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14. ABSTRACT

Manganese (Mn) and iron are essential metals for normal growth and development that compete for and share the same transporters. Thus, during periods of low dietary iron intake, the transport and deposition of Mn in the brain are increased. Conversely, high-risk populations for Mn intoxication, namely Mn miners and welders, may benefit from iron supplementation, which may lower their central nervous system (CNS) Mn burden. For the first 3 years, we proposed to determine the temporal brain Mn deposition pattern using magnetic resonance imaging (MRI). We have completed the imaging and atomic absorption spectroscopy (AAS) phases. Both iron and Mn content in six discrete brain regions have been determined, along with ascertainment of blood and plasma metal levels. Data analysis is progressing for both the brain images and R1 values from the MRI, and several manuscripts have been submitted, both addressing Mn and Fe modeling in the brain, as well as the relationship between Mn and Fe brain depositions.

15. SUBJECT TERMS
Manganese, neurotoxicology, iron deficiency, welding, manganese mining, nutrition

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Introduction

Manganese (Mn) is an essential metal for normal growth and development. Recent work demonstrates that Mn and iron (Fe) compete for and share the same transporters. Thus, during periods of low dietary Fe intake, the transport and deposition of Mn in the brain are increased. Conversely, high-risk populations for Mn intoxication, namely Mn miners and welders, may benefit from Fe supplementation, which may lower their central nervous system Mn burden. Given the potential health risks associated with Mn neurotoxicity, we proposed using a non-invasive *in vivo* technique, magnetic resonance imaging (MRI), to monitor the accumulation of brain Mn when dietary levels of Fe are modulated. However, before Fe levels could be experimentally manipulated in animal models, it was first necessary to determine the usefulness of MRI in monitoring brain Mn levels in the absence of changes in dietary Fe.

Body

STUDY 1 – A Chronic Iron-Deficient/High-Manganese Diet in Rodents Results in Increased Brain Oxidative Stress and Behavioral Deficits in the Morris Water Maze

Iron deficiency (ID) is especially common in pregnant women and may even persist following childbirth. This is of concern in light of reports demonstrating that ID may be sufficient to produce homeostatic dysregulation of other metals, including manganese (Mn). These results are particularly important considering the potential introduction of the Mn-containing gas additive, methyl cyclopentadienyl manganese tricarbonyl (MMT), in various countries around the world. In order to model this potentially vulnerable population, we fed female rats fed either control (35 mg Fe/kg chow; 10 mg Mn/kg chow) or low iron/high manganese (IDMn; 3.5 mg Fe/kg chow; 100 mg Mn/kg chow) diet, and examined whether these changes had any long-term behavioral effects on the animals' spatial abilities, as tested by the Morris water maze (MWM). We also analyzed behavioral performance on auditory sensorimotor gating utilizing prepulse inhibition (PPI), which may be related to overall cognitive performance. Furthermore, brain and blood metal levels were assessed, as well as regional brain isoprostane production. We found that treated animals were slightly ID, with statistically significant increases in both iron (Fe) and Mn in the hippocampus, but statistically significantly less Fe in the cerebellum. Additionally, isoprostane levels, markers of oxidative stress, were increased in the brain stem of IDMn animals. Although treated animals were indistinguishable from controls in the PPI experiments, they performed less well than controls in the MWM. Taken together, our data suggest that vulnerable ID populations exposed to high levels of Mn may indeed be at risk of potentially dangerous alterations in brain metal levels which could also lead to behavioral deficits.

STUDY 2 – Differential deposition of manganese in the rat brain following subchronic exposure to manganese: a T_1-weighted MRI study

Manganism is a central nervous system (CNS) disorder caused by toxic exposure to manganese (Mn). Manganism has been related to occupational exposures, liver diseases, prolonged parenteral nutrition and abuse of illicit drugs. Initially manifested by a reversible neuropsychiatric syndrome (locura manganica), the main symptoms and signs of manganism are emotional lability, compulsive behavior and visual hallucinations. Locura manganica is followed by an irreversible extrapyramidal syndrome, the onset of which occurs years after chronic exposure. The objective of the present study was to characterize the regional distribution of Mn in the rat brain after subchronic exposure to Mn. This animal model holds special clinical relevance, reflecting the earlier clinical stages of manganism, before chronic exposure to Mn exerts its irreversible effects. Sprague-Dawley rats were intravenously injected with $MnCl_2$ weekly, for a total of 14 weeks – approx. 1/10 of the lifetime of the rat. T_1-weighted MRI was employed as a sensitive tool for detecting the distribution of Mn deposition in brain tissues, as evidenced by areas of T_1-weighted hyperintense signals. A consistent region-specific pattern of T1-weighted hyperintensities was observed in the brains of Mn treated rats. Cortical hyperintensities were prominent in the hippocampus and dentate gyrus. Hyperintensities were also observed in the olfactory bulbs, pituitary gland, optic nerves and chiasma , pons, midbrain tegmentum, habenula, lentiform and caudate nuclei, thalamus, chorioid plexus and cerebellar hemispheres. Prominent Mn depositions, evidenced by T_1-weighted hyperintensities in the hippocampus after subacute exposure to Mn, are compatible with the clinical picture of manganism during its early stages; and may explain its pathophysiology.

STUDY 3 – A Model for the Analysis of Competitive Relaxation Effects of Manganese and Iron *in Vivo*
This study completes the objective of our proposal.

The precise mechanisms by which Mn and Fe are delivered into the brain, and how Mn and Fe affect each other's disposition, are not clear. Mn and Fe share similar transporter systems that allow the metals to enter cells (e.g. transferrin, divalent metal transporter-1. When the concentrations of the metals are high, there may be competition for the transporter or other targets between the ions so that changes in one metal may influence the distribution of the other. For example, Zheng et al. found that brain Fe metabolism was changed in rats exposed to high doses of Mn. Fe deficiency leads to increased Mn accumulation in the brain, while high Fe in the diet decreases Mn absorption. There is also evidence that Mn and Fe may enter the brain through independent transport systems. It is well know that, in simple solutions, the water NMR relaxation rates (R1 and R2) are directly proportional to the concentration of paramagnetic ion. This simple linear relationship has been widely used to estimate tissue levels of Mn or Fe alone using MRI. However, Mn and Fe are both paramagnetic, and they may compete or affect each other in biological systems. Thus the relationship between water relaxation rates and the concentrations of these paramagnetic ions together may not be as simple as assumed by the linear model. A more complex model may be to addreass the interaction between Mn and Fe binding and storage systems, and their combined effects on water relaxation times.

To address these questions, and to illustrate the need for more sophisticated analyses of imaging data, we studied the variations in MR relaxation rates as a function of metal content in groups of rats subjected to different combinations of dietary Fe and intravenous Mn. Our goal was to induce varying levels of both metals and to use quantitative MRI relaxometry to determine the Mn deposition pattern in different brain regions in rats exposed to this metal. In attempting to relate MR relaxation changes to tissue metal levels, we have developed a model that incorporate competitive mechanisms between the metal ions. Such a model is essential to explain our relaxation measurements.

Animals were scanned at the 14^{th} week (24 hours after manganese injection). All experiments were acquired using a 4.7T, 31 cm bore Varian INOVA magnet with actively shielded gradients and a 63 mm transmit/receive quadrature imaging volume coil. Rat brains were scanned from both axial (FOV=40 X 40mm, 30 slices) and coronal (FOV=40 X 50mm, 20 slices) directions with 0.75mm slice thickness.

Before the imaging procedure, animals were first anesthetized with isoflurane (2%) and placed in a stereotaxic support cradle with the head secured. The cradle was then put in the volume coil to make sure the head of the animal was located at the center of the coil. Isoflurane was lowered to 1.5-1.75% and was maintained throughout the imaging experiment. Temperature (36.5-37.5^{o}C) of the animal was monitored (SA Instruments) and maintained via a flow of warm air which is controlled by a rectal temperature probe (SA Instrument). Respiration rate were externally monitored and maintained at 50-70 breaths per minute throughout the imaging session. All procedures were in compliance with and approved by the Institutional Animal Care and Use Committee of Vanderbilt University.

T_1 was measured using 2-D Fast Low Angle Shot sequences (FLASH) and different flip angles with parameters as follows: TR/TE=489/6.59 ms; flip angle=10, 30, 55, 70; 2 acquisitions. The image matrix was 256 X 256. T_2 was measured using a multislice fast spin echo (FSE) sequence: TR=5100 ms; Echo train length=8; k-space center=4; TE=5, 6.7, 10, 13, 15 ms; 2 acquisitions; Image matrix=128 X 128;

T_1 and T_2 maps were calculated by fitting the series of T_1 and T_2 dependent images to the appropriate theoretical expressions using 2 parameter least squares fits. The parametric maps were then coregistered to a high resolution rat template and resliced using SPM (http://www.fil.ion.ucl.ac.uk/spm). Based on the rat brain template, multislice regions of interest (ROIs) were chosen for seven brain regions including cerebellum, brain stem, midbrain, striatum, hippocampus and cortex. Averaged T_1 and T_2 values within each of the ROIs were calculated for each rat and used in further analyses.

Multiple $MnCl_2$ phantoms with similar T_1 and T_2 values as the rat brain tissues were also made (pH =7). Their T_1 and T_2 values were measured at 37^{o}C using an inversion recovery method and by changing TE in a spin echo sequence respectively.

Comparison of the competition model with the linear models

The fits to the data for the linear and competition models have been compared. Also, the regressions for all brain regions for all four models (competition model, linear model with both Mn and Fe considered, linear model with only Mn considered, linear model with only Fe considered) were analyzed. The competition model and the linear model with Mn and Fe were fitted together to provide the best r^2 in all of the brain regions. In some brain regions the fits derived by these two models are significantly improved over the other two linear models in which only one ion is considered. For example, for striatum the correlation coefficients derived by the competition and the linear model with both Mn and Fe considered are 0.87 and 0.85 respectively, while the values for the other two linear model with either Mn or Fe considered are 0.69 and 0.02 with 95% confidence interval of [0.36, 0.86] and [-0.42, 0.45] respectively. Other brain regions like brainstem and midbrain also provide evidence that the competition and the linear model taking account of both Mn and Fe provide better fitting results than one or both of the other two linear models. There is no significant difference between the correlation coefficients of the competition model and the linear model with both Mn and Fe counted. However, as shown above, the relaxivities of Fe fitted with the linear model are significantly negative in almost all of the brain regions (Table 5) which does not have any physical meaning. Thus, considering one metal alone at a time or both acting independently fails either to provide physically interpretable fits or to fit the data very successfully. Only by considering the competitive model are reasonable fits that have physical meanings offered.

A diet with Fe overload is known to increase Fe accumulation in the plasma. The increased brain Mn concentration accompanied by decreased Fe concentration with Fe supplements is surprising. This may be due to the fact that the added Mn that is on board in the FeSMnT group is more readily taken into the brain, offsetting the Fe overload in the diet.

It likely is associated with the inability of the regulatory machinery to accurately reflect the brain's Fe requirements due to transport and storage of Fe in ferritin. Normally, in the presence of exceedingly high levels of Fe, the regulatory pathway perceives the CNS as Fe deficient despite excessive Fe accumulation and Fe uptake into the brain continues. When Fe regulatory protein-1 (IRP1) and IRP2 bind to the Fe regulatory element (IRE) in the 3′-untranslated region of transferrin receptor (TfR) or DMT1 mRNA, the transcript is stabilized, translation proceeds, and the proteins are synthesized. Thus, a high IRP binding activity reflects low body Fe stores and results in up-regulation of DMT1 and TfR. Vice versa, high intracellular Fe concentrations would have an opposite effect. Nonetheless, it is possible that the down-regulation of DMT1 and TfR is associated with up-regulation of transporters that are Mn-specific. Thus even in the presence of high Fe, the uptake of Mn may unabatedly continue. It has also been reported by Chua and Morgan that iron overload and deficiency led to increased brain Mn.

Regional variation of the relaxivities and combined influence of Mn and Fe on MRI signal

The effects of Mn and Fe on MR relaxation rates have been studied before in isolation and without consideration of the potential interaction between the two. Thus, when studies are designed to examine the effect of Mn on relaxivities, no other metal ions are usually considered. Conversely, other studies that focus solely on Fe have failed to take into account the interrelationship of this metal with Mn. As a result, most researchers have used a linear model to explain the influence of paramagnetic ions on the MRI signal. Our results show that when only one paramagnetic ion concentration change occurs, the simple linear model may appear to explain the relationship between ion concentration and relaxation rates. Thus the relaxation rate measured by MRI can be used as an indicator of ion concentration for this case. However, even with this simple linear model, the relaxivities vary among regions, implying that different brain regions should be treated separately rather than taking the whole brain as a single homogeneous region. To our knowledge, our study is the first one to examine the regional variation of the relaxivities.

Although Mn and Fe are commonly studied together in the toxicology field, no MRI study has been found in the literature that reports the combined influence of Mn and Fe on MRI signals. Our study is the first one to investigate this effect. Our results reveal that when more than one paramagnetic ion concentration is changing, the linear model does not describe the effects properly. As a result, a more complicated model must be applied. We propose an interacting model based on the fact that Mn and Fe may compete in vivo and both of them will affect MRI signals. The regional variation effect is still very apparent in the interacting model.

Conclusion:

It has been shown above, when Mn and Fe concentrations are both altered in a biological system, that their combined influence on MRI signals is complicated. In such a case, the simple linear model for explaining the relationship between MRI signal and a single changed paramagnetic ion will not be suitable to explain the change in MRI relaxation rates. Regional variations are apparent in both the experimental data and the model. Although some limitations and uncertainty exist in our model such as the presence of several free parameters in the fit, this represents a first attempt to explain the interacting relationship of two paramagnetic ions and their influence on the MRI signals. Our model correctly predicts the nonlinear relationship between relaxation rates and ion contents. This model may be useful for interpreting MR results when more than one paramagnetic species is involved.

Key Research Accomplishments

- Rats were fed normal diets, with treated animals receiving weekly injections of $MnCl_2$ for 14 weeks, with no significant toxicity.
- Both control and treated groups were scanned prior to start of treatment to obtain baseline MR images. Scanning continued at weeks 1, 3, 5, 7, 9, 11, 13 and 14. This allowed for successful comparison of images between groups as well as within groups at various time points.
- Blood samples were taken at weeks 8 and 14 for determination of both Mn and Fe levels.
- At the conclusion of the study, animals were humanely euthanized and brains were removed and dissected into the following regions: cerebellum, brain stem (pons and medulla), midbrain, hippocampus, striatum and cortex.
- Metal analysis, as determined by atomic absorption spectroscopy (AAS), has been completed for each brain region.
- A model was developed to assess the relationship between brain Mn and Fe deposition.
- Our model correctly predicts the nonlinear relationship between relaxation rates and ion contents.
- The effects of Mn and Fe on rat behavior were assessed.
- When Mn and Fe concentrations are both altered in a biological system, that their combined influence on MRI signals is complicated.

Reportable Outcomes

Manuscript: Supported by this project (last 2 years)

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Book Chapters Supported by this Project (last 2 years)

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14. ABSTRACT

During this project we i) reviewed methods for the evaluation of neurological health end points and to develop recommendations for a core set of evaluation methods, and ii) developed a comprehensive exposure database tool for collection and storage of manganese exposure data. The review of neurological health end points concluded that there was a high variability in methodological quality and tests and that more rigorous research methodologies and testing procedures are required.

A prototype of the database for Manganese occupational exposure data (Manganex) was developed together with a draft guidance document on collecting and storing occupational exposure data, in particular the use of Manganex. The database tool was trialled at two member companies of IMnI and feedback obtained. Final amendments are currently made to Manganex and the final version is expected in April 2009. Manganex will be disseminated through IMnI members and the MHPR showcase event in June 2009.

15. SUBJECT TERMS
Manganese, occupational exposure, databases

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Introduction

Information on manganese exposure is routinely collected by several companies within the manganese production industry (IEH/IOM, 2004; Searl, 2007). Many different approaches are used for exposure measurements, including differences in the use of personal and static monitoring, collection of different size fractions (respirable, inhalable, total), different analysis methods, (gravimetric, ICP, species) and differences in data metrics, storage, traceability and quality assurance procedures. Often, little contextual data is retained. These differences limit the utility of such data for exposure assessment and exposure reconstruction required for high quality epidemiology, particularly where a multi-centre approach is being developed.

Diverse approaches are also used to characterise the assessment of health end points in health surveillance programs.

The aims of this project are:

(i) to review methods for the evaluation of neurological health end points in the manganese industry and in other industries where these end points are considered relevant and to develop recommendations for a core set of evaluation methods to be used for the evaluation of these end points; and

(ii) to identify, develop and evaluate a set of methods, guidelines and tools to enable manganese producer companies to routinely collect valid, appropriate and comparable information relating to manganese exposure, applicable to current and future, as yet unplanned, epidemiological studies.

The first aim was completed during 2006 and a full report was submitted to MHRP last year. During the last 12 months the database tool and guidance have been revised and trials were carried out at two manganese manufacturing companies. This report will describe the results of the trials. The database tool and guidance are nearly completed with the final report, which will include a copy of the database and guidance expected before the end of April 2009.

Body

Database design and development

A prototype exposure assessment database was developed for the consistent collection, storage and analyses of manganese exposure data (Manganex). This relational database has been implemented in Microsoft Access. The following features have been incorporated into the database, standardised as far as possible.

- Adoption of EU & US standards for exposure data elements
- Common terms and definitions for manganese industry data items, parameters, characteristics and exposure survey and assessment data
- Agreement on appropriate contextual data to be included – to help describe & explain exposure
- Overall compatibility and uniformity in data formats – to facilitate the common methodology for data collection, storage and analysis, and potentially the transfer, amalgamation and sharing of (anonymised) data.

Other features of the database include:

- User friendly interface design with menus and forms for data entry and management
- Data collection forms for exposure surveys
- Help & guidance materials

- Ability to produce reports and data summaries
- Output of exposure data for analysis by other applications

The data for manganese exposure assessments are arranged in three main related areas:

1) Company, with information on
    - Company details; Employees; Jobs

2) Plant / Premises, covering
    - Processes ; Workplaces; Tasks

3) Exposure surveys with details of
    - Survey planning
        - Methods & strategy - sampling and analysis schemes;
        - Processes and workplaces surveyed
    - Sample collection and analysis results details
        - Sample type; jobs/operations; task; and additional contextual data
        - Analytical results; agents and concentrations

A generalised schematic of these is shown in Figure 1 below.

