat hippocampal neurons for 48 hr. Length of axon and neurites is expressed in μ m. Results represent the mean ($\pm$ SD) of three separate experiments. *Significantly different from control, $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

The effects of Mn in astrocytes are due to oxidative stress. When astrocytes were exposed to MnCl₂ (200 uM) in the presence of the antioxidants melatonin (200 uM) or N-t-butyl-alpha-phenylnitron (PBN; 100 uM), and then co-cultured with hippocampal neurons, the effect of Mn was totally antagonized (Table 2). A similar result was obtained when astrocyte glutathione (GSH) levels were increased by treatment with GSH ethyl ester (GSHee, 2.5 mM, for 3 h) (Table 2). This treatment increased intracellular GSH levels from 17.4 ± 0.9 to 27.5 ± 0.9 nmol/mg protein ($n=3$, $p < 0.05$). In contrast, depletion of astrocytic GSH with buthionine sulfoxime (BSO, 25 uM for 24 h, which decreased GSH levels from 17.4 ± 0.9 to 5.3 ± 0.3 nmol/mg protein; $n=3$, $p < 0.05$), potentiated the effect of Mn (Table 3). These findings suggest that Mn-induced oxidative stress may be involved in its ability to impair the neuritogenic action of astrocytes. Mn had been previously shown to cause oxidative stress in astrocytes, and this was confirmed in the present study (not shown).

Table 2. Antioxidants prevent manganese-induced inhibition of astrocyte-promoted neuritogenesis of hippocampal neurons

Treatment	Average Axon Length	Average neurite Length	Number of Neurites/Cell
Control	158.2 ± 13.3	15.2 ± 2.3	8.0 ± 1.2
MnCl ₂ 200 uM	92.4 ± 13.5**	9.3 ± 1.4*	5.3 ± 0.9*
Melatonin 200 uM	156.5 ± 7.6	17.7 ± 3.3	8.3 ± 0.7
MnCl ₂ + melatonin	147.6 ± 8.4	14.3 ± 2.3	7.8 ± 0.7
PBN 100uM	169.9 ± 11.7	15.0 ± 1.7	8.6 ± 1.3
MnCl ₂ + PBN	139.7 ± 16.8	13.9 ± 3.1	7.0 ± 1.1
GSHee 2.5mM	167.8 ± 6.8	17.9 ± 1.1	8.6 ± 1.5
MnCl ₂ + GSHee	154.2 ± 10.7	14.5 ± 1.5	8.5 ± 1.6

Astrocytes were incubated in the presence of manganese alone or pre-incubated for 3 hr with different antioxidants: melatonin, GSH ethylester (GSHee), or N-t-butyl-alpha-phenylnitron (PBN). After 24 hr, astrocytes were washed out and incubated with freshly prepared rat hippocampal neurons for 48 h. Length of axon and neurites is expressed in μ m. Results represent the mean ($\pm$ SD) of three separate experiments. *Significantly different from control (untreated astrocytes), $p < 0.05$; ** $p < 0.01$.

Table 3. Glutathione depletion potentiates manganese-induced inhibition of astrocyte-promoted neuritogenesis of hippocampal neurons

Treatment	Average Axon Length	Average neurite Length	Number of Neurites/Cell
Control	161.4 ± 14.6	14.3 ± 1.8	9.1 ± 1.5
MnCl ₂ 200 uM	104.9 ± 22.1*	8.7 ± 1.4*	6.2 ± 1.5*
BSO 25 uM	158.2 ± 4.5	14.6 ± 2.2	9.6 ± 1.4
MnCl ₂ + BSO	57.2 ± 9.9**	4.0 ± 1.0**	3.2 ± 0.7*#

Astrocytes were pre-incubated for 24 hr with the GSH synthase inhibitor buthionine sulfoximine (BSO). After an additional 24 hr of manganese treatment, astrocytes were washed out and incubated with freshly prepared rat hippocampal neurons for 48 hr. Length of axon and neurites is expressed in μ m. Results represent the mean ($\pm$ SD) of three separate experiments. *Significantly different from control, $p < 0.05$; #Significantly different from MnCl₂-treated astrocytes, $p < 0.05$.

Mn decreases the expression of fibronectin in astrocytes. The ability of astrocytes to induce neuronal differentiation is most likely mediated by the release of neurite-promoting molecules. A proteomics analysis of astrocyte secretome identified 160 proteins that can be characterized as extracellular, a number of which are involved in neurite outgrowth (Moore et al. 2009). We focused on an extracellular matrix glycoprotein, fibronectin, because of its reported permissive role in neurite outgrowth (Guizzetti et al. 2008). Fibronectin was identified in astrocytes and in the astrocyte medium. Furthermore, when astrocytes were incubated with an activity-inhibiting fibronectin antibody during their co-incubation with neurons, the ability of astrocytes to promote neuritogenesis in hippocampal neurons was inhibited. Fig. 2 shows that $MnCl_2$ decreased the expression of fibronectin protein in astrocytes lysate (Fig. 2 A,B) and in astrocyte medium (Fig. 2D). Confocal analysis of astrocytes confirmed that $MnCl_2$ (200 μM) decreased fibronectin levels in astrocytes (Fig. 2C). Furthermore, $MnCl_2$ also decreased fibronectin mRNA levels (Fig. 2E).

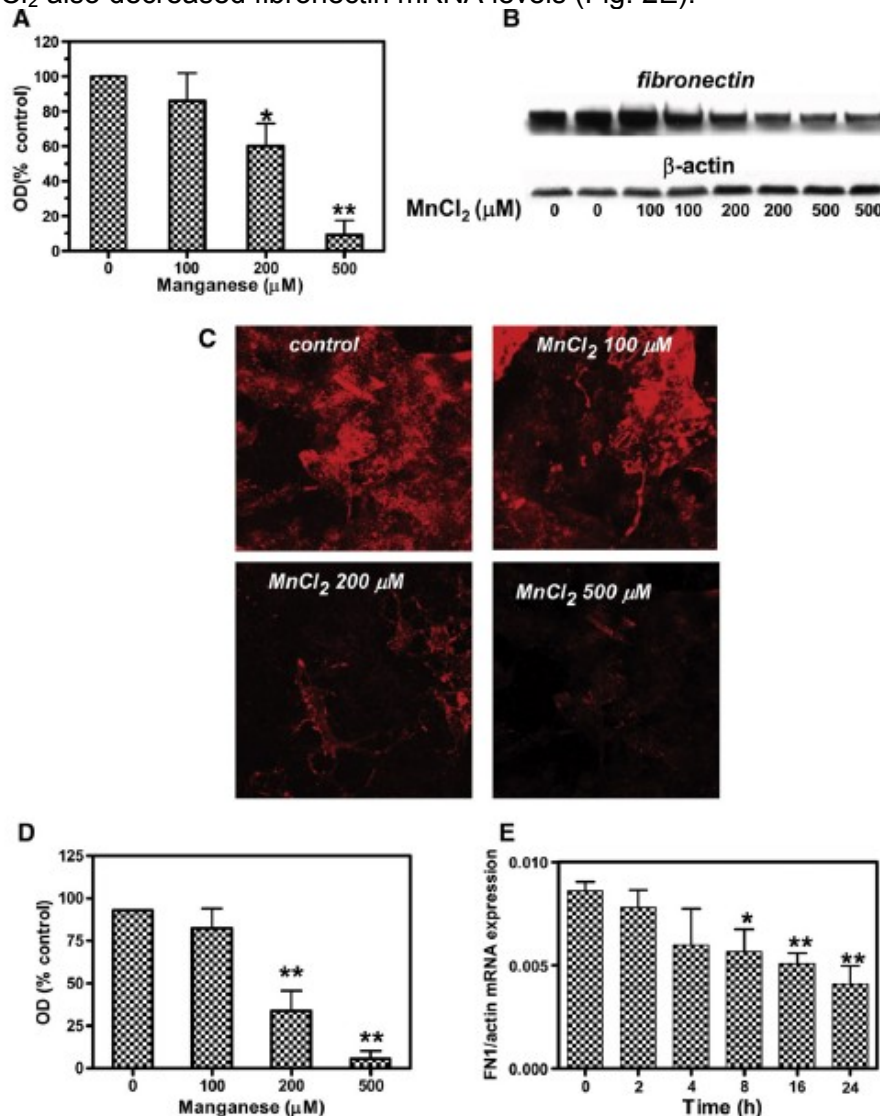


Fig. 2. Effect of manganese on fibronectin expression in rat cortical astrocytes.

(A,B) Rat astrocytes were incubated with different concentrations of manganese for 24 h. Cells were then washed out and collected for Western blot analysis in the lysate. Beta actin was used as loading control. In (A) quantification of Western blot shown in (B). In (C) astrocytes were fixed and stained with a fibronectin antibody and the nuclear dye Hoechst 33342 and analyzed by confocal microscopy. In (D) the medium was collected and processed as described in Methods. Glyceral and comassie staining were used as loading controls. (E) Astrocytes were exposed to $MnCl_2$ (200 μM) for the indicated times, and fibronectin mRNA levels were determined by RT PCR as described in Methods. Results are expressed as mean $\pm$ SD of at least three different experiments performed in duplicate. * $p < 0.05$; ** $p < 0.01$ vs control.

The effect of Mn on fibronectin are mediated by oxidative stress. Two antioxidants (melatonin, 200 μ M) and PBN (100 μ M) inhibited the decrease of fibronectin levels in astrocytes and their medium and the decrease in fibronectin mRNA levels caused by $MnCl_2$ (Fig. 3). Similarly, increasing astrocyte GSH levels by treatment with GSHee (2.5 mM, 3 h), antagonized the effect of $MnCl_2$ on fibronectin levels in cell lysate and in medium, and on fibronectin mRNA levels (not shown). In contrast, upon depletion of GSH with BSO (25 μ M, 24 h), the effect of $MnCl_2$ on fibronectin levels in astrocyte lysate and medium was increased (not shown). These findings suggest that Mn-induced oxidative stress may mediate its inhibitory effect on fibronectin levels.

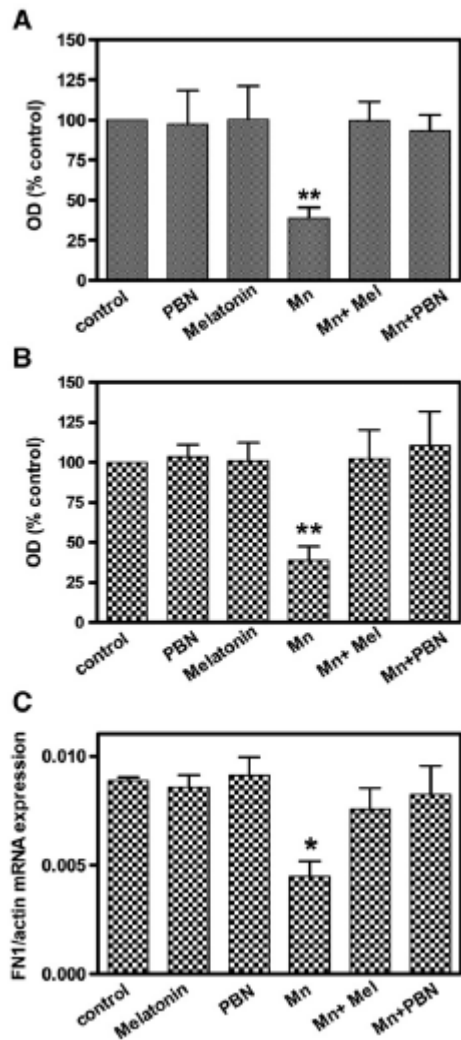


Fig. 3. Antioxidants inhibit the effect of manganese on fibronectin in astrocytes. Astrocytes were incubated for 24 hr with manganese (200 μ M) alone, or after a 3 h pre-incubation with melatonin (200 μ M) or PBN (100 μ M) and fibronectin levels were assessed by Western blot in cell lysate (A) or in medium (B). (C) Effect of Mn and antioxidants on fibronectin mRNA levels, assessed at 24 h by RT PCR. Data represent the mean ($\pm$ SD) of three separate experiments done in duplicate. * $p < 0.05$ vs control. ** $p < 0.001$ vs control.

Oxidative stressors inhibit the ability of astrocytes to promote neuritogenesis in hippocampal neurons by decreasing fibronectin levels. To further probe the hypothesis that oxidative stress may underlie the observed effects of Mn, astrocytes were exposed for 24 h to hydrogen peroxide (H_2O_2) or 2,3-dimethoxy-1,4-naphthoquinone (DMNQ) before wash-out, and an additional 48 h co-culture with hippocampal neurons. As shown in Table 4, both compounds significantly impaired the ability of astrocytes to promote neurite outgrowth in hippocampal neurons at concentrations which did not cause any cytotoxicity (not shown). Furthermore, both H_2O_2 and DMNQ caused a significant decrease of fibronectin levels in astrocytes lysate and their medium (Fig. 4).

Table 4. Quantitative morphometric analysis of the effect of oxidant-treated astrocytes on rat hippocampal neurons

Treatment	Average Axon Length	Average Neurite Length	No. of Neurites/Cell
Control	165.2 ± 24.0	11.0 ± 2.3	7.0 ± 1.4
MnCl ₂ 200 uM	91.0 ± 10.0**	6.1 ± 1.5*	4.1 ± 1.1*
H ₂ O ₂ 5 uM	119.1 ± 16.0*	8.2 ± 1.0	5.4 ± 1.2
H ₂ O ₂ 10 uM	81.8 ± 10.0**	5.4 ± 1.1*	3.6 ± 1.0*
DMNQ 2.5 uM	147.6 ± 11.0	10.4 ± 2.0	7.8 ± 0.7
DMNQ 10 uM	112.3 ± 6.2*	7.1 ± 0.9	4.7 ± 0.5

Rat astrocytes were incubated in the presence or absence of MnCl₂, H₂O₂ or DMNQ at the indicated concentrations for 24 h. Cells were washed out and incubated with freshly prepared rat hippocampal neurons for 48 hr. Length of axon and neurites is expressed in μ m. Results represent the mean ($\pm$ SD) of three separate experiments. *Significantly different from control, $p < 0.05$; ** $p < 0.01$.

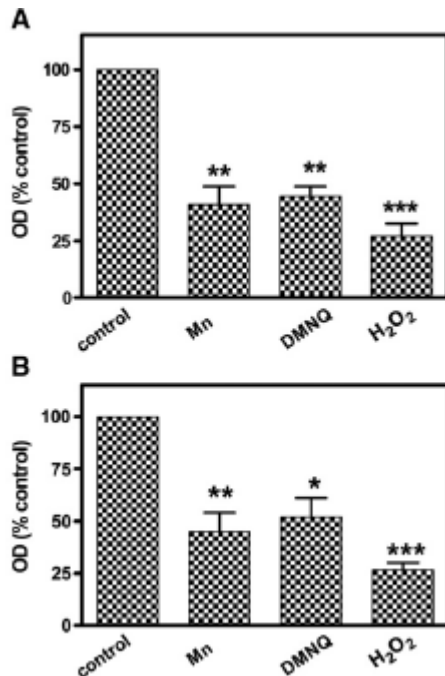


Fig. 4. Oxidative stressors decrease fibronectin levels in astrocytes. Astrocytes were incubated for 24 hr with manganese (200 μ M), H₂O₂ (10 μ M) or DMNQ (10 μ M), and fibronectin levels were assessed by immunoblot in cell lysate (A) or in medium (B). Results represent the mean ($\pm$ SD) of three separate experiments done in duplicate. * $p < 0.05$; ** $p < 0.01$ vs control ; *** $p < 0.001$ vs control.

Direct exposure of hippocampal neurons to manganese does not alter neurite outgrowth. Inhibition of neurite outgrowth by Mn may have resulted from a direct effect on neurons caused by residual Mn after astrocyte wash-out, or by Mn leaking from astrocytes during their 48 h incubation with hippocampal neurons. To address this possibility, hippocampal neurons were cultured for 24 h in the absence of astrocytes, at which time MnCl₂ (1, 10 or 100 μ M) was added for an additional 48 h. Table 5 shows that Mn did not affect axon length, average neurite length and number of extensions/cell. At the highest concentration tested (100 μ M), Mn actually increased axon length, confirming a previous observation in PC12 cells. This concentration of Mn also caused minimal but significant cytotoxicity (Table 5).

Table 5. Quantitative morphometric analysis of the direct effect of manganese on rat hippocampal neurons

Treatment	Average Axon Length	Average Neurite Length	No. of Neurites/Cell	Cytotoxicity
Control	107.8 ± 12.5	20.8 ± 2.3	3.4 ± 0.1	100 ± 5
MnCl ₂ 1 μ M	131.0 ± 16.3	20.0 ± 3.1	3.2 ± 0.1	95 ± 6
MnCl ₂ 10 μ M	130.3 ± 12.5	18.9 ± 1.8	3.2 ± 0.1	92 ± 2
MnCl ₂ 100 μ M	141.2 ± 13.7*	19.3 ± 1.6	3.4 ± 0.1	86 ± 5*

Hippocampal neurons were plated in glass coverslips, cultured for 24 h, then treated with different concentrations of MnCl₂ for an additional 48 h. At the end of the treatment neurons were immunostained with a neuron specific-tubulin antibody and pictures were taken with a fluorescence microscope. Length of axon and neurites is expressed in μ m. Cytotoxicity was assessed by the MTT assay and is expressed as percent of untreated neurons. Data represent the mean ($\pm$ SD) of three separate experiments done in duplicate. *p<0.05 vs control.

Conclusion:

Glial-neuronal interactions are increasingly being recognized as playing a primary role in normal brain function and development. Our results show that exposure of astrocytes to Mn impairs their ability to promote differentiation of hippocampal neurons. Astrocytes are known to act as a “sink” for Mn. At concentrations that do not alter astrocyte viability, Mn affects their ability to promote neurite outgrowth in hippocampal neurons. This effect of Mn in astrocytes is most likely mediated by its ability to induce oxidative stress in these cells, and involves an effect of Mn on fibronectin, an extracellular matrix protein which has a neurite-promoting action. Other compounds causing oxidative stress also caused the same astrocyte-mediated impairment of neuritogenesis and of fibronectin expression. The effect of Mn was due solely to its action on astrocytes as direct exposure of hippocampal neurons to Mn did not affect neuritogenesis. These results show that by targeting astrocytes, Mn can alter an important aspect of glial-neuronal interactions, contributing to its overall neurotoxicity and developmental neurotoxicity.

Key Research Accomplishments

- Exposure of rat cortical astrocytes to Mn, followed by wash-out, decreased their ability to promote neurite outgrowth in hippocampal neurons.
- This effect of Mn was observed at concentrations that did not alter the viability of astrocytes and neurons.
- Anti-oxidants reversed the effect of Mn, while GSH depletion potentiated its effect, suggesting an involvement of Mn-induced oxidative stress in astrocytes.
- Mn caused a decrease in the levels of fibronectin protein and mRNA, which was also antagonized by antioxidants.
- Other oxidative stress-inducing compounds caused effects similar to Mn
- Results indicate that by targeting astrocytes, Mn impairs their ability to promote neuronal differentiation.

Reportable Outcomes

Manuscripts Supported by this project

- Giordano G, Pizzurro D, VanDeMark K, Guizzetti M, Costa LG. Manganese inhibits the ability of astrocytes to promote neuronal differentiation. *Toxicol. Appl. Pharmacol.* 240: 226-235, 2009.

Book Chapters Supported by this project

Not applicable

Abstracts Supported by this project

- Costa LG, Pizzurro D, Dao K, Guizzetti M, Giordano G. Manganese impairs the ability of astrocytes to promote neurite outgrowth in rat hippocampal primary neurons. Society of Toxicology Annual Meeting, Baltimore, MD, March 2009 (*Toxicologist* 108: 40, 2009).

- Pizzurro D, Giordano G, Guizzetti M, Costa LG. Manganese impairs fibronectin and plasminogen activator inhibitor-1 (PAI-1) expression in astrocytes. Pacific Northwest Association of Toxicologists (PANWAT) Annual Meeting, Seattle, WA, September 2009.

Current faculty receiving support from the grant:

- Lucio G. Costa, PhD

References

Guizzetti M, Moore NH, Giordano G, Costa LG. Modulation of neuritogenesis by astrocyte muscarinic receptors. J. Biol. Chem. 283: 31884-31897, 2008.

Moore NH, Costa LG, Shaffer SA, Goodlett DR, Guizzetti M. Shotgun proteomics implicates extracellular matrix proteins and protease systems in neuronal development induced by astrocyte cholinergic stimulation. J. Neurochem. 108: 891-208, 2009.

AD _____

(Leave blank)

AWARD NUMBER: W81XWH-05-1-0239, HRPO A-12931.10

TITLE: A Study of the Nervous System in Welders

PRINCIPAL INVESTIGATOR: Dag G Ellingsen, MD, PhD

CONTRACTING ORGANIZATION: National Institute of Occupational Health
Gydas vei 8
P.O. Box 8149 Dep
N-0033 Oslo, Norway

REPORT DATE: January 2011

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Material Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT:

- ☒ Approved for public release; distribution unlimited
- ☐ Distribution limited to U.S. Government agencies only;
report contains proprietary information

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

REPORT DOCUMENTATION PAGE				<i>Form Approved</i> <i>OMB No. 0704-0188</i>	
Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Department of Defense, Washington Headquarters Services, Directorate for Information Operations and Reports (0704-0188), 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302. Respondents should be aware that notwithstanding any other provision of law, no person shall be subject to any penalty for failing to comply with a collection of information if it does not display a currently valid OMB control number. PLEASE DO NOT RETURN YOUR FORM TO THE ABOVE ADDRESS.					
1. REPORT DATE (DD-MM-YYYY) 25-01-2011		2. REPORT TYPE Annual		3. DATES COVERED (From - To) 01/02/10 – 31/01/11	
4. TITLE AND SUBTITLE A Study of the Nervous System in Welders				5a. CONTRACT NUMBER W81XWH-05-1-0239	
				5b. GRANT NUMBER	
				5c. PROGRAM ELEMENT NUMBER	
6. AUTHOR(S) Dag G Ellingsen, MD, PhD				5d. PROJECT NUMBER	
				5e. TASK NUMBER	
				5f. WORK UNIT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) National Inst. of Occupational Health Gydas vei 8, P.O. Box 8149 Dep N-0033 Oslo, Norway				8. PERFORMING ORGANIZATION REPORT	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) Vanderbilt University Medical Center Nashville, TN 37203, USA				10. SPONSOR/MONITOR'S ACRONYM(S) VUMC	
				11. SPONSOR/MONITOR'S REPORT NUMBER(S)	
12. DISTRIBUTION / AVAILABILITY STATEMENT					
13. SUPPLEMENTARY NOTES Not applicable					
14. ABSTRACT Inhalation of high manganese (Mn) concentrations may result in serious irreversible neurological disease (manganism). The exposure level associated with an increased risk of acquiring subtle neurological disturbances is currently not known. Welding fumes contain Mn. In this study 332 subjects have been examined with neurobehavioral methods, of whom 137 are currently exposed welders and 137 are referents. Among these, 63 welders and 65 referents were examined with the same methods six years earlier. Also 34 patients diagnosed with manganism (17 also examined six years earlier) and 25 with idiopathic Parkinson's disease (PD) were examined. Positron Emission Tomography examinations were carried out in eight PD-patients, eight manganism cases and six referents. Biological samples and personal welding fume samples are currently being analyzed in Norway. Currently the statistical work is being carried out. The laboratory and statistical work is presumably finalized this summer. When finalized the scientific publication process will start.					
15. SUBJECT TERMS Manganese, neurotoxicology, welders, manganism, idiopathic Parkinson's disease					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE Dag G Ellingsen, MD, PhD
a. REPORT	b. ABSTRACT	c. THIS PAGE			19b. TELEPHONE NUMBER (include area code) + 47 23 19 53 77

Table of Contents

	<u>Page</u>
Cover	1
SF 298	2
Table of contents	3
Introduction.....	4
Body.....	4
Key Research Accomplishments.....	6
Reportable Outcomes.....	6
Conclusion.....	6
References.....	7

Introduction

Manganese (Mn) is an essential trace element in man, but inhalation of high Mn concentrations has been associated with irreversible neurological disease (manganism). Welding fumes may contain high amounts of Mn, and cases of manganism among welders are reported every year in Russia. Welders are by number the most important group of workers occupationally exposed to Mn. Exposure to Mn in lower concentrations can result in subtle motor disturbances. The exposure level associated with an increased occurrence of such disturbances is currently not sufficiently known.