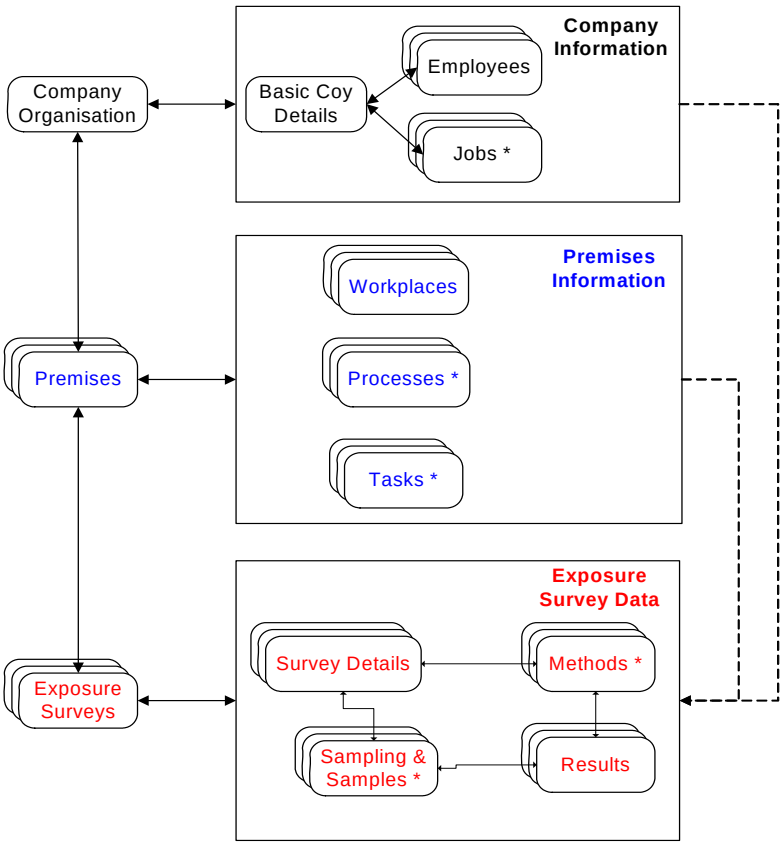

Figure 1: General schematic of data areas in Manganex

Data contents and definitions

Adapted from earlier IOM chemical exposure database work (Ritchie et al., 2004) a generalised conceptual data model of the database was produced to accommodate all of the data elements

required, and the relationships between them. The overall conceptual model was subsequently further developed and refined using entity-relationship modelling techniques to design the relational database, and then its implementation in Microsoft Access tables, and accessory lookup tables.

A key attribute of the database design is the ability to link related data both within, and between, the main areas. This allows links to be made for example between data at the company level (eg an employee and their current job), and data at the sample level (eg the job and tasks being done by the worker during sampling). In this way a bank of basic “reference” data about the company and premises is entered once, and referred (linked) to during sampling and exposure measurement, without necessarily having to repeat the data entry of the premises data.

A diagram of the database entity-relationship model in the current prototype implementation is shown below in Figure 2. This is a simplified view of the more complex model within each of the three areas in Manganex and does not resolve sub-entities, many to many relationships, etc, at this point.

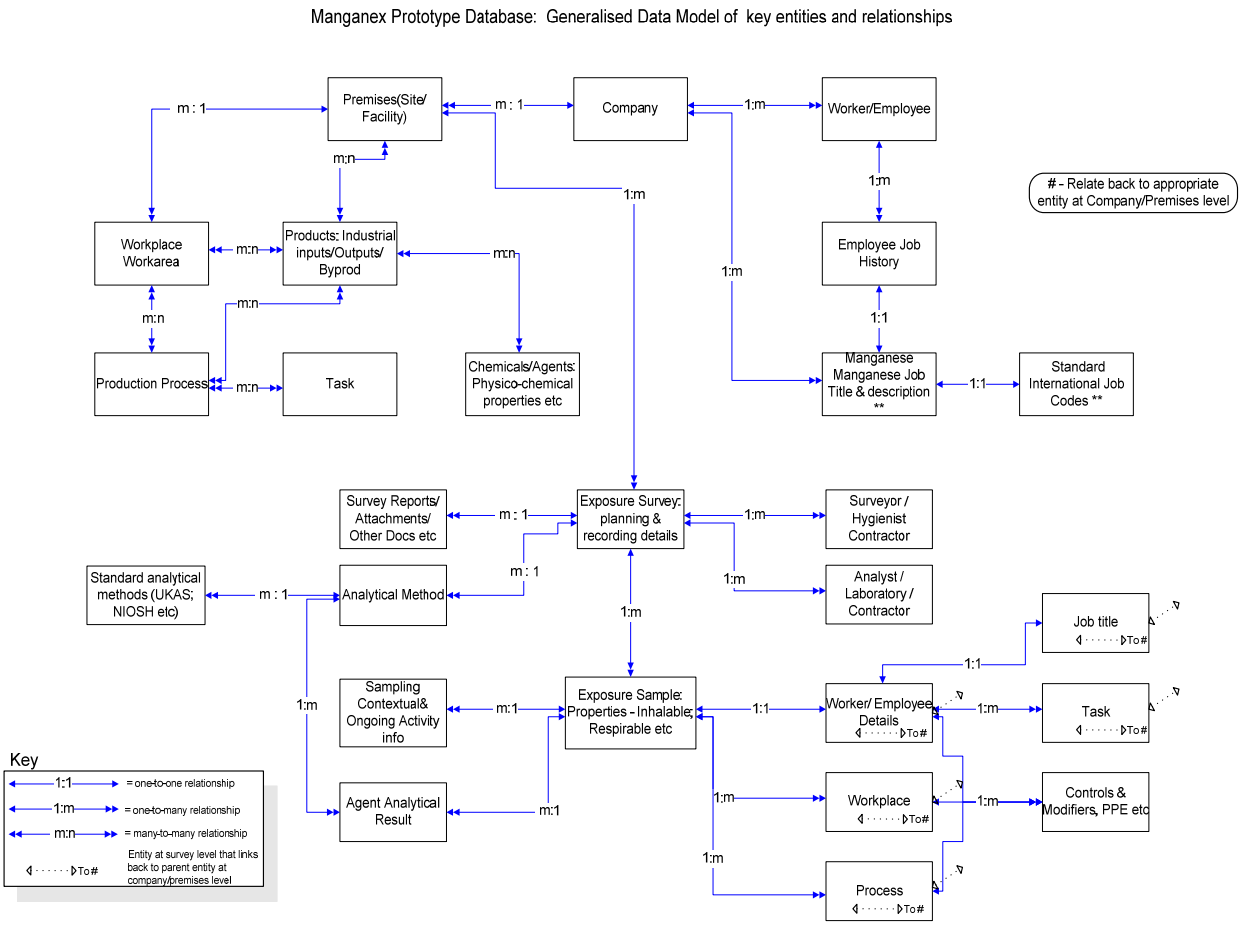

Figure 2: Overall entity relationship diagram for prototype Manganex database

The full data model is used to define a physical schema for the data base that define the actual tables used to implement the data entities. Coding or “lookup” tables are used to implement coding lists and drop down lists for the database interface.

Interface development

The following diagrams demonstrate the layout and look of the database. Figure 3 shows the main menu of Manganex, while Figures 4 to 9 show examples of the database interface for entering company information (Figure 4), information for production sites (Figure 5), process information (Figure 6), information on the measurement survey (Figure 7), information on the measurements (Figure 8) and for entering the results of the measurements (Figure 9).

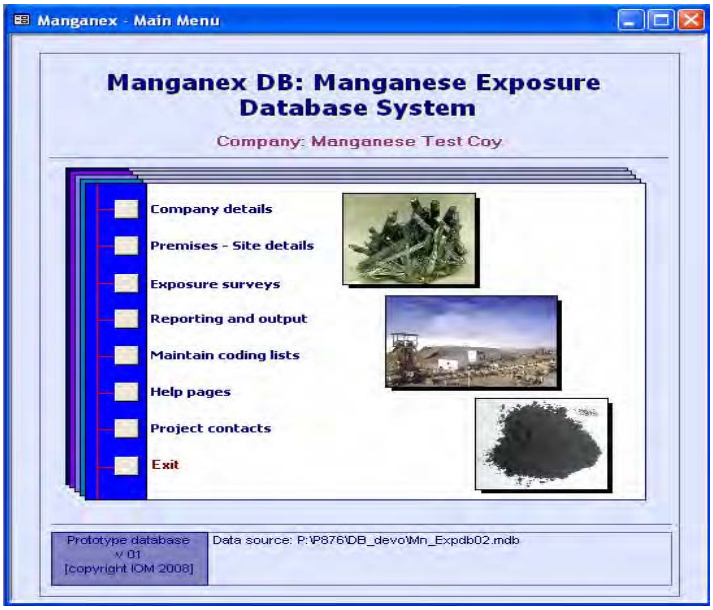

Figure 3 Main menu of Manganex exposure database.

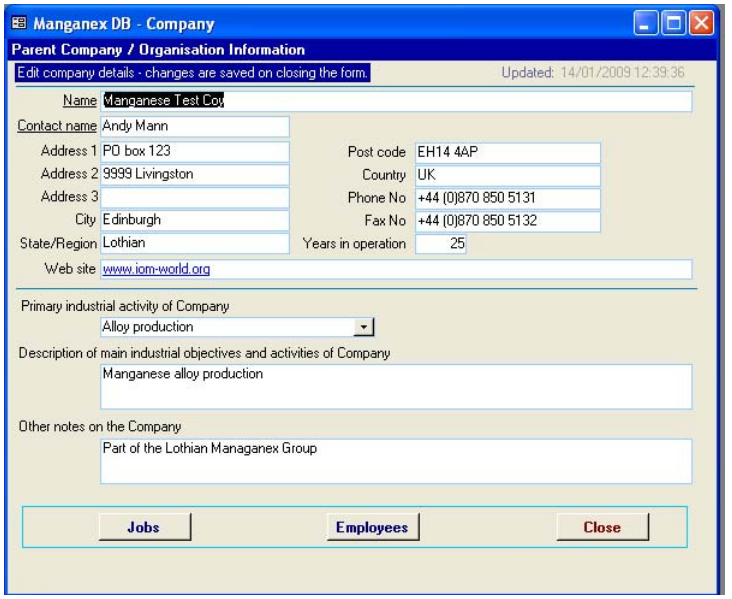

Figure 4 Company information interface

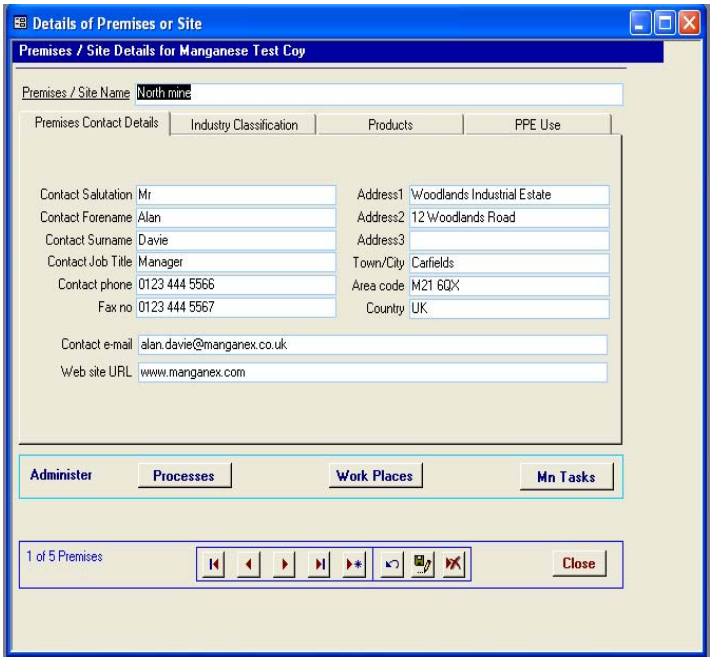

Figure 5 Interface for entering information on sites

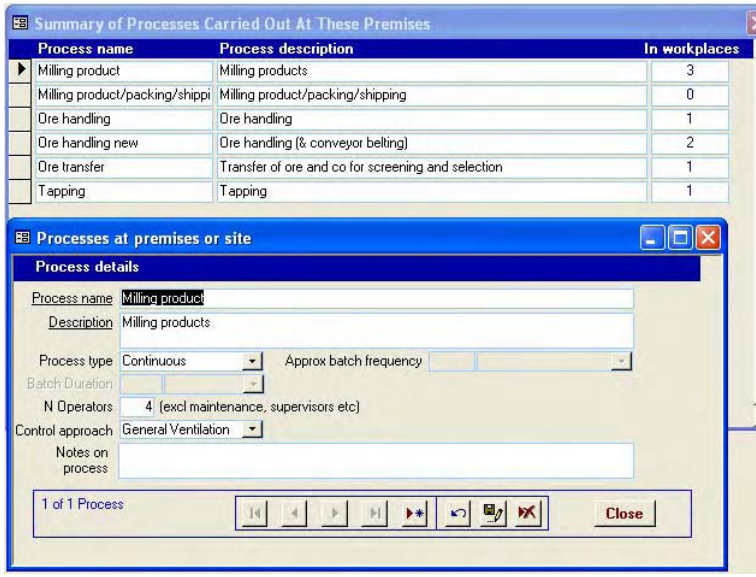

Figure 6 Interface for entering information on processes

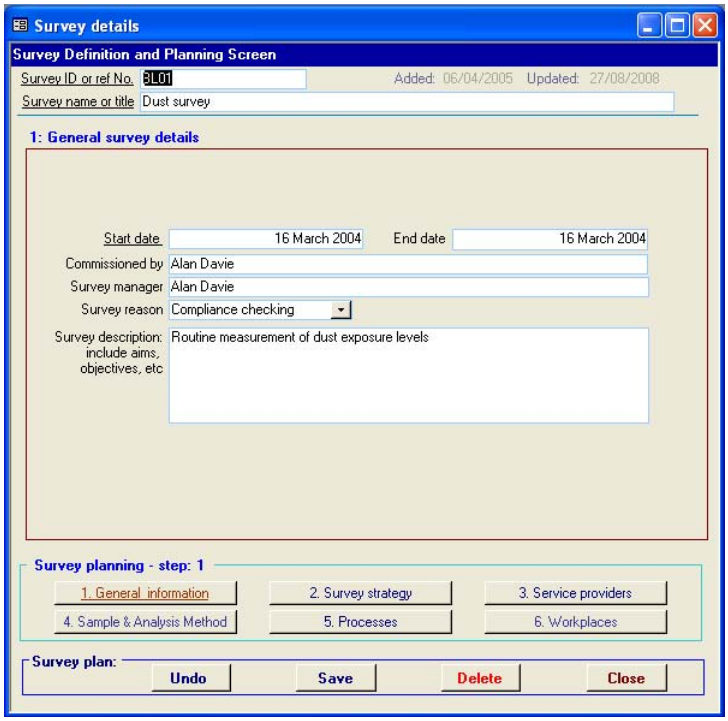

Figure 7 Interface for entering information on the measurement survey

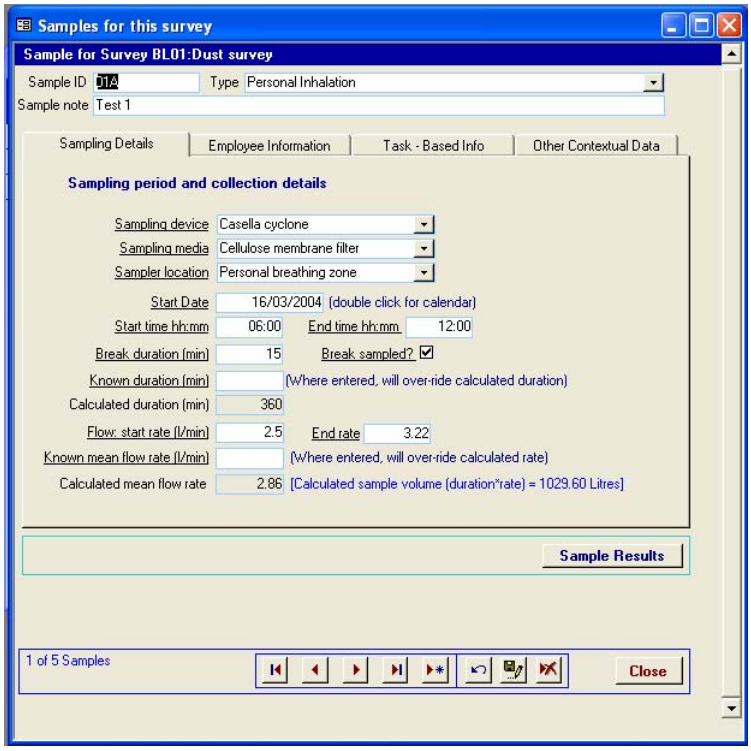

Figure 8 Interface for entering information on the sample collection

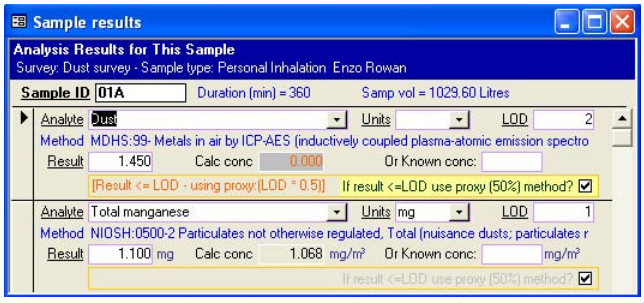

Figure 9 Interface for entering results

Field trials and Feedback

Field trials were carried out at two manganese alloys manufacturing sites (Site A and B). In addition, comments were received after presentation of the Manganex database to the International Manganese Institute (IMnI). Site A was visited on two occasions, once for a demonstration of the prototype of the database and once for a trial. Site B was visited on only one occasion. Several other facilities were contacted during the course of the project, but unfortunately we were not able to arrange any further field trials. The field trial at site A took place over a two day period, which included a detailed presentation of the database and a trial with dummy data. The field trial at site B was condensed into one day to accommodate staff availability. This included a presentation / demonstration of the database, and a dummy data collection exercise that ran through the facilities and capabilies of the database.

Two members of IOM facilitated the field trials and key company personnel responsible for the collection and outputs from Mn exposure data participated. Comments raised were recorded on a specially developed feedback form.

Both companies recognised the need for a detailed database tool that allowed contextual information to be stored together with the results of the measurements. Both companies were impressed with the comprehensive nature of Manganex.

The feedback is summarised in the following main areas.

1) **Software system**. The fact that the database was developed in Microsoft Access was considered a limitation, as it might restrict the possibility for integration with other corporate systems, especially for some larger companies. Any future developments of Manganex should consider using software such as SQL Server to allow for greater global implementation. It was also reported that an import facility for data stored in other software packages, such as Excel, would be advantageous.

2) **Guidance and definitions.** During the trials the guidance document was not available. Both companies expressed the need for guidance on using the database and navigation between the various interface modules. In addition, the guidance documents should provide sufficient clarification of terminology and definitions of the variables.

3) **Coding lists**. Both companies commented that there is a need to expand various coding lists for processes, tasks, control measures, measurement methods, etc. However, with regard to a standard coding list for job titles, there was a difference of opinion between the companies. One company felt that 'job titles' should be updated to reflect occupations rather than workplaces and that descriptions and guidance should be included to define the various jobs. In contrast, the other company stated that it would be difficult to have a predefined list of job titles and that most organisations would prefer to use job titles names specific for their own company. However, in order to be able to compare results of measurements across different companies, it is important to standardise job titles or exposure scenarios across different sites and companies.

4) **Data confidentiality.** It was reported that there may be problems with confidentiality and data protection, especially when including names of employees. However, this will only be an issue when the data are combined for several companies. Currently, Manganex is designed as a standalone system. It was also raised that employee details would most likely be held on some other system and there should be the facility to quickly and easily transfer data from one system to another.