Neurobehavioral tests are applied, parameters for iron status are determined and an extensive exposure assessment is carried out in this study. Also PET-scan examinations are carried out in a limited number of subjects. The main objective is to assess the value of selected neurobehavioral tools in an epidemiological study, in order to investigate their sensitivity for detecting subtle neurological functional changes.

Body

A contract was signed between the National Institute of Occupational Health (Norway) and Vanderbilt University (USA) on January 31, 2007, to carry out "A Study of the Nervous System in Welders". In the letter from Vanderbilt University Medical Center to the National Institute of Occupational Health in Norway dated March 8, 2007, the fully executed original of the contract was received, this date representing the start of the project.

After the original contract was received, preparations for examining the participants were started. All necessary sampling equipment for the collection of biological samples and air was purchased and transported to Russia. More than 300 air filters was weighed on a micro-weight in order to prepare them to be mounted into the filter cassettes as a preparation for the exposure assessment. The equipment for the neurobehavioral examinations were shipped to Russia as well. However, due to software problems in the CATSYS test system, it had to be transported back to the producer in Denmark for adjustments. This resulted in a delay in the progress of the study of around about 3 months. Our neuropsychologist was in Russia for the final preparations before starting data collection. She has a supervision role for the testing, and videotapes were made for training and supervision/standardisation purposes of the neurobehavioral testing.

In this investigation 137 welders and 137 age-matched referents have been examined with neurobehavioral test in a study with cross-sectional design. Further, 25 patients with newly diagnosed idiopathic Parkinson's disease (PD) and 34 patients diagnosed with manganism were examined. The latter group consists of former welders only. Among the welders and referents, 63 and 65 subjects respectively, had been examined with the same neurobehavioral test battery around six years earlier. Thus, this part of the investigation has a prospective study design.

Also 17 of the manganism patients had been examined previously. In 22 subjects, of whom eight had PD, eight had manganism and six were referents, PET scan examinations have also been carried out. Whole shift air samples for the determination of welding fume components were collected among the welders. Samples of whole blood, serum and urine were collected among all participants. These samples have been shipped to Norway for the laboratory analysis. The determination of trace elements in whole blood with an ICP-MS has been completed at the National Institute of Occupational Health in Oslo (Norway).

Table 1 shows some key data of the welders and the referents who were examined in

2002/2003 and re-examined in 2008/2009. Data from the original group that was examined in 2002/2003 have been published (Ellingsen et al., 2006; Ellingsen et al., 2007; Ellingsen et al., 2008). The follow up time was nearly identical in the two groups, being slightly shorter than six years. The referents were older than the welders. This age difference must be accounted for in the statistical analysis. The contrast in B-Mn between these welders and the referents is quite large, the mean concentration difference being 5.6 µg/L. At the first examination, six years previously, the difference in the mean B-Mn concentrations was 1.6 µg/L between the same subjects, suggesting a higher personal exposure to welding fumes at follow up than at baseline six years earlier. Data on Pb, Hg, Co and some other trace elements in whole blood are not shown. The neurobehavioral data are currently treated statistically. Preliminary results suggest that the welders may have a larger decline in the grooved pegboard test than the referents. It may also further be noticed that a few welders had a substantial decrease in their finger tapping test scores, far beyond what was observed among the referents.

Table 1. Background variables recorded in the subjects that were examined in 2002/2003 and followed up in 2008/2009. Data at follow up.

	Welders (N=63)	Referents (N=65)
	Mean[#] (range)	Mean (range)
Age	42.7(26-70)	45.8(22-70)
Months of follow up	70.8(59-90)	70.7(61-80)
Years of welding	19.5(7-45)	-
B-Mn (µg/L)	13.6	8.0 [†]

[#] Arithmetic mean; [†] Eight subjects declined blood sampling

Table 2 shows some key data of all welders (N=137) and referents (N=137) that were examined in the current study. The two groups are nearly identical with respect to age. The level of B-Mn in whole blood is substantially higher in the welders. The statistical analysis of the neurobehavioral data has started. Preliminary results indicate significant group differences with respect to several of the applied neurobehavioral tests. The differences are mainly observed for the grooved pegboard and finger tapping tests, the digit symbol test and the reporting of subjective symptoms. We have also applied the simple reaction time test, where the welders perform significantly poorer than the referents as a whole. However, further statistical analysis need to be carried out before firm conclusions can be drawn, and in particular to assess potential dose-response associations.

Table 2. Background variables among all welders and referents examined in 2008/2009

	Welders (N=137)	Referents (N=137)
	Mean[#] (range)	Mean (range)
Age	39.9(19-70)	40.1(19-70)
Years of welding	16.6(1-45)	-
B-Mn (µg/L)	13.9(5.9-40.3)	8.2(4.1-13.9) [†]

[#] Arithmetic mean; [†] Fourteen subjects declined blood sampling

Altogether 25 subjects diagnosed with PD and 34 subjects (all welders) diagnosed with welding related manganism were examined. One PD patient had in the medical history information about a cerebral ischemic event, and were thus excluded. These subjects underwent the same neurobehavioral test battery as the welders and referents. Again,

preliminary results indicate substantial differences between the two groups. Crudely it can be summarized that the PD patients had more tremor, but also different quality of tremor when compared to the manganism patients. This was mainly a shift in the tremor frequency pattern towards lower frequencies, a more regular (pathological) tremor, and a smaller dispersion in the tremor frequencies. The mangansim patients, on the other hand, had more postural sway. There is apparently more side difference in the clinical manifestations in the PD-patients as compared to the manganism patients with respect to neurobehavioral performance. However, the data analysis has not yet been completed, and the results must be regarded as preliminary.

Eight patients with PD, eight diagnosed with manganism and six referents have been examined with PET-scan. The data treatment has not yet been completed, but preliminary results indicate that the manganism patients predominantly have reduced glucose activity in the head of the nucleus caudatus. This is also the case in the PD patients, but they also have alterations in other brain areas such as thalamus and amygdalae. It could be of some interest that the preliminary data indicate an association between certain neurobehavioral test scores and the reduction of glucose activity in the nucleus caudatus.

Before the statistical work can be completed, the remaining laboratory work needs to be finalized. Currently, the urine samples are being analyzed with ICP-MS for the presence of a range of trace elements, in particular manganese, at the National Institute of Occupational Health in Oslo (Norway). The collected whole shift air samples will also be analyzed at this laboratory together with the element concentrations in the serum of the participants. The welding fume samples are planned to be dissolved in a Hatch solution (artificial lung fluid) in order to get estimates of the amount of soluble welding fume components and less soluble components. In addition, some clinical chemical analysis will be carried out in the serum samples.

Key Research Accomplishments

None so far

Reportable Outcomes

None so far

Conclusion

The enrollment of subjects to the study has been completed. Altogether 332 subjects have been examined with neurobehavioral methods. Whole blood samples have been analyzed with ICP-MS for trace elements in 315 subjects (14 referents and 3 PD patients declined blood sampling). Currently urine samples are being analyzed for trace elements, while the remaining laboratory analysis are planned to be carried out soon. The statistical analysis is currently carried out, and will probably be completed this spring/summer. After that time the scientific publication will commence.

References

Ellingsen DG, Dubeikovskaya L, Dahl K, Chashchin M, Chashchin V, Zibarev E, Thomassen Y. Air exposure assessment and biological monitoring of manganese in welders. *J Environ Monit* 2006;8:1078-1086.

Ellingsen DG, Chashchin V, Haug E, Chashchin M, Tkachenko V, Lubnina N, Bast-Pettersen R, Thomassen Y. An epidemiological study of reproductive function biomarkers in male welders. *Biomarkers* 2007;12:497-509.

Ellingsen DG, Konstantinov R, Bast-Pettersen R, Merkurjeva L, Chashchin M, Thomassen Y, Chashchin V. A neurobehavioral study of current and former welders exposed to manganese. *NeuroToxicology* 2008;29(1):48-59.

AWARD NUMBER: W81XWH-05-1-0239

TITLE: Water-Borne Manganese Exposure and Motor Function in Young Adults

PRINCIPAL INVESTIGATOR: Joseph Graziano, PhD

CONTRACTING ORGANIZATION: Vanderbilt University Medical Center
Nashville, TN 37203

REPORT DATE: February 2011

TYPE OF REPORT: Progress report

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: (Check one)

- ☒ Approved for public release; distribution unlimited
- ☐ Distribution limited to U.S. Government agencies only;
report contains proprietary information

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

REPORT DOCUMENTATION PAGE				Form Approved OMB No. 0704-0188	
Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Department of Defense, Washington Headquarters Services, Directorate for Information Operations and Reports (0704-0188), 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302. Respondents should be aware that notwithstanding any other provision of law, no person shall be subject to any penalty for failing to comply with a collection of information if it does not display a currently valid OMB control number. PLEASE DO NOT RETURN YOUR FORM TO THE ABOVE ADDRESS.					
1. REPORT DATE (DD-MM-YYYY) 31-02-2011		2. REPORT TYPE Progress Report		3. DATES COVERED (From - To) 1/1/2010-12/31/2010	
4. TITLE AND SUBTITLE Water-Borne Manganese Exposure and Motor Function in Young Adults				5a. CONTRACT NUMBER W81XWH-05-1-0239	
				5b. GRANT NUMBER	
				5c. PROGRAM ELEMENT NUMBER	
6. AUTHOR(S) Graziano, Joseph, PhD Wasserman, Gail, PhD Liu, Xinhua, PhD				5d. PROJECT NUMBER	
				5e. TASK NUMBER	
				5f. WORK UNIT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) The Trustees of Columbia University in the City of New York 630 168 th Street, Box -49 New York, NY 10032				8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) Vanderbilt University Medical Center Nashville, TN 37232				10. SPONSOR/MONITOR'S ACRONYM(S) VUMC	
				11. SPONSOR/MONITOR'S REPORT NUMBER(S)	
12. DISTRIBUTION / AVAILABILITY STATEMENT					
13. SUPPLEMENTARY NOTES Not applicable					
14. ABSTRACT The neurotoxicity of Mn in adults with occupational inhalation exposure is well established. The syndrome known as "manganism" is characterized by a Parkinson-like condition with weakness, anorexia, apathy, slowed speech, emotionless facial expression, and slow movement of the limbs. Many issues remain to be determined however, including dose-response relationships, the contribution from non-inhalation sources of Mn exposure, and the impact of nutritional status – particularly iron – on susceptibility to neurologic disease. We propose here to expand an ongoing study in Bangladesh, investigating the consequences of water-borne Mn exposure on motor functioning in young children, 7-9 years of age, to include young adults, 18-21 years of age, i.e., an age group that is representative of young U.S. military personnel. To do this, we will use a well-standardized, individually-administered test of motor function that is normed for children, adolescents and young adults from 4- 21 years of age, i.e., the Bruininks Oseretsky Test, 2 nd Edition. Field work and laboratory analyses for the study have just been completed and statistical analyses will begin shortly.					
15. SUBJECT TERMS Manganese, neurotoxicology, motor function, dose-response, water-borne, nutrition					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON Joseph Graziano, PhD
a. REPORT	b. ABSTRACT	c. THIS PAGE			19b. TELEPHONE NUMBER (include area code) (212) 305-1678

Table of Contents

Cover.....	1
SF 298.....	2
Table of Contents.....	3
Body.....	4
Key Research Accomplishments.....	6
Reportable Outcomes.....	6
References.....	6

Body

The primary aim of this study is to contribute to the knowledge base concerning dose-response relationships between Mn exposure and motor functioning in 18-21 year old men and women, i.e., an age group that is representative of young U.S. military personnel.

Specifically, our stated specific aims were as follows:

1. We will recruit 100 young men and 100 young women, 18-21 years of age, who will be interviewed and evaluated in our existing medical field clinic in Araihaazar, Bangladesh. The study participants will be selected from the Northwestern region of Araihaazar, Bangladesh, where the drinking water is As-free, but where water Mn ranges from 1-3900 ug/L. (The EPA and WHO drinking water guidelines for Mn are 300 and 400 ug/L, respectively.) To maximize statistical power, participants will be recruited such that half consume water < 300 ug/L and half consume water $\geq$ 300 ug/L. A validated dietary survey questionnaire (3) will also provide an estimate of dietary Mn intake.
2. After informed consent is obtained, we will evaluate motor function in each participant, using the Bruininks Oseretsky Test (BOT), 2nd edition (2). At the same time, a structured, validated interview instrument will be employed to gather information on occupational, medical, demographic, exercise and dietary histories. In addition, a blood sample will be obtained for the measurement of Mn, serum ferritin, iron and total iron binding capacity (TIB).

In 2009, progress for this study had been severely restricted because of slow IRB approval by the Bangladesh Medical Research Council (BMRC). However, all necessary IRB, BMRC and DOD approvals were ultimately obtained in 2010 and progress has been extraordinary. We completed the recruitment of study participants on August 31, 2010, by which time we had recruited 182 participants (100 men and 82 women). After the signing of informed consent, the BOT-2 was administered, questionnaires were completed, and blood samples were drawn. All study materials now reside at Columbia University, where analyses are ongoing. The analyses of blood samples is now completed and the results are tabulated in Table 1 below:

Table 1: Summary of blood analyses in study participants:

ASSAY	TOTAL			MALE		FEMALE	
	N	Mean (SD)	Range	N	Mean (SD)	N	Mean (SD)
Serum Fe (ug/dl)	152	141 (39.4)	51-243	84	149 (40.3)	68	130 (35.7)
Serum TIBC (ug/dl)	152	272 (32.8)	194-361	84	276 (35.0)	68	268 (29.4)
Serum Ferritin (ng/ml)	179	49.6 (28.1)	5.6-164.1	97	60.1 (30.0)	82	37.1 (19.4)
Hemoglobin (g/L)	182	12.9 (1.4)	9.6-16.7	100	13.7 (1.0)	82	11.9 (1.0)
Blood Mn (ug/L)	182	14.1 (3.3)	7.0-25.2	100	13.2 (2.6)	82	15.2 (3.7)
Blood Pb (ug/L)	182	103 (45.7)	22-238	100	120 (42.0)	82	82 (41.5)
Blood Se (ug/L)	182	141 (20.0)	87-204	100	145 (18.5)	82	136 (20.9)
Blood As (ug/L)	182	5.9 (4.6)	1.2-28.5	100	6.5 (4.6)	82	5.3 (4.6)

In addition, the water Mn concentrations of all well water samples have been compiled and are presented in Table 2. Household well water Mn concentrations covered a very wide range, from 10 to 7450 ug/L. The distribution of water Mn levels is depicted in Table 3. Roughly one-third of households had water Mn levels less than 300 ug/L, while one-third had levels exceeding 1340 ug/L.

Table 2: Average water Mn of participant's household wells:

Average Water Manganese Concentrations, Overall, and by Gender		
Overall water Mn conc. (µg/L) (range) (N=182)	Male 55 % (100)	Female 45% (82)
1032 (10-8960)	953 (20-8960)	1129 (10-7450)

Table 3: Distribution of Water Mn Concentrations, Overall and by Gender

Levels of Mn. Conc. (µg/L)	Water Mn. Distribution % (N=182)	Male 55 % (100)	Female 45% (82)
10-300	35 (64)	40 (40)	29 (24)
>300-1340	35 (64)	34 (34)	37 (30)
>1340	30 (54)	26 (26)	34 (28)

Results of the BOT-2 motor function tests are currently being compiled; scores are being generated for each of the four sub-tests as well as a Total Score. In addition, a database has been created by our Database Management Core in the department of Biostatistics for the entry of questionnaire data concerning occupational, medical, demographic, exercise and dietary histories. Data entry is about to begin.

Thus, during the coming months we will be able to test our hypothesis that total Mn exposure may be associated with deficits in motor function in young adults. With regard to Mn, a strength of the current study in young adults is that we also have information on dietary sources of Mn, thereby allowing us to estimate total Mn intake.

We note that we have recently completed a separate study of young children, funded by the NIEHS Superfund Research Program, which tested the hypothesis that Mn and/or As might have adverse effects on motor function in children 7-9 years of age. To our surprise, As but not Mn was adversely associated with two of the four sub-tests of the BOT-2, i.e., fine motor control and body coordination. The As finding is robust and highly significant, and a dose-dependent relationship is very apparent regardless of which marker of exposure is included in the regression models, i.e., blood As, urine As, or water As. There was no interaction between As and Mn exposure and motor function. These findings have been summarized in a manuscript that is about to be submitted for publication in Environmental Health Perspectives.

In summary, the field work and laboratory analyses for this project have been completed. Data entry for the exercise and dietary questionnaires are being entered into our database. Once this is completed, we will begin the statistical analyses and prepare a manuscript describing the findings.

Key Research Accomplishments

None yet

Reportable Outcomes

None yet.

Current faculty who will be receiving support from the grant:

- Joseph Graziano, PhD
- Gail Wasserman, PhD
- Xinhua Liu, PhD

References

Not applicable

AD _____
(Leave blank)

AWARD NUMBER: VUMC34541

TITLE: Effects of manganese in welding fumes on cognitive function

PRINCIPAL INVESTIGATOR: Tomas Guilarte Ph.D. and Alison Geyh Ph.D.

CONTRACTING ORGANIZATION: Johns Hopkins Bloomberg School of Public Health
Baltimore, Maryland 21205

REPORT DATE: February 2011

TYPE OF REPORT: Progress Report

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: (Check one)

- ☒ Approved for public release; distribution unlimited
- ☐ Distribution limited to U.S. Government agencies only;
Report contains proprietary information

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

REPORT DOCUMENTATION PAGE				Form Approved OMB No. 0704-0188	
Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Department of Defense, Washington Headquarters Services, Directorate for Information Operations and Reports (0704-0188), 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302. Respondents should be aware that notwithstanding any other provision of law, no person shall be subject to any penalty for failing to comply with a collection of information if it does not display a currently valid OMB control number. PLEASE DO NOT RETURN YOUR FORM TO THE ABOVE ADDRESS.					
1. REPORT DATE (DD-MM-YYYY) 01-02-2011		2. REPORT TYPE Progress Report		3. DATES COVERED (From - To) 1/1/2010-2/1/2011	
4. TITLE AND SUBTITLE Effects of manganese in welding fumes on cognitive function				5a. CONTRACT NUMBER VUMC34541	
				5b. GRANT NUMBER	
				5c. PROGRAM ELEMENT NUMBER	
6. AUTHOR(S) Tomás R. Guilarte, PhD Alison Geyh, PhD				5d. PROJECT NUMBER	
				5e. TASK NUMBER	
				5f. WORK UNIT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Johns Hopkins Bloomberg School of Public Health Baltimore, MD 21205				8. PERFORMING ORGANIZATION REPORT	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) Vanderbilt University Medical Center Nashville, TN 37232				10. SPONSOR/MONITOR'S ACRONYM(S) VUMC	
				11. SPONSOR/MONITOR'S REPORT NUMBER(S)	
12. DISTRIBUTION / AVAILABILITY STATEMENT					
13. SUPPLEMENTARY NOTES Not applicable					
14. ABSTRACT Emerging experimental evidence in humans indicates that Mn-induced effects on cognitive function occur at much lower levels of Mn than those needed to affect motor function. However, despite this information, there is very limited knowledge on the molecular mechanisms responsible for Mn-induced effects on cognition and the extent to which deficits in cognitive function occur. The aim of this study is to provide new information in an experimental animal model of Mn exposure from welding fumes and its effects in cognitive domains mediated by the glutamatergic system in the hippocampus and cerebral cortex. During the past few years, we have completed both rounds each of a 10 week welding fume exposure, as well as the spatial learning tests. Some further detailed analysis is done, but also still continues. A full analysis of Metal analysis on collected particle mass samples, as well as in blood and brain samples from the mice are in the process of being completed.					
15. SUBJECT TERMS Manganese, neurotoxicology, iron deficiency, welding, manganese mining, nutrition					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON Tomás R. Guilarte, PhD
a. REPORT	b. ABSTRACT	c. THIS PAGE			19b. TELEPHONE NUMBER (include area code) (212) 305-3959

Table of Contents

Cover.....	1
SF 298.....	2
Table of Contents.....	3
Introduction.....	4
Experimental Design and Methods.....	4
Progress-To-Date.....	8
References.....	13

Study: Effects of manganese in welding fumes on cognitive function

Introduction:

There is a substantial body of evidence indicating that increased accumulation of manganese (Mn) in the central nervous system produces neurological disease. Humans can increase their body burden of Mn as a result of environmental or occupational exposures including welding, from certain medical conditions related to liver disease and parenteral nutrition. There is also increasing concern from the scientific community that the long-term use of gasoline containing the Mn additive methylcyclopentadienyl manganese tricarbonyl (MMT) can increase Mn exposure to the general population and thus increase the burden of neurological disease.

The goal of this study is to determine if exposure to Mn in welding fumes produces alterations in tests of cognitive function. In addition, neurochemical changes that could account for Mn-induced effects on cognitive function will be examined. The study design is to determine if exposure to Mn in welding fumes at different concentrations produces deficits in spatial learning and to determine if exposure to these different levels of Mn in welding fumes alters brain markers.

Experimental Design and Methods:

Description of Welding Fume Exposure: Fume generation is accomplished using a MillerMatic 251 Metal Inert Gas (MIG) all-in-one wire welding system (Miller Electric, Appleton, WI). The MIG system uses 0.045 mm diameter welding wire that contains either 0.5-2.5% Mn (Radnor/Airgas Baltimore MD) or 12-14% Mn (Stulz & Sickel, Elizabeth NJ) and an inert cover gas composed of 75% argon and 25% carbon dioxide (Airgas, Baltimore, MD). The wire is welded onto steel plates with 2% Mn content (Durrett Sheppard Steel Company, Baltimore, MD). Welding is conducted according to the 60 percent duty cycle of the MillerMatic, which allows for six minute-welds followed by four minutes of rest.