5) **Other exposures.** It was felt that it would be a benefit if Manganex could be expanded to include other substances as both companies did not wish to use one system for Mn surveys and a separate system for other hazardous agents.

6) **Additional information.** The inclusion of fields to be able to provide details of the environmental conditions at the time of the survey; operators / static monitor's position near key exposure sources and time spent and; overall number of operators working for that particular monitoring period were raised.

7) **Sample record sheets and reporting.** Sample record sheets can be produced which contain information input during the sampling strategy development and which allow the occupational hygienist to record key information during the survey, for example, flow rates, times etc. It was considered that space should also be available to allow details of the environmental conditions at the time of sampling to be recorded (i.e. temperature, relative humidity) and that the database should be modified to allow the input of such information.

8) **Reporting**. The design of the report was felt to be too crowded and that the information provided in this should be limited.

Dissemination
With the assistance of the International Manganese Institute a workshop to present the Manganex database to member companies of IMnI was organised and scheduled for 4 December 2008. Unfortunately, due to a low take-up and several late cancellations, it was decided to cancel the workshop. Instead, we will be presenting the Manganex database at the next OHES meeting of IMnI on 17 February in Paris. In addition, copies of the Manganex database will be made available on memory sticks to members of the OHES and other interested companies. We will also send the database to the two companies that participated in the field trials.

The Manganex Database will also be presented at the MHRP Showcase Conference June 24-25 2009, Lansdowne Resort, US. We aim to prepare at least one manuscript for publication in a peer-reviewed scientific journal.

Key Research Accomplishments

1) The health endpoints and methods to determine these have been reviewed and a report produced.
2) A comprehensive and detailed exposure database tool was developed and trialled at two manganese producers.
3) A guidance document has been developed.

Reportable Outcomes

A tested occupational exposure database tool with a guidance document has been developed and is available, free of charge, to the manganese industry.

Conclusions

A tool for the systematic collection and storage of occupational exposure data in the manganese industry has been developed. This database tool was initially developed for use in large scale, international epidemiological studies of manganese exposure. In addition, the database will form a useful resource for regulatory risk assessments, such as REACH in Europe. The exposure database tool has not been populated with existing exposure data, although a project is currently underway funded by the International Manganese Institute (IMnI), during which exposure data will be collected from several production sites. The exposure data will be stored in this Manganex database.

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Searl A (2007) Review of the availability of exposure and toxicological data for manganese and its compounds relevant to the purposes of REACH. IOM Ltd Consultancy (611-00282).

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

TITLE: A Study of the Nervous System in Welders

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Nashville, TN 37203, USA

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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. 150 welders are planned to be compared to 150 referents in a cross-sectional study. Also 50 patients diagnosed with manganism are compared to 25 patients with idiopathic Parkinson's disease (PD). Ninety-six welders, 96 referents and 27 patients with manganism were studied in 2003, and their re-examination has a prospective design. Neurobehavioral tests are applied, parameters for iron status are measured and personal exposure assessed. 21 subjects will be examined with Positron Emission Tomography as well. So far 24 PD and 32 manganism patients have been examined with neurobehavioral methods in addition to 59 welders and 21 referents. Blood and urine samples have been collected from the participants together with welding fume samples from two consecutive days from each welder.

15. SUBJECT TERMS
Manganese, neurotoxicology, welders, manganism, Parkinson's disease

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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.

In this investigation 150 welders are planned to be compared to 150 non-exposed referents in a cross-sectional study design. Neurobehavioral tests are applied, parameters for iron status are determined and an extensive exposure assessment is carried out. The main objective is to assess the value of these neurobehavioral tools in an epidemiological study, in order to investigate their sensitivity for detecting subtle neurological functional changes. In addition 50 patients who have received the diagnosis of manganism is planned to be assessed with the same clinical examinations and compared with 25 patients with newly diagnosed idiopathic Parkinsons disease (PD). A small subsample of 21 subjects (7 patient with manganism, 7 with PD and 7 referents will be examined with Positron Emission Tomography (PET-Scan) as well. Ninety-six welders, 96 referents and 27 patients with manganism were studied in 2003, and thus a part of this study is to follow up as many as these subjects as possible in a prospective study design. Results from that study have been published (Ellingsen et al., 2006; Ellingsen et al., 2007; Ellingsen et al., 2008).

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.

One of the most difficult parts of this study is the sampling and examination of patients with newly diagnosed idiopathic Parkinson’s Disease (PD), because the time-lag between diagnosis and the start of medications is very short. We therefore concentrated the examinations of PD patients at the first stage of the study. As of February 10, 2009, 24 PD patients have been included into the study. Totally 25 PD patients are planned to be included. The purpose of investigating PD patients is to compare their neurobehavioral performance with the performance of former welders diagnosed as having manganism. Initially we planned to examine 50 such patients, of whom 27 were examined in 2003 as well. Up until now 32 patients with manganism have been examined with neurobehavioral methods. Of the 27 patients that were examined in 2003, 14 patients have been re-examined so far. We assume that some more subjects with manganism will be enrolled at a later stage, and also that the target of examining 25 PD patients will be achieved.

The follow-up part of welders and referents that were examined in 2003 is also challenging. Thus this task has been prioritized at this stage. We therefore started at one of the two plants that were investigated in 2003. In the first of these plants (a shipyard) 28 welders out of 40 that were also

examined in 2003 have so far been examined. The focus is currently on the second of these plants (a heavy machinery construction plant), and so far 15 welders have been examined. We have also so far re-examined 21 referents. Also 16 new welders have been examined. In the study protocol subjects (not examined before) from a third plant will also be examined. This task has been regarded by us as the easiest part of the study to perform. We have therefore not yet started the examinations there. Further, the part of the study involving PET-scan examinations has not yet been started.

In total 136 subjects have been examined so far with neurobehavioral examinations. Blood and urine samples have been collected and two air samples have been collected for each welder. We have also established databases as a prerequisite for carrying out statistical analysis.

A continuing review of the study was conducted by the end of September 2008. A renewed continuing review date was established for October 1, 2009.

Key Research Accomplishments

None so far

Reportable Outcomes

None so far

Conclusion

So far 136 subjects have been examined, of whom 78 also were examined in 2003. The aim of examining newly diagnosed PD patients has nearly been fulfilled. We are currently examining subjects that participated in the study of 2003. Some extra effort has to be put into this task, because tracing of some of the subjects is challenging. We have not yet started to examine subjects in the third plant to be studied, because this is regarded as the easiest part of the whole study. This part is planned for the autumn. Also the examination of subjects with PET-scan is planned for the autumn.

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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.

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AWARD NUMBER: W81XWH-05-1-0239

TITLE: ROLE OF MANGANESE IN PRION DISEASE PATHOGENESIS

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CONTRACTING ORGANIZATION: Iowa State University, Ames, IA 50011

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Manganese-Induced Upregulation of Prion Proteins and its Relevance to Prion Diseases

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14. ABSTRACT

Prion disease is a devastating neurodegenerative disorder that causes fatalities 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. The cellular function of this protein remains to be elucidated but studies have suggested it is a metalloprotein with a binding affinity for divalent cations. 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 confirmation and metabolism of prion proteins. Therefore, the long term objective of our project is to determine whether 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. During the current funding period, we continued to study the interaction of manganese with prion protein and made several interesting observations. Mn treatment in mouse brain slice cultures upregulated prion protein by stabilizing the protein in a time-dependent manner. Since manganese has been suggested to compete with copper for binding to the octapeptide repeat region of prion protein, we examined whether Cu treatment upregulates and stabilizes the prion protein as it does with Mn. We found copper treatment not only upregulated prion protein but also caused the shedding of prion proteins into the extracellular milieu. Additional studies with another divalent metal cadium (Cd) revealed that the metal can potently inhibit proteasomal activity, which results in greatly increased formation of high molecular weight ubiquitinated proteins. Immunohistochemical analysis also revealed a dramatic increase in the formation of oligomers after Cd treatment, which leads to ubiquitinated PrP, but did not lead to formation of PK-resistant PrP. Further, we examined Mn-neurotoxicity in scrapie-infected neuronal cells and found that infected cells are more resistant to both Mn-induced cytotoxicity and Mn-induced apoptosis. We partially attribute this response to the inability of manganese to access its binding site in the scrapie protein. The Mn-binding site may be masked due to the presence of the scrapie prion protein in the oligomeric form and/or aggregates. Current studies are underway to determine the role of octapeptide repeats in manganese-induced stabilization of prion protein and its relevance to the pathogenesis of prion diseases.

15. SUBJECT TERMS

Manganese, neurotoxicology, prion diseases, oxidative stress, and protein aggregation

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Body

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). 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 (Mouillet-Richard et al., 2000; Schmitt-Ulms et al., 2001; Chiarini et al., 2002; Nishimura et al., 2004). 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, 2003). Additional studies have shown that altered Mn content was observed in prion diseases including the human prion disease known as Crueztfelt-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). 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; suggesting that normal cellular prion interacts with manganese and protects cells from manganese neurotoxicity at early stages of exposure (Choi et al., 2007). 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).

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:

Study 1 – Manganese treatment upregulates prion protein by increasing the stability of the protein: 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. To extend our research closer to brain tissue, we examined the effect of manganese on prion protein in mouse brain slice culture models. Mouse brain slices were prepared and cultured for 1, 3, and 7 days in the presence or absence of manganese (300 µM). Following the treatment, tissues were lysed and prion proteins were measured by Western blot analysis. As shown in **Fig. 1,** a time-dependent increase in PrP expression was observed in manganese-treated slices as compared to the untreated control slices. There was no significant change in the actin protein level, which was used as a loading control.

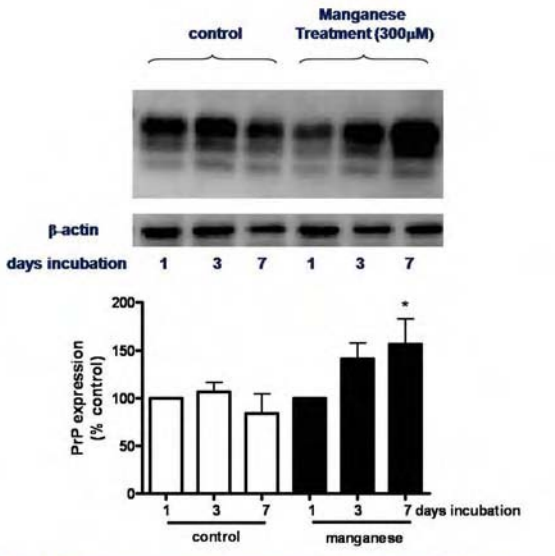

Fig 1. Manganese treatment results in increased PrP levels in mouse brain slices. Representative western blot analysis of total PrP levels in mice brain treated with manganese and untreated are shown at top. Quantification of band intensity of PrP normalized with β-actin levels.

Next we examined if the increased PrP expression in Mn-treated brain slices was a result of increased Mn uptake. Mouse brain slices were collected following treatment and processed for measurement of metals by ICP-MS. Since manganese alters the binding of other divalent cations with prion protein, concentrations of copper, iron, manganese, and zinc were analyzed in mouse brain slice samples. As seen in **Fig. 2,** manganese contents were significant increased in samples treated with manganese starting at Day 1. However, levels of other divalent cations such as iron, copper and zinc were not significantly altered during manganese treatment, and remained constant through 7 days of manganese treatment.

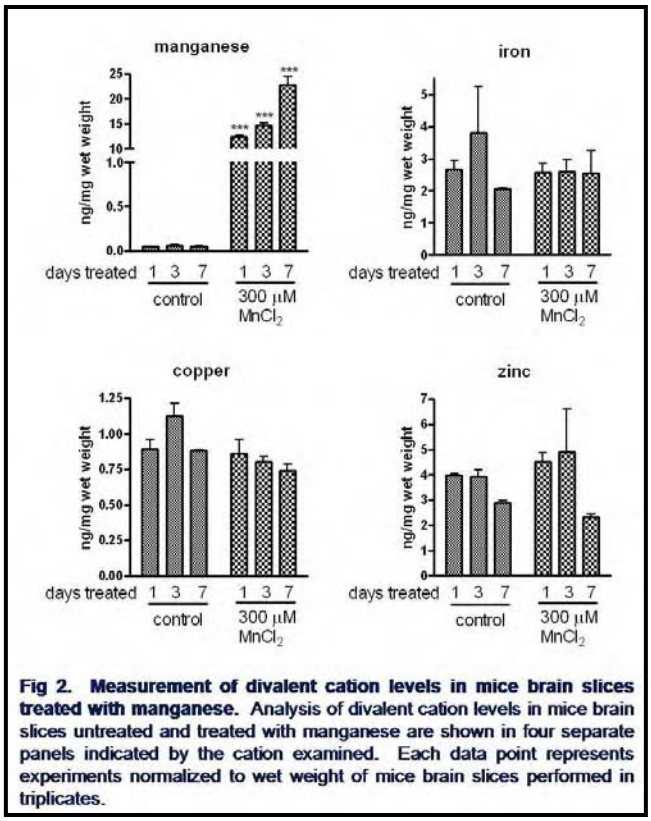

Fig 2. Measurement of divalent cation levels in mice brain slices treated with manganese. Analysis of divalent cation levels in mice brain slices untreated and treated with manganese are shown in four separate panels indicated by the cation examined. Each data point represents experiments normalized to wet weight of mice brain slices performed in triplicates.

Conclusions: Together, the results from Study 1 demonstrate that the increase in PrP^C levels and increased Mn-uptake following manganese treatment can be observed in mouse brain slice cultures. These results are consistent with our previous observations in cell culture models in which we reported that manganese upregulated prion protein by stabilizing the protein by possibly binding to the metal binding site. The mRNA level and its stability were not altered during manganese treatment. Thus, increased PrP^c stability and altered PK-resistance upon manganese exposure could play a key role in the formation of aggregated PrP^{sc} in prion diseases.

Study 2: Copper upregulates and stabilizes the prion protein similar to manganese: Since manganese has been suggested to compete with copper for binding to the octapeptide repeat region of prion protein, we examined whether copper treatment upregulates and stabilizes the prion protein like manganese. First, we examined the effect of copper on cell viability in PrP^{KO} and PrP^{C} expressing cells. The cells were exposed to various copper concentrations ranging from 0 to 10,000 μM for 24 hr and the extent of cell death was determined by the trypan blue exclusion method. **Fig 3A** shows a dose-dependent increases in cytotoxicity with increasing concentrations of copper. The cell viability curve shows that PrP^{KO} cells were more sensitive to copper toxicity as compared to PrP^{C} cells, with EC_{50} of 186 μM and 245 μM respectively. Furthermore, Cu-treated cells also had higher amounts of GSH and higher SOD activity **(Fig. 3B and 3**C). These results support the idea that PrP^{C} when bound to copper exhibits antioxidant-like activity. Correspondingly, an increase in SOD activity following copper treatment of PrP^{C} cells would explain the observed increase in intracellular glutathione levels. Also, to further characterize the difference in copper uptake between PrP^{C} and PrP^{KO} cells, copper content in the two cell lines was assessed. Measurement of intracellular levels of copper using ICP-MS revealed that PrP^{C} cells contained higher amounts of intracellular copper than PrP^{KO} cells: 23.86 ± 5.2 versus 7.31 ± 2.9 ng/mg protein, respectively. Determination of PrP levels in whole cell lysates of copper- (100μM) treated cells showed time dependent increases in prion protein levels as compared to untreated cells **(Fig. 4A)**. Interestingly, analysis of extracellular media showed a dramatic increase in prion protein levels during copper treatment, suggesting that the shedding of prion to the extracellular milieu occurs during copper treatment **(Fig 4B).** PrP levels were slightly increased in the membrane prion protein **(Fig 4C)**. The mRNA levels were not altered during copper treatment. However, copper treatment significantly reduced the amount of PrP turnover relative to the untreated control. Additionally, treatment with copper decreased the susceptibility of PrP to proteolytic digestion with proteinase-K **(Fig 5) .** No change in the proteolytic digestion rate of actin was noted, demonstrating the specific action of copper on prion protein proteolysis. Pulse analysis data showed that copper impairs the turnover rate of prion protein and there by increases the stability (Fig 6).

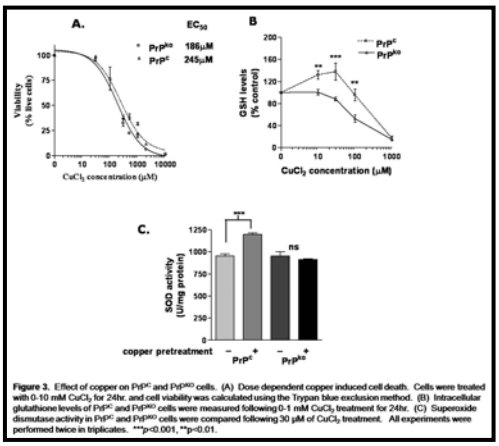

Figure 3. Effect of copper on PrP^{C} and PrP^{KO} cells. (A) Dose dependent copper induced cell death. Cells were treated with 0-10 mM $CuCl_2$ for 24hr. and cell viability was calculated using the Trypan blue exclusion method. (B) Intracellular glutathione levels of PrP^{C} and PrP^{KO} cells were measured following 0-1 mM $CuCl_2$ treatment for 24hr. (C) Superoxide dismutase activity in PrP^{C} and PrP^{KO} cells were compared following 30 μM of $CuCl_2$ treatment. All experiments were performed twice in triplicates. ***p<0.001, **p<0.01.

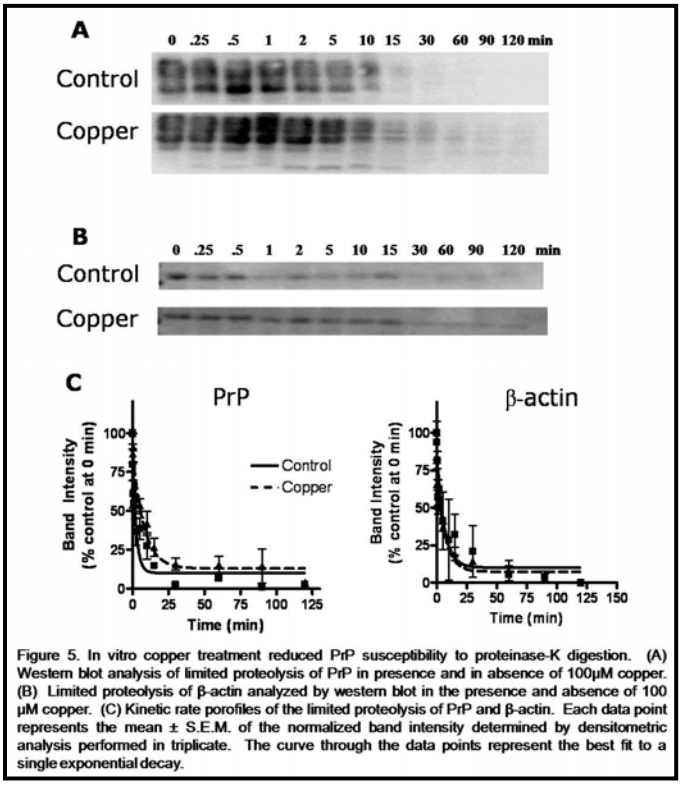

Figure 5. In vitro copper treatment reduced PrP susceptibility to proteinase-K digestion. (A) Western blot analysis of limited proteolysis of PrP in presence and in absence of 100μM copper. (B) Limited proteolysis of β-actin analyzed by western blot in the presence and absence of 100 μM copper. (C) Kinetic rate porofiles of the limited proteolysis of PrP and β-actin. Each data point represents the mean ± S.E.M. of the normalized band intensity determined by densitometric analysis performed in triplicate. The curve through the data points represent the best fit to a single exponential decay.