The welding fume generation was conducted in a one cubic meter chemical fume hood. The exposure chamber is interfaced to the output of the fume hood via a PVC manifold connected to a GAST pump (30 L/min). Two exposure chambers were used for each experiment. Each chamber is 12.5" x 12.5" x 12.5" and made of stainless steel and glass. The rear wall of each chamber includes two ports to allow for connection to the manifold and a connection to a low flow pump (5 to 9 L/min), which pulls fume through the chamber providing between 10 – 20 air exchanges per hour.

During exposure sessions, an active personal DataRAM (pDR 1000AN, Thermo Electron, Franklin, MA) was used to simultaneously record continuous (10 sec interval) mass concentrations and to collect particle mass samples using a 37 mm Teflon filter contained within a 37mm polystyrene filter cassette (Pall Gelman Laboratory, Ann Arbor, MI). In order to reduce over-loading of the filters, in each chamber filters were replaced at the end of every second 6 minute exposure period. This resulted in a total of 3 filters per exposure plus one blank per chamber for analysis of Mn and other metals associated with welding fume generation. Each biweekly welding session lasted approximately one hour, this includes 36 minutes of active welding fume generation, and approximately 24 minutes of down time for the MillerMatic to recover from the duty cycle.

Experimental Exposure (see Figure 1 and Table 1): Each experiment included 3 different groups of animals defined by the welding fume exposure conditions, and a total of two experiments were conducted. The three exposure groups of animals for the first experiment were: 1) clean air control; 2) exposed to welding fumes generated from 2% Mn content wire (Low Mn group (2%S)) and; 3) exposed to welding fumes generated from 14% Mn content wire (High Mn group(14%S)). After assessing the data from this experimental round, it was decided to use only the 14% Mn content wire and increase the total mass concentrations (mg/m^3) between the exposure groups (an approximate 10 fold difference between the two groups). The three exposure groups of animals for the second experiment were: 1) clean air control; 2) exposed to welding fumes generated from 14% Mn content wire with high PM concentration (High Mn group (14%H)); 3) exposed to welding fumes generated from 14% Mn content wire with very high PM concentrations generated (Very High Mn group (14%VH)). We have the ability to expose a total of 5 animals in each chamber and there are 3

chambers (one clean air and 2 with welding fumes) for the first experimental group, and 4 chambers (2 clean air and 2 with welding fumes) for the second experimental group. One exposure group (i.e. 2% Mn wire) was exposed during the morning and the other exposure group (i.e. 14% Mn wire) was exposed during the afternoon of the same day for a total of 10 animals per exposure group/day. All animals were exposed to their specific exposure conditions for a total of approximately 60 minutes, 2 times per week (Tuesday/Thursday) for a total of 10 weeks.

At the beginning of each exposure day, all pumps were turned on and allowed to warm up for approximately 1 hr. At the end of this period, flow rates through the chambers and through the particle samplers were calibrated. All animals were introduced to the chambers once the flow rate measurements had been completed. Welding fume generation was conducted manually. Total particle concentrations (mg/m³) reported by the pDR1000AN were monitored to ensure adequate particle levels during the exposure. Welding fume generation start and stop time, as well as filter start and stop time were recorded on study log sheets. At the end of the exposure period, the animals were removed from the chambers and the chambers were cleaned in preparation for the next exposure group. Preparation for all exposure groups followed the same steps as described above.

Figure 1: Experimental Design. Round 1 of exposure was composed of 2% Mn content wire (Low Mn group (2%S)) in the morning sessions and 14% Mn content wire (High Mn group (14%S)) in the afternoon sessions. Round 2 of exposure was composed of 14% Mn content wire (High Mn group (14%H)) in the morning sessions and 14% Mn content wire (Very High Mn group (14%VH)) in the afternoon sessions.

Morning Exposure

<u>Week 0</u>	<u>Chamber 1-Clean Air</u> 5 mice	<u>Chamber 2-Mn Exp.</u> 5 mice	<u>Chamber 3-Mn Exp.</u> 5 mice
<u>Week 5</u>	-1 mouse (Brain/Blood Mn levels @ 5wks) +1 "filler" mouse	-1 mouse (Brain/Blood Mn levels @ 5wks) +1 "filler" mouse	-1 mouse (Brain/Blood Mn levels @ 5wks) +1 "filler" mouse
<u>Week 10</u>	-1 mouse (Brain/Blood Mn levels @ 5wks) -1 mouse (Brain/Blood Mn levels @ 10wks) -3 mice (behavior & neurochem. studies)	-1 mouse (Brain/Blood Mn levels @ 5wks) -1 mouse (Brain/Blood Mn levels @ 10wks) -3 mice (behavior & neurochem. studies)	-1 mouse (Brain/Blood Mn levels @ 5wks) -1 mouse (Brain/Blood Mn levels @ 10wks) -3 mice (behavior & neurochem. studies)

Afternoon Exposure

<u>Week 0</u>	<u>Chamber 1-Clean Air</u> 5 mice	<u>Chamber 2-Mn Exp.</u> 5 mice	<u>Chamber 3-Mn Exp.</u> 5 mice
<u>Week 5</u>	-1 mouse (Brain/Blood Mn levels @ 5wks) +1 "filler" mouse	-1 mouse (Brain/Blood Mn levels @ 5wks) +1 "filler" mouse	-1 mouse (Brain/Blood Mn levels @ 5wks) +1 "filler" mouse
<u>Week 10</u>	-1 mouse (Brain/Blood Mn levels @ 5wks) -1 mouse (Brain/Blood Mn levels @ 10wks) -3 mice (behavior & neurochem. studies)	-1 mouse (Brain/Blood Mn levels @ 5wks) -1 mouse (Brain/Blood Mn levels @ 10wks) -3 mice (behavior & neurochem. studies)	-1 mouse (Brain/Blood Mn levels @ 5wks) -1 mouse (Brain/Blood Mn levels @ 10wks) -3 mice (behavior & neurochem. studies)

Table 1. Summary characteristics of the experimental exposure groups.

	Round 1		Round 2	
	Group 1	Group 2	Group 1	Group 2
Dates of exposure	February 26 – May 5, 2009		October 6, 2009 – December 10, 2009	
Number of Exposure Days	20	20	20	20
Welding Wire %Mn Content	2%	14%	14%	14%
Particulate Matter Exposure Level	Standard (S)	Standard (S)	High (H)	Very High (H)
Number of Exposure Mice	5 + 1 filler = 6	5 + 1 filler = 6	5 + 1 filler = 6	5 + 1 filler = 6
Number of Control Mice	5 + 1 filler = 6		5 + 1 filler = 6	5 + 1 filler = 6
Number of Filters Collected (including blanks)	172	172	160	160
Number of MIE records collected	~17,392	~14,894	~12,383	~15,106

Filter analysis: All filters are weighed before and after sampling for determination of total particle mass. Filters are weighed in a room controlled for temperature and humidity (21°C ± 2°C; 35% ± 5%, respectively) using a Mettler T5 microbalance with precision of ± 0.001 mg (Mettler-Toledo, Columbus OH). For each mass measurement filters are weighed twice with the average of the two measurements recorded as the mass value for that weighing. If the difference between the first and the second measurement are greater or less than 0.003 mg the filter is re-weighed and the two closest measurements averaged.

Preparation and analysis of filters for Mn, Pb, Fe, V, Se, As, Cr, Co, content: The sample preparation method has been adapted from Kinney et al. 2002 EHP 110 (S4) 539 - 546. Samples are acid digested using a Mars5 Xpress microwave system (CEM Corporation, Matthews NC). Prior to digestion, the polyolefin support ring is removed. In order to minimize sample loss from the filter, the filter is layered between Kimwipes (3.5 cm x 3.5 cm) (Kimberly Clark, New Milford CT) and wetted with 100 uL of ethanol. The polyolefin ring is then removed using a stainless steel scalpel and the Teflon membrane and Kimwipes are transferred to a 7mL Teflon digestion microwave vessel (CEM Corporation, Matthews NC), and 160 uL of ultrapure water (Millipore, Billerica MA) and 1.35 mL concentrated optima grade nitric acid (Fisher Scientific, Columbia MD) are added. The samples are initially digested using a two-stage ramp-to-temperature method with a maximum temperature of 165 °C and a hold time of 30 min. Following the first digestion, 100 uL of concentrated optima nitric acid and 55 uL of concentrated optima grade hydrofluoric acid (Fisher Scientific, Columbia MD) are added and a second digestion performed according to the same ramp-to-temperature method. At the completion of the second digestion, the Teflon membrane is removed and an aliquot of the digestate is diluted with 1% optima grade nitric acid + 0.5% hydrochloric acid (Fisher Scientific, Columbia MD) for elemental analysis by inductively coupled plasma-mass spectrometry (ICP-MS). Internal standard, 50 ug/L Li, Ge, Sc, Tb, Bi, Y, In (CPI International, Santa Rosa CA), is added to each sample to monitor for instrument drift over analysis time. For every batch of 21 samples, 3 samples of the NIST

standard reference material 1648a Urban Particulate Matter (National Institutes of Standards and Technologies, Rockville MD) is digested and analyzed, as well as 3 reagent blanks for quality control. Total elemental analysis was performed using an Agilent 7500ce Inductively Coupled Plasmas Mass Spectrometer (Agilent Technologies, Santa Clara CA). An 8 point calibration curve is determined for each element and considered acceptable when the actual concentration is within $\pm 5\%$ of the expected value. The analytical limit of detection (LOD), as calculated by 3 times the standard deviation of the lowest detectable calibration standard (1 ug/L), was determined for each element analyzed. For samples with values that were below the analytical LOD, $\frac{1}{2}$ the LOD was substituted. Quality Control and Quality Assurance is maintained by using a NIST Standard Reference Material (1648a) in the microwave digestion procedure as well as replicate sample analysis at predetermined intervals. Percent recovery of the SRM is to be within an acceptable range of 85-115%.

Analysis of Mn levels in tissue and blood: At 5 and 10 weeks of exposure, 1 animal from each exposure chamber was euthanized for blood and brain Mn analysis (see Figure 1). In order to maintain the exposure dynamics for each group the same throughout the 10-week exposure period, we used “filler” mice at the 5-week time point when one of the animals per group was euthanized for Mn analysis. Animals were given pentobarbital (50mg/kg i.p. injection) to anesthetize them, and blood samples were obtained via heart puncture. Animals were then decapitated and their brains were dissected for analysis in the following regions: hippocampus, cerebral cortex, striatum, cerebellum, and brain stem. These samples will be analyzed using high resolution ICP-MS in Dr. Tore Syversen’s laboratory in Norway.

Spatial Learning (Morris Water Maze): The day following the last exposure session, a spatial learning assessment on all remaining animals using the Morris Water Maze was performed. Briefly, mice swim in a circular pool filled with 25°C water made opaque by dissolution of soluble, non-toxic, white paint. A transparent platform is submerged 1 cm below the surface of the opaque water, and large designs in visible areas around the room serve as distal cues. An automated video tracking system (Videomex-V Image Analyzer, Columbus Instruments, Columbus, OH) is used to record the swim path, distance, time to platform (latency) and the time spent in each quadrant of the pool. We followed a protocol similar to that used by Xu et al. (2009). This protocol consists of three phases: (1) visible platform training (cue tests); (2) hidden platform training and probe test 1; and (3) reversed platform and a second probe test.

For the first three trial days, mice were trained to find a visible platform, marked with a “flag”. All mice were released from the same quadrant for all trials, and the platform was rotated through the remaining three quadrants for each trial (for a total of 3 trials each day). Each mouse was allowed to swim for a maximum of 60 seconds, and if the visible platform was not found within those 60 seconds the mouse was guided to the platform. Every mouse was allowed to remain on the platform for 15 seconds after each trial.

Mice were trained to find a hidden platform (submerged in the opaque water and not marked with a “flag”) during trial days 4-7. A submerged platform was placed in a fixed location, which was the center of a quadrant different than that used for the visible platform training. Each mouse was given 3 trials per day, and each trial was a maximum time of 60 seconds. Starting points for each trial were randomly rotated through the remaining 3 quadrants not containing the hidden platform. Mice were allowed to rest on the platform for 15 seconds after each trial. The first probe test was conducted 24 hours after the last trial of the hidden platform training. During the probe test the mice all started in the same quadrant and were allowed to swim for 60 seconds without the platform in the tank.

For the reverse platform training, the hidden platform was moved to the quadrant opposite that used for the hidden platform training without changing any visual cues. Mice were then trained with 3 trials per day to find this new platform location, with starting points changed every trial as before. Reverse platform training was conducted on trial days 8-10. The second probe test was performed 3 days after the final reversal day.

Progress-To-Date:

Two rounds of exposure experiments have been completed.

Round 1 Exposure:

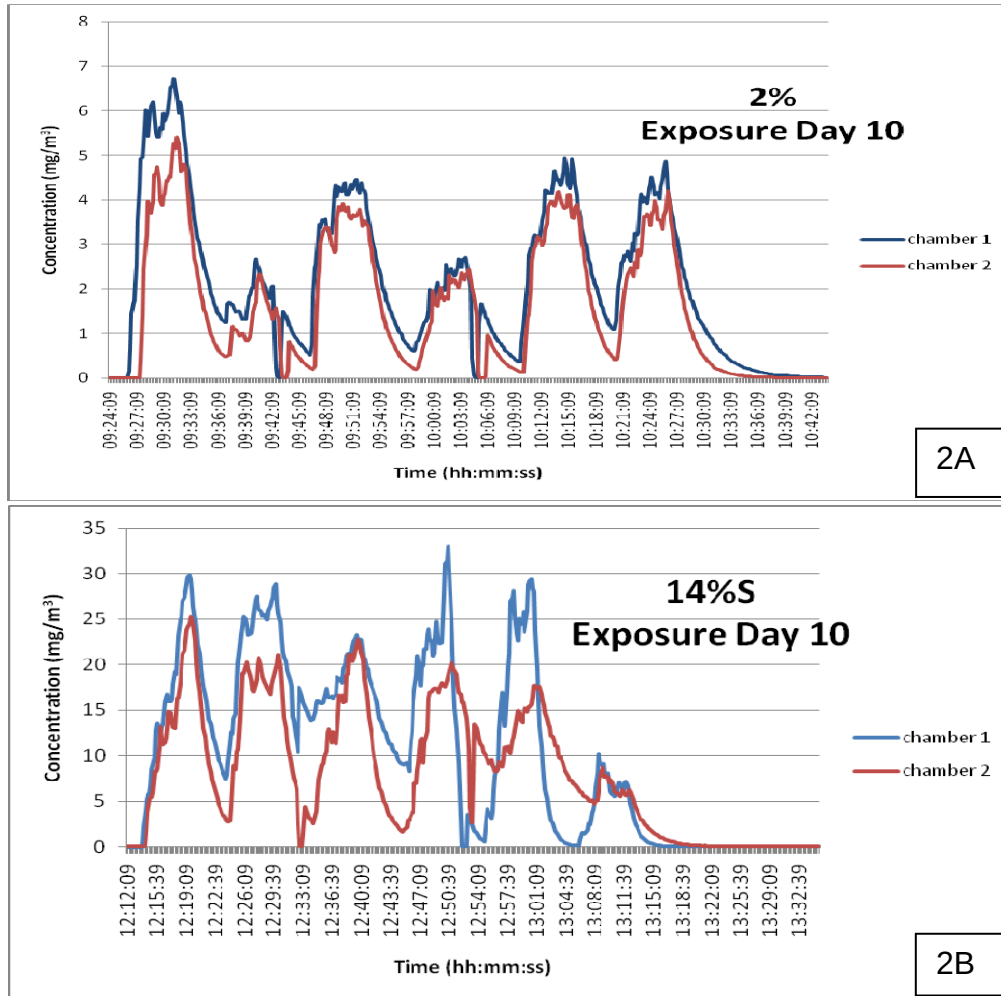


Figure 2 (left). Real time concentration (mg/m^3) as reported by the pdr1000AN for each chamber in a given exposure group. A) 2% Mn wire exposure group B) 14%S Mn wire exposure group.

Round 1 was conducted from February 26, 2009 to May 5, 2009. A total of 12 mice were exposed to low particle concentration conditions using 2% Mn wire (2%S) and 12 mice were exposed to high particle concentration conditions using 14% Mn wire (14%S); 12 mice were not exposed and acted as controls. One (1) mouse per exposure group was sacrificed at 6 ½ weeks (April 9, 2009) and was replaced with a “filler” mouse. A second mouse from each group was sacrificed at week 10 (May 5, 2009). A total of 344

filters were generated for Mn analysis along with 40 continuous mass concentration data sets. Of the 344 filters collected all have been digested and analyzed for total metals (Table 1).

As each exposure group consisted of two exposure chambers, the difference in total mass concentration was assessed to indicate potential differences in welding fume exposure between the two chambers using the pdr1000AN real-time concentration readings. These real time total mass concentration (mg/m^3) readings for each chamber for a specific exposure day are shown in Figure 2a, 2b. To characterize the difference in average total mass concentration between the two exposure groups, the total average mass concentration (mg/m^3) as determined from the mass loading of the Teflon filters has been analyzed (Figure 3a,b). For the 2% group, average total mass concentrations ranged from $0.674 \text{ mg}/\text{m}^3$ to $12.998 \text{ mg}/\text{m}^3$ with a mean of $5.548 \text{ mg}/\text{m}^3$. For the 14%S group, average total mass concentrations ranged from $3.724 \text{ mg}/\text{m}^3$ to $28.802 \text{ mg}/\text{m}^3$ with a mean of $13.743 \text{ mg}/\text{m}^3$. To characterize the difference in average Mn concentration between the two exposure groups, average Mn concentration as determined by ICP-MS analysis have been analyzed (Figure 4a,b). For the 2% group average Mn mass concentrations ranged from $103.94 \text{ mg}/\text{m}^3$ to $3,483.92 \text{ mg}/\text{m}^3$ with a mean of $998.78 \text{ mg}/\text{m}^3$. For the 14%S group average Mn mass concentrations ranged from $1,492.94 \text{ mg}/\text{m}^3$ to $94,139.53 \text{ mg}/\text{m}^3$ with a mean of $13,006.24 \text{ mg}/\text{m}^3$. Analysis to relate exposure of welding fume to cognitive function as measured by the Morris Water Maze is ongoing.

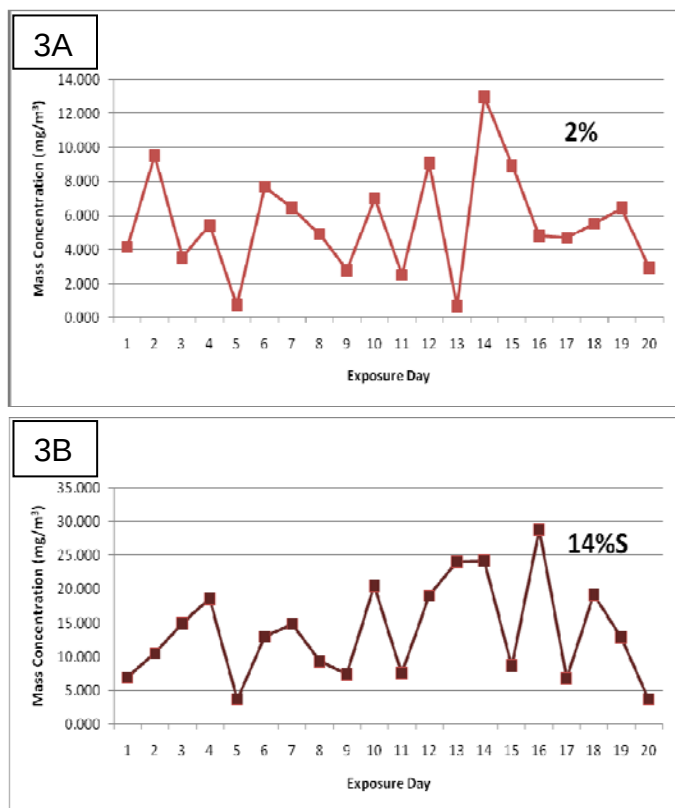


Figure 3 (above). Average total mass concentration (mg/m³) as determined from the Teflon filters. A) 2% Mn wire exposure group B) 14%S Mn wire exposure group.

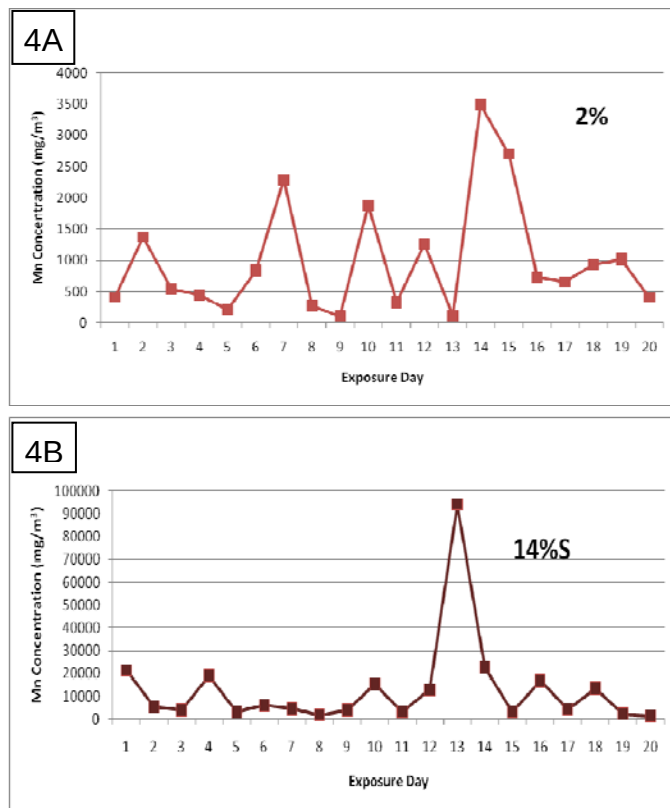


Figure 4 (above). Average Mn mass concentration (mg/m³) as determined from the Teflon filters. A) 2% Mn wire exposure group B) 14%S Mn wire exposure group.

Round 2 Exposure:

Round 2 was conducted from October 6, 2009 to December 10, 2009. A total of 12 mice were exposed to moderate particle concentration conditions using 14% Mn wire (14%H) and 12 mice were exposed to very high particle concentration conditions using 14% Mn wire (14%VH); 24 mice were not exposed and acted as controls. One (1) mouse per exposure group was sacrificed at 6 ½ weeks (November 17, 2009) and was replaced with a “filler” mouse. A second mouse from each group was sacrificed at week 10 (December 10, 2009). A total of 320 filters were generated for Mn analysis along with 40 continuous mass concentration data sets. Of the 320 filters collected all have been digested and analyzed for total metals content (Table 1).

As each exposure group consisted of two exposure chambers, the difference in total mass concentration was assessed to indicate potential differences in welding fume exposure between the two chambers using the pdr1000AN real-time concentration readings. These real time total mass concentration (mg/m³) readings for each chamber for a specific exposure day are shown in Figure 5a, 5b. To characterize the difference in average total mass concentration between the two exposure groups, the total average mass concentration as determined from the mass loading of the Teflon filters has been analyzed (Figure 6a,b). For the 14%H group, average total mass concentrations ranged from 7.130 mg/m³ to 112.270 mg/m³ with a mean of 18.201 mg/m³. For the 14%VH group, average total mass concentrations ranged from 3.724 mg/m³ to 28.802 mg/m³ with a mean of 13.743 mg/m³. To characterize the difference in average Mn concentration between the two exposure groups, average Mn concentration as determined by ICP-MS analysis have been analyzed (Figure 7a,b). For the 14%H group average Mn mass concentrations ranged from 2,126.63 mg/m³ to 13,909.91 mg/m³ with a mean of 5,604.32 mg/m³. For the 14%VH group average Mn mass concentrations ranged from 1,492.94 mg/m³ to 94,139.53 mg/m³ with a mean of 13,006.24 mg/m³.

Analysis to relate exposure of welding fume to cognitive function as measured by the Morris Water Maze is ongoing.

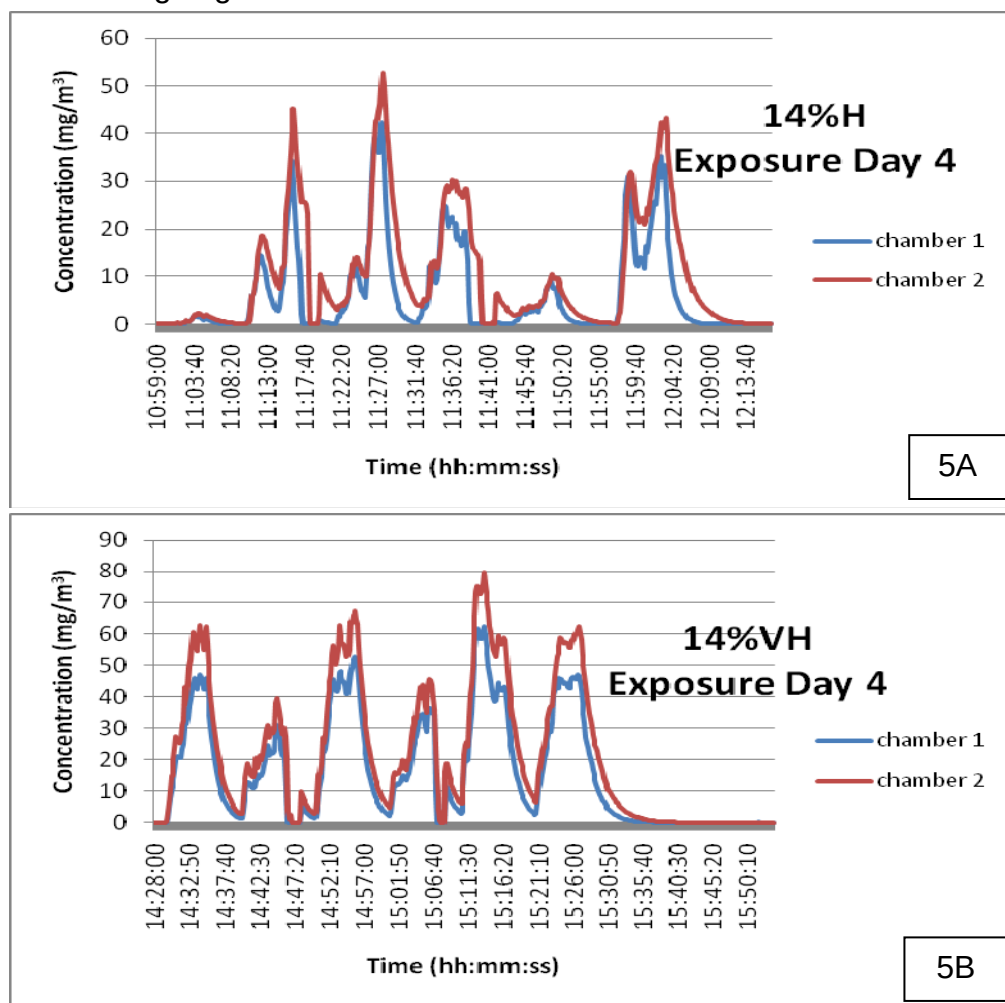


Figure 5 (left). Real time concentration (mg/m³) as reported by the pdr1000AN for each chamber in a given exposure group. A) 14%H Mn wire exposure group B) 14%VH Mn wire exposure group.

Behavioral Analysis: After 2 rounds of 10 weeks exposure, there were no physical symptoms of Mn exposure observed in any of the groups. Mice were weighed prior to each exposure period. There was no difference in weights between any of the exposure groups at the start of the experiment or over the exposure period of 10 weeks. Analysis of the Morris Water Maze data shows no differences between exposure groups

in training phase or in the probe tests. Data for Daily Average Latency and Proximity Average are presented in Figure 8. For the ease of presentation, data for the 2 middle exposure groups (14%S and 14%H) are combined for the water maze analysis. Daily average latency is the time (in secs) to find the platform averaged for 3 trials for each animal each day of training. Proximity average is a measurement described by Gallagher (et al., 1993) as a more accurate analysis to allow for the comparison of animals independent of their swim speed, age, etc. This data is also expressed as the average for 3 trials for each animal each day of training. More extensive analysis of this data is ongoing.

Brain and blood samples have been collected and analysis of metal content is ongoing by our collaborators. Further neurochemical analysis of the glutamatergic system using autoradiography and HPLC is also still in process. Immunohistochemistry for astrocyte and microglia activation has begun on the brain tissue and is in the process of analysis. Once this data is collected, we hope to correlate the results with the exposure data.

Data Management for Exposure Data for both Round 1 and Round 2: Logsheet Data

Each exposure session included three log sheets: one general logsheet for welding information, and one for each exposure chamber. These logsheets included information pertaining the filter start and stop times, filter pre and post flow rates, chamber pre and post flow rates, and welding start and stop times. All data from these logsheets was entered in a master database using Microsoft Excel. Filter pre and post weight averages were added to this database. The filter ID was used as the unique identifier for all analyses.

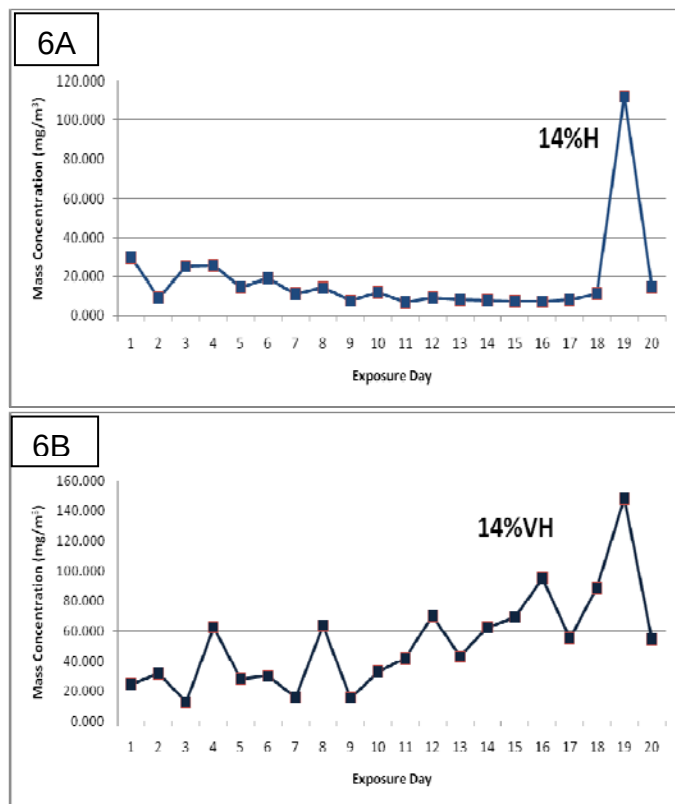


Figure 6 (above). Average total mass concentration (mg/m³) as determined from the Teflon filters. A) 14%H Mn wire exposure group B) 14%VH Mn wire exposure group.

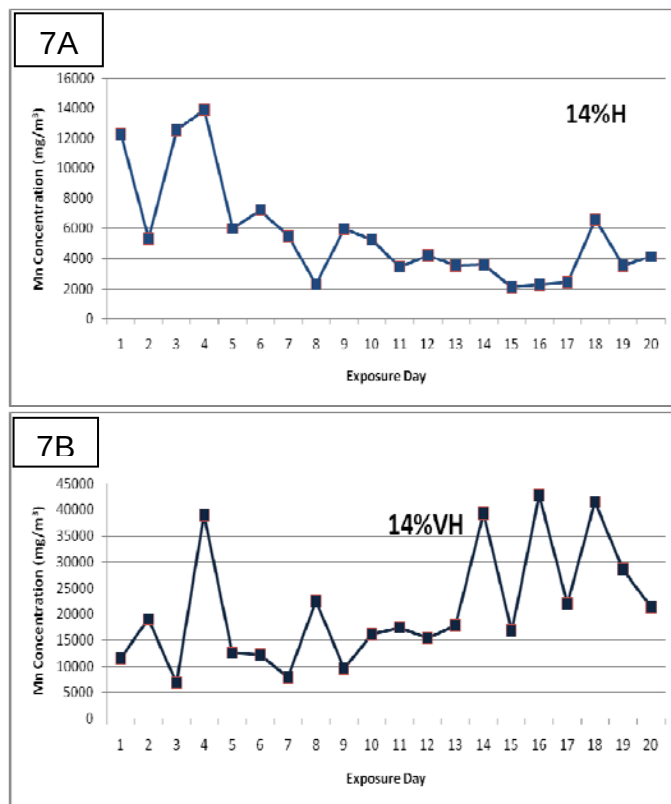


Figure 7 (above). Average Mn mass concentration (mg/m³) as determined from the Teflon filters. A) 14%H Mn wire exposure group B) 14%VH Mn wire exposure group.

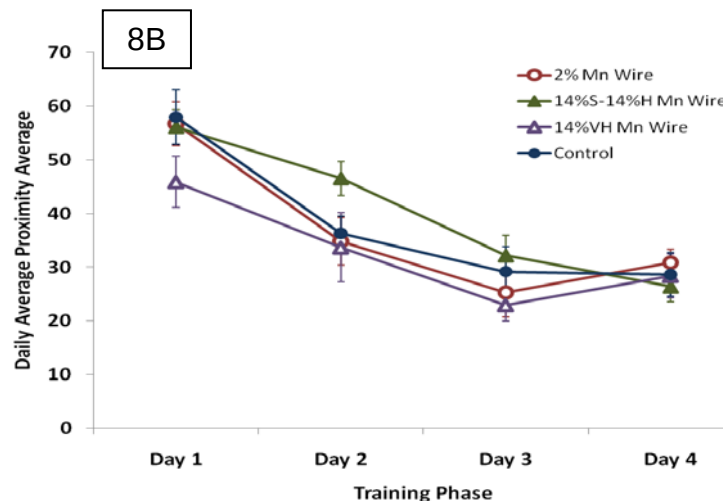
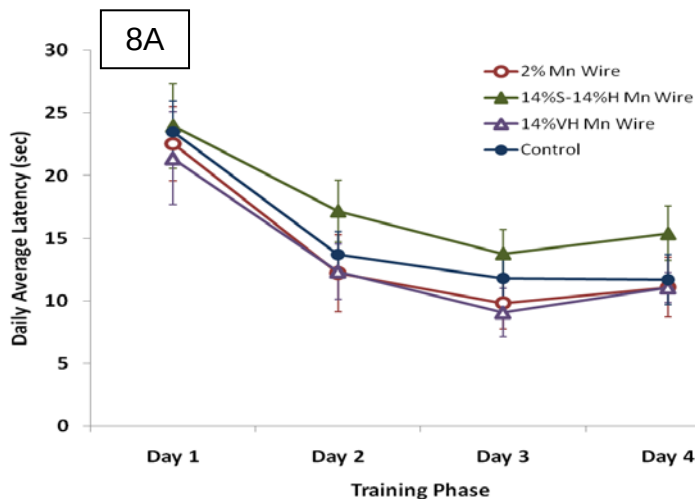


Figure 7 (above). Morris Water Maze Training Phase data for all exposure groups. There are no significant differences between the exposures groups, so for ease of presentation the 2 middle exposure groups (14%S and 14%H) have been combined. (n=6-12 in each group) A) Latency to platform (secs) averaged for 3 trials for each animal on each day of the training phase. B) Proximity average averaged for 3 trials for each animal on each day of the training phase.

pdr1000AN Data

pdr1000AN data was downloaded daily using pDR-COM software (Thermo Fisher Scientific, Frankling MA). The data were then converted to Comma-Separated-Value files and opened in Excel for data processing. Data were adjusted to local standard time and truncated to correspond with the time period of the 3 filters collected per chamber for each exposure session and adjusted to local

standard time. Each 10 second interval file was then assigned the filter ID to which it corresponded and the data were imported into the master spreadsheet.

Table 2. Descriptive Statistics for Exposure Metal Analysis

ROUND	Exposure Group	Mass (mg)	N*	Mean	Std Dev	Min	25th Pctl	75th Pctl	Max
1	2%	Total	131	0.5300	0.4000	0.0100	0.2700	0.6900	2.1800
		V	131	0.1258	0.7818	0.0013	0.0013	0.0100	5.8141
		Cr	131	1.2188	4.6097	0.0027	0.0396	0.9769	49.0403
		Mn	131	100.4736	146.6731	0.0018	26.9376	103.2983	1236.2772
		Fe	131	268.0000	226.5350	0.0017	104.4401	357.4617	1173.7814
		Co	131	0.0514	0.0623	0.0015	0.0100	0.0694	0.2411
		Zn	131	0.3533	0.7242	0.0071	0.0220	0.3961	5.1778
		As	131	0.0163	0.0187	0.0034	0.0034	0.0192	0.1379
		Se	131	0.0050	0.0031	0.0042	0.0042	0.0042	0.0276
		Pb	131	0.0291	0.0615	0.0006	0.0071	0.0278	0.4822
	14%S	Total	126	0.0013	0.0011	-0.0008	0.0004	0.0021	0.0042
		V	126	1.1281	4.2206	0.0013	0.0033	0.0198	29.7196
		Cr	126	17.1226	16.2383	0.0027	4.2417	24.7703	93.5693
		Mn	126	1236.8882	2258.9291	0.0018	140.1341	1325.0693	16263.8989
		Fe	126	88.6859	80.3444	0.0017	22.1033	135.4350	293.3317
		Co	126	0.0130	0.0135	0.0015	0.0015	0.0187	0.0722
		Zn	126	2.1783	2.0123	0.0071	0.5173	3.4839	7.8430
		As	126	0.0336	0.0289	0.0034	0.0107	0.0461	0.1505
		Se	126	0.0058	0.0040	0.0042	0.0042	0.0042	0.0271
		Pb	126	0.1773	0.1627	0.0006	0.0414	0.2877	0.6977
2	14%H	Total	114	0.0011	0.0027	-0.0224	0.0007	0.0013	0.0098
		V	114	0.0352	0.2120	0.0013	0.0046	0.0204	2.2710
		Cr	114	9.0671	5.5562	0.0027	5.2077	13.4504	24.1241
		Mn	114	538.5450	480.0696	0.0018	262.8805	680.6180	2907.5923
		Fe	114	136.1759	90.2460	0.0017	71.4770	188.8189	517.5588
		Co	114	0.0384	0.2081	0.0015	0.0072	0.0247	2.2267
		Zn	114	1.0370	1.5197	0.0071	0.2859	0.8473	10.1016
		As	114	0.0665	0.2101	0.0034	0.0205	0.0723	2.2487
		Se	114	0.0337	0.2130	0.0042	0.0042	0.0149	2.2771
		Pb	114	0.2393	0.2897	0.0006	0.1216	0.2572	2.3139
	14%VH	Total	118	0.0046	0.0042	0.0008	0.0022	0.0056	0.0278
		V	118	0.0313	0.0227	0.0013	0.0146	0.0453	0.0998
		Cr	118	33.7929	28.7536	0.0027	15.2601	39.4906	149.6207
		Mn	118	1716.9940	1088.1483	0.0018	969.2115	2214.3368	5321.0314
		Fe	118	407.2390	382.0095	0.0017	219.2159	444.1731	2015.3651
		Co	118	0.0380	0.0265	0.0015	0.0185	0.0515	0.1218
		Zn	118	2.4264	1.8062	0.0071	1.0902	3.5112	8.4257
		As	118	0.1087	0.0658	0.0034	0.0583	0.1576	0.2756
		Se	118	0.0252	0.0286	0.0042	0.0042	0.0392	0.1626
		Pb	118	0.8456	0.6099	0.0006	0.3727	1.2375	2.6771

*N= number of samples after blank correction.

Blank Correction

Each chamber had 4 filters per exposure session, 1 blank filter, and 3 filters which collected particle samples. Total mass was blank corrected by chamber and exposure day. Once blank corrected, data were tested for outliers and the 99th percentile were retained. For total metals mass (ng), each metal was blank corrected in a similar fashion by subtracting the blank value by chamber and exposure day from each of the 3 particle sample filters. Once blank corrected, metals data were tested for outliers and the 95th percentile were retained.

Preliminary exposure metal analysis for each exposure group is presented in Table 2. All statistical analyses were conducted using SAS9.2 (SAS Institute Incorporated, Cary NC).

References

Gallagher M, Burwell R, Burchinal M (1993) Severity of spatial learning impairment in aging: development of a learning index for performance in the Morris water maze. *Behav Neurosci* 107(4):618-26.

Kinney PL, Chillrud SN, Ramstrom S, Ross J, Spengler JD (2002) Exposures to multiple air toxics in New York City. *Environ Health Perspect* 110 (S4):549-46.

Xu J, Zhu Y, Contractor A, Heinemann S (2009) mGluR5 has a critical role in inhibitory learning. *J Neurosci* 29(12):3676-3684.

AWARD NUMBER:
VUMC31527-R

TITLE:
Exposure to Welding Fume and Parkinson's Disease: a feasibility study.

PRINCIPAL INVESTIGATOR:
Prof Jon Ayres

CONTRACTING ORGANIZATION:
Institute of Occupational and Environmental Medicine,
University of Birmingham,
Edgbaston,
Birmingham
UK.
B15 2TT

REPORT DATE:
April 2010

TYPE OF REPORT:
Final Report

PREPARED FOR:
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: (Check one)

- ☒ Approved for public release; distribution unlimited
- ☐ Distribution limited to U.S. Government agencies only;
report contains proprietary information

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

REPORT DOCUMENTATION PAGE				Form Approved OMB No. 0704-0188	
Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Department of Defense, Washington Headquarters Services, Directorate for Information Operations and Reports (0704-0188), 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302. Respondents should be aware that notwithstanding any other provision of law, no person shall be subject to any penalty for failing to comply with a collection of information if it does not display a currently valid OMB control number. PLEASE DO NOT RETURN YOUR FORM TO THE ABOVE ADDRESS.					
1. REPORT DATE (DD-MM-YYYY) 09/04-2010		2. REPORT TYPE Final Report		3. DATES COVERED (From - To) 1/1/2006-9/5/2010	
4. TITLE AND SUBTITLE Exposure to Welding Fume and Parkinson's Disease: a feasibility study.				5a. CONTRACT NUMBER VUMC31527-R	
				5b. GRANT NUMBER	
				5c. PROGRAM ELEMENT NUMBER	
6. AUTHOR(S) McMillan, Grant; Jackson, Craig; Nicholl, David; Pramstarra, Peter; Ayres, Jon.				5d. PROJECT NUMBER	
				5e. TASK NUMBER	
				5f. WORK UNIT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Institute of Occupational and Environmental Medicine, University of Birmingham, Edgbaston, Birmingham UK. B15 2TT				8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) Vanderbilt University Medical Center Nashville, TN 37203				10. SPONSOR/MONITOR'S ACRONYM(S) VUMC	
				11. SPONSOR/MONITOR'S REPORT NUMBER(S)	
12. DISTRIBUTION / AVAILABILITY STATEMENT					
13. SUPPLEMENTARY NOTES Not applicable					
14. ABSTRACT This feasibility study conducted interviews with a small number of males diagnosed as having Parkinson's Disease. A method was devised to interview the participants to assess their previous occupational exposures to manganese and welding work, and to ascertain if such exposed patients differed from non-exposed patients in terms of age of onset and diagnosis. This study has provided two useful research tools and statistical indications of the absence of evidence of risk of Parkinson's disease being caused or accelerated by exposure to welding. We have shown that it would be feasible to conduct the main phase of this study as proposed over a period of a further three years providing sufficient funds were available over and above the previous funding application to allow us to employ at least one full-time Research Assistant. We have shown through the literature review that the knowledge we sought to acquire in the proposed main phase of the study, has been provided satisfactorily by others since this feasibility study started. In consequence of this, no matter how feasible it may be, we cannot now justify an argument for the cost and time required to undertake the previously proposed main phase.					
15. SUBJECT TERMS Manganese, neurotoxicology, iron deficiency, welding, manganese mining, nutrition, Parkinson's disease, movement					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT UU	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON Craig Jackson PhD
a. REPORT U	b. ABSTRACT U	c. THIS PAGE U			19b. TELEPHONE NUMBER (include area code) +44 772 511 5254

Table of Contents

Background	5
Aim & Objectives	7
Methods	8
Results	11
Discussion	14
Conclusions	15
References	16
Appendices	17

REPORT OF THE FEASIBILITY PHASE OF A PROPOSED CLINICAL CASE-CONTROL STUDY TO DETERMINE IF EMPLOYMENT AS A WELDER AFFECTS THE AGE OF ONSET OF PARKINSON'S DISEASE

Grant McMillan, Honorary Clinical Senior Lecturer,
Institute of Occupational & Environmental Medicine, University of Birmingham

Craig Jackson, Professor of Occupational Health Psychology,
Division of Psychology, Birmingham City University

David Nicholl, Consultant Neurologist,
Dept. of Neurology, Queen Elizabeth Hospital, Birmingham

Peter Pramstarra, Consultant Neurologist,
Dept. of Neurology, Queen Elizabeth Hospital, Birmingham

Jon Ayres, Professor of Occupational & Environmental Medicine
Institute of Occupational & Environmental Medicine, University of Birmingham

Acknowledgements

The authors wish to express their thanks to Miki Aschner and Anne Tremblay for their support and guidance in the development of this feasibility study. The authors also thank the USAMRMC for their support and funding of this study.

Introduction

This is the report of the feasibility phase of a proposed epidemiological study to determine if having been employed as a welder affects the age of onset of Parkinson's disease.

Background

Electric arc welding is a ubiquitous metal joining process at the very heart of industry worldwide. Hundreds of thousands of workers in the United States of America and Europe classify it as their principal full-time occupation. Worldwide there may be over a million people employed as welders with three to five million others using welding as part of their occupational tasks. Many other men and women work with and around welders, sharing their working environment. All these workers are at risk of exposure to the fumes and gases which are emitted by arc welding processes. The overall numbers are likely to increase steadily in line with production of steel, the most commonly welded material, as developing countries industrialise. In addition, many others are employed in the large and sophisticated industry researching, developing, manufacturing and supplying welding materials.