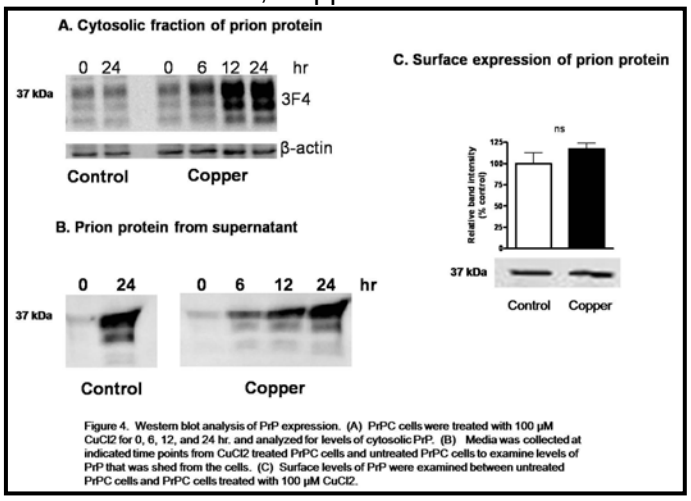

Figure 4. Western blot analysis of PrP expression. (A) PrPC cells were treated with 100 μM CuCl2 for 0, 6, 12, and 24 hr. and analyzed for levels of cytosolic PrP. (B) Media was collected at indicated time points from CuCl2 treated PrPC cells and untreated PrPC cells to examine levels of PrP that was shed from the cells. (C) Surface levels of PrP were examined between untreated PrPC cells and PrPC cells treated with 100 μM CuCl2.

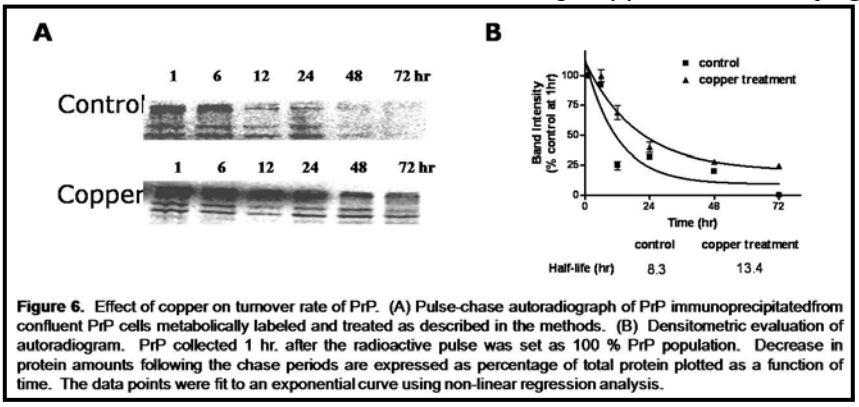

Figure 6. Effect of copper on turnover rate of PrP. (A) Pulse-chase autoradiograph of PrP immunoprecipitatedfrom confluent PrP cells metabolically labeled and treated as described in the methods. (B) Densitometric evaluation of autoradiogram. PrP collected 1 hr. after the radioactive pulse was set as 100 % PrP population. Decrease in protein amounts following the chase periods are expressed as percentage of total protein plotted as a function of time. The data points were fit to an exponential curve using non-linear regression analysis.

Conclusions: This study demonstrates copper, similar to manganese, upregulates prion protein by stabilizing the protein. Taken together with the results of manganese treatment, the data suggest that copper-bound PrP shows altered susceptibility to PK digestion, which strengthens our hypothesis that divalent Cu and Mn interact with PrP. Additionally, the results demonstrate that low dose copper can also enhance the antioxidant capacity of prion protein by increasing SOD activity and intracellular glutathione content at early time points. Also, copper induces the shedding of prion protein into the extracellular milieu.

Study 3: Manganese neurotoxicity in scrapie-infected SN56 cell line: In order to gain insight into the role of manganese in cellular mechanisms of prion protein aggregation as well as the possible pathogenesis of prion diseases, we determined the effect of manganese in an infectious model of prion diseases, i.e. the scrapie-infected SN56^{sc} cell model. First, we examined the effect of manganese on scrapie-infected SN56^{sc} cells. Treatment with varying doses produced a dose-dependent cell death in both infected and uninfected SN56 cells. However, scrapie-infected cells showed resistance to manganese toxicity. The EC_{50} for $MnCl_2$ in scrapie-infected SN56 cells was roughly twice that of uninfected cells as determined by MTT cell death assay (Fig. 7). Further, we examined whether manganese treatment induces apoptosis in SN56^{sc} cells. Caspase-3 activity was assessed as a marker of apoptosis. Although the difference between infected and uninfected cells was not significant, a slight increase in caspase 3 activity in uninfected cells was a distinct trend (Fig. 8). The mechanism underlying the resistance of scrapie-infected cells to manganese toxicity is yet to be characterized but we suggest that the resistance to manganese toxicity may be due in part to altered divalent cation binding characteristics of PrP^{Sc}. To further explicate the consequence of manganese binding to PrP^{Sc}, we aimed to determine the effect of manganese on aggregation of scrapie-infected protein. In order to achieve that goal, we first examined whether manganese had any effect on proteasomal activity in Sn56 and SN56^{sc} cells. The results showed no significant difference between the PrP^{C} and PrP^{Sc} SN56 cells following manganese treatment (Fig 9). Also basal levels of proteasome activity in untreated PrP^{C} and PrP^{Sc} SN56 cells was unchanged. These results are consistent with the results obtained with PrP^{ko} and PrP^{C} expressing cells. Presently, we are determining whether manganese accelerates the oligomerization and aggregation of scrapie infected prion proteins.

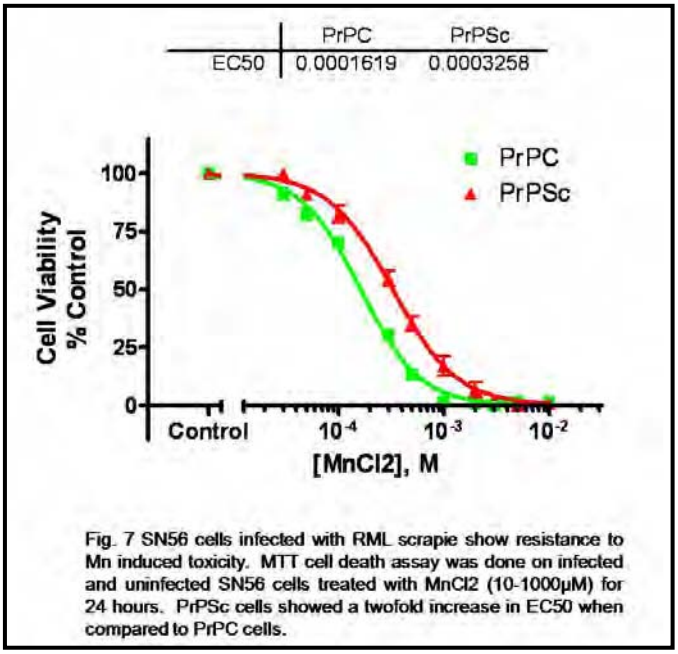

Fig. 7 SN56 cells infected with RML scrapie show resistance to Mn induced toxicity. MTT cell death assay was done on infected and uninfected SN56 cells treated with MnCl2 (10-1000μM) for 24 hours. PrPSc cells showed a twofold increase in EC50 when compared to PrPC cells.

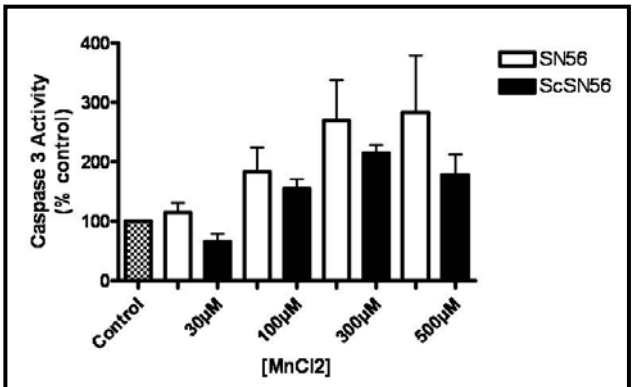

Fig. 8 Caspase-3 activity in Sn56 and ScSN56 cells treated with manganese. Cells were treated with MnCl2 for 24 hours and lysate was incubated with Ac-DEVD-AFC caspase-3 specific substrate for 1 hour at 37°C. Fluorescence was then read by plate reader and normalized to mg protein. Activity is expressed as percent control.

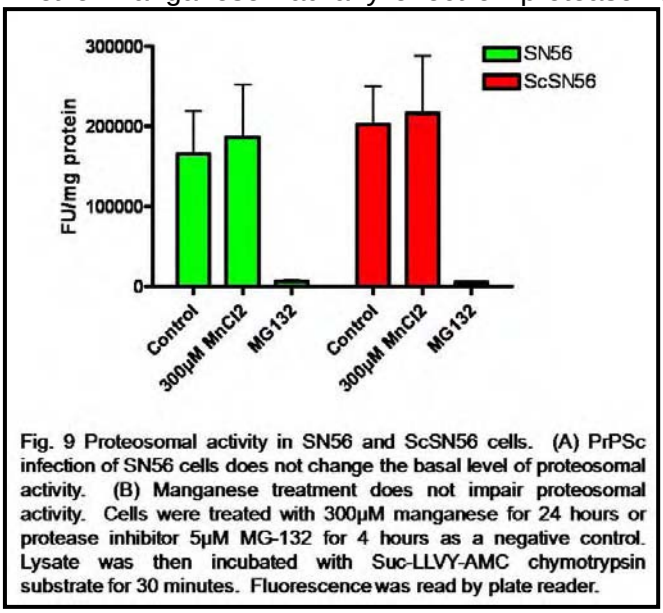

Fig. 9 Proteosomal activity in SN56 and ScSN56 cells. (A) PrPSc infection of SN56 cells does not change the basal level of proteosomal activity. (B) Manganese treatment does not impair proteosomal activity. Cells were treated with 300μM manganese for 24 hours or protease inhibitor 5μM MG-132 for 4 hours as a negative control. Lysate was then incubated with Suc-LLVY-AMC chymotrypsin substrate for 30 minutes. Fluorescence was read by plate reader.

Conclusions: The data suggest that scrapie-infected neuronal cells are more resistant to Mn-induced cytotoxicity as well as Mn-induced apoptosis. We partially attribute this response to the inability of manganese to access its binding site in the scrapie protein. The Mn-binding site may be masked due to the presence of the scrapie prion protein in the oligomeric form and/or aggregates.

Study 4: Cadmium, not manganese, impairs the neuronal proteasomal system leading to formation of protein aggregation and ubiquitinated prion proteins: To determine whether the neurotoxic effects of Mn on prion protein were specific to Mn, we tested another divalent metal cadmium. Exposure of cadmium to PrPc neuronal cells caused both dose- and time-dependent cell death in mouse neuronal cells, as measured by the trypan blue exclusion method **(Fig. 10A and B)**. Dose response analysis revealed cadmium was more toxic than manganese and copper. The EC50 value of $CdCl_2$ was approximately 12μM after 24 hr of treatment. Phase-contrast images and Sytox, green nucleic acid staining further confirmed the neurotoxic effect of cadmium in this cell model **(Fig 10C).** Next, we examined the enzymatic activity of the 20S/26S proteasome following manganese and cadmium treatments after 24 and 48h.

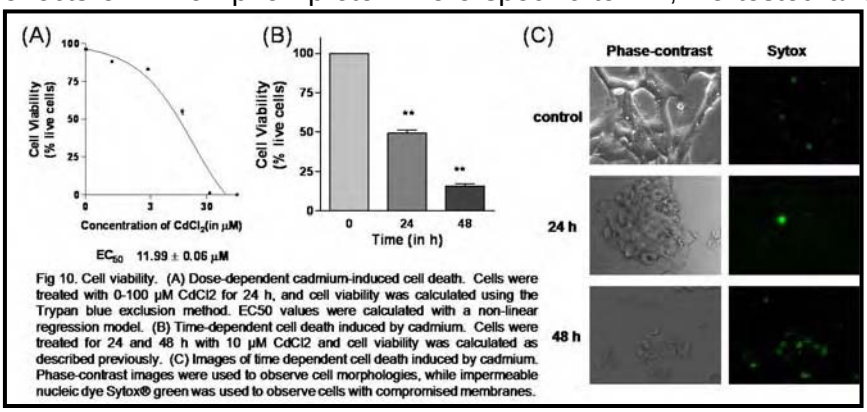

Fig 10. Cell viability. (A) Dose-dependent cadmium-induced cell death. Cells were treated with 0-100 μM CdCl2 for 24 h, and cell viability was calculated using the Trypan blue exclusion method. EC50 values were calculated with a non-linear regression model. (B) Time-dependent cell death induced by cadmium. Cells were treated for 24 and 48 h with 10 μM CdCl2 and cell viability was calculated as described previously. (C) Images of time dependent cell death induced by cadmium. Phase-contrast images were used to observe cell morphologies, while impermeable nucleic dye Sytox® green was used to observe cells with compromised membranes.

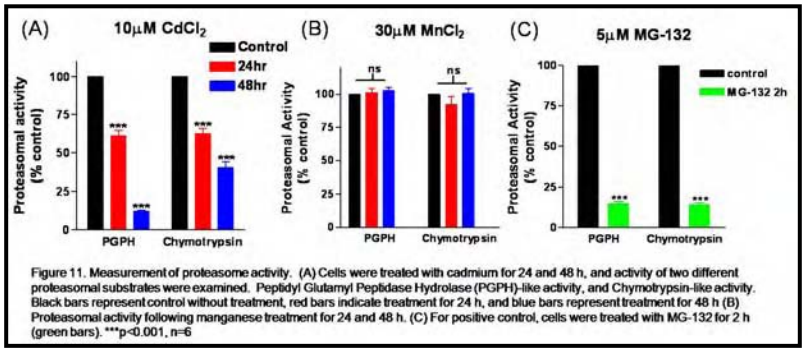

Figure 11. Measurement of proteasome activity. (A) Cells were treated with cadmium for 24 and 48 h, and activity of two different proteasomal substrates were examined. Peptidyl Glutamyl Peptidase Hydrolase (PGPH)-like activity, and Chymotrypsin-like activity. Black bars represent control without treatment, red bars indicate treatment for 24 h, and blue bars represent treatment for 48 h (B) Proteasomal activity following manganese treatment for 24 and 48 h. (C) For positive control, cells were treated with MG-132 for 2 h (green bars). ***p<0.001, n=6

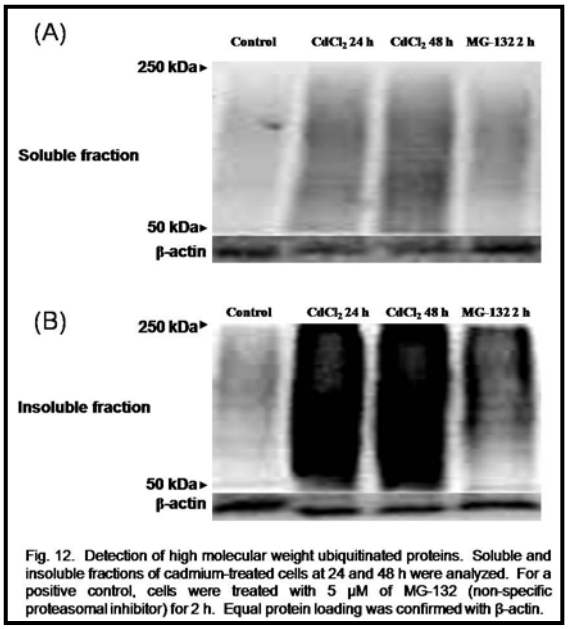

Fig. 12. Detection of high molecular weight ubiquitinated proteins. Soluble and insoluble fractions of cadmium-treated cells at 24 and 48 h were analyzed. For a positive control, cells were treated with 5 μM of MG-132 (non-specific proteasomal inhibitor) for 2 h. Equal protein loading was confirmed with β-actin.

Fluorogenic substrates for peptidyl glutamyl peptide hydrolase-(PGPH) like activity and chymotrypsin-like activity were used to evaluate the proteasome activity as shown in **Fig 11A and B.** Treatment with 10μM $CdCl_2$ resulted in a highly significant decrease ($p<0.001$) in both PGPH- and chymotrypsin-like activity at 24 and 48 hr. In contrast, treatment with 30 μM $MnCl_2$ did not result in inhibition of proteasome activity in any of the substrates. As a positive control, treatment with 5 μM MG-132 for 2 hr resulted in a significant decrease ($p<0.001$) in proteasomal activities (**Fig. 11C)**. Furthermore, cadmium-induced impairment of proteasome activity resulted in the accumulation of high molecular weight ubiquitinated proteins in both soluble and insoluble fractions after cadmium treatment (**Fig 12).** Compared to the control group, both 24 and 48 hr treatments with cadmium resulted in a significant increase in ubiquinated proteins (second and third lanes, respectively) in both soluble and insoluble fractions. Treatment with MG-132 also resulted in increased ubiquitinated proteins in both soluble and insoluble fractions (fourth lane). Surprisingly, cadmium-treated samples showed more ubiquitinated protein than MG-132, indicating that cadmium is a potent inhibitor of proteasome activity in cell culture models of prion diseases.

Next we examined whether Cadmium induces formation of soluble oligomers and proteinase-K-resistant prion proteins. The recent development of A11 anti-oligomer antibody that recognizes amino acid sequence-independent oligomers of proteins has enabled us to determine the soluble protein oligomers in a cell culture model of prion disease. PrPc cells were treated with cadmium for 24 and 48 hr and then oligomeric proteins were detected by immunostaining with A11 antibody **(Fig 13).** Additional dot blot analysis of whole cell lysate further confirmed the formation of oligomeric proteins during cadmium treatment **(Fig. 14A).** To verify the cellular location of oligomer formation, nuclear and cytoplasmic fractions were obtained from the samples and dot blot analysis revealed no observable difference in the nuclear fraction of control or cadmium-treated samples **(Fig. 14B)**. However, analysis of the cytoplasmic fraction indicates a clearly significant difference in the amount of oligomers that are detected in the cytoplasm with cadmium treatment **(Fig. 14B).** Furthermore, cadmium treatment did not result in formation of prion proteins that were resistant to proteinase-K digestion as determined by limited proteolysis when compared to the control. Together, these data show that cadmium treatment results in increased formation of soluble oligomers but does not induce the formation of PK resistant prion protein.