Much had been written over the years about the possible adverse effects of welding on health, especially respiratory health, when in 2001 it was reported that there appeared to be a risk of harm to the central nervous system. Those who had conducted a small clinical cross-sectional case-referent study had concluded that employment as a welder was associated with a significantly younger age of onset of typical idiopathic Parkinson's disease than had been found in the control group; 46 years for welders compared with 63 years for those in the control group [1].

Parkinson's disease is a neurological disorder affecting mood and movement. It is a common disease, said to affect one in 500 of the general population and 1% of those over the age of 65. There is no proven causal agent. Studies have suggested that environmental factors, notably exposure to pesticides, may be causally linked to the disease, possibly through interactions with some genetic susceptibility.

The 2001 finding raised the possibility that some exposure resulting from working as a welder caused or accelerated the onset of this disease, provoked debate in legal and occupational health circles, and caused anxiety among welders and in the welding industry. Injury litigation was initiated. Despite uncertainty or ignorance about the bioavailability, transport and effects of compounds present in welding fume, and with a paucity of exposure data or good epidemiology, the putative hazard was identified as manganese compounds in fume from welding steel.

Manganese is an essential trace element for humans. Derived from processed mined ore, its compounds are constituents of several industrial processes. The greatest amount by far is used in steelmaking as it is an essential constituent of all types of steel. In consequence, it is found in particles in the fume emitted from welding steel, that being derived mainly from the vaporisation of consumable electrodes or other filler metal used in the joining process. Evidence from other occupational exposures has shown that excess exposure to manganese and its compounds in dusts and fumes may result in its accumulation in the brain where it is neurotoxic. This may cause the rare mood and movement disorder of "manganism". There is thought to be a sub-clinical form of that disease detectable by neurobehavioural tests.

Manganism and Parkinson's disease are similar but separate disorders with different underlying cellular pathologies. The superficial similarities can lead easily to diagnostic confusion and, in turn, to incorrect assignation of cases in epidemiological studies. The disorders can, however, be distinguished clinically by thorough careful examination and investigation, strict discipline in defining signs and symptoms, then making the correct inferences from the observations backed, if necessary, by special examinations such as MRI and PET scans.

One of the authors of this report (GM) had maintained a special interest in the health of welders and had reviewed related literature since the 1970s. He became increasingly concerned as risk assessments and reports of central nervous system damage occurring in workers said to be welders were promulgated as at least some of the assessments had been developed by possibly inappropriate extrapolation from other work situations and from case reports and epidemiological investigations which appeared to be flawed significantly by imprecision in diagnosis and use of the term "welder". He conducted a systematic review of the literature and concluded that it was impossible to accept or dismiss the contention that employment as a welder, with consequent exposure to manganese compounds in welding fume, enhanced susceptibility to or caused Parkinson's disease in welders and that this matter merited more precise investigation [2].

We came to agree that this further work should include a case-referent study of sufficient power and precision to detect a significant excess incidence of a history of work as a welder joining metal in men diagnosed with Parkinson's disease using defined and strict applied clinical criteria. We set out to conduct this in Birmingham, a city at the heart of industrial England. We resolved that should this excess be found then retrospective assessment of exposure should be attempted to provide a dose-response curve.

As the most challenging aspects of this study would be case definition and selection of appropriate referents it was thought prudent to explore these aspects in a feasibility study before embarking on a large scale investigation. The study aim and design of the feasibility phase were supported by the International Manganese Institute which brought the proposal to the attention of the US Department of Defence, a major employer of workers who weld steel, and USMRMC agreed to fund the feasibility phase, reported here, and, provisionally, the main study.

Aim and objectives

The feasibility phase was designed to define means and determine the feasibility of conducting a definitive case-referent study to determine if there was a significant excess of employment as a welder or in other work with exposure to manganese containing metal fumes in men diagnosed with Parkinson's disease.

The objectives of the feasibility study were to:

1. Develop and use suitable study tools for diagnosis of Parkinson's disease and to elicit a full occupational history within a National Health Service clinic environment.
2. Collect information in the clinic during the development of these tools and seek to use it to test two hypotheses;
 - a. A significantly higher proportion of those diagnosed as having undisputed PD have been welders of steel, or otherwise exposed to manganese-containing fumes.
 - b. Within those diagnosed as having PD, the age of onset is lower among those who have been occupationally exposed to manganese than those who have not.
3. Investigate sources and the process of recruiting sufficient referents suitable to test the hypothesis that the proportion of men in the Parkinson's disease group who have been welders of steel or otherwise exposed to manganese-containing fumes is significantly greater than in the general population i.e. among men in a control group matched for age, family history and other known risk factors but who do not have Parkinson's disease.
4. Maintain a review of the literature to inform the study and, following its completion, assist in determining if there was a persisting knowledge requirement to undertake the main study.

Ethical approval

Ethical approval was obtained from the Solihull NHS Local Research Ethics Committee in August 2007 and USMRMC in October 2007. This was a more time-consuming exercise than we had anticipated but valuable experience was gained in crafting the study design to meet the requirements of each authority.

Methods

Objective 1: Development of research tools

The reliability and validity of the diagnosis of a “case” of Parkinson’s disease is central to the precision of the case-referent study. This diagnosis is not straightforward as there are no biological markers for ante-mortem diagnosis and the decision depends on the presence and progression of clinical features. Misdiagnosis has been shown to be common, particularly in the early stages of the disease. This is a factor which has been suggested as limiting the usefulness of several epidemiological studies investigating the causes of Parkinson’s disease. We decided to overcome this difficulty by applying precisely defined diagnostic criteria in our selection of cases.

A number of sets of diagnostic criteria have been proposed, including presence of at least two “cardinal signs”, Parkinson’s disease Staging Scale and the UK Parkinson’s Disease Society Brain Bank. Drawing on these and other sources we produced a Clinical Record Sheet to be used by two of us (DN & PP) to record the absence or presence of defined Cardinal Features, Supportive Criteria and Exclusion Criteria in patients presenting at their Movement Disorder Clinic on study days. This was completed in the presence of the patient following, rather than during, their usual questioning and examination. This allowed consistency and transparency of allocation of men to the Parkinson’s disease group without interrupting the flow of the consultation.

This Clinical Record also had the potential to provide a record of the clinical history, findings and diagnosis for controls, whether they be the other men who had attended the clinic but were found not to have Parkinson’s disease or a completely separate group. A copy of the Clinical Record Sheet can be found in Appendix 1.

The second research tool developed was the Occupational History Record. This took the form of the template for a structured interview of each subject which could be undertaken by a trained Research Assistant and reduce the costs of a main study. A copy of the Occupational History Record can be found in Appendix 2.

Objective 2: Data collection

In the British National Health Service, Consultants (the senior specialists) in each clinical specialty hold clinics, usually lasting half a day, which are attended by appointment by patients referred by their family general practitioner or perhaps another hospital doctor for diagnosis and/or advice on management or treatment. The study was conducted at the Movement Disorder Clinics (held by DN & PP).

It had been intended that, to ensure that each patient had time to consider their decision to participate, the study would be explained to the patient and their consent obtained at one clinic and the Occupational History Record interview conducted when they next attended. It was decided by the team that separation

of explanation and obtaining consent by a month or more was not sensible and moreover would pose serious practical difficulties. The postal information letter procedure outlined below was substituted.

All males due to attend that clinic were identified each week the study was undertaken and a nominal list prepared on a data base. Access to this and all other study documents was restricted to the four named investigators. An information letter was individualized for each patient by name and appointment date, a clinician member of study team and sent to the patient at his home address. In this the clinician asked the patients to consider taking part in a brief occupational history interview with a psychologist immediately following their next consultant appointment.

Enclosed with it was a further letter, from the chief investigator, setting out details of the study and what would be required of participants, a consent form, and proof of ethical approval. A copy of the Information Letter and Consent Form can be found in Appendices 3 & 4 respectively. Patients were asked to contact the chief investigator or their consultant neurologist with any questions or concerns they had. A contact telephone number was provided and CJ took the calls or responded to voice-mail messages daily. Patients were also requested to bring their consent form with them on the day of their appointment for completion by them and the interviewing psychologist.

At the clinic each patient had a clinical consultation with the consultant neurologist. He completed the study Clinical Record in respect of those he diagnosed as having Parkinson's disease and then discussed the study with the patient, encouraging him to be interviewed by the chief investigator (CJ) and, when practicable, made that introduction.

These interviews were conducted between January and September 2008. They were held in private rooms in the Movement Disorders Clinic. At the outset he checked that the Clinical Record showed that the subject met the prescribed diagnostic criteria then provided the patient with the information already given in the letter sent to their home and responded to questions about this as necessary. The patient, now a potential subject, was shown an example of both data collection sheets and that the top section containing their personal identification details would be cut off and thus physically separated from all the other information which had been recorded. It was then emphasised to them that the risk of a breach of confidentiality would be negligible as confidential information could be linked to them only by the study number, only the chief investigator would hold the list which allowed this to be done and he would prepare that list personally before destroying the cut off top portion of the form. The patient was then asked to sign the consent form to signify having given informed consent for participation.

Using a standardised procedure and the Occupational History Record form as a template, he gathered from the patient details of demography, work history, diet, and lifestyle. If participants confirmed they had been employed in jobs involving manganese exposures, further questions were asked about such jobs.

For welders this would include details such as welding type, ventilation, and exposure details. Data was made anonymous with no personal or identifying details retained, and was stored on a secure and encrypted hard disc, only accessible through password.

Objective 3: Identification of potential controls

Three sources of future controls were explored. The first was those patients who attended that clinic or another specialty clinic and were not found to have Parkinson's disease. The second was to ask each subject at the end of the Occupational History questioning if they would be willing to recruit a male friend of similar age as a control. The third was to seek to recruit the cooperation of the patient's general practitioner to identify another patient who met control criteria to match with our already recruited subject.

Objective 4: Literature review

The literature review initiated prior to the study was continued throughout this feasibility phase and to date. It formed the basis of a paper published in Toxicological Review in 2006 [2].

Statistical analysis

All data obtained from the occupational history questionnaires and the clinical record sheets was entered into SPSS v16 for descriptive and inferential analyses. Analysis of Variance and T-tests were used to make comparison between patients with and without manganese or welding exposures. For categorical comparisons between patient groups, Chi square analysis was made, using Yates's correction for continuity of small samples.

Results

Gaining ethical approval

Delay in gaining ethical approval from USMRMC was engendered by the University not having a current and updated Federal Wide Assurance cover (reference no FWA00008367) which had to be updated by the University. This was eventually re-established and was put in place until renewal would be required again by 30th May 2010.

An additional delay was incurred by the University not having current and updated OHRP Human Subjects Assurance Training certificates for each of the investigators in the study – as this is not standard requirement of researchers in the UK. This was established for each member of the research team as soon as possible.

Provision of information and obtaining consent

All patients attending for interview were found to have received the invitation and information letter sent to their home. In most cases there proved to be a need to revisit the explanation to be confident that they understood the study objectives and methods, and were thus equipped to make an informed decision about participating. All subjects interviewed were content with arrangements for the study, including those to safeguard the confidentiality of information, and gave their informed consent.

Data collection tools

The clinicians found the Clinical Record sheet proved to be easy to use in a clinical setting to provide a permanent record of the presence or absence of pre-defined diagnostic features of Parkinson's disease. They considered that completing it did not add significantly to the duration of the consultation.

It also allowed the clinical findings to be compared to the diagnostic criteria set for the study (defined on the form as an aide memoir) and thus identify the patients with confirmed Parkinson's disease. The Occupational History interviewer did not need any clinical knowledge to check that the patient sent to him satisfied the diagnostic criteria. He needed only to check that the *number* of features marked as present met the numerical criteria for that section of the form.

The Occupational History Record proved to be a useful template to ensure disciplined and thus consistent structure for the interview and to guide the questioning to cover all possible exposures relevant to the interviewee, ensuring a comprehensive occupational history. Interviews lasted between 5 and 20 minutes, depending on the answers given by participants. There was noteworthy advantage in having the man's wife in attendance as, although the interview took longer, the women proved to have better memories than their husbands and provided much additional useful information about their employment. The standardised structure of the Occupational Health Record appeared to be suitable for use by a trained Research Assistant without a clinical background.

Data analysis

Fifteen patients with confirmed Parkinson's disease were identified using the Clinical Record. Three records were destroyed in a domestic accident before they could be added to the database. Thus 12 records were available for analysis.

The mean age of participants was 70 years $\pm$ 10.8, with the youngest being 50 and the oldest being 85. The mean age upon diagnosis of PD for the participants was 62.9 years $\pm$ 13.7, with the youngest being 37 and the oldest being 78. At the point of interview, the mean time elapsed since diagnosis was made was 7.4 years $\pm$ 9.2, with the shortest time being 1 year and the longest time being 31 years.

The first hypothesis to be tested using this limited data set was that a significantly higher proportion of those patients diagnosed as having undisputed Parkinson's disease had been welders of steel, or otherwise exposed to manganese-containing fumes. It was found that none of the men with the disease had worked as welders, although 2 (16%) had worked in jobs involving some occasional welding; one as an apprentice engineer with some welding work for 4 years and the other as a central lathe turner with occasional welding for 50 years. A third participant had worked as a car mechanic for 44 years and divulged that he frequently worked in the presence of colleagues who welded. These three participants who confirmed they had workplace welding exposures had a mean exposure period of 32.6 years $\pm$ 25.

The second hypothesis to be tested was that within those diagnosed as having Parkinson's disease the age of onset is lower among those who have been occupationally exposed to manganese. Inferential analysis showed that the mean age of Parkinson's disease diagnosis for welding-exposed participants (n=3) was 64 years $\pm$ 14.5, compared with 62.5 years $\pm$ 14.3 for non welding-exposed participants (n=9), although this was not significantly different (F=0.02, P=0.88). The mean years elapsed since Parkinson's disease was diagnosed was 2.3 years $\pm$ 1.5 for the three welding-exposed participants compared with 9.1 years $\pm$ 10.1 for the nine non-welding-exposed participants (n=9). This difference is not statistically significant different (F=1.23, P=0.29).

This analysis was repeated in respect of the two men who had worked as welders. The mean age of PD diagnosis for them was 63.5 years $\pm$ 20.5 compared with 62.8 years $\pm$ 13.5 for those ten men who had not been welders. The difference in age of diagnosis is not significantly different (F=0.00, P=0.95). The mean years elapsed since PD diagnosis for participants who worked as welders was 1.5 years $\pm$ 0.7, compared with 8.6 years $\pm$ 9.7 for the others. Again, this difference is not significantly different (F=0.98, P=0.34).

Selection of controls

All participants said that they would be willing to try to recruit a friend of a similar age to act as a control. It was observed that a substantial number of men referred to the clinic were not diagnosed as having Parkinson's disease.

Literature review

Three well-designed and implemented studies of employment as a welder being a risk factor for Parkinson's disease published since we embarked on our investigation have been identified in the literature. The earliest of these, published in 2007, identified 767 cases of the disease in five European countries [3]. A standard definition was used. No association suggestive of a causative role was reported with regard to exposure to copper, iron or manganese exposure. In 2009 Stampfer reported on a study examining mortality from Parkinson's disease and other neurodegenerative diseases in 107,773 men in the United States who had had welding-related occupations [4]. After sophisticated analysis (adjusting for attained age, race, place of residence and year of death) the data did not support an association between welding occupations and death from Parkinson's disease or other neurodegenerative disease, nor that welders are at increased risk of dying from Parkinson's disease at a younger age. Also in 2009, Tanner and colleagues reported on their case-control study of the risk of Parkinsonism in occupations including welding, specific job tasks and toxicant exposures putatively associated with parkinsonism [5]. This was conducted in eight movement disorder centres in North America. Having analysed findings in 519 cases and 511 controls they found that welding was not associated with increased risk of Parkinsonism or with younger age at diagnosis.

Discussion

During this study we have developed two research tools and gained experience in arranging ethical approval to meet the needs of authorities in UK and USA. It has proved that the methods we have devised would make it be feasible for us to recruit subjects and use National Health Service clinics and the two research tools to conduct an epidemiologically sound, ethically approved study of the incidence and age of onset of confirmed cases of Parkinson's disease among those with a history of work of occupational exposure or no occupational exposure to welding fumes.

The small number of Occupational History interview records completed was sufficient to test our methods and identify how these should be improved by ensuring the availability of an interviewer at all the clinic sessions; this had not been possible during this study. In a main study we would employ and train a Research Assistant to attend every Movement Disorder Clinic held by the clinicians in the team, organise patient attendance, provide information and obtain consent, and conduct the structured interview to obtain the Occupational History. Whereas sufficient cases were studied to test our methods there were too few to test the hypotheses rigorously. That, however, is the primary task for the main study should this be done. Analysis of data from the 12 attendees did not support the hypothesis that there is a causal association between work as a welder or exposure to welding fumes and the development of Parkinson's disease prematurely or at all. Selection of controls for a large study will prove challenging. We had considered asking the general practitioner of each patient with confirmed Parkinson's disease to match him with another of his patients and invite him to permit his contact details to be provided to the study team. This method would have to be covered by an umbrella ethical approval - which should not present a problem. We have been advised, however, that we would be expected to cover the GP's costs and that these might prove to be appreciable. It would also make the study's administration more labour intensive. Having considered these factors we have chosen not to include that method in the design of the main study. We are attracted to recruiting as controls the patients attending the Movement Disorder Clinic who are not found to have Parkinson's disease and secondly patients attending a hospital clinic for a condition which is not neurological. This would be entirely feasible.

Moving to the literature review, we embarked on this study because, while no convincing evidence to support the hypothesis that either exposure to manganese or employment as a welder causes or is a risk factor for causation of Parkinson's disease had emerged from a large body of epidemiologic and clinico-pathological investigations of widely varying quality, we could not easily dismiss welding fume as a possible risk factor which might facilitate the onset of the disease.² As reported above, that is no longer the situation as we have identified in the literature review three sound studies where investigators have sought and failed to find evidence to support the contentions that welding is a causal factor for Parkinson's disease or of its premature onset.

Conclusions

We conclude that:

- This study has provided two useful research tools and statistical indications of the absence of evidence of risk of Parkinson's disease being caused or accelerated by exposure to welding.
- We have shown that it would be feasible to conduct the main phase of this study as proposed over a period of a further three years providing sufficient funds were available over and above the previous funding application to allow us to employ at least one full-time Research Assistant.
- We have shown through the literature review that the knowledge we sought to acquire in the proposed main phase of the study, has been provided satisfactorily by others since this feasibility study started. In consequence of this, no matter how feasible it may be, we cannot now justify the cost and time required to undertake the previously proposed main phase.

References

1. Racette BA, McGee-Minnich L, Moerlein SM, Mink JW, Videen RO, Perlmutter JS. Welding-related parkinsonism: Clinical features, treatment, and pathophysiology. *Neurology*, 56, 8-13, 2001.
2. McMillan G. Is electric arc welding linked to manganism or Parkinson's disease? *Toxicol Rev* 2006;24(4):237-57.
3. Dick FD, De Palma G, Ahmadi A, Scott NW, Prescott GJ, Bennett J, Semple S, Dick S, Counsell C, Mozzoni P, Haites N, Wettinger SB, Mutti A, Otelea M, Seaton A, Söderkvist P, Felice A; Geoparkinson study group. Environmental risk factors for Parkinson's disease and parkinsonism: the Geoparkinson study. *Occup Environ Med*. 2007 Oct;64(10):666-72.
4. Stampfer MJ. Welding occupations and mortality from Parkinson's disease and other neurodegenerative diseases among United States men, 1985-1999. *J Occup Environ Hyg*. 2009 May;6(5):267-72.
5. Tanner CM, Ross GW, Jewell SA, Hauser RA, Jankovic J, Factor SA, Bressman S, Deligtisch A, Marras C, Lyons KE, Bhudhikanok GS, Roucoux DF, Meng C, Abbott RD, Langston JW. Occupation and risk of parkinsonism: a multicenter case-control study. *Arch Neurol*. 2009 Sep;66(9):1106-13.

Appendices

Appendix 1	Clinical Data Sheet
Appendix 1	Occupational History Questionnaire (talked-through version)
Appendix 1	Patient Information Sheet accompanying their appointment letter
Appendix 1	Consent Form

Appendix 1



Surname Initials¹

D.O.B
/ /

Hospital no.

Survey no.

Cut here-----

CONFIDENTIAL ¹		
SURVEY FORM 1: POTENTIAL CASE CLINICAL RECORD SHEET ²		
Examined by:	Grade:	Date / /
Please tick box against features found / not found on history / examination.	Y	N
Cardinal features (Must have first one + at least one other)		
Slowness of movement (bradykinesia)		
Stiffness (muscular rigidity)		
Rest tremor (4-6Hz)		
Postural instability ³		
Supportive criteria (Three or more required)		
Unilateral onset		
Rest tremor present		
Progressive disorder		
Persistent asymmetry affecting side of onset most		
Excellent response to levodopa		
Severe levodopa-induced chorea		
Levodopa response for over 5 years		
Clinical course of over 10 years		
Exclusion criteria		
Repeated strokes with stepwise progression		
Repeated head injury		
Anti-psychotic or dopamine depleting drug		
Definite encephalitis and/or oculogyric crisis on no drug treatment		
More than one affected relative		
Sustained remission		
Negative response to large doses of levodopa ⁴		
Strictly unilateral features after 3 years		
Other neurological features. Circle on list below. ⁵		
Presence of cerebral tumour or communicating hydrocephalus on neuroimaging		
Exposure to MPTP		
ACCEPTED as a case? (To be completed by Dr Jackson)		
Date of Clinical Diagnosis / /		
Date of Form Completion / /		

¹ Once completed in full or part this form is available only to survey staff as per protocol.

² Based on Parkinson's Disease. National clinical guideline for diagnosis and management in primary and secondary care. RCP London 2006.

³ Unrelated to primary visual, cerebellar, vestibular or proprioceptive dysfunction.

⁴ If malabsorption excluded.

⁵ Supranuclear gaze palsy, cerebellar signs, early severe autonomic involvement, Babinski sign, early severe dementia with disturbance of language, memory or praxis.



UNIVERSITY OF
BIRMINGHAM

Parkinson's Disease & Manganese Project Occupational History Interview

Private and Confidential

For Medical Research Only

Dr Grant McMillan
Honorary Clinical Senior Lecturer
Institute of Occupational and Environmental Medicine
University of Birmingham

Dr C.A. Jackson
Honorary Senior Lecturer in Occupational Psychology
Institute of Occupational and Environmental Medicine
University of Birmingham

February 2006

A. Date of Birth _____ / _____ / _____

B. Sex *Male / Female*

C. Have you ever been employed in any of the following occupations or worked in any factory concerned with these jobs or processes?

- | | |
|---|----------|
| 1. The manufacture of rubber and rubber products | Yes / No |
| 2. Cable manufacturing | Yes / No |
| 3. The manufacture of dyes and dyestuffs | Yes / No |
| 4. Manufacture and professional use of solvents | Yes / No |
| 5. Leather work | Yes / No |
| 6. Welding of metals | Yes / No |
| 7. Manufacture or professional use of paints | Yes / No |
| 8. Gasworks and coke ovens | Yes / No |
| 9. Rodent or pest extermination | Yes / No |
| 10. Sewage works | Yes / No |
| 11. Laboratory technician | Yes / No |
| 12. Medicine or Nursing | Yes / No |
| 13. Textile printing and dyeing | Yes / No |
| 14. Manufacture of plastics | Yes / No |
| 15. Hairdressing or Beauty therapy | Yes / No |
| 16. Metal casting | Yes / No |
| 17. Printing | Yes / No |
| 18. Metal smelting | Yes / No |
| 19. Professional use or manufacture of pesticides | Yes / No |

D. Please give details if you have answered "Yes" to any part of "C". Give the name of the firm, describe the work you carried out and give the years you worked.

DATES

NAME OF FIRM	JOB TITLE	JOB DESCRIPTION	FROM	TO
.....				
.....				
.....				

E. We are interested in any metal welding work you may have done.

1. What was your job title? _____

2. How many hours a week were you working as a welder? _____

3. How many years in total did you work as a welder for? _____

4. What type of metal were you welding?

Stainless Steel (____ %) *Mild Steel* (____ %) *Other* (____ %)

5. Can you remember what type of electrode you used most? _____

6. What type of welding technique did you use?

Manual Metal Arc (MMA) (____ %)

Tungsten Inert Gas (TIG) (____ %)

Metal Inert Gas (MIG) (____ %)

7. What kind of engineering control was used?

General Ventilation (____ %)

Local Exhaust Ventilation (LEV) (____ %)

Only Natural Ventilation (____ %)

8. If LEV was used, how far was the hood from the welding area? _____

9. Did you wear a welding visor when welding? Yes (____ %) No

10. What immediate surroundings were you in mostly when welding?

Indoor (____ %)

Outdoor (____ %)

Enclosed space (e.g. inside a tank) (____ %)

11. When welding, were you near other welders? Yes (____ %) No

12. Over an average 8-hour shift, how much time did you spending arcing ____ hrs

F. We are interested in your diet and smoking habits

1. Have you ever smoked cigarettes regularly (at least once a week)? Yes / No
2. Which year did you start smoking regularly? _____
3. Number smoked per day
Less than 10/day ☐ 10-20/day ☐ More than 20/day ☐
4. Which year did you stop smoking regularly? _____
5. How many years have you smoked for? _____
6. Which of the following have you eaten regularly (at least once a week)?
Spinach ☐ Lentils ☐ Liver ☐ Poultry ☐
Whole grains ☐ Cereals ☐ Red meat ☐
7. Do you currently take daily vitamin supplements? Yes / No
8. Which vitamins? _____
9. When did you start taking daily vitamins? _____
10. Have you ever taken any of the following substances?
"Angel dust" ☐ "Ozone" ☐ "Wack" ☐ "Rocket fuel" ☐
"Killer joints" ☐ "Crystal supergrass" ☐ PCP ☐ Phencyclidine ☐

G. Finally, we would ask for some extra information about you.

1. What is your marital status? _____
2. How many children do you have? _____
3. What is the highest education achievement you have made?
School-leaving exams ☐ O-levels ☐ A-levels ☐ Diplomas ☐
University degree ☐ Certificates ☐ Post graduate degree ☐

Thank you for your time and cooperation



UNIVERSITY OF
BIRMINGHAM

Parkinson's Disease & Manganese Project

Information Sheet

Dear Sir

As you are being offered an appointment at the Movement Disorders Clinic in the Department of Neurology at Queen Elizabeth Hospital Birmingham, we are passing this information sheet to you to ask you to consider taking part in a small study concerning the development of Parkinson's Disease. We are approaching everyone who is offered an appointment in the department, so please do not feel you have been "singled out". We wish to investigate the possible link between Parkinson's Disease and some occupations, especially jobs involving welding work. We hope to publish the results of this study in a medical journal for other health professionals to read – your personal details will never be disclosed.

We would like to invite you to speak to one of our research team for 10-15 minutes when you are in the department for your next appointment. This would be for a brief and confidential interview in a private room where we would like to ask you some questions about any jobs you may have had in the past. That is all that will be required of you, and you will not be approached again for any other details. In your appointment, your neurologist will perform three brief clinical evaluations of your movements – which will take only a few moments. Everything will be kept confidential, and we will not even need to know your name. **We are interested in hearing about any occupations, but we are especially interested in speaking to people who have worked as welders of metal.** Even if you have not "worked" as a welder, but have done some welding work in the past, we would still be keen to hear from you.

It is important for you to know that taking part is entirely voluntary and if you decide not to take part, your appointment and treatment will not be altered in any way. If you do not wish to take part, you need do nothing further and we will not trouble you again.

If you feel that you would like to take part in this research study, please return the consent form that is printed on the reverse of this letter, and return it to the address provided on the freepost envelope. If you would like to speak to the research team with any questions you may have, please feel free to call on **0121 331 5338** and the lead researcher will be happy to speak to you.

Yours faithfully

Dr Craig. A. Jackson BSc MSc PhD C.Psych
Honorary Senior Lecturer in Occupational Psychology
Institute of Occupational and Environmental Medicine
University of Birmingham, Edgbaston. B15 2TT

Appendix 4



UNIVERSITY OF
BIRMINGHAM

Parkinson's Disease & Manganese Project

Consent Form

Please read below and tick the necessary boxes if you wish to participate in the study

"I confirm that I have read and understood the participant information sheet for this study, and I have had the opportunity to ask questions."

☐

"I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason, and without my medical and legal rights being affected."

☐

"I agree to take part in the above study."

☐

Name of participant

Date

Signature

Name of researcher

Date

Signature

1 copy for participant
1 copy for researcher
1 copy for hospital notes

AWARD NUMBER: W81XWH-05-1-0239

TITLE: ROLE OF MANGANESE IN PRION DISEASE PATHOGENESIS

PRINCIPAL INVESTIGATOR: Anumantha Kanthasamy, Ph.D.

CONTRACTING ORGANIZATION: Iowa State University, Ames, IA 50011

REPORT DATE: January 2011

TYPE OF REPORT: Final report

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: (Check one)

- ☒ Approved for public release; distribution unlimited
- ☐ Distribution limited to U.S. Government agencies only;
report contains proprietary information

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

REPORT DOCUMENTATION PAGE			Form Approved OMB No. 0704-0188		
Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Department of Defense, Washington Headquarters Services, Directorate for Information Operations and Reports (0704-0188), 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302. Respondents should be aware that notwithstanding any other provision of law, no person shall be subject to any penalty for failing to comply with a collection of information if it does not display a currently valid OMB control number. PLEASE DO NOT RETURN YOUR FORM TO THE ABOVE ADDRESS.					
1. REPORT DATE (DD-MM-YYYY) 02/13-2008		2. REPORT TYPE Final Report		3. DATES COVERED (From - To) 04/15/2008-01/31/2010	
4. TITLE AND SUBTITLE Manganese-Induced Upregulation of Prion Proteins and its Relevance to Prion Diseases			5a. CONTRACT NUMBER W81XWH-05-1-0239		
			5b. GRANT NUMBER		
			5c. PROGRAM ELEMENT NUMBER		
6. AUTHOR(S) Anumantha G Kanthasamy, PhD			5d. PROJECT NUMBER		
			5e. TASK NUMBER		
			5f. WORK UNIT NUMBER		
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Iowa State University Ames, IA 50011			8. PERFORMING ORGANIZATION REPORT NUMBER		
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) Iowa State University Ames, IA 50011			10. SPONSOR/MONITOR'S ACRONYM(S) ISU		
			11. SPONSOR/MONITOR'S REPORT NUMBER(S)		
12. DISTRIBUTION / AVAILABILITY STATEMENT					
13. SUPPLEMENTARY NOTES Not applicable					
14. ABSTRACT Prion disease is a devastating and fatal neurodegenerative disorder in animals and humans. Unlike conventional infectious diseases, prion diseases are caused by an abnormally folded host-encoded prion protein that accumulates in the central nervous system. Prion diseases result from misfolding of normal cellular prion (PrP ^C) into an abnormal form of scrapie prion (PrP ^{Sc}). The cellular mechanisms underlying the misfolding of PrP ^C are not well understood. Emerging studies have shown that prion proteins contain octapeptide-repeat regions that bind to several divalent metals, including manganese (Mn) and copper (Cu), and that the metal binding may influence the conformation and metabolism of prion proteins. Therefore, the long term objective of our project is to determine whether the divalent metal Mn plays any role in the pathogenesis of prion diseases. During the previous funding period, we reported that normal prion protein impairs manganese transport and protects the cells from manganese-induced oxidative stress, mitochondrial dysfunction, cellular antioxidant depletion, and apoptosis. We also reported that Mn treatment results in increased prion protein levels in mouse neuronal cells and in mouse brain slice cultures by upregulating and stabilizing prion protein in a time-dependent manner. In order to test the specificity of Mn-induced prion protein upregulation, another divalent metal cadmium (Cd2+) was used. Cd treatment did not upregulate or stabilize the prion protein as Mn did. Manganese-induced PrP ^C upregulation was independent of mRNA transcription or stability. Additionally, manganese treatment did not alter the PrP ^C degradation by either proteasomal or lysosomal pathways. Limited proteolysis studies with proteinase-K further demonstrated that manganese increases the stability of PrP ^C . Furthermore, manganese exposure to an infectious prion cell model, mouse RML-infected CAD5 cells, significantly increased prion protein levels. Collectively, our results demonstrate for the first time that the divalent metal manganese can alter the stability of prion proteins in both normal and infectious prion models and suggest that manganese-induced stabilization of prion protein may play a role in prion protein misfolding and prion disease pathogenesis.					
15. SUBJECT TERMS Manganese, neurotoxicology, prion diseases, oxidative stress, and protein aggregation					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT UU	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON Anumantha G. Kanthasamy, PhD
a. REPORT U	b. ABSTRACT U	c. THIS PAGE U			19b. TELEPHONE NUMBER (include area code) (515) 294-2516

Table of Contents

COVER.....	1
SF 298.....	2
Table of Contents.....	3
Introduction.....	4
Body	5
Conclusions.....	5
Key Research Accomplishments.....	6
Reportable Outcomes.....	6-7
References.....	8

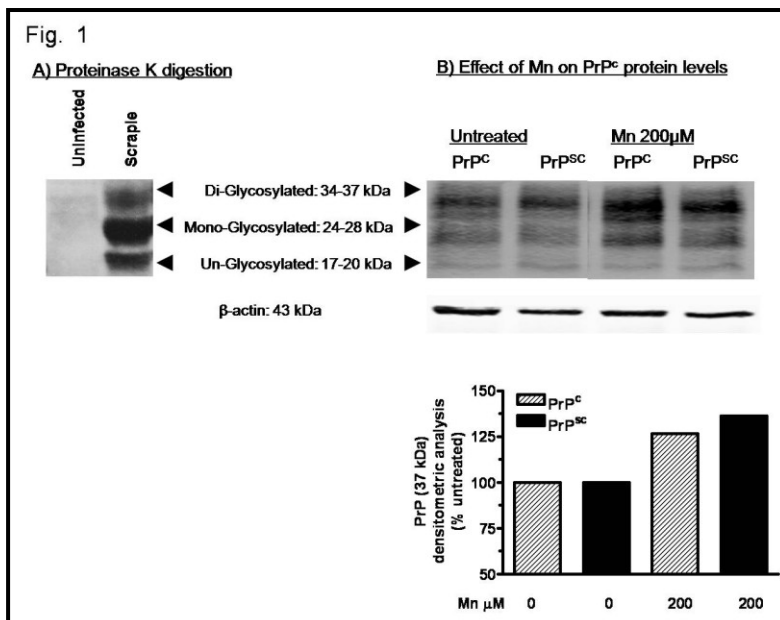
Introduction

Prion diseases are severe neurodegenerative diseases affecting animals and humans. The major pathophysiological change associated with this devastating disease is the aberrant processing of normal cellular prion protein (PrP^C) into the pathological form (PrP^{Sc}) (Prusiner, 1982; Bae et al., 2009). PrP^C is highly conserved in mammals and is expressed predominantly in the brain. The biological function of the normal prion protein in the central nervous system has not been fully elucidated, but studies have suggested that the prion protein can function as a metal binding protein, an antioxidant, a cellular adhesion molecule and a signal transducer (Schmitt-Ulms et al., 2001; Chiarini et al., 2002; Nishimura et al., 2004; Mouillet-Richard et al., 2005). The four-six octapeptide repeat sequences toward the N-terminus of the protein can bind to divalent cations including copper, zinc, and manganese, with varying degrees of affinity (Hornshaw et al., 1995; Brown et al., 1997; Viles et al., 1999; Garnett and Viles, 2003). Also, the brains of prion knockout mice had lower concentrations of these metals than the brains of normal mice (Brown et al., 2002). Additional studies have shown that altered Mn content was observed in prion diseases including the human prion disease known as Cruetzfeldt-Jacob Disease (CJD) (Wong et al., 2001). The pathological form of prion protein PrP^{Sc} tends to aggregate into plaques, which are highly resistant to digestion with proteinase K (Hay et al., 1987). Binding of Mn to the normal prion protein has been suggested to result in partial resistance to protease digestion and possibly conformational changes of the infectious PrP^{Sc} (Brown et al., 2000). Also, chronic Mn exposure may result in altered manganese binding to PrP^C and may increase the likelihood of conversion of PrP^C to the proteinase-resistant PrP^{Sc} (Choi et al., 2006; Choi et al., 2007). Recently, we reported that normal prion protein impairs manganese transport and protects the cells from manganese-induced oxidative stress, mitochondrial dysfunction, cellular antioxidant depletion, and apoptosis. We attributed this to normal prion protein binds to manganese by acting as a metal-sink, thereby reducing manganese transport and protecting the cells from manganese-induced neurotoxicity at early stages of exposure (Choi et al. 2007; Choi et al., 2006). However, over time the binding of manganese to prion protein may promote the conversion of normal PrP^C to PrP^{Sc}, which results in the loss of the protective function associated with normal prion protein (Choi et al., 2006; Choi et al., 2007). We also reported that PrP^C protects against apoptotic cell death during oxidative stress but exacerbates apoptosis during endoplasmic reticular stress (Anantharam et al., 2008). While studying the role of prion protein in metal neurotoxicity, we unexpectedly found that manganese exposure upregulated cellular prion protein in neuronal cell models. During the last funding period, we also reported that manganese treatment upregulated cellular prion levels independently of transcription. Manganese also increased the stability of prion protein, as determined by limited proteolysis studies. During the current funding period, we continued to study the interaction of manganese with prion protein. We made several interesting discoveries that are summarized below:

that are summarized below:

Study 1. Manganese induces upregulation of prion protein in a scrapie-infected cell culture model of prion disease

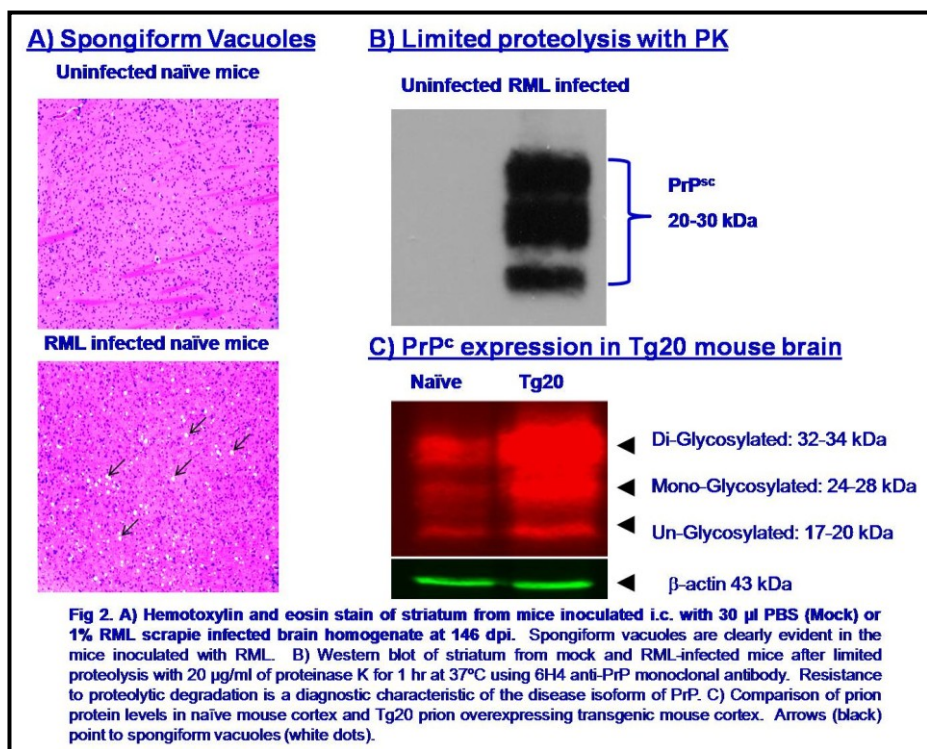
In last year's progress report, we reported that interaction of prion protein with manganese results in increased prion protein levels through decreased protein turnover rates in mouse neural cell lines as well as in mouse brain slices. To extend our research closer to disease pathology, we examined the effect of manganese on prion protein in mock-infected and RML scrapie-infected Cath.A Differentiated (CAD5) mouse neuronal cell lines. We also examined whether Mn is capable of



upregulating PrP levels in the presence of the infectious form of prion protein, PrP^{Sc}. This cell model of infectious prion disease was obtained from Dr. Charles Weissmann at Scripps Institute, Florida, who recently demonstrated that RML-infected CAD5 cells make an excellent cell culture model of infectious prion disease because these cells propagate PrP^{Sc} infection through multiple passages without the need for reinfection (Mahal et al., 2007). We first demonstrated the presence of PK-resistant PrP^{Sc} prion protein in scrapie-infected CAD5 cells by performing a limited proteolysis assay with proteinase K (PK). Prion proteins were stained with 6H4 antibody from uninfected and scrapie-infected CAD5 cells and then the proteolytic susceptibility was monitored. As shown in **Fig 1A**, PK resistant PrP^{Sc} protein was present in scrapie-infected CAD5 cells but not in uninfected CAD5 cells. RML scrapie-infected and uninfected CAD5 cells were exposed to 200 μ M Mn for 12 h. Cells were harvested and subjected to Western blot analysis for PrP protein expression levels. As shown in **Fig 1B**, manganese induced similar increases in PrP protein levels in both uninfected and RML scrapie-infected CAD5 cells, suggesting that manganese treatment can upregulate PrP expression levels even during the progression of prion disease in infected cell culture models.

Study 2. Animal models of prion diseases: PK-resistant PrP^C and neuropathology

In order to study the role of manganese in animal models of prion disease, we recently established the C57 black mouse model of prion disease, which has become a classic model of prion disease and is used extensively by prion researchers throughout the world. C57 black mice were infected with 1% RML mouse scrapie by intracerebraventricular injection and the disease progression was followed for 146 days post-infection. Brains were harvested and subjected to histological staining with hematoxylin/eosin to determine spongiform vacuoles and limited proteolysis experiments were performed to determine the levels of PrP^{Sc} (infectious form of PrP^C). Microscopic examination



expression in Tg20 prion overexpressing transgenic mouse brain. As shown in Fig. 2C, Western blot analysis revealed almost 6-fold higher PrP^C levels in brain tissues of Tg20 prion overexpressing mice compared to normal mice.

Conclusions: Since the normal cellular form of prion protein PrP^C serves as a seed for conversion of the diseased form of prion protein, PrP^{Sc}, manganese-induced upregulation of PrP^C could provide more substrate for spontaneous conversion of PrP^C into PrP^{Sc}. The structural changes within the protein induced by manganese binding to the octapeptide repeats may explain the altered PK resistance and increased stability observed in this study. Whether manganese exposure alone can

cause prion pathology remains to be investigated. However, the presence of higher levels of PK-resistant nonpathogenic prion protein can serve as a seed for PrP^C conversion to pathogenic PrP^{Sc}, resulting in the acceleration of the prion disease progression. We have also established the animal models of prion disease in our laboratory. Next we propose to evaluate the effect of manganese exposure on prion protein expression, PrP^{Sc} formation and spongiform degeneration in these mouse models of prion diseases by using molecular, cellular, and neurochemical approaches. Together, this study points to the possibility of intracellular manganese impacting the availability of the PrP^C substrate for conversion to PrP^{Sc}, and thereby contributing to the pathogenesis of prion diseases. Understanding the role of prion protein in manganese neurotoxicity will provide new insights into manganese neurotoxicity as well as the pathogenesis of prion diseases.

Data analysis and statistics: Data were analyzed with Prism 4.0 software (GraphPad Software, San Diego, CA). Bonferroni's post-hoc multiple comparison testing was used to delineate significant differences between treatment groups. For densitometric analysis of limited proteolysis, band intensity was normalized to control bands at 0 min. $p < 0.05$ was considered significant and differences are indicated with asterisks.

Key Research Accomplishments

- Established an infectious prion cell culture model, which retains the infection for a longer period of time.
- Manganese-induced toxicity in the scrapie-infected CAD5^{Sc} cell model of prion disease but the toxicity was less than the toxicity induced in uninfected cells.
- Manganese treatment upregulated PrP expression levels even during the progression of prion disease in infected cell culture models.
- Established the RML-scrapie infected mouse model of prion disease for manganese neurotoxicity studies. Limited proteolysis with 20 µg/ml proteinase K, revealed high levels of protease resistant PrP^{Sc} in RML-infected mouse brain compared to uninfected mouse brain.
- Microscopic examination revealed extensive spongiform neurodegeneration in different RML scrapie-infected brain regions in hematoxylin/eosin stained sections.
- Tg20 prion overexpressing transgenic mouse brain expressed almost 6-fold higher PrP^C levels in brain tissues compared to normal mice.

Reportable Outcomes

Manuscripts/Abstracts

Choi CJ, Anantharam V, Martin DP, Nicholson EM, Richt JA, Kanthasamy A, Kanthasamy AG. (2010) Manganese upregulates cellular prion protein and contributes to altered stabilization and proteolysis: relevance to role of metals in pathogenesis of prion disease. *Toxicol Sci.* 115(2):535-46.

Choi, C.J., Kanthasamy, A., Anantharam, V., Kanthasamy, A.G. (2006), Interaction of metals with prion protein: possible role of divalent cations in the pathogenesis of prion diseases. *Neurotoxicol.*, 27: 777-87.

Choi, C.J., Anantharam, V., Saetveit, N.J., Houk, R.S., Kanthasamy, A., Kanthasamy AG. (2007), Normal cellular prion protein protects against manganese-induced oxidative stress and apoptotic cell death. *Toxicol. Sci.*, 98: 495-509.