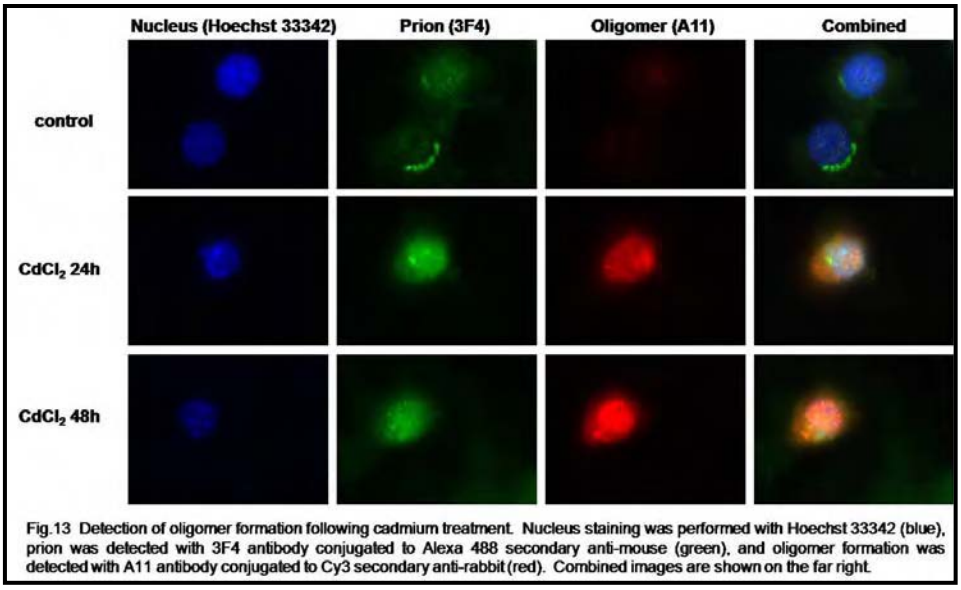

Fig.13 Detection of oligomer formation following cadmium treatment. Nucleus staining was performed with Hoechst 33342 (blue), prion was detected with 3F4 antibody conjugated to Alexa 488 secondary anti-mouse (green), and oligomer formation was detected with A11 antibody conjugated to Cy3 secondary anti-rabbit (red). Combined images are shown on the far right.

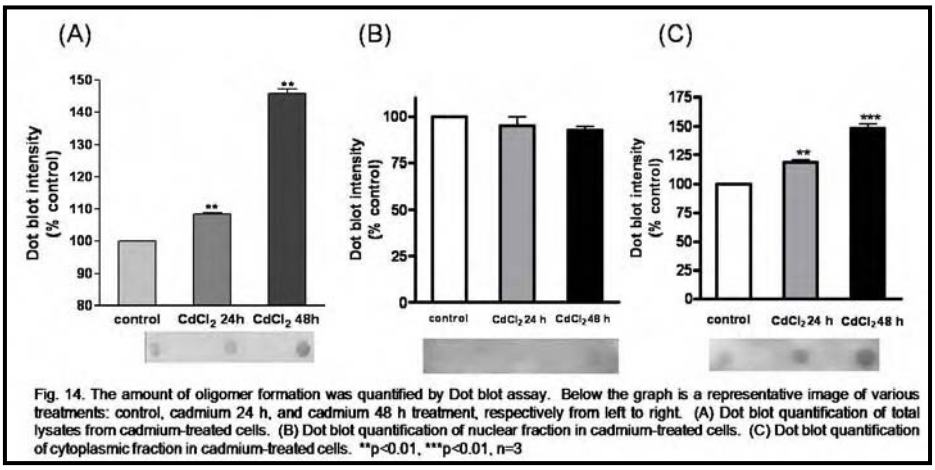

Fig. 14. The amount of oligomer formation was quantified by Dot blot assay. Below the graph is a representative image of various treatments: control, cadmium 24 h, and cadmium 48 h treatment, respectively from left to right. (A) Dot blot quantification of total lysates from cadmium-treated cells. (B) Dot blot quantification of nuclear fraction in cadmium-treated cells. (C) Dot blot quantification of cytoplasmic fraction in cadmium-treated cells. **p<0.01, ***p<0.01, n=3

Conclusions: Together, our results suggest that certain divalent metals such as cadmium could impair proteasomal function in neuronal cells leading to increased ubiquitination of proteins and cell death. However, manganese does not alter proteasome function. Taken together, manganese enhances prion accumulation by increasing the stability of prion protein, whereas cadmium causes oligomerization and aggregation of prion proteins by impairing the proteasomal degradation machinery.

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, and single-phase exponential decay was fit to the data. $P<0.05$ was considered significant and differences are indicated with asterisks.

Key Research Accomplishments

- Manganese upregulated prion protein by increasing the stability of the protein,
- Manganese neither altered the prion mRNA levels nor inhibited proteasomal function,
- Manganese reduced the proteinase-K dependent proteolysis of prion protein.
- Similar to manganese, copper upregulated prion protein independent of transcriptional activation and proteasomal degradation.
- Low dose copper enhances the antioxidant capacity of prion protein.
- Copper also reduced the proteinase-K dependent proteolytic rate of PrP^{c}, suggesting a common mechanism of protein stability by manganese and copper.
- Copper induces the shedding of prion protein into the extracellular milieu.
- Manganese-induced toxicity in the scrapie-infected $SN56^{sc}$ cell model of prion disease but the toxicity was less than the toxicity induced in uninfected cells.
- Manganese did not alter the proteasome activity in uninfected and scrapie-infected cells.
- Another divalent metal, cadmium, was a potent inhibitor of proteasome function and caused oligomerization and aggregation of prion protein.

Reportable Outcomes

Manuscripts/Abstracts

- Choi C. J., Anantharam, V., Nicholson, EM., Richt, J.A, Kanthasamy, A. and Kanthasamy, A.G (2009). Manganese stabilizes cellular prion protein and alters the Rate of Proteinase-K-Dependent Limited Proteolysis in Neuronal cells. Prepared for submission to Tox. Sci
- Choi C. J., Anantharam, V., Nicholson, E.M., Richt, J.A, Kanthasamy, A. and Kanthasamy, A.G (2006). Copper upregulates cellular Prion proteins and inhibits the rate of Proteinase-K dependent limited proteolysis in neuronal cells. Under preparation.
- Choi C. J., Anantharam, V., Nicholson, EM., Richt, J.A, Kanthasamy, A. and Kanthasamy, A.G (2009). Manganese stabilizes cellular prion protein and alters the Rate of Proteinase-K-Dependent Limited Proteolysis. Presented at the 2008 Neuroprion meeting at Madrid, Oct 8-10, 2008.
- 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.

Current faculty receiving support from the grant:

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

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PRINCIPAL INVESTIGATOR: Sophie Rocks, PhD
(role formally undertaken by Prof Leonard Levy, PhD and Philip Holmes)

CONTRACTING ORGANIZATION: Institute of Environment and Health, First Floor, Building 63, Cranfield University, Cranfield, Bedfordshire MK43 0AL, UK

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14. ABSTRACT

IEH has worked — in collaboration with the International Manganese Institute (IMnI) and their subcontractor Intendance Limited — since November 2005 on the provision of knowledge management services (Research Core 6) to provide the research and regulatory communities with web-based information on developments relevant to assessing adverse health effects of manganese. Fields considered comprise of: epidemiology; human exposure assessment; toxicological mechanisms; human susceptibility; and treatment of patients exposed to excessive levels of manganese. During the period 1st January 2008 to 31st December 2008, another four quarterly reports have been posted on the MHRP website; it is anticipated that such reports will continue to be produced at 3 monthly intervals throughout the duration of the project. A Research Overview Report discussing the significance of new knowledge gained through research published between March 2007 and February 2008, was also posted on the MHRP website in September 2008. A further annual report (for March 2008 - February 2009) is scheduled for publication in March 2009. IEH and collaborators have also established the Database of Global Research Activity on Manganese (Dogram) as an up-to-date source of ongoing research activities. This currently contains information on 35 projects and 58 researchers.

15. SUBJECT TERMS
Manganese; Health; Published information

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Table of Contents

Introduction

While manganese is an essential trace element, associations with a number of adverse human health effects have been identified at high occupational or environmental exposures, and the health effects of inorganic forms of manganese published before 2002 was comprehensively reviewed in a recent Criteria Document (CD) (IEH, 2004). Since the 2002 assessment was produced, there has been - and continues to be - extensive research activity (including that embodied within the current MHRP research program) into aspects as diverse as occupational and environmental exposures, epidemiology, mechanisms of toxicity, and the development and implementation of new medical approaches to the treatment of excessive manganese exposures.

Given the scale of current research (with, for example, over 500 references relating to inorganic forms published in 2002 alone), this project (Research Core 6), undertaken by the Institute of Environment and Health (IEH) at Cranfield University, in collaboration with the International Manganese Institute (IMnI) and their subcontractor Intendance Limited, was designed to provide readily available sources by which researchers and those sponsoring research programs could readily access up-to-date information on recently reported and ongoing projects, so as to avoid duplication of efforts and thereby proactively enhance efficiency. In addition, the data sources generated in this project were intended to facilitate and encourage multicentre and multidisciplinary research collaborations through facilitating networking and to provide both the technical and general viewer with readily understandable and freely available sources of information on the latest developments in understanding.

Following the retirement, in September 2008, of the then Principal Investigator, Prof Leonard Levy OBE, and the impending departure of the current PI Philip Holmes from IEH at Cranfield University, Dr Sophie Rocks has – as of January 2009 – taken up this responsibility. Dr Rocks, a toxicologist specializing in nanotoxicology, is currently a Toxicologist within IEH and has also been recently appointed as a member of the UK Advisory Committee on Hazardous Substances (ACHS). Dr Rocks, along with Prof Simon Pollard in Cranfield University's School of Applied Sciences, has also established an EPSRC/ESRC/NERC/Defra Collaborative Centre of Excellence in Understanding and Managing Natural and Environmental Risks (Risk Centre) at Cranfield University, where Dr Rocks is Centre Manager. She has degrees in Biochemistry and Toxicology from the University of Surrey and a PhD from Barts and The London, Queen Mary's School of Medicine and Dentistry, University of London, and has had a varied research career to date with interests in inflammatory diseases and biomaterials. She also has experience of materials science gained within the internationally-renowned Microsystems and Nanotechnology Centre at Cranfield University where she was involved in the production and characterization of nanoparticles and functional ceramics. More recently, Dr Rocks has managed projects considering the risk assessment approaches for manufactured nanomaterials and their suitability, as well as the risk associated with emerging nanotechnologies. The work that will now fall to Sophie Rocks relates to: overall project management responsibility for delivery of this project; scientific scrutiny and editing of the quarterly updates of published literature that will continue to be produced over the remainder of the project; deciding - in conjunction with our Information Scientist (Lini Ashdown) - on the correct key wording of DOGRAM entries; and preparation of the annual research overview (the next of which is due to start preparation in March 2009). Indeed, Dr Rocks was the main author of the latest of these annual overviews published on the MHRP website in September 2008. It is however envisaged that Prof Leonard Levy OBE will continue to provide additional scientific expertise to the program, particularly in relation to the annual research report.

Body

OVERALL OBJECTIVES

The objectives of the Core 6 research project is to:

- create a database of information on recently completed and ongoing research projects;
- provide a current awareness service on a quarterly basis; and
- provide short annual "state of the science" reviews, identifying recent key scientific papers.

The intention has been to provide these services – on a free-access basis – via a specially constructed web-site, containing this and other information relevant to the study of the health effects of manganese.

PROJECT ELEMENT 1 – Establishment of a Database of Information

Following a detailed analysis of functionaility and specification requirements focussing on ease of use and access to the information by users, the project team developed a database and website using Microsft database and Internet server technologies (including .Net, SQL Server, XML). The database, known as *"Database of Global Research Activity on Manganese*" (DOGRAM), is available on the website at: http://www.manganese-health.org/home. The database was designed to be viewed using Browse facilities **(**by Project, Researcher, Research organization and Funder) and by use of Search facilities (using a customized 4-level thesaurus based on defined keywords as well as by a free text search facility).

Information for entry onto DOGRAM is gathered by a dedicated project team using email prompts to potential researchers and research groups around the world (identified from the output of the quarterly update report; see below) or through researchers obtain the questionnaire in electronic form and returning it either as hardcopy by post or as an email attachment.

Thus, DOGRAM is a fully searchable inventory of current research activities relating to the potential health effects of, and methods of controlling and treating, exposure to manganese. As such, its scope is intended to encompass investigation of both inorganic and organic forms of manganese, and includes:

- Estimation of the contributions of environmental and occupational manganese exposure to health, disease and dysfunction;
- Investigation of the physiological and biochemical mechanisms (including toxicokinetic considerations);
- Investigation of the physiological mechanisms that govern manganese accumulation within the brain, with special emphasis on the role of olfactory transport of the metal;
- Assessment of the influence of factors, such as age, nutritional deficiencies, pre-existing disease and genetics, that influence individual susceptibility to manganese;
- Investigation of the roles and mechanisms of manganese toxicity, including its role in neurodegenerative disease;
- Measurement and/or modeling of occupational or environmental exposure;
- Identification of existing and novel biomarkers of exposure or adverse effects; and
- Development and implementation of new medical approaches to the treatment of excessive manganese exposure.

DOGRAM thus provides in an ongoing manner an effective and powerful tool for identifying current and recently completed research activities and the key workers in relevant fields. As such, it is of value to stakeholders and researchers, providing a means of minimizing the risk of duplication of effort, and over time should facilitate the identification of changing patterns of research activity, gaps in programs, opportunities for collaboration, and emerging areas of concern.

PROJECT ELEMENT 2 – Current Awareness Services

Using a comprehensive structured search strategy (see Tabulations below) literature searches have been performed at approximately 13 week intervals on Medline (1966+), Embase (1974+), Pascal (1990+), Biosis (+1969) and Toxfile (1966+), using the host Dialog DataStar. The search terms used to denote for manganese substance (see Set 1), CAS (see Set 2) and toxicity (see Set 3) are listed below.

Set 1

Substance – title, abstract, descriptors
Braunite
Cianciulliite
Ferromanganese or ferro manganese - FeMn
Ferrosiliconmanganese or ferro silicon manganese
Manganese ore$1
Manganese oxide$1
Manganese sulphate or manganese sulphate
Manganese with steel – (title, abstract)
Manganous salt$1

Manganous Manganic Oxide or Hausmannite – Mn_3O_4
Polianite
Pyrochroite
Pyrolusite (manganese oxide)
Ramsdellite (manganese oxide)
Siliconmanganese or silicon manganese
Sodium manganate – Na_2MnO_4
Manganese
Manganese carbonate – $MnCO_3$
Manganese chloride or Manganese (II) chloride – $MnCl_2$
Manganese (III) fluoride – MnF_3
Manganese oxide or Manganese tetroxide – Mn_3O_4
Manganese (II) oxide – MnO
Manganese (III) oxide – Mn_2O_3
Manganese dioxide or Manganese (IV) oxide – MnO_2
Manganese nitrate or Manganese (II) nitrate – $Mn(NO_3)_2$
Manganese sulphate or Manganese (II) sulphate – $MnSO_4$
Manganese sulphide or Manganese (II) sulphide – MnS
Manganese oxide – MnO
Barium manganate – $BaMnO_4$
Potassium manganate – K_2MnO_4
Potassium permanganate or Potassium (VII) manganate –$KMnO_4$

Set 2

CAS No.	Substance
7439-96-5	Manganese
598-62-9	Manganese carbonate – $MnCO_3$
13446-34-9	Manganese chloride tetrahydrate
7773-01-5	Manganese chloride or Manganese (II) chloride – $MnCl_2$
7783-53-1	Manganese (III) fluoride – MnF_3
1317-35-7	Manganese oxide/Manganese tetroxide – Mn_3O_4
1344-43-0	Manganese (II) oxide – MnO
1317-34-6	Manganese (III) oxide – Mn_2O_3
1313-13-9	Manganese dioxide or Manganese (IV) oxide – MnO_2
10377-66-9	Manganese nitrate or Manganese (II) nitrate – $Mn(NO_3)_2$
15710-66-4	Manganese (II) nitrate hydrate
7785-87-7	Manganese sulphate or Manganese (II) sulphate – $MnSO_4$
18820-29-6	Manganese sulphide or Manganese (II) sulphide – MnS
1344-43-0	Manganese oxide – MnO
7787-35-1	Barium manganate - $BaMnO_4$
10294-64-1	Potassium manganate – K_2MnO_4
7722-64-7	Potassium permanganate or Potassium VII manganate -$KMnO_4$

Set 3

Medline, Toxline	**Embase**	**Biosis, Pascal**
Carcinogen$5.ti,de,ab.	Carcinogen$5.ti,de,ab.	Carcinogen$5.ti,de,ab.
Tumor-markers-biological#	Carcinogen-testing#	Mutagen$5.ti,de,ab.
Carcinogenicity-tests#	Carcinogenic-activity#	Genotoxic$5.ti,de,ab.
Carcinogens-environmental#	Carcinogen-dna-interaction#	Cytotox$5.ti,de,ab.
Mutagen$5.ti,de,ab.	Mutagen$5.ti,de,ab.	Epidemiology.ti,de,ab.
Mutagenecity-tests#	Mutagenic-agent#	
Genotoxic$5.ti,de,ab.	Chemical-mutagen#	
dna-damage#	Promutagen#	
Cytotox$5.ti,de,ab.	Mutagen-testing#	
Epidemiologic-factors#	Chemical-mutagenesis#	
Epidemiologic-methods#	Environmental-mutagen#	
Epidemiology#	Mutagenic-activity#	
Effect-modifiers-epidemiolgy#	Genotoxic$5.ti,de,ab.	
Epidemiolog$2.ti,de,ab.	Cytotox$5.ti,de,ab.	
	Cytotoxic-agent#	
	Cell-mediated-cytotoxicity#	

	Cytotoxicity-test#	
	Epidemiology#	
	Cancer-epidemiology#	

The terms/phrases were searched for in abstracts, descriptors and titles; Truncation was used where appropriate.

Set 1 and 2 were then combined using the Boolean operator 'OR', and the results from the Set 1/2 were then combined with Set 3, using the Boolean operator 'AND'.

Based upon this exhaustive search of the published information, relevant English-language papers on manganese were identified and categorized by an experienced toxicologist, before preparation of a summary report which is then posted on the MHRP website. Abstracts are categorized into the following sections:

Section 1 - EXPOSURE MEASUREMENT AND MODELLING: Papers relating to the measurements or modelling of environmental and occupational manganese exposure, the development of biomarkers of exposure or effect.

Section 2 - HEALTH EFFECTS: Papers on the influence of manganese on health, disease and dysfunction.

Section 3 - MECHANISMS: Papers on the physiological, biochemical and cellular mechanisms underlying the toxic effects of manganese.

Section 4 - HUMAN SUSCEPTIBILITY: Papers relating to assessment of the influence of genetic and epigenetic factors on human susceptibility to the effects of manganese.

Section 5 - TREATMENT AND IMAGING: Papers on the development and implementation of new medical approaches to the treatment of excessive manganese exposure.

Section 6 - MISCELLANEOUS: Other papers considered of interest or potential relevance to the study of the health effects of manganese.

To date, a total of 11 reports identifying published information (covering January 2002 to November 2008) have been posted on the MHRP website.

PROJECT ELEMENT 3 – Production of state of the science reviews on manganese

A Research Overview Report discussing the significance of new knowledge gained through research published between 2002 (publications before this were addressed in the IEH Criteria Document) and February 2007 (time of publication of the 4th awareness update report) was posted on the MHRP website in December 2007. This has been followed by a further report published in September 2008 that related to papers published between March 2007 and February 2008. Each report summarises established thinking on the toxicology of manganese and its inorganic compounds, and considered the implications of the recently published studies that have appeared in the scientific literature and are identified on the MHRP database. Each report was prepared for use by a wide readership (including researchers, interested scientists and health professionals) but is also intended to be of value to any laypersons who may wish to have an overview of manganese toxicity and recently published research.

In summary, the most recent September 2008 publication considered studies on both environmental and occupational exposures to manganese that were reported over a 12 month period. Examples of exposures to manganese at concentrations greater than the WHO guideline value of 400 µg/l were noted to have been reported in a number of different areas during the period including in samples of commercially-available bottled water in Italy and in food stuffs grown in areas surrounding industrial plants. One occupational study of welders highlighted the fact that manganese concentration in welding fumes has been shown to be affected by voltage (increased voltage lead to increased manganese concentration). This observation is in keeping with other studies of welding fumes that have consistently shown that welding fume concentrations can be markedly affected by the applied voltage and that a reduction of fume is best achieved by keeping the voltage at low as possible consistent with a good work-piece weld.

After human exposure to manganese, the concentrations in urine, blood, saliva were shown in papers to be increased. This increase in these manganese biomarkers was correlated with the increase in the MRI signal intensity in the globus pallidus, and concentrations in tooth enamel and hair, although these are not quantifiable. These biological manganese concentrations were shown to decrease over time after the cessation of exposure.

Papers on a number of potential mechanisms for manganese transfer across the blood brain barrier were also published; transport mechanisms considered included facilitated diffusion; active transport; DMT-1-mediated transport; ZIP8-mediated transport; DAT-mediated transport; and Tf-dependant transport. However despite these recent insights, the predominant mechanism is not yet clearly established, although it appears that certain mechanisms (e.g. DMT-1 mediated transport) may not be as important as others. There is some evidence that low molecular weight species, such as manganese citrate, may pass directly across the blood-brain barrier without need of a transport facilitator.

Toxicological studies have shown that manganese exposure via inhalation may affect a range of biomarkers in different brain regions (e.g. striatal GABA and dopamine expression). The alteration in neuronal metabolic function and regulation has also been shown to differ significantly in the early phase of manganese neurotoxicity and this may be important in determining the severity of subsequent cellular injury.

Papers published during the period under consideration, have provided further information on the potential associations between manganese exposure/accumulation and effects on neurobehavioural, motor control, childhood behaviour and development, neuropsychological behaviour and respiratory function, as well as the controversial potentially-increased likelihood of Parkinson's Disease development (although there are conflicting reports) and a speculative potential link with CJD. The respiratory effect of occupational exposure may be due to the inhalation of particulate matter, but has been suggested to be linked to subclinical neurological symptoms and blood manganese concentrations.

Pre-existing metabolic disorders have been shown to affect manganese accumulation, which could in turn elicit secondary symptoms, and it has been suggested that liver transplantation may be an appropriate route to treat severe manganese accumulation and manganism, if all other options become exhausted.

Concerns have also been expressed that data from the experimental manganese administration in animal models may be unrepresentative of the human manganese neurotoxicity that may occur at low concentrations, raising concerns that existing animal models are of limited relevance for the risk assessment of chronic low-level exposure to humans. These concerns, if vindicated, would directly impact on the scientific background used to establish current guidelines (e.g. the WHO guideline value for manganese in drinking water [400 µg/l)). Furthermore, some authors have suggested that the increasing number of studies reporting associations between neurologic symptoms and manganese exposure in children warrant a re-evaluation of this guidance value.

A third report (to focus on publications in the period March 2008 - February 2009) is scheduled for publication in March or April 2009.

IMPACT

The MHRP website and in particular the database provides information on the objectives, scope and methods being employed in ongoing research projects, together with insight into funders with an interest in this field (information that would not be readily available in a consolidated format through other means). As such this facilitates the development of an understanding of the ongoing profile of research, an aspect that cannot be identified from the existing databases on published literature which are, by their very nature, retrospective, and may be several years following completion of investigations. It is designed to be of value to a wide audience, drawing as it does on a wide rnage of study types of potential relevance to addressing the potential health effects of manganese. These include topic areas such as: occupational and environmental epidemiological studies, clinical case reports, experimental volunteer studies, and *in vivo* and *in vitro* mechanistic studes (including any application of *–omic* technologies). Together with the awareness update reports, produced at 13

week intervals and the overview scientific assessments, this project has succeded in establishing a valuable information resource to researchers, funders and regulators and the wider communittee.

Conclusions

As part of the research programme supported by the Manganese Health Research Program (MHRP), the Institute of Environment and Health (IEH) at Cranfield University, UK, has undertaken — in collaboration with the International Manganese Institute (IMnI) and their subcontractors Intendance Limited, UK (that designed and maintains the MHRP website) — the provision of knowledge management services (identified as Research Core 6: Provision of Research Activity Awareness Services in the MHRP Phase 1 research programme) for the MHRP. As discussed above, as from January 2009 Dr S Rocks will succeed to the position of PI for this project, and will thus be responsible for fulfilling the role and responsibilities vacated by Prof L Levy and P Holmes.

Our collaborative activities have been directed towards providing the MHRP, and the wider research and regulatory community, with easily accessible (web-based) information on the latest developments in the fields of: epidemiology; human exposure assessment; toxicological mechanisms and the study of human susceptibility; and the treatment of patients exposed to excessive levels of manganese (see: http://www.manganese-health.org/home).

The Year 1 activities on the project were initiated at IEH in November 2005, with the regular production of 3-monthly (quarterly) updates and annual overview reports, together with maintenance and expansion of the DOGRAM database, continuing thereafter throughout the remainder of the project.

Key Research Accomplishments

This service focused project has achieved all its objectives, namely:

- Creation of a database (DOGRAM) of information on recently completed and ongoing research projects;
- Provision of a current awareness service of published papers on a 13-week (quarterly) basis; and
- Publication of short annual “state of the science” review reports, identifying recent key scientific papers and findings.

Reportable Outcomes

Creation of a database (DOGRAM) of information on recently completed and ongoing research projects;

Provision of a current awareness service of published papers on a 13-week (quarterly) basis; and

Publication of short annual “state of the science” review reports, identifying recent key scientific papers and findings.

These outputs are all accessable via: http://www.manganese-health.org/home

Current faculty receiving support from the grant:

- Philip Holmes
- Lini Ashdown
- Sophie Rocks

References

Not applicable

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AWARD NUMBER: W81XWH -05-1-0239

TITLE: Manganese Research Health Project (CFDA No. 12.420): Provision of Research Activity Awareness Services

PRINCIPAL INVESTIGATOR: Sophie Rocks, PhD

CONTRACTING ORGANIZATION: Institute of Environment and Health, First Floor, Building 63, Cranfield University, Cranfield, Bedfordshire MK43 0AL, UK

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Not applicable

14. ABSTRACT

On 15 January 2009, Cranfield Univeristy and Vanderbilt University signed a contract to provide further resource support for the Research Activity Awareness Services (Research Core 6) undertaken by IEH in connection with the MHRP research program. The various activities in the work program have been ongoing — in collaboration with the International Manganese Institute (IMnI) and their subcontractor Intendance Limited — since November 2005 using funding from another contract with Vanderbilt Univeristy (drawn from Grant Number VUMC 31529), and the intention is for this new funding stream to ensure continuity of support for the service following exhaustion of the original grant. It is anticipated that activities under this grant will commence within 3-6 months of its establishment.

15. SUBJECT TERMS
Manganese; Health; Published information

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					19b. TELEPHONE NUMBER *(include area code)* +44 (0) 1234 758511

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Introduction

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Given the scale of current research (with, for example, over 500 references relating to inorganic forms published in 2002 alone), the initial project (Research Core 6) undertaken by the Institute of Environment and Health (IEH) at Cranfield University, in collaboration with the International Manganese Institute (IMnI) and their subcontractor Intendance Limited, was designed to provide readily available sources by which researchers and those sponsoring research programs could readily access up-to-date information on recently reported and ongoing projects, so as to avoid duplication of efforts and thereby proactively enhance efficiency. In addition, the data sources generated by the earlier project were intended to facilitate and encourage multicentre and multidisciplinary research collaborations through facilitating networking and to provide both the technical and general viewer with readily understandable and freely available sources of information on the latest developments in understanding. It is the intention of the current project to continue the full range of activities that have been ongoing since November 2005 so as to ensure continuity of service provision.

Following the retirement, in September 2008, of the intended Principal Investigator (PI), Prof Leonard Levy OBE, and the impending departure of the then-nominated PI, Philip Holmes from IEH at Cranfield University, Dr Sophie Rocks has – as of 15 January 2009 – taken up the responsibility for this and the earlier project. Dr Rocks, a toxicologist specializing in nanotoxicology, is currently a Toxicologist within IEH and has also been recently appointed as a member of the UK Advisory Committee on Hazardous Substances (ACHS). Dr Rocks, along with Prof Simon Pollard in Cranfield University's School of Applied Sciences, has also established an EPSRC/ESRC/NERC/Defra Collaborative Centre of Excellence in Understanding and Managing Natural and Environmental Risks (Risk Centre) at Cranfield University, where Dr Rocks is Centre Manager. She has degrees in Biochemistry and Toxicology from the University of Surrey and a PhD from St. Barts and The London, Queen Mary's School of Medicine and Dentistry, University of London, and has had a varied research career to date with interests in inflammatory diseases and biomaterials. She also has experience of materials science gained within the internationally-renowned Microsystems and Nanotechnology Centre at Cranfield University where she was involved in the production and characterization of nanoparticles and functional ceramics. More recently, Dr Rocks has managed projects considering the risk assessment approaches for manufactured nanomaterials and their suitability, as well as the risk associated with emerging nanotechnologies. The work to be conducted under the current contract will fall to Dr Rocks (overall project management responsibility for delivery of this project; scientific scrutiny and editing of the quarterly updates of published literature), in conjunction with our Information Scientist (Lini Ashdown). It is also envisaged that Prof Len Levy OBE will continue to provide additional scientific expertise to the program, particularly in relation to the annual research report.

Body

OVERALL OBJECTIVES

The objectives of the Core 6 research project is to:

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- provide a current awareness service on a quarterly basis; and
- provide short annual "state of the science" reviews, identifying recent key scientific papers.

The intention is to provide these services – on a free-access basis – via a specially constructed web-site, containing this and other information relevant to the study of the health effects of manganese.

PROJECT ELEMENT 1 – Maintenance of a Database of Information

The project team will maintain and seek to expand the information held on the previously developed database *"Database of Global Research Activity on Manganese*" (DOGRAM),available on the website at: http://www.manganese-health.org/home. The database is designed to be viewed using Browse facilities **(**by Project, Researcher, Research organization and Funder) and by use of Search facilities (using a customized 4-level thesaurus based on defined keywords as well as by a free text search facility).

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Manganese oxide$1
Manganese sulphate or manganese sulphate
Manganese with steel – (title, abstract)
Manganous salt$1
Manganous Manganic Oxide or Hausmannite – Mn_3O_4

Polianite
Pyrochroite
Pyrolusite (manganese oxide)
Ramsdellite (manganese oxide)
Siliconmanganese or silicon manganese
Sodium manganate – Na_2MnO_4
Manganese
Manganese carbonate – $MnCO_3$
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Medline, Toxline	**Embase**	**Biosis, Pascal**
Carcinogen$5.ti,de,ab.	Carcinogen$5.ti,de,ab.	Carcinogen$5.ti,de,ab.
Tumor-markers-biological#	Carcinogen-testing#	Mutagen$5.ti,de,ab.
Carcinogenicity-tests#	Carcinogenic-activity#	Genotoxic$5.ti,de,ab.
Carcinogens-environmental#	Carcinogen-dna-interaction#	Cytotox$5.ti,de,ab.
Mutagen$5.ti,de,ab.	Mutagen$5.ti,de,ab.	Epidemiology.ti,de,ab.
Mutagenecity-tests#	Mutagenic-agent#	
Genotoxic$5.ti,de,ab.	Chemical-mutagen#	
dna-damage#	Promutagen#	
Cytotox$5.ti,de,ab.	Mutagen-testing#	
Epidemiologic-factors#	Chemical-mutagenesis#	
Epidemiologic-methods#	Environmental-mutagen#	
Epidemiology#	Mutagenic-activity#	
Effect-modifiers-epidemiolgy#	Genotoxic$5.ti,de,ab.	
Epidemiolog$2.ti,de,ab.	Cytotox$5.ti,de,ab.	
	Cytotoxic-agent#	
	Cell-mediated-cytotoxicity#	
	Cytotoxicity-test#	

	Epidemiology#	
	Cancer-epidemiology#	

The terms/phrases are searched for in abstracts, descriptors and titles; Truncation is used where appropriate.

Set 1 and 2 are combined using the Boolean operator 'OR', and the results from the Set 1/2 then combined with Set 3, using the Boolean operator 'AND'.

Based upon this exhaustive search of the published information, relevant English-language papers on manganese are selected and categorized by an experienced toxicologist, before use to prepare a summary report which is then posted on the MHRP website. Abstracts are categorized into the following sections:

Section 1 - EXPOSURE MEASUREMENT AND MODELLING: Papers relating to the measurements or modelling of environmental and occupational manganese exposure, the development of biomarkers of exposure or effect.

Section 2 - HEALTH EFFECTS: Papers on the influence of manganese on health, disease and dysfunction.

Section 3 - MECHANISMS: Papers on the physiological, biochemical and cellular mechanisms underlying the toxic effects of manganese.

Section 4 - HUMAN SUSCEPTIBILITY: Papers relating to assessment of the influence of genetic and epigenetic factors on human susceptibility to the effects of manganese.

Section 5 - TREATMENT AND IMAGING: Papers on the development and implementation of new medical approaches to the treatment of excessive manganese exposure.

Section 6 - MISCELLANEOUS: Other papers considered of interest or potential relevance to the study of the health effects of manganese.

PROJECT ELEMENT 3 – Production of state of the science reviews on manganese

A Research Overview Report discussing the significance of new knowledge gained through research published between 2002 (publications before this were addressed in the IEH Criteria Document) and February 2007 (time of publication of the 4th awareness update report) was posted on the MHRP website in December 2007. This has been followed by a further report published in September 2008 that related to papers published between March 2007 and February 2008, and it is anticipated that a further report – addressing the period March 2008 – February 2009 will be prepared under the previous contract. Subsequent to this, additional Research Overview Reports will be produced annual throughout the course of this project.

Each report will summarise established thinking on the toxicology of manganese and its inorganic compounds, and considered the implications of the recently published studies that have appeared in the scientific literature and are identified on the MHRP database. Reports are prepared in a manner suitable for use by a wide readership (including researchers, interested scientists and health professionals), including laypersons who may wish to have an overview of manganese toxicity and recently published research.

IMPACT

It is anticipated that the current project will maintain and extend the benefits gained under the previosu contract, and provide continuity of information to support the ongoing MHRP programme.

Conclusions

Under a previous contract and as part of the research programme supported by the Manganese Health Research Program (MHRP), the Institute of Environment and Health (IEH) at Cranfield University, UK, has undertaken — in collaboration with the International Manganese Institute (IMnI) and their subcontractors Intendance Limited, UK (that designed and maintains the MHRP website) — the provision of knowledge management services (identified as Research Core 6: Provision of Research Activity Awareness Services in the MHRP Phase 1 research programme) for the MHRP.

Under the leadership of Dr S Rocks, this newly established contract will facilitate the continued services of IEH to the programme into the future.

Key Research Accomplishments

The activities proposed to be maintained under this contract may be summarized as:

- Maintenance and expansion of a database (DOGRAM) of information on recently completed and ongoing research projects;
- Provision of a current awareness service of published papers on a 13-week (quarterly) basis; and
- Publication of short annual “state of the science” review reports, identifying recent key scientific papers and findings.

Reportable Outcomes

Contained maintenance of a database (DOGRAM) of information on recently completed and ongoing research projects;

Provision of a current awareness service of published papers on a 13-week (quarterly) basis; and

Publication of short annual “state of the science” review reports, identifying recent key scientific papers and findings.

These outputs will all be accessable via: http://www.manganese-health.org/home

Current faculty receiving support from the grant:

- Lini Ashdown
- Sophie Rocks

References

Not applicable

AD_____
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AWARD NUMBER: W81XWH-05-1-0239

TITLE: Molecular Mechanisms Underlying Manganese Neurotoxicity

PRINCIPAL INVESTIGATOR: BethAnn McLaughlin, PhD

CONTRACTING ORGANIZATION: Vanderbilt University Medical Center
Nashville, TN 37203

REPORT DATE: January 2009

TYPE OF REPORT: Final report

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

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McLaughlin, BethAnn, PhD

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14. ABSTRACT

Manganese is an essential nutrient, integral to proper metabolism of amino acids, proteins and lipids. Excessive environmental exposure to manganese can produce symptoms similar to those observed in Parkinson's disease. Using in vivo and in vitro models to examine cellular and circuitry alterations induced by manganese exposure. Primary mesencephalic cultures were treated with 10-800μM manganese chloride ($MnCl_2$) which resulted in dramatic changes in the neuronal cytoskeleton even at subtoxic concentrations. In our in vivo system, we observed a 20% reduction in TH-positive neurons in the substantia nigra pars compacta (SNpc) following manganese treatment. Quantification of Nissl bodies revealed a widespread reduction in SNpc cell numbers. Other areas of the basal ganglia were also altered by manganese as evidenced by the loss of GAD67 in the striatum. These studies suggest that acute manganese exposure induces cytoskeletal dysfunction prior to degeneration and that chronic manganese exposure results in neurochemical dysfunction with overlapping features to PD.

15. SUBJECT TERMS
Manganese, neurotoxicology, iron deficiency, welding, manganese mining, nutrition

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					19b. TELEPHONE NUMBER *(include area code)* (615) 936-3847

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Prescribed by ANSI Std. Z39.18

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ABSTRACT

Manganese is an essential nutrient, integral to proper metabolism of amino acids, proteins and lipids. Excessive environmental exposure to manganese can produce symptoms similar to those observed in Parkinson's disease. Using in vivo and in vitro models to examine cellular and circuitry alterations induced by manganese exposure. Primary mesencephalic cultures were treated with 10-800μM manganese chloride ($MnCl_2$) which resulted in dramatic changes in the neuronal cytoskeleton even at subtoxic concentrations. In our in vivo system, we observed a 20% reduction in TH-positive neurons in the substantia nigra pars compacta (SNpc) following manganese treatment. Quantification of Nissl bodies revealed a widespread reduction in SNpc cell numbers. Other areas of the basal ganglia were also altered by manganese as evidenced by the loss of GAD67 in the striatum. These studies suggest that acute manganese exposure induces cytoskeletal dysfunction prior to degeneration and that chronic manganese exposure results in neurochemical dysfunction with overlapping features to PD.