Choi C. J., Anantharam, V., Kanthasamy, A. and Kanthasamy, A.G. (2008) Cadmium, not Manganese, impairs Neuronal Proteasomal Systems Leading to Formation of Protein Aggregation and Ubiquitinated Prion Proteins. *Manuscript in preparation.*

Choi, C.J., Anantharam, V., Kanthasamy, A., & Kanthasamy, (2005). Neurotoxic Effect of Manganese and Neuroprotective Effect of Copper in a Cell Culture Model of Prion Diseases. A.G. Abstract No. 633. 44th Annual Meeting of Society of Toxicology, March 6-10, 2005, New Orleans, LA.

Choi, C.J., Anantharam, V., Kanthasamy, A., & Kanthasamy, A.G. (2005). Effect of Prion Protein on Manganese-induced oxidative insult and mitochondrial Dysfunction. Abstract No. P101. Environment and Neurodevelopmental Disorders, Neurotoxicology Conference Series, September 11-14, 2005, Research Triangle Park, NC.

Choi, C.J., Anantharam, V., Kanthasamy, A., & Kanthasamy, A.G. (2006). Effect of Divalent Metals Manganese and Cadmium on Ubiquitin-Proteasome Function and Protein Aggregation in Cell Culture Models of Prion Disease: Possible Role of Metals in Prion Disease Pathogenesis. 45th Annual Meeting of Society of Toxicology, March 5-9, 2006, San Diego, CA.

Choi C. J., Anantharam, V., Nicholson, E.M., Richt, J.A, Kanthasamy, A. and Kanthasamy, A.G (2006). Manganese upregulates cellular Prion proteins and inhibits the rate of Proteinase-K dependent limited Proteolysis in Neuronal cells. 36th Annual Meeting of the Society for Neuroscience, Oct. 14-18, 2006, Atlanta, GA.

Kanthasamy, A.G., Choi, C.J., Anantharam, V and Kanthasamy, A (2007) Manganese Exposure Enhances Prion Protein Turnover and Proteinase-K Resistance: Possible Role in Pathogenesis of Prion Diseases. SETAC Europe 17th Annual Meeting, 20 - 24 May 2007, Porto, Portugal.

Invited talk:

A.G. Kanthasamy, Role of Metals in Prion Protein Upregulation and Aggregation, presented at the 26th Neurotoxicology Meeting 26th International Neurotoxicology Conference held June 6-10, 2010 in Portland.

Grants:

Role of Prion Protein in Manganese Neurotoxicity; Principal Investigator: Anumantha Kanthasamy, Ph.D.; RO1 ES019267-01, NIEHS, 07/10 to 06/15

The overall goal of this project is to determine the effect of manganese on prion protein upregulation and stabilization in cell culture and animal models and to examine the prion like neurodegenerative mechanisms in metal neurotoxicity.

Current faculty receiving support from the grant: All the funding was used up 01/31/10. The following faculty received support in 2009.

- Anumantha G. Kanthasamy, Ph.D.
- Vellareddy Anantharam, Ph.D.
- Arthi Kanthasamy, Ph.D.

References:

- Anantharam V, Kanthasamy A, Choi CJ, Martin DP, Latchoumycandane C, Richt JA, Kanthasamy AG (2008) Opposing roles of prion protein in oxidative stress- and ER stress-induced apoptotic signaling. *Free Radic Biol Med* 45:1530-1541.
- Bae SH, Legname G, Serban A, Prusiner SB, Wright PE, Dyson HJ (2009) Prion proteins with pathogenic and protective mutations show similar structure and dynamics. *Biochemistry* 48:8120-8128.
- Brown DR, Schmidt B, Kretzschmar HA (1997) Effects of oxidative stress on prion protein expression in PC12 cells. *Int J Dev Neurosci* 15:961-972.
- Brown DR, Nicholas RS, Canevari L (2002) Lack of prion protein expression results in a neuronal phenotype sensitive to stress. *J Neurosci Res* 67:211-224.
- Brown DR, Hafiz F, Glasssmith LL, Wong BS, Jones IM, Clive C, Haswell SJ (2000) Consequences of manganese replacement of copper for prion protein function and proteinase resistance. *Embo J* 19:1180-1186.
- Chiarini LB, Freitas AR, Zanata SM, Brentani RR, Martins VR, Linden R (2002) Cellular prion protein transduces neuroprotective signals. *Embo J* 21:3317-3326.
- Choi CJ, Kanthasamy A, Anantharam V, Kanthasamy AG (2006) Interaction of metals with prion protein: possible role of divalent cations in the pathogenesis of prion diseases. *Neurotoxicology* 27:777-787.
- Choi CJ, Anantharam V, Saetveit NJ, Houk RS, Kanthasamy A, Kanthasamy AG (2007) Normal cellular prion protein protects against manganese-induced oxidative stress and apoptotic cell death. *Toxicol Sci* 98:495-509.
- Garnett AP, Viles JH (2003) Copper binding to the octarepeats of the prion protein. Affinity, specificity, folding, and cooperativity: insights from circular dichroism. *J Biol Chem* 278:6795-6802.
- Hay B, Prusiner SB, Lingappa VR (1987) Evidence for a secretory form of the cellular prion protein. *Biochemistry* 26:8110-8115.
- Hornshaw MP, McDermott JR, Candy JM, Lakey JH (1995) Copper binding to the N-terminal tandem repeat region of mammalian and avian prion protein: structural studies using synthetic peptides. *Biochem Biophys Res Commun* 214:993-999.
- Mahal SP, Baker CA, Demczyk CA, Smith EW, Julius C, Weissmann C (2007) Prion strain discrimination in cell culture: the cell panel assay. *Proc Natl Acad Sci U S A* 104:20908-20913.
- Mouillet-Richard S, Pietri M, Schneider B, Vidal C, Mutel V, Launay JM, Kellermann O (2005) Modulation of serotonergic receptor signaling and cross-talk by prion protein. *J Biol Chem* 280:4592-4601.
- Nishimura T, Sakudo A, Nakamura I, Lee DC, Taniuchi Y, Saeki K, Matsumoto Y, Ogawa M, Sakaguchi S, Itohara S, Onodera T (2004) Cellular prion protein regulates intracellular hydrogen peroxide level and prevents copper-induced apoptosis. *Biochem Biophys Res Commun* 323:218-222.
- Prusiner SB (1982) Novel proteinaceous infectious particles cause scrapie. *Science* 216:136-144.
- Schmitt-Ulms G, Legname G, Baldwin MA, Ball HL, Bradon N, Bosque PJ, Crossin KL, Edelman GM, DeArmond SJ, Cohen FE, Prusiner SB (2001) Binding of neural cell adhesion molecules (N-CAMs) to the cellular prion protein. *J Mol Biol* 314:1209-1225.
- Viles JH, Cohen FE, Prusiner SB, Goodin DB, Wright PE, Dyson HJ (1999) Copper binding to the prion protein: structural implications of four identical cooperative binding sites. *Proc Natl Acad Sci U S A* 96:2042-2047.
- Wong BS, Brown DR, Pan T, Whiteman M, Liu T, Bu X, Li R, Gambetti P, Olesik J, Rubenstein R, Sy MS (2001) Oxidative impairment in scrapie-infected mice is associated with brain metals perturbations and altered antioxidant activities. *J Neurochem* 79:689-698.

AWARD NUMBER: W81XWH-05-1-0239

TITLE: Biomarkers of Early Onset of Manganese Neurotoxicities among
Occupationally Exposed Chinese Workers

PRINCIPAL INVESTIGATOR: Wei Zheng, Ph.D.

CONTRACTING ORGANIZATION: Purdue University School of Health Sciences
West Lafayette, IN 47907

REPORT DATE: January 28, 2011

TYPE OF REPORT: Final Report

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: (Check one)

- ☒ Approved for public release; distribution unlimited
- ☐ Distribution limited to U.S. Government agencies only;
report contains proprietary information

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

REPORT DOCUMENTATION PAGE				Form Approved OMB No. 0704-0188	
Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Department of Defense, Washington Headquarters Services, Directorate for Information Operations and Reports (0704-0188), 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302. Respondents should be aware that notwithstanding any other provision of law, no person shall be subject to any penalty for failing to comply with a collection of information if it does not display a currently valid OMB control number. PLEASE DO NOT RETURN YOUR FORM TO THE ABOVE ADDRESS.					
1. REPORT DATE (DD-MM-YYYY) 28-01-2011		2. REPORT TYPE Final Report		3. DATES COVERED (From - To) January - December 2010	
4. TITLE AND SUBTITLE Biomarkers of Early Onset of Manganese Neurotoxicities among Occupationally Exposed Chinese Workers				5a. CONTRACT NUMBER W81XWH-05-1-0239	
				5b. GRANT NUMBER	
				5c. PROGRAM ELEMENT NUMBER	
6. AUTHOR(S) Wei Zheng, PhD				5d. PROJECT NUMBER	
				5e. TASK NUMBER	
				5f. WORK UNIT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Purdue University School of Health Sciences West Lafayette, IN 47907				8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) Vanderbilt University Medical Center Nashville, TN 37203				10. SPONSOR/MONITOR'S ACRONYM(S) VUMC	
				11. SPONSOR/MONITOR'S REPORT NUMBER(S)	
12. DISTRIBUTION / AVAILABILITY STATEMENT					
13. SUPPLEMENTARY NOTES Not applicable					
14. ABSTRACT The central hypothesis to be tested in this competitive continuing application is that the external and internal manganese (Mn) exposure indices among a well-established smelter cohort are associated with changes in worker's brain magnetic resonance imaging (MRI), and Mn-elicited neuronal damage can be mechanistically explored by magnetic resonance spectroscopy (MRS). In Aim 1, we will test the sub-hypothesis that the prevalence of the increased MRI signal intensity correlates with both external and internal Mn exposure indices as well as the subtle changes in neurobehavioral functions among smelting workers. We will recruit 15 subjects from each of the existing groups (total 45) for the MRI study. We will collect additional blood samples and air samples for determination of concentrations of other metals in the biological system and in working environment. In Aim 2, we will test the sub-hypothesis that Mn exposure may alter the status of brain metabolites and neurotransmitters; a non-invasive MRS technique can detect these changes and therefore suggest the damaged cell types in humans. We plan to use the subjects as in Aim 1 and apply the MRS technique to determine changes N-acetyl-aspartate (NAA), choline (Cho), creatine (Cr) and myo-inositol (MI), and of the neurotransmitters such as GABA and glutamate (Glu). Changes in MRS among subjects will be correlated to their environmental, epidemiological and clinical experimental outcomes.					
15. SUBJECT TERMS Manganese, neurotoxicology, biomarker, iron metabolism, MRI, MRS, human study, smelters					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT UU	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON Wei Zheng, Ph.D.
a. REPORT U	b. ABSTRACT U	c. THIS PAGE U			19b. TELEPHONE NUMBER (include area code) (765) 496-6447

Table of Contents

COVER.....	1
SF 298.....	2
Table of Contents.....	3
Introduction.....	4
BODY.....	4
Key Research Accomplishments	8
Reportable Outcomes	8
Conclusions	8
References	9

Introduction

The central hypothesis to be tested in this competitive continuing application is that the external and internal manganese (Mn) exposure indices among a well-established smelter cohort are associated with changes in worker's brain magnetic resonance imaging (MRI), and Mn-elicited neuronal damage can be mechanistically explored by magnetic resonance spectroscopy (MRS). The study will help develop a useful strategy in combining MRI and other biomarkers of Mn exposure for assessing early onset of Mn neurotoxicities. More importantly, the study, by using a novel approach of MRS, may reveal for the first time in human studies the primary brain cell type that is injured by Mn intoxication.

In our previous study, which is funded by the Manganese Health Research Program (MHRP), we have characterized, by exposure assessment, three unique study populations with airborne Mn levels less than 0.01 mg/m³ (control), between 0.01 and 0.1 mg/m³ (low-exposed group), or higher than 0.3 mg/m³ (high-exposed group). Among this study cohort, we have recruited a total of 328 study subjects, from whom the epidemiological questionnaires were obtained, biological samples (i.e., saliva, hair, serum, whole blood, and urine) were collected, and physical examinations, particularly neurological examination, were performed. In addition, we have conducted neurobehavioral testing among these workers by using Purdue Grooved Pegboard (for visual-motor coordination) and the Nine Hole Steadiness Tester (for intentional tremor). By far, we have finished phase-I occupational exposure assessment study and phase-II epidemiological and clinical investigation. Currently the study is in phase-III laboratory and statistical analysis. The progress of this study will be discussed in Section C: Preliminary Studies. A well-characterized study population, plus the large quantities of original data that cover the exposure assessment, epidemiological survey, physiological examination, neurobehavioral testing, and clinical chemistry, provides a unique opportunity for further exploring an additional novel idea, i.e., using brain MRI to study the dose-dependent effect of Mn toxicity in smelters. Together with other biomarkers originally proposed to investigate, this study will help understand the potential health effects of metal fumes on exposed workers and define a better strategy for early diagnosis of Mn-induced neurotoxicity. In addition, we will use the MRS technique to investigate the damaged brain cell types among Mn-exposed smelters. Our specific aims are:

Aim 1: To test the sub-hypothesis that the prevalence of the increased MRI signal intensity correlates with both external and internal Mn exposure indices as well as the subtle changes in neurobehavioral functions among smelting workers. First, we plan to recruit 15 subjects from each of the existing groups (total 45) for the MRI study. By using the subjects who had carried the personal air samplers, we will be able to link directly MRI signal changes to air levels of Mn or other metals in the working environment. Second, we plan to collect additional blood samples and air samples for determination of concentrations of other metals in the biological system and in working environment. Finally, we plan to conduct a statistical analysis to seek for the correlations between MRI signal changes and the collected environmental, epidemiological and clinical parameters, such as levels of external and internal exposure, neurobehavioral outcomes, and age/gender effect.

Aim 2: To test the sub-hypothesis that Mn exposure may alter the status of brain metabolites and neurotransmitters; a non-invasive MRS technique can detect these changes and therefore suggest the damaged cell types in humans. We plan to use the same subjects as in Aim 1 and apply the MRS technique to determine changes in the neurochemical spectra of the markers for cell integrity, such as N-acetyl-aspartate (NAA), choline (Cho), creatine (Cr) and myo-inositol (MI), and of the neurotransmitters such as GABA and glutamate (Glu). Changes in MRS among subjects will be correlated to their environmental, epidemiological and clinical experimental outcomes.

Body of the Final Report

1. Human Study Logistics

The IRB protocol entitled "Biomarkers of Early Onset of Manganese Neurotoxicities among Occupationally Exposed Chinese Workers" (Ref#04-655) was re-approved by the Committee on the Use of Human Research Subjects, Institutional Review Board of Purdue University, on 24 May 2010.

The initial application for IRB approval was sent to the Human Subjects Research Review Board (HSRRB) of the U.S. Army Medical Research and Materiel Command (AMRMC) on 26 Aug 2005. The application was suggested for full review and subsequently reviewed by AMRMC HSRRB on 12 Oct 2005. The protocol was approved by the Committee on 22 Feb 2006 and reapproved on 6 May 2008 and valid for 5 years. The human research assurance number was approved and granted to the ZMC by U.S. DHHS on 13 Jan 2006. The protocol was approved by ZMC IRB for 5 years.

2. Trips to China for Human Studies

The first trip to Zunyi city was made between April 4-9, 2005. Drs. Zheng (team leader and neurotoxicologist), Rosenthal (expert in exposure assessment), and McGlothlin (expert in industrial hygiene and epidemiology) at Purdue and Dr. Jie Liu of NIH/NCI (expert in bioassays) joined the visit. The purpose was (1) to consolidate working relationship with Chinese counterpart, (2) to establish the direct communication channels between the investigators from the US and China, (3) to clearly define and assign the responsibility to each researcher in this multinational team, (4) to train the researchers on the site for how to use the equipment we brought to ZMC, and (5) to discover the potential problems and to solve them on the site. The visit resulted in a signed Research Agreement between Purdue University and ZMC.

The 2nd trip to Zunyi was made between April 17-20, 2006. Dr. Michael Aschner, the Program Director, Dr. Wei Zheng, the PI of this project, and Mr. Dallas Cowan, doctoral student in Zheng group, participated in this site visit. The tasks were for Dr. Aschner to meet the research team and to oversee the progress (Aschner, Zheng), to examine if the human research conduct follows the IRB and other protocols (Zheng, Aschner), to conduct neurobehavioral testing on the subjects (Zheng, Cowan), to monitor laboratory experiments and assays (Zheng, Cowan), to bring some biological samples back to the US for quality control (Cowan), and to discuss the exchange scholar for training propose (Aschner, Zheng). During the trip, six subjects were recruited to the research center. Mr. Cowan trained the researchers for neurobehavioral test, and Dr. Zheng supervised the administration of questionnaires, physical examination, and obtaining biological samples (blood, saliva and hair).

The 3rd trip to Zunyi was made between Nov 10-14, 2006. Dr. Zheng performed the on-site inspection of data storage, confidentiality compliance, and analytical quality control. Dr. Zheng also had the meeting with Chinese team to discuss the progress of the project, technical help needed for sample analysis, and financial issues. During the meeting, the issue was raised on the underestimation of the budget for reagents, consumables, and effort compensation.

The 4th trip to Zunyi was made between Oct 30-Nov 3, 2007. Dr. Zheng and Dr. Aschner visited the ZMC, listened the report by Dr. Qiyuan Fan, inspected all clinical and laboratory records, and checked the storage of all biological samples. Dr. Aschner expressed his expectation on this important human research. Dr. Zheng reported the initial data analysis on all subjects, the problems encountered, the solution sought and the time line for the final phase of this research. Prof. Jingshan Shi, the President of ZMC and Dr. Chen, Director of Quzhou Institute of Occupational Safety and Health, met with Drs. Zheng and Aschner. During the meeting, the next phase of MRS study was discussed and planned.

The 5th trip to Zunyi was made between April 2-4, 2008. The purpose was to explore the possibility to conduct MRI/MRS study on the subjects in ZMC cohort. Dr. Zheng visited the Guangxi Medical University ZMC, met Dr. Yueming Jiang and Dr. L. Long, Director of Radiation Dept, inspected MIR equipment, forged the verbal agreement on collaboration among three institutes, and planned the formal study in Fall, 2008.

The 6th trip to China was made between Sept 19-23, 2008. Dr. Zheng, Dr. Ulrike Dydak and Dr. Aschner visited Guangxi Medical University in Nanning. During the visit, the subjects in ZMC cohort were transported from Zunyi City to Nanning City. Dr. Zheng, along with Dr. Jiang, coordinated the clinical examination and questionnaire collection. Dr. Dydak conducted MRI/MRS examination on the subjects. Dr. Aschner inspected the quality of research conduct. Two manuscripts are currently under writing.

The last trip to China was made between May 26 – 30, 2010. Dr. Ulrike Dydak of Purdue University, USA and Dr. Enrico Pira of Tulin University, Italy joined the research. The purpose was to perform neurological exam by Dr. Pira and to obtain GABA sequences from the Mn-exposed subjects and controls by Dr. Dydak. The data obtained in combination with previous studies were the basis for a research paper submitted to EHP in October 2010.

3. Summary of Research Achievement by January 2010

Background: Exposure to excessive manganese (Mn) is known to lead to motor and psychological disorders. Understanding the vulnerability of glutamatergic and GABAergic systems to manganese (Mn) toxicity should provide insights into the mechanisms of Mn-induced neurotoxicity.

Objectives: Our goal was to study whether brain levels of gamma aminobutyric acid (GABA), glutamate and other brain metabolites in smelters were altered as a consequence of Mn exposure as compared to healthy controls, and to investigate their potential as biomarker.

Methods: High-resolution 3D MRI was used to quantify Mn deposition in the brain by the pallidal index, and the single voxel Magnetic Resonance Spectroscopy (MRS) was used to investigate brain concentrations of active metabolites in the globus pallidus, putamen, thalamus and frontal cortex of a well-established cohort of

10 male Mn-exposed smelters and 10 male age-matched control subjects. In addition, the MEGA-PRESS sequence was used to determine GABA levels in the basal ganglia.

Results: Seven out of ten exposed subjects showed clear T1-hyperintense signals in the globus pallidus. A significant increase of GABA/tCr by 82% ($p < 0.01$) was found in the basal ganglia region due to Mn exposure and NAA/tCr was significantly decreased in the frontal cortex of exposed subjects (9%, $p < 0.05$). Using both, the GABA level and the pallidal index, the logistic regression model allows for differentiation of the exposed from the non-exposed subjects with 91% accuracy.

Conclusions: We demonstrated for the first time that GABA levels in the human basal ganglia are elevated in Mn-exposed smelters. In combination with the pallidal index, the GABA level may be a powerful non-invasive biomarker for Mn exposure.