Introduction

Manganese (Mn^{2+}) is a naturally occurring essential element with an environmental prevalence second only to iron. Manganese is crucial for maintaining proper cellular function and contributes to biological processes including maintenance of redox status, ensuring appropriate protein conformation, modulating ion and energy homeostasis and signal transduction [1-4]. Dietary intake is the largest source of manganese in the human body under normal circumstances, but airborne manganese particulates comprise the most prevalent source of excessive manganese exposure [5, 6]. Manganese is used in numerous industries including welding, mining, steel production, and formulating gasoline additives. Chronic manganese overexposure results in a neurological irreversible phenomena referred to as manganism [6-10]. The factors that influence vulnerability to manganese and onset of manganism remain ill defined. The motor symptoms of the disorder are, however, strikingly similar to those observed in Parkinson's disease [11-13].

Manganese is capable of having direct actions on neurons and glia within the central nervous system. Manganese is readily transported into the brain, either as a free ion species or as a nonspecific protein-bound species [14]. We have previously shown that primary astrocytic cultures are highly vulnerable to manganese and undergo apoptotic cell death involving mitochondrial dysfunction [15], a finding which is consistent with the work of Maynard and Cotzias who demonstrated preferential sequestration of this element in the mitochondria [16, 17]. Bioenergetic studies have shown that neurons are even more intensely dependent upon intact mitochondria for respiration. The majority of mitochondria are located in dendrites [18, 19] where the density of excitatory inputs necessitates a high respiratory capacity due to the need to maintain Na^+/K^+ gradients during neural activation [20]. If mitochondria are an essential target organelle of manganese, one would predict that changes in neural processes may proceed nuclear or soma dysfunction and thus contribute to circuit level dysfunction. Indeed, cytoskeletal changes may be essential to mediating neurodegeneration in Parkinson's disease and other neurological disorders. Tau, tubulin and neurotransmitter releasing proteins have been documented in these disorders [21-31].

The purpose of this work was to extend our previous studies in astrocytes to dopaminergic neurons and *in vivo* systems to address the neurotoxic potential of manganese and to determine if nigrostriatal pathways are uniquely vulnerable to manganese exposure. The ability to define mechanisms of toxicity and cellular features, which increase cellular vulnerability, would enhance our ability to treat manganism and potentially provide essential insight into vulnerability of the basal ganglia in this and other disorders.

Body

Project Goals

1. Surveying the molecular damages caused by Mn exposure in primary neuronal cultures and in animal model.

Our first experiments examined the relative vulnerability of mesencephalic cells to manganese by exposing primary cultures to increasing concentrations of the metal continuously for 24hr. We used neuron-enriched cultures (50% neurons) that were dissected mid-gestation to promote survival of TH-positive cell populations.

Our cultures typically had 5-10% TH-positive neurons with the other population of neurons (identified with MAP2 staining) expressing the GABAergic marker GAD67. Cell death was assessed by measuring LDH release by dead and dying cells into the exposure media and by visually inspecting cells for signs of cell death including loss of neurites, soma shrinkage and presence of cellular debris in media. We found that $MnCl_2$ induced cell death in a concentration-dependent manner with an LD_{50} of 909μM. Many phase-bright neurons were present in control cultures, which grow on top of a bed of glia. With increasing concentrations of $MnCl_2$, soma volume shrinkage resulting in a stippled, less smooth appearance (800μM), and extensive neuronal cell death can be seen at 3mM $MnCl_2$.

2. Evaluate the mechanism of Mn induced cell death, effects of Mn on cellular redox status and the role of apoptotic pathways.

For this project goal, we chose to focus on the consequences of pathophysiologically relevant concentrations of chronic manganese exposure at 100 – 800μM [32]. These concentrations were not overtly neurotoxic, but could alter cell structure in ways that might influence the circuitry of the basal ganglia and contribute to manganese-induced motor dysfunction. For this work, we surveyed the changes in the structural protein tau, the synapse specific marker synapsin and the cytoskeletal marker tubulin. Neurons were treated with vehicle, 100μM $MnCl_2$ or 800μM $MnCl_2$. We observed that increasing manganese concentrations lead to a progressive loss of cohesive tau staining and an increase in cytoskeletal abnormalities as reflected by the >60% decrease in tau-positive neuritis. Similarly, synapsin was altered with both 100μM $MnCl_2$ and 800μM $MnCl_2$ exposure resulting in a ~75% decrease in synapsin staining. However, no changes in DNA were evident from our Hoechst stain suggesting that preapoptotic asymmetric chromatin formations were not induced and changes were confined to regions outside the nucleus.

3 and 4. Evaluate and validate the specific factors (e.g. genetic and neurochemical background) which contribute to vulnerability to Mn.

In our previous goals we determined that Mn induced produced profound changes in cultures which contained dopaminergic cells, However, mesencephalic cultures contain both GABAergic and dopaminergic populations. Based on the symptomology of manganism, there appears to be a stronger dopaminergic dysfunction. To test the hypothesis that unique genetic and environmental profile of dopaminergic cells renders them at higher vulnerability to manganese, we used a combination of *in vitro* and *in vivo* systems. We first stained manganese-exposed cultures with TH, the rate-limiting step in dopamine biosynthesis and a specific marker of dopaminergic neurons. We observed that manganese exposure resulted in shortening of TH-positive neurites with increasing exposure and loss of neurite integrity in distal processes.

We next used cultures derived from TH:RFP reporter mice and counted TH-positive and TH-negative neurons before and after exposure to $MnCl_2$. Cells containing this dopamine synthetic enzyme comprised 12 ± 3% of the total neuronal population. Manganese induced a similar concentration-dependent toxicity as in the cultures from the non-transgenic animals. The TH-positive cells were, however, more vulnerable than the other neuron populations in the mesencephalic cultures to $MnCl_2$ at both 300 and 800μM doses. This was evidenced by cell counts performed before and after $MnCl_2$ exposure where we observed a progressive increasing in death of TH-positive neurons between concentrations of 300μM to 1mM, which was significantly greater than that of TH-negative neurons.

5. Validate the pathways which contribute to Mn induced dysfunction using biochemical and genetic tools.

To address this goal, we moved to an *in vivo* model of chronic manganese exposure to determine if systemic administration of manganese in any way recapitulated alterations in the direct and indirect circuits of the basal ganglia that have been observed in PD and PD models. Animals were given IP injections (5 mg/kg body mass, intraperitoneal) of $MnCl_2$ or vehicle daily for 30 days. Coronal brain sections were stained with TH antibody and cell counts were performed. Cell counts revealed a significant decrease in TH-positive cells in the SN but not in the adjacent ventral tegmental area (VTA) ($n=5$; $p< 0.05$ paired t test).

To determine if the loss of TH staining was caused by a loss of the dopamine synthetic enzyme itself or by a loss of cellular viability, we undertook cell counts of the SN and VTA. We found that the loss of TH staining was associated with cell loss in the SN as there was a 20% decrease in cresyl violet-stained cell numbers in this region, which was not evident in the VTA. These data suggest that dopaminergic cells are highly vulnerable to Mn intoxication, but exposure to Mn also impairs the function of other essential neurotransmitter systems.

6. Develop a comprehensive model by identifying factors rendering cells vulnerable to Mn exposure

Our final experiments to fulfill the objectives of this grant were to provide a comprehensive understanding of factors which may render cells vulnerable to Mn exposure and were done in vivo using animals chronically exposed to $MnCl_2$ or vehicle daily for 30 days as above. We evaluate the consequences of chronic manganese exposure on the basal ganglia by evaluating the GABA synthetic enzyme, glutamic-acid-decarboxylase (GAD67), staining within this circuit. We observed appreciable GAD67 loss in the CPu which receives the dopaminergic projections of the SNpc in manganese treated animals compared to controls. GAD67 immunoreactivity was not altered in the SNpr, which was consistent with our *in vitro* findings suggesting that dopaminergic dysfunction occurs prior to GABAergic dysfunction in this region. We also observed a loss of GAD67 staining in the GP in manganese-treated animals compared to controls. Cell counts of several other regions within and outside the basal ganglia showed no significant difference, suggesting that the loss of GAD-positive cells was limited to the striatum (STR) and globus pallidus (GP), but not in the dentate gyrus or anterior cingulate cortex ($n=5$; $p< 0.05$ paired *t* test).

Key Research Accomplishments

- Using primary mesencephalic cultures, we concluded that acute *in vitro* manganese exposure is neurotoxic and produces more profound cytoarchitectural dysfunction and death in TH-positive neurons than other neuronal populations.

- tau and synapsin have been shown to be altered in PD and PD models as well as by environmental and genetic stress, but neither has been evaluated following manganese treatment. Our immunohistochemical analysis and quantification support the hypothesis that acute manganese exposure induces early and profound changes in neurite length and integrity at concentrations that are not overtly neurotoxic (100µM).

- The loss of synapsin staining observed with manganese treatment is consistent with data supporting an essential role for appropriate manganese content in maintaining neurotransmission and synaptic function and the role of PD associated proteins in regulating synaptic function.

- Our data strongly support a preferential loss of TH-positive neurons in mesencephalic cultures at concentrations of manganese which are not overtly toxic to other populations.

- In our chronic *in vivo* manganese exposure paradigm, we also observed cell loss in the TH-rich SN, but damage was not restricted solely to this region. Changes in GAD or loss of GABA content are not present in the post mortem striatum in PD, however we did observe subtle but significant loss of in GAD-immunoreactive neuron number in the STR and GP in our animals chronically exposed to manganese.

- On net, our data provide some explanation for the motor manifestations of manganese intoxication but do not support a model of selective dopaminergic dysfunction *in vivo*. These data suggest that circuit level influences are essential to mediating manganese induced degeneration in the adult brain.

Reportable Outcomes

Publications: Supported by this project (last 2 years)

Stankowski, J., Leitch, D., Aschner, M., McLaughlin, B. and Stanwood, G. D. *Selective vulnerability of dopaminergic systems to Manganese: Relevance to occupational exposure.* Neurotoxicology And Teratology, 2008. 30(3): p. 259-259.

Stanwood, G.D., Leitch, D.B., Savchenko, V. Wu, J. Fitsanakis, V.A., Anderson, D.J., Stankowski, J.N, Aschner, M. and McLaughlin, B. *Manganese Exposure is Cytotoxic and Alters Dopaminergic and GABAergic Neurons within the Basal Ganglia.* Submitted 1/2009

Current faculty receiving support from the grant:

- Michael Aschner, PhD
- BethAnn McLaughlin, PhD

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AWARD NUMBER: W81XWH-05-1-0239

TITLE: Coordinator of the Mn Health Research Program Steering Committee & Administrator of the Research Activity Awareness Services

PRINCIPAL INVESTIGATOR: Anne Tremblay

CONTRACTING ORGANIZATION: International Manganese Institute
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75001 Paris, France

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Not applicable

14. Abstract

The primary activities of the MHRP Steering Committee are to select the projects to be included in the MHRP and to review their progress. The role of the Chair and Coordinator of the Steering Committee is to ensure that the projects selected are of irreproachable scientific worth, but also take into account the primary concern of the industry and the US Department of Defense: preserving the health of their workers. The composition of the Steering Committee (a mix of academics, scientists, and qualified industry representatives), along with the active participation of the program's principal investigator, Dr. Michael Aschner, ensure that these goals are being met.

Administering the Research Activity Awareness Services (RAAS) implies working in tandem with Dr. Leonard Levy and his team so that his RAAS project is made available on a MHRP-dedicated web site: www.manganese-health.org The web site, launched in Feb. 06, contains information about the MHRP, manganese, along with useful contacts and news.

15. SUBJECT TERMS
Manganese, manganese health, neurotoxicology, iron deficiency, welding, manganese mining, nutrition.

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Chair & Coordinator of the Mn Health Research Program Steering Committee

The MHRP Steering Committee is chaired by Anne Tremblay, Secretary General of the International Manganese Institute (IMnI). Its members in 2008 were Dr. Barbara Beck of Gradient Corporation, Dr. Tomas Guilarte of the Johns Hopkins University, Dr. Joan Cranmer of the University of Arkansas, Dr. Leonard Levy, Cranfield University (UK), Dr. Harry Roels, Professor Emeritus at the Catholic University of Louvain (Belgium), Dr. W Les Dees, Professor at Texas A & M University, Dr. Jerry Roper of Afton Chemicals, Mr. Pierre Rousseau of Eramet, Mr. Dirk van Niekerk of BHP Billiton. The Steering Committee works in close tandem with Dr. Michael Aschner, Professor at Vanderbilt University and the Principal investigator for the entire program.

The MHRP Steering Committee (SC) met by telecon several times in 2008.
Besides keeping abreast of the MHRP-funded studies, the focus of these meetings was largely to plan and organize a Conference in 2009 to showcase the MHRP-funded projects.
The SC Chair met both with Dr. Aschner and John Hilbert (Kinghorn, Hilbert & Associates) to discuss the conference and also visited a few possible venues around DC.

Tracking Welding Issues for the MHRP

Manganese is a component of coated welding rods and various steel alloys. As a result, there can be significant exposures to a finely divided dust/fume in welding operations, and massive exposures which have been associated with a debilitating neurological disease. Welding is one of the primary industrial activities in defense department activities common to all of the armed forces. For this reason, Anne Tremblay continued to track the litigation cases aiming to prove that Mn in welding rod fumes causes Parkinson's disease.

She maintained regular contact with Charles Read, Senior Partner with O'Melveny & Myers LLP, a law firm with offices in Washington, DC and Los Angeles, which is representing the defendants in many of these cases.

Keeping the Metals Industry Informed of the MHRP

During 2008, Anne Tremblay met with a number of metals associations to inform them of progress on the MHRP. These included: the North American Metals Council, Worldsteel (formerly the International Iron and Steel Institute—IISI), Eurometaux, Euroalliages and the International Chromium Development Association.

Conclusions

The Steering Committee is fully functional. Throughout 2008, it continued to track the 24 MHRP studies underway, and began organizing the MHRP Showcase Conference to take place near Washington, DC, June 24-25, 2009.

References

Not applicable

Administrator of the Research Activity Awareness Services

The International Manganese Institute is funded to provide an MHRP dedicated web site to house the Research Activity Awareness Services. The site is also designed to include general information about the MHRP, including project descriptions, along with background on manganese and its uses, useful contacts, news & developments.

Reportable Outcomes

The MHRP web site (www.manganese-health.org) was launched on February 10th, 2006. During the course of 2007 it underwent a major updating and plans for another were discussed at the end of 2008, and will be put into effect in early 2009.

References

Not applicable

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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

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14. ABSTRACT

Exposure to Mn occurs in both civilian and military settings. To search the biomarker for early diagnosis of Mn-induced toxicity, we proposed a cross-sectional design to evaluate the associations between airborne Mn levels and biomarkers of exposure (concentrations of Mn, Fe, and Fe regulatory proteins) in biological matrices, and between the levels of biomarkers and early signs of neurological deficits. The cohort is located in Zunyi City in China, with industries involving Mn mining, refining, smelting, processing, and ferroalloy production and with the existing Mn intoxication cases. We propose to recruit total 300 subjects into the project. The IRB protocol was officially approved by Zunyi Medical College (ZMC) on 20 Aug 2005 (valid for 5 years), reapproved by Purdue Univ. on 3 July 2008, and by the U.S. AMRMC Human Subjects Research Review Board on 6 May 2008. The Human Research Assurance Number of the ZMC was awarded by U.S. DHHS on 13 Jan 2006. The study was initiated in spring 2006 during and after Drs. Zheng and Aschner visited the studying site between April 17-20, 2006, Oct 30-11/04/07, and Sep 19-223, 2008. To this report date, all 323 recruitments are finished. Samples have been analyzed. Two manuscripts have been published. Additional field study with more air samples is in progress.

15. SUBJECT TERMS

Manganese, neurotoxicology, biomarker, iron metabolism, manganese mining, human study

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Introduction

The early onset of Mn intoxication is usually subtle and progressive. The initial signs may be categorized as the nonspecific neurological manifestations, psychiatric symptoms, and extrapyramidal signs. The exposed workers may complaint asthenia, anorexia, apathy, insomnia or drowsiness, malaise, somnolence, or diminished libido or impotence. Psychiatric symptoms are more specifically indicative of Mn toxicity, including disorientation, emotional instability, compulsive acts, hallucinations, illusions, delusions, and slurring and stuttering speech with diminished voice. These are followed by selective extrapyramidal disorders such as imbalance in walking or on arising, finger coordination, and tremor.

Since Mn induced neuronal damage is irreversible, the early diagnosis becomes crucial for prevention of Mn toxicity in occupational and environmental exposure scenarios. Based on a recent study on 97 welders in Beijing, China, Dr. Zheng and his colleagues in China has found that serum concentrations of ferritin and transferrin were increased among welders, while serum transferrin receptor levels were significantly decreased in comparison to controls. Moreover, this group found that serum transferrin receptor levels were inversely associated with serum manganese concentrations ($p<0.05$). Thus, these iron regulatory proteins along with Fe itself may serve as the potential useful biomarker for early diagnosis of Mn toxicity.

We propose a three-phase, three-year, cross-sectional study to test the hypothesis that occupational exposure to airborne Mn is associated with health disorders among exposed workers in a time-dose dependent manner. More specifically, we aim to see if airborne Mn levels are positively correlated with levels of Mn in blood, urine, saliva, or hair, and Fe or Fe regulatory proteins in serum, one of which can be used as the biomarker to assess Mn exposure. In addition, we aim to study whether Mn concentrations in biological matrices (blood, urine, or hair) are associated with early signs of health disorders among exposed workers.

In Phase I, the principal task is to further characterize the study sites and to conduct exposure assessment in the environment from which the study subjects will be recruited. During this period, the instruments for air sampling, questionnaires for epidemiological study, documents for data storage, and methodology for laboratory assays (AAS) for Mn, etc., shall be fully prepared or developed.

The Phase II aims to study the biological outcomes of exposure. We will collect biological samples, conduct physical examinations, and determine Mn and biomarker concentrations. Biological samples from all workers will be obtained at the time of physical examination within 10-12 months. The time frame for data collection will be approximately 12 months and the lab analyses will take longer time.

In Phase III, we will put much our effort on statistical analysis to draw the conclusions on our hypotheses. We estimate a 9-12 month period, for we may revisit some of the sampling spots or subjects to verify the data.

Body of Progress 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 3 July 2008.

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. The further approved is in progress. 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 multination 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 Quizhou 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.