4. Overall Achievements for the Funded Period 2005-2010

4.1. Manganese exposure among smelting workers: blood manganese–iron ratio as a novel tool for manganese exposure assessment

Unexposed control subjects ($n = 106$), power distributing and office workers ($n = 122$), and manganese (Mn)-exposed ferroalloy smelter workers ($n = 95$) were recruited to the control, low and high groups, respectively. Mn concentrations in saliva, plasma, erythrocytes, urine and hair were significantly higher in both exposure groups than in the controls. The Fe concentration in plasma and erythrocytes, however, was significantly lower in Mn-exposed workers than in controls. The airborne Mn levels were significantly associated with Mn/Fe ratio (MIR) of erythrocytes (eMIR) ($r = 0.77$, $p < 0.01$) and plasma (pMIR) ($r = 0.70$, $p < 0.01$). The results suggest that the MIR may serve as a useful biomarker to distinguish Mn-exposed workers from the unexposed, control population. (Cowan et al., Biomarkers 2009;14:3)

4.2. Manganese exposure among smelting workers: Relationship between blood manganese–iron ratio and early onset neurobehavioral alterations

A total of 323 subjects were recruited to control ($n = 106$), low-exposure (122), and high-exposure (95) groups. The test battery consisted of standard testing procedures including the nine-hole and groove-type steadiness tester, Benton visual retention test, and Purdue pegboard coordination test. No significant health problems or clinically diagnosed neurological dysfunctions were observed. Benton test did not reveal any abnormal memory deficits among Mn-exposed smelters, nor did the groove and nine-hole tests detect any abnormality in dynamic and static steadiness in tested subjects. Purdue pegboard test showed a remarkable age-related decline in fine movement coordination among all study participants regardless of the Mn-exposure condition. Mn exposure significantly exacerbated this age-related deterioration. Statistical modeling revealed that the plasma and erythrocyte MIR (i.e., pMIR and eMIR, respectively) were associated with Purdue pegboard scores. Among all subjects whose MIR were above the cut-off value (COV), pMIR was significantly correlated with pegboard scores ($r = -0.261$, $p = 0.002$), whereas for those subjects over the age of 40, the eMIR, but not pMIR, was associated with declined pegboard performance ($r = -0.219$, $p = 0.069$). When both factors were taken into account (i.e., age > 40 and MIR $>$ the COV), only pMIR was inversely associated with pegboard scores. Combining their usefulness in Mn-exposure assessment, we recommend that the blood Mn–Fe ratio may serve as a reasonable biomarker not only for assessment of Mn exposure but also for health risk assessment. (Cowan et al., NeuroToxicology 2009;30:1214)

4.3. Manganese exposure among smelting workers: In Vivo Measurement of Brain GABA Concentrations by Magnetic Resonance Spectroscopy

The goal of this study was to study whether in-vivo brain levels of gamma-aminobutyric acid (GABA), N-acetylaspartate (NAA) and other brain metabolites in smelters were altered as a consequence of Mn exposure. T1-weighted MRI was used to visualize Mn deposition in the brain. Magnetic resonance spectroscopy (MRS) was used to quantify concentrations of NAA, glutamate and other brain metabolites in globus pallidus, putamen, thalamus, and frontal cortex from a well-established cohort of 10 male Mn-exposed smelters and 10 male age-matched control subjects. The MEGA-PRESS MRS sequence was used to determine GABA levels in a region encompassing the thalamus and adjacent parts of the basal ganglia (“GABA-VOI”). Our data show that seven out of ten exposed subjects showed clear T1-hyperintense signals in the globus pallidus indicating Mn accumulation. We found a significant increase (82%; $p = 0.014$) of GABA/tCr in the GABA-VOI of Mn-exposed subjects, as well as a distinct decrease (9%, $p = 0.04$) of NAA/tCr in frontal cortex that strongly correlated ($R = -0.93$, $p < 0.001$) with cumulative Mn exposure. In summary, we demonstrated elevated GABA levels in the thalamus and adjacent basal ganglia and decreased frontal cortex NAA levels, indicating neuronal dysfunction in a brain area not primarily targeted by Mn. Therefore, the non-

invasive in vivo MRS measurement of GABA and NAA may prove to be a powerful tool for detecting presymptomatic effects of Mn neurotoxicity. (Dydak et al., EHP, 2011, in press)

4.4. Relationship Between Changes in Brain MRI and 1H-MRS, Severity of Chronic Liver Damage, and Recovery After Liver Transplantation

We extended our DoD study to use magnetic resonance imaging (MRI) and 1H magnetic resonance spectroscopy (1H-MRS) have been used in clinics for diagnosis of chronic liver diseases. This study was designed to investigate the relationship between MRI/MRS outcomes and the severity of liver damage. Of 50 patients examined, the MRI signal intensity in the globus pallidus as determined by pallidus index (PI) increased as the disease severity (scored by Child Pugh ranking) worsened ($r = 0.353$, $P < 0.05$). The changes in PI values were also linearly associated with Mn concentrations in whole blood (MnB) ($r = 0.814$, $P < 0.01$). MRS analysis of four major brain metabolites (i.e., Cho, ml, Glx, and NAA) revealed that the ratios of Cho/Cr and ml/Cr in cirrhosis and CHE patients were significantly decreased in comparison to controls ($P < 0.05$), whereas the ratio of Glx/Cr was significantly increased ($P < 0.05$). The Child Pugh scores significantly correlated with ml/Cr (20.484, $P < 0.01$) and Glx (0.369, $P < 0.05$), as well as MnB (0.368, $P < 0.05$), but not with other brain metabolites. Three patients who received a liver transplant experienced normalization of brain metabolites within 3 months of post-transplantation; the MR imaging of Mn in the globus pallidus completely disappeared 5 months after the surgery. Taken together, this clinical study, which combined MRI/MRS analysis, autopsy exam and liver transplant, clearly demonstrates that liver injury-induced brain Mn accumulation can reversibly alter the homeostasis of brain metabolites Cho, ml and Glx. Our data further suggest that liver transplantation can restore normal brain Mn levels. (Long et al., *Exp Biol Med* 2009;234:1075)

4.5. Chelation therapy of manganese intoxication with para-aminosalicylic acid (PAS) in Sprague–Dawley rats

While this study was not originally designed in our DoD study, the collaboration built based on the DoD funding made it possible for the PI to strengthen the relationship with Dr. Yueming Jiang for Mn patient therapeutic treatment. Para-aminosalicylic acid (PAS), an FDA-approved anti-tuberculosis drug, has been used successfully in the treatment of severe manganese (Mn)-induced Parkinsonism in humans. This study was conducted to explore the capability of PAS in reducing Mn concentrations in body fluids and tissues of Mn-exposed animals. Sprague–Dawley rats received daily intraperitoneally (i.p.) injections of 6 mg Mn/kg, 5 days/week for 4 weeks, followed by a daily subcutaneously (s.c.) dose of PAS (100 and 200 mg/kg as the PAS-L and PAS-H group, respectively) for another 2, 3 or 6 weeks. Mn exposure significantly increased the concentrations of Mn in plasma, red blood cells (RBC), cerebrospinal fluid (CSF), brain and soft tissues. Following PAS-H treatment for 3 weeks, Mn levels in liver, heart, spleen and pancreas were significantly reduced by 25–33%, while 3 weeks of PAS-L treatment did not show any effect. Further therapy with PAS-H for 6 weeks reduced Mn levels in striatum, thalamus, choroid plexus, hippocampus and frontal cortex by 16–29% ($p < 0.05$). Mn exposure greatly increased iron (Fe) and copper (Cu) concentrations in CSF, brain and liver. Treatment with PAS-H restored Fe and Cu levels comparable with control. These data suggest that PAS likely acts as a chelating agent to mobilize and remove tissue Mn. A high-dose and prolonged PAS treatment appears necessary for its therapeutic effectiveness. (Zheng et al., *Neurotox* 2009;30:240)

4.6. HPLC analysis of para-aminosalicylic acid and its metabolite in plasma, cerebrospinal fluid and brain tissues

Para-aminosalicylic acid (PAS), an approved drug for treatment of tuberculosis, is a promising therapeutic agent for treatment of manganese (Mn)-induced parkinsonian syndromes. Lack of a quantifying method, however, has hindered the clinical evaluation of its efficacy and there upon new drug development. This study was aimed at developing a simple and effective method to quantify PAS and its major metabolite, N-acetyl-para-aminosalicylic acid (AcPAS), in plasma, cerebrospinal fluid (CSF) and tissues. Biological samples underwent one-step protein precipitation. The supernatant was fractionated on a reversed phase C18 column with a gradient mobile system, followed by on-line fluorescence detection. The lower limits of quantification for both PAS and AcPAS were 50 ng/ml of plasma and 17 ng/g of tissues. The intra-day and inter-day precision values did not exceed 5% and 8%, respectively, in all three matrices. The method was used to quantify PAS and AcPAS in rat plasma and brain following a single iv injection of PAS. Data showed a greater amount of PAS than AcPAS in plasma, while a greater amount of AcPAS than PAS was found in brain tissues. The method has been proven to be sensitive, reproducible, and practically useful for laboratory and clinical investigations of PAS in treatment of Mn Parkinsonism. (Hong et al., *J Pharmaceut Biomed Analysis* 2010;54:1101)

Key Research Accomplishments

- For the first time in literature, we proposed biological measurable values that may truly reflect Mn exposure status in humans. These values (i.e., eMIR and pMIR) are a composite of the blood index of Mn exposure and the biological consequence of such an exposure. It may be useful for clinical diagnosis of Mn intoxication as well as for risk assessment of Mn toxicity in general population.
- High-resolution 3D T1-weighted MR imaging allows to trace the regions of Mn accumulation in high detail to the most interior parts of the GP and other involved structures. Two pallidal indices, PIWM and PIMU, can be used to identify Mn-exposed subjects with 70-80% accuracy.
- We show for the first time a significant increase in GABA concentration in the thalamic regions of Mn-exposed subjects. Overall this study demonstrates the feasibility of measuring GABA in the basal ganglia of highly Mn exposed subjects.
- We found that PAS can be used to reduce brain concentrations of Mn.
- We have also developed the HPLC method for PAS quantification. This paves the way for future kinetic study to produce the parameters needed for its approval by IRB for clinical trial in the US.
- Local Chinese researchers have been trained along with the progress of this project. They have now had a better sense on the quality of data collection, proper conduct of human study, respect of subject's privacy, and scientific and objective interpretation of data. Data safety monitoring meets the strict guideline of DoD requirement.

Reportable Outcomes

- Abstracts already presented:
Yes (see References for papers and abstracts supported by this Grant).
- Current faculty receiving support from the grant:
 - o Wei Zheng, PhD
 - o Frank Rosenthal, PhD
 - o Ulrike Dydak
- Current students receiving training from participation on projects related to this grant:
 - o Sherleen Fu

Conclusions

Manganese (Mn), upon absorption, is primarily sequestered in tissue and intracellular compartments. For this reason, blood Mn concentration does not always accurately reflect Mn concentration in the targeted tissue, particularly in the brain. The discrepancy between Mn concentrations in tissue or intracellular components means that blood Mn is a poor biomarker of Mn exposure or toxicity under many conditions and that other biomarkers must be established. For group comparisons of active workers, blood Mn has some utility for distinguishing exposed from unexposed subjects, although the large variability in mean values renders it insensitive for discriminating one individual from the rest of the study population. Mn exposure is known to alter iron (Fe) homeostasis. The Mn/Fe ratio (MIR) in plasma or erythrocytes reflects not only steady-state concentrations of Mn or Fe in tested individuals, but also a biological response (altered Fe homeostasis) to Mn exposure. Recent human studies support the potential value for using MIR to distinguish individuals with Mn exposure. Additionally, magnetic resonance imaging (MRI), in combination with noninvasive assessment of γ -aminobutyric acid (GABA) by magnetic resonance spectroscopy (MRS), provides convincing evidence of Mn exposure, even without clinical symptoms of Mn intoxication. For subjects with long-term, low-dose Mn exposure or for those exposed in the past but not the present, neither blood Mn nor MRI provides a confident distinction for Mn exposure or intoxication. While plasma or erythrocyte MIR is more likely a sensitive measure, the cut-off values for MIR among the general population need to be further tested and established. Considering the large accumulation of Mn in bone, developing an X-ray fluorescence spectroscopy or neutron-based spectroscopy method may create yet another novel non-invasive tool for assessing Mn exposure and toxicity.

Publications Resulted from DoD Grant

Peer-Reviewed Publications Supported by the DoD Fund (03/2005-01/2011)

- Jiang, Y, and Zheng, W* (2005). Cardiovascular toxicities upon manganese exposure. *Cardiovasc. Toxicol.* 5:345-354.
- Jiang, Y-M, Zheng, W*, Long, L-L, Zhao, W-J, Li, X-G, Mo, X-A, Lu, J-P, Fu, X, Li, W-M, Liu, S-F, Long, Q-Y, Huang, J-L, and Pira, E (2007). Brain magnetic resonance imaging and manganese concentrations in red blood cells of smelting workers: Search for biomarkers of manganese exposure. *NeuroToxicology* 28:126-135. (doi:10.1016/j.neuro.2006.08.005)
- Aschner, M, Nass, R, Guilarte, TR, Schneider, JS, and Zheng, W* (2007). Manganese: Recent advances in understanding its transport and neurotoxicity. *Toxicol. Appl. Pharmacol.* 221(2):131-147. (doi:10.1016/j.taap.2007.03.001)
- Jiang YM, Long LL, Zhu XY, Zheng H, Fu X, Ou SY, Wei DL, Zhou HL, and Zheng W* (2008). Evidence for altered hippocampal volume and metabolites in workers occupationally exposed to lead: A study by magnetic resonance imaging and ¹H magnetic resonance spectroscopy. *Toxicol Letters* 181: 118-125. (doi: 10.1016/j.toxlet.2008.07.009)
- Aschner M, Santos AP, Erikson KM and Zheng W* (2008). Manganese transport into the brain: Putative mechanisms. *Metal Ions Biol Med.* 10:695-700.
- Wang, DX, Du, XQ, and Zheng, W* (2008). Alteration of saliva and serum concentrations of manganese, copper, zinc, cadmium and lead among career welders. *Toxicol Letters* 176:40-47. (doi:10.1016/j.toxlet.2007.10.003)
- Kalia K, Jiang W, and Zheng W* (2008). Manganese accumulates primarily in nuclei of cultured brain cells. *NeuroToxicology* 29(3):466-470. (DOI: 10.1016/j.neuro.2008.02.012)
- Wang XQ, Li J, and Zheng W* (2008). Efflux of iron from the cerebrospinal fluid to the blood at the blood-CSF barrier: Effect of manganese exposure. *Exp Biol Med* 233:1561-1571 (doi: 10.3181/0803-RM-104)
- Wang XQ, Miller DS, and Zheng W* (2008). Intracellular trafficking of metal transporters in intact rat choroid plexus following in vitro treatment of manganese or iron. *Toxicol Appl Pharmacol* 230:167-174. (doi:10.1016/j.taap.2008.02.024)
- Zheng W*, Jiang YM, Zhang YS, Jiang W, Wang X, and Cowan DM (2009). Chelation Therapy of manganese intoxication by para-aminosalicylic acid (PAS) in Sprague-Dawley rats. *NeuroToxicology* 30: 240-248 (doi:10.1016/j.neuro.2008.12.007)
- Cowan DM, Fan QY, Zou Y, Shi XJ, Chen J, Rosenthal FS, Aschner M, and Zheng W* (2009). Manganese exposure among smelting workers: Blood manganese-iron ratio as a novel tool for manganese exposure assessment. *Biomarkers* 14(1): 3-16. (doi:10.1080/13547500902730672)
- Cowan DM, Zheng W*, Zou Y, Shi XJ, Chen J, Rosenthal FS, and Fan QY (2009). Manganese exposure among smelting workers: Relationship between blood manganese-iron ratio and early onset neurobehavioral alternations. *Neurotoxicology* 30:1214-1222 (doi:10.1016/j.neuro.2009.02.005)
- Long LL, Li XR, Huang ZK, Jiang YM, and Zheng W* (2009). Brain MRI and ¹H-MRS of patients with chronic hepatic diseases: Relation to the severity of liver damage and recovery after liver transplantation. *Exp Biol Med* 234:1075-1085. (doi: 10.3181/0903-RM-118)
- Zheng W*, Fu SX, Dydak U, and Cowan DM (2011). Biomarkers of manganese intoxication. *NeuroToxicology* 32(1):1-8. (doi:10.1016/j.neuro.2010.10.002)
- Hong L, Jiang W, Zheng W, and Zeng S (2011). HPLC analysis of para-aminosalicylic acid and its metabolite in plasma, cerebrospinal fluid and brain tissues. *J Pharmaceut Biomed Analysis* 54:1101-1109. (doi:10.1016/j.jpba.2010.11.031)
- Dydak U, Jiang YM, Long LL, Zhu H, Chen J, Li WM, Edden RAE, Hu SG, Fu X, Long ZY, Mo XA, Meier D, Harezlak J, Aschner M, Murdoch J, and Zheng W (2010). In vivo measurement of brain GABA concentrations by magnetic resonance spectroscopy in smelters occupationally exposed to manganese. *Env Health Persp* (in press) 119:000–000 (doi:10.1289/ehp.1002192)

Abstracts Presented and Published

Year 2006

- Zheng, W (2006). Discovery of biomarkers of manganese exposure in humans. *Toxicol Sci* suppl 90(S-1), 1847.
- Yang, LZ, Jiang, YM, and Zheng, W (2006). Erythrocytes as a useful biological matrix for assessment of manganese exposure among smelting Workers. *Toxicol Sci* supplement 90(S-1), 191.

- Jiang, YM, Mo, XA, Du, FQ, Gao, HY, Xie, JL, Lia, FL, Pira, E, and Zheng, W (2006). Effective treatment of manganese-induced occupational Parkinsonism with p-aminosalicylic Acid (PAS-Na): A case of 17-year follow-up study. *Toxicol Sci supplement* 90(S-1), 1767.
- Mo, XA Jiang, YM, Long, LL, Zhao, WJ, Li, XR, Su, SH, Zheng, W (2006). Brain magnetic resonance imaging and blood levels of trace elements among manganese-exposed steel workers. *Toxicol Sci supplement* 90(S-1), 191.
- Rutchik, JS, Mo, XA, Jiang, YM, and Zheng, W. (2006). Manganism or Parkinson's disease: Indications from six cases among Chinese welders and steel workers. *International Conference on Industrial Medicine*, Rome, Italy.

Year 2007

- Zheng, W, Jiang, Y, Jiang, W, Zhang, Y, Wang, X, and Cowan, DM (2007). Chelation therapy of manganese (Mn) intoxication with p-aminosalicylic acid (PAS) in Sprague-Dawley rats. *Toxicol Sci supplement* 96(S-1), 1087. (Charlotte, NC)
- Zheng, W, Miller, GW, Pira, E, Rutchik, JS, and Zhang, J (2007). Advances in causation and therapy of parkinsonian diseases: Views from toxicologists and clinicians. *Toxicol Sci supplement* 96(S-1), 1328. (Workshop in Charlotte, NC)
- Zheng, W, and Jiang, Y (2007). Efficacy and Selectivity of Metal Chelators in Treatment of Manganese Parkinsonism. *Toxicol Sci supplement* 96(S-1), 1333. (Workshop in Charlotte, NC)
- Cowan, DM, Fan, Q, Shi, X, Zou, Y, Rosenthal, FS, Aschner, M, and Zheng, W (2007). Manganese (Mn) in saliva as an indicator for occupational exposure in Chinese smelting workers. *Toxicol Sci supplement* 96(S-1), 1086. (Charlotte, NC)
- Zheng, W, Wang, D, Du, X (2007). Alteration of manganese, copper, zinc, cadmium, and lead in saliva of career welders. *The 11th International Congress of Toxicology*, Montreal, Canada, July 15-19.
- Zheng, W, Pira, E, and Jiang, Y (2007). Alternation of the oxidative stress status in erythrocytes and sera among manganese-exposed smelting workers. *The 11th International Neurotoxicology Association Meeting*, Pacific Grove, CA, June 10-15.

Year 2008

- Cowan DM, Fan QY, Zou Y, Shi XJ, Rosenthal FS, Aschner M, and Zheng W (2008). Manganese exposure in 328 smelting workers: Relationship among external/internal markers and neurological/psychomotor examinations. *Toxicol Sci supplement* 102(1), 708. (Seattle)
- Zheng W, Zhang YS, Jiang YM, and Jiang W (2008). Removal of tissue manganese by p-aminosalicylic acid (PAS) in manganese-exposed rats in vivo. *Toxicol Sci supplement* 102(1), 2102. (Seattle)
- Zheng W, Cowan DM, Zou Y, Shi XJ, Chen J, and Fan QY (2008). Use of manganese-iron ratio in blood for assessment of Mn exposure among smelting workers. *The 20th International Conference on Epidemiology in Occupational Health (EPICOH-2008)* (June 9-11) and the *10th International Symposium on Neurobehavioral Methods and Effects in Environmental and Occupational Health (NEUROEH-2008)* (June 11-13). Costa Rica June 9-13, 2008. *Occ Env Med* 65(Suppl): 149.

Year 2009

- Zheng W (2009). A single parameter combining multiple bio-indices as a new approach to discover biomarkers of metal toxicities: A case study with manganese. *SOT meeting in Baltimore*
- Dydak U, Jiang Y, Long L, Chen J, Li W, Murdoch J, Meier D, Aschner M, and Zheng W (2009). Assessment of manganese exposure by 3D high-resolution T1-weighted MRI. *SOT meeting in Baltimore*
- Cowan DM and Zheng W (2009). Relationship between blood manganese-iron ratio and early onset neurobehavioral alterations. *SOT meeting in Baltimore*
- Fan Q, Zou Y, Liu J, Yu C, Chen J, Shi X, Zhang Y, and Wei Zheng (2009). Decreased DMT1, TF and hepcidin gene expressions in leucocyte of manganese exposed workers. *SOT meeting in Baltimore*
- Dydak U, Long L, Zhu H, Li WM, Jiang Y, Chen J, Fu X, Hu S, Edden RAE, Meier D, Aschner M, Murdoch J, and Zheng W (2009). Assessment of neurotransmitter concentrations in occupational manganese exposure as measured by MRS. *The ISMRM 17th Scientific Meeting & Exhibition*, Honolulu, Hawaii, USA 18-24 April 2009.
- Dydak U, Jiang Y, Long L, Chen J, Li W, Murdoch J, Meier D, Aschner M, and Zheng W (2009). Assessment of manganese exposure by 3D high-resolution T1-weighted MRI. *Toxicol Sci supplement* 108(1):362 (Baltimore)

Dydak U, Yoder K. How to Measure Neurotransmitters with MRS and PET: Focus on GABA, Glutamate, and Dopamine (Educational Exhibit). Radiological Society of North America Annual Meeting, Chicago, IL, USA, Nov 29-Dec 4 2009.

Year 2010

- Monnot AD, Ho S, and Zheng W* (2010). Regulation of copper (Cu) homeostasis at the brain barriers: Effects of Fe-overload and Fe-deficiency. *Toxicol Sci suppl.* 114(1): 983. (Salt Lake City).
- Fu X, Zhang Y, Jiang W, Behl M, Monnot AD, and Zheng W (2010). Increased P-glycoprotein expression at the blood-cerebrospinal fluid barrier following acute lead exposure. *Toxicol Sci suppl.* 114(1): 984.
- Yoshimoto Ninamango EQ, Zhao C, Yung K-T, Zheng W, Ackley E, Dager S, VanMeter J, Dydak U, Heberlein K, Tsai S-Y, Lin F-H, Wald L, Van Der Kouwe A, Bustilo J, Posse S. 3D High Spatial Resolution Short TE Proton-Echo-Planar-Spectroscopic-Imaging (PEPSI) at 3T in Clinically Feasible Measurement Times. ISMRM 18th Scientific Meeting & Exhibition, Stockholm, Sweden, May 1-7, 2010.
- Zheng W, Monnot AD, Choi B, and Zhang Y (2010). Regulation of copper homeostasis in brain and cerebrospinal fluid: Effect of Manganese exposure. *Toxicol Sci suppl.* 114(1): 1365.
- Behl M, Zhang Y, Shi Y, Cheng J, and Zheng W (2010). Activation of protein kinase C in lead-induced accumulation of β -amyloid in the choroid plexus: Relationship to subcellular relocation of low density lipoprotein receptor protein-1 (LRP1). *Toxicol Sci suppl.* 114(1): 1842.
- Zhang Y, Fan Q, Behl M, Jiang W, Fu S, Hong L, Monnot AD, and Zheng W (2010). Age-dependent transport of copper (Cu) at the blood-cerebrospinal fluid barrier. *Toxicol Sci suppl.* 114(1): 2163.
- Li GJ, Jing HM, Wei KH, Yang E, Gao WH, Zhao CY, Ma L, Liu JZ, Zhang T, and Zheng W (2010). Ultrastructural and toxicoproteomic studies of structure and function of the blood-CSF barrier in manganese-exposed rat model. *Toxicol Sci suppl.* 114(1): 2173.
- Dydak U, Jiang Y, Long Z, Long L, Chen J, Harezlak J, Zhu H, Aschner M, Murdoch J, and Zheng W (2010). GABA increases in basal ganglia in manganese exposed smelters. *Toxicol Sci suppl.* 114(1): 1000.