3. Summary of Research Achievement by January 2009

Reliable biomarkers for manganese (Mn) exposure are not available at present. The purpose of this cross-sectional study was to establish a distinct value that distinguishes Mn-exposed subjects from the general, Mn-unexposed healthy population. Mn-exposed ferroalloy smelters (n=95), power distributing and office workers (122), and unexposed control subjects (106) were recruited to the high, low and control groups, respectively. Airborne Mn levels were 0.003 mg/m3, 0.03 mg/m3 and 0.18 mg/m3 for control, low and high exposure groups, respectively. Mn concentrations in saliva, plasma, erythrocytes, urine and hair were significantly higher in both exposure groups than those in controls. The Fe concentration in plasma and erythrocytes, however, was significantly lower in Mn-exposed workers than in controls. A concept of the Mn/Fe ratio (MIR) was developed with the numerator (Mn) reflecting Mn exposure and the denominator (Fe) indicating a biological alteration. The MIRs for erythrocytes (eMIR) and plasma (pMIR) exhibited significant exposure-group related increases. Linear regression analysis revealed that the airborne, inhalable Mn level was significantly associated with the eMIR ($r=0.77$, $p<0.01$) and pMIR ($r=0.70$, $p<0.01$). Among all determinants, only the eMIR and the pMIR were significantly associated with smelters' years of employment. The cut-off value (COV), above which workers were considered to be Mn-exposed was established using the receiver-operator characteristic analysis. At the eMIR COV of 8.8, about 88% of the high exposure smelters had an eMIR above the COV, while 87% of controls had an eMIR below the COV. Taken together, this study suggests that chronic occupational exposure to Mn in smelters increases Mn and decreases Fe concentrations in erythrocytes and in plasma. The eMIR exhibits a good correlation between workers' years of employment and airborne Mn levels. Using a cut-off eMIR value of 8.8, we are accurately able to distinguish Mn-exposed workers from the unexposed, control population.

A biomarker for detection of early-onset neurobehavioral alterations in manganism remains unknown. The purpose of this study was to use a neurobehavioral test battery to identify subtle changes in Mn-induced motor and memory dysfunction and to relate the quantifiable neurological dysfunction to an established Mn exposure index such as blood manganese-iron ratio (MIR). A total of 323 subjects were recruited to control

(n=106), low (122), and high (95) exposure 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.

Exposure to excessive manganese (Mn) leads to psychological and motor disorders, indicating a permanent damage to certain brain structures. Yet, there has been no reliable means for early, pre-symptomatic diagnosis and assessment of Mn intoxication in clinics. The aim of this study was to use magnetic resonance imaging (MRI) as a noninvasive method to distinguish Mn-exposed subjects from the control subjects. A group of 10 smelting workers were recruited from a Mn-Fe allay manufacturer in Zunyi city, China, and the other 10 control subjects with no history of Mn exposure were recruited from the same area. All study subjects were subjected to physiological examination, blood testing, and MRI examination. High-resolution 3D T1-weighted isotropic images were acquired in addition to conventional axial T1-weighted slices and analyzed by visual inspection. The Pallidal Index (PI) was calculated based on (1) the signal ratio between a region of interest (ROI) within the globus pallidus (GP) and white matter in the frontal cortex (PIwm) and (2) the signal ratio between the same ROI in the GP and a ROI in the muscle (PImu). Results showed that 3D T1weighted MRI was much more sensitive than axial T1-weighted method in identifying Mn deposit in brain. Of the 10 subjects occupationally exposed to Mn, PImu-based and PIwm-based 3D TI-MRI had 90% and 70%, respectively, success rate to identify Mn exposed smelters. Thus, it appeared that PImu was more accurate than PIwm in distinguishing Mn exposed smelters from the control workers. In addition, the 3D imagine allowed to trace the regions where Mn accumulated in brain structures. Further analysis by MRS of changes in brain metabolites is in progress.

Although it was not included in the original grant, we have conducted a pilot study in treatment of Mn using para-aminosalicylic acid (PAS), an FDA-approved anti-tuberculosis drug. PAS has been used successfully in the treatment of severe manganese (Mn)-induced Parkinsonism in humans (Jiang et al., JOEM 48:644, 2006). 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 d/wk for 4 wks, followed by a daily subcutaneously (sc.) dose of PAS (100 and 200 mg/kg as the PAS-L and PAS-H group, respectively) for another 2, 3 or 6 wks. 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 wks, Mn levels in liver, heart, spleen and pancreas were significantly reduced by 25 to 33%, while 3 wks of PAS-L treatment did not show any effect. Further therapy with PAS-H for 6 wk reduced Mn levels in striatum, thalamus, choroid plexus, hippocampus and frontal cortex by 16 to 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.

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.
- This study has been successfully accomplished with regards to the subject recruitment and examination, exposure monitoring, laboratory sample analysis, and data record, entry and analysis. Two manuscripts have been accepted for publication. We are in the final stage of analyzing MRI/MRS data for another manuscript.

- 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

- Current faculty receiving support from the grant:
  - Wei Zheng, PhD
  - Frank Rosenthal, PhD
  - Ulrike Dydak

- Current students receiving training from participation on projects related to this grant:
  - Xue Fe

Conclusions

With the support of this grant, we have determined 15 parameters in saliva, plasma, erythrocytes, urine and hair were examined for their utility as a biomarker for Mn exposure. These biochemical values have been correlated to Mn concentrations in air, subjects' age, working year, neurobehavioral testing outcomes, and other social factors. For the first time in literature, we established the concept of Mn/Fe ratio (MIR) in biological matrices for its applicability to assess Mn exposure. The erythrocyte MIR (eMIR) exhibits good correlations with worker's employment years, airborne Mn levels, and neurobehavioral outcomes. A cut-off eMIR value 9.0 may be useful for assessment of Mn exposure in general populations. In addition, we have embarked MRI/MRS study to use the non-invasive method to study the changes of brain metabolites among Mn-exposed workers. The results may create new avenue in Mn neurotoxicological research.

Peer-Reviewed Publications

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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.

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Year 2007

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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)

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Year 2008

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Cowan DM and Zheng W (2009). Relationship between blood manganese-iron ratio and early onset neurobehavioral alterations. SOT meeting in Baltimore

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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, Hawai'i, USA 18-24 April 2009.

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PRINCIPAL INVESTIGATORS: West Virginia University: Richard Dey PhD; NIOSH: James Antonini PhD, Diane Miller PhD, James O'Callaghan PhD, Krishnan Sriram PhD

CONTRACTING ORGANIZATION: Vanderbilt University Medical Center
Nashville, TN 37203

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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 via olfactory transport after inhalation in short-term studies. Consistent with the observed accumulation of manganese in the brain, intratracheal instillation of welding fumes differentially elicited neuroinflammatory responses in the olfactory bulb, striatum, and midbrain. Longer-term inhalation exposure studies in animals are ongoing to make more definite conclusions about the potential neurotoxicity of welding fume.

15. SUBJECT TERMS
Manganese, welding, neurotoxicity, inhalation

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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/m^3 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 m. Additional nanometer-sized particles in the range of 0.010-0.10 m as well as larger, coarse particles with diameters >1.0 m in size also were collected. The mass median aerodynamic diameter was calculated to be approximately 0.31 m. 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 (Il1β, Tnfα) 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 neuroinflammatory 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 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, Tnfα, Il1α, Il6, Ccl2 and Cxcl2, have been implicated as etiological factors in several neurodegenerative diseases. In the brain, these factors are elaborated by activated microglia and play a key role in the glial response to neuronal injury. 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 Tnfα (1.5-fold), Il6 (1.8-fold) and Cxcl2 (Mip2; 2.1-fold) in STR. Similarly, exposure to MMA-HS also induced Tnfα (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 Tnfα (1.8-fold) in the midbrain. Collectively, these observations suggest that exposure to manganese-containing welding fumes could potentially cause dopaminergic neurotoxicity. Whether such exposures can lead to progressive dopaminergic cell loss and induce Parkinson-like pathology remains to be elucidated.

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.

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, Albini 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 TB, Schwegler-Berry D, Jefferson AM, 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 after Ten Days of Inhalation by Rats, in preparation.

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.

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 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 neuroinflammatory 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. Whether such exposures can lead to progressive dopaminergic cell loss and induce Parkinson-like pathology remains to be elucidated.

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14. ABSTRACT

Excessive accumulation of manganese (Mn) from occupational and environmental sources in regions of the basal ganglia may lead to neurological symptoms similar to those of Parkinson's Disease. Many tissue fractionation studies and studies of other types have shown Mn accumulation in the mitochondria in the tissue in which accumulation occurs in the brain. In past work, our laboratory and other laboratories have shown that Mn^{2+} is readily sequestered by the mitochondrial Ca^{2+} uniporter and can efflux mitochondria through the Na+-independent Ca^{2+} efflux mechanism. We have also shown that Mn^{2+} transport via the dominant Ca^{2+} efflux mechanism in heart and brain mitochondria, the Na+-dependent mechanism, is very slow or nonexistent. However, we don't know whether Mn^{2+} can be transported via the RaM mechanism which is very important in activating ATP production. We also don't know whether Mn^{2+} transport via the Na+-dependent mechanism occurs at all or is simply very slow or whether mitochondria can transport Mn^{3+} at all. We propose to investigate these modes of transport.

15. SUBJECT TERMS
Manganese, neurotoxicology, mitochondria, transport mechanisms

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Introduction

Manganese (Mn) accumulation in areas of the basal ganglia, particularly in the globus pallidus and striatum, correlates with a form of toxicity known as manganism which shows symptoms somewhat like those of Parkinsonism. Our work and the work of a number of other investigators has strongly suggested that mitochondria play a significant role in Mn toxicity[1-15]. Therefore, it is vital to understand the transport of Mn into and out of mitochondria as well as the effects of intramitochondrial Mn. Mn^{2+} behaves similarly to Ca^{2+} in mitochondria, binds to essentially every Ca^{2+} binding site often more strongly than Ca^{2+} itself, and is transported into and out of mitochondria via Ca^{2+} transport mechanisms. There are at least 2 mechanisms of Ca^{2+} influx (the uniporter and the RaM) into mitochondria and 2 mechanisms of efflux (the Na^{+}-dependent and the Na^{+}-independent mechanisms)[2, 16] . In addition, in some types of mitochondria such as heart mitochondria there is the possibility of a mitochondrial ryanodine receptor and in all vertebrate mitochondria there is the possibility of opening the mitochondrial permeability transition pore (PTP) which makes the inner membrane leaky to all small ions and molecules[16]. From past work, we know that Mn^{2+} can enter mitochondria via the Ca^{2+} uniporter[2, 16]. We also know that Ca^{2+} uptake via the RaM mechanism is capable of transporting enough Ca^{2+} into mitochondria to activate oxidative phosphorylation; however, we do not know whether Mn can be transported via the RaM mechanism. It has been shown that Mn^{2+} is readily transported out of mitochondria via the Na^{+}-independent Ca^{2+} efflux mechanism and that transport via the Na^{+}-dependent mechanism is minimal but we don't know whether or not the Na^{+}-dependent mechanism transports Mn^{2+} at all. We also don't know whether mitochondria transports Mn^{3+} at all. We proposed to look into these questions; however, since this MHRP money has only been available to us for 7 months, these projects are not completed and the discussion below is a progress report. Carrying out each of these measurements accurately requires very careful techniques.

Also, during the past year, another group has challenged the work showing uptake of Mn by mitochondria in cells, claiming that such uptake is minimal and that most of the Mn in cells is found in the nucleus. Since such a claim undermines all of our work and ideas about how Mn affects cells, we had no choice but to respond to this publication and have submitted a short paper containing relevant data to Neurotoxicology, the journal which published the challenging paper, to refute these claims. This work was supported by this grant and will be briefly discussed below.

In addition, another group published results in Nature claiming to have identified a component of the mitochondrial uniporter and potentially the RaM. By giving a seminar to the local Mitochondrial Research Interest Group on the many problems with the data in this Nature paper, we initiated an effort which was led by Dr. Paul Brookes to correct these problematic claims in the Nature paper. This led to additional data taken by several laboratories and a letter to Nature, written by a group of the world's experts in this field, in an effort to correct the misconceptions in the original Nature paper. A reference to our letter to Nature is given below.

Body

Study 1 Is manganese transported via the RaM mechanism?

The mitochondrial Ca^{2+} uniporter does not transport Ca^{2+} at external concentrations less than 200 to 250 nM; however, the RaM will. Our work on Ca^{2+} transport via the RaM mechanism shows that this is a very unusual mechanism which transports a limited amount of Ca^{2+} from cytosolic Ca^{2+} pulses into mitochondria. However, this transported Ca^{2+} is sufficient to activate ATP production by these mitochondria[16-21]. When [Ca^{2+}] is low (below 140 nM), this mechanism will transport Ca^{2+} from a Ca^{2+} transient very rapidly into mitochondria until binding of Ca^{2+} from the pulse to an external inhibition site stops the transport. We believe that this behavior allows sufficient Ca^{2+} for activation of ATP production to get in but closes the mechanism before

enough Ca^{2+} is sequestered to open the PTP. We do not know whether Mn^{2+} will bind to the external inhibition site and close the mechanism as Ca^{2+} does and if it does, we do not know the Mn^{2+} concentration necessary to close this mechanism. We have hypothesized that the RaM and the uniporter represent the same transport proteins in two different conformational forms, and that inhibition of the RaM by binding of Ca^{2+} to the external inhibition site actually converts it into the uniporter conformation. The reason that this is important for Mn^{2+} toxicity is that since Mn^{2+} is not present in the cytosol at concentrations as high as those of Ca^{2+} during Ca^{2+} transients, mitochondrial uptake of Mn^{2+} may actually be through the RaM conformation and not through the uniporter conformation.

In order to determine whether Mn^{2+} is transported via the RaM and also see if external Mn^{2+} closes the RaM as Ca^{2+} does, we propose to set up Mn^{2+} pulses similar to the Ca^{2+} pulses that we generated using a computer controlled automatic pipetter to carry out the original investigation of the RaM. The difficulty in doing this is that with Ca^{2+} we could set up the pulses by calculating the concentrations of Ca^{2+} buffers (EGTA and others) using the software Maxchelator and then check it with [Ca^{2+}] measurements using the fluorescent indicator fura2. However, Mn^{2+}, which is paramagnetic, quenches the fluorescence of fura2 and other fluorescent indicators. Therefore, we have to be able to set up the Mn^{2+} pulses using Maxchelator and the thermodynamic parameters for Mn^{2+} binding to the buffers alone. At the highest concentrations, these calculations can be checked using EPR, but this is time consuming and not as accurate as the fluorescence measurements of Ca^{2+}. That is where this project is now. We have been setting up pulses for several months, but we are still trying to be sure that their concentrations are correct. When we are sure about this, we should be able to complete the experiment relatively quickly since we have probably carried out more Ca^{2+} and Mn^{2+} uptake experiments with mitochondria than anyone else.

Study 2 Is Mn^{2+} transported at al by the Na^+-dependent efflux mechanism?

Carrying out this set of experiments is essentially a question of setting up the most sensitive experiments possible to see if any Mn^{2+} can efflux mitochondria via the Na^+-dependent mechanism and then working more carefully to get the best possible results. In order to do this, we need to work with a system in which Na^+-dependent efflux is dominant, as it is in heart mitochondria and also one where we can get enough good quality mitochondria to carry out a sufficient number of experiments from the same mitochondrial preparation. The Na^+-dependent efflux mechanism is about 15 times more active in heart mitochondria than in liver mitochondria. It is essential to use very good quality mitochondria for this study because poor quality mitochondria undergo the permeability transition (MPT) easily and induction of the MPT in the latter stages of these experiments can show up as false positives in the data. Heart mitochondria are the best system to use for this work. Knowing whether any Mn^{2+} efflux can occur via Na^+-dependent transport is important because the Na^+-dependent system is by far the dominant Ca^{2+} efflux mechanism in heart and brain mitochondria. If even a small amount of Na^+-dependent Mn^{2+}efflux exists, it could greatly decrease the Mn^{2+} exposure time in heart or brain mitochondria.

During the past year, we have mastered the techniques required to isolate really good quality heart mitochondria for oxidation rate experiments and we are currently ready to apply these techniques to the question of Na^+-dependent efflux. We will carry out these experiments using techniques similar to those that were used in Wingrove and Gunter[22] with $^{54}Mn^{2+}$. After uptake of a range of amounts of $^{54}Mn^{2+}$ by the heart mitochondria, first Na^+-independent efflux will be measured and then varying amounts of Na^+ will be added and the rate of efflux again measured by the appearance of isotope in the external medium. The Na^+-independent rate will be subtracted from the rate in the presence f Na^+ to obtain the Na^+-dependent rate. This will be plotted in an Eadie Hofstee plot as was done in Wingrove and Gunter[22].

Study 3: Is Mn^{3+} transported across the mitochondrial inner membrane on the citrate transporter (tricarboxylic acid)?

While oxidation by Mn^{3+} has been suggested both as being produced by free radicals in mitochondria and as a mechanism through which Mn could damage cells, we don't know whether Mn^{3+} can be transported across the mitochondrial inner membrane. We think that if Mn^{3+} does cross the mitochondrial inner membrane, it probably does so via the tricarboxylic acid exchanger along with citrate. This is because Mn^{3+} has been suggested to form a relatively stable complex with citrate. We have recently set up Mn^{2+} citrate solutions in conjunction with a procedure to make Mn^{3+} transferrin (Mn^{3+}Tf). The process has successfully made Mn^{3+}Tf which has the characteristic blackish color of Mn^{3+} and is very different from the light pink color of Mn^{2+}. In conjunction with this, we have developed EPR and optical/uv techniques for analyzing Mn^{2+} and Mn^{3+} left in these solutions and separation techniques for separating Mn^{3+} protein complexes. While the separation techniques must be different, we will now use these techniques to see if Mn^{3+} can form a stable complex with citrate and other TCA cycle intermediates, and if so, we will assay mitochondrial uptake of a complex of Mn^{3+}citrate or Mn^{3+} with other TCA cycle intermediates.

***Study 4: The case for mitochondrial uptake of Mn in cells and in vivo*.**

Kalia et al al[23] have recently published a paper in which they claim to have fractionated neuronal cells after treatment with Mn^{2+} and found that hardly any is found in the mitochondrial fraction. They found most of the Mn in the nuclear fraction. They conclude from this that nuclei "may represent the preferential targets for Mn accumulation and toxicity." They also maintain that "there has been no direct evidence -- on subcellular distribution of Mn" and that a recent report by Morello et al[24] agrees with them that nuclei are the preferential targets of intracellular Mn.

We have submitted a short paper to Neurotoxicology which makes the following points:

1. In fact, a number of other studies[1, 3-6] have determined the subcellular distribution of Mn after tissue fractionation following treatment with Mn *in vivo*, and all demonstrated treatment-related increases in intramitochondrial Mn.

2. In addition, other workers have found that Mn decreases energy metabolism *in vivo* and *in vitro*, including decreases in the activities of mitochondrial enzymes, in membrane potential, and ATP production[7-15, 25]. This would be virtually impossible without mitochondrial interaction with Mn.

3. Using electron energy-loss spectroscopy with electron microscopy, Morello et al[24] reported that although nuclei contained more Mn than mitochondria, treatment-related increases were greater in the mitochondria. They concluded that "the relevant distribution of Mn is *not limited* to the mitochondria."

4. Consideration of why the results of Kalia et al differ from those of other distribution studies requires an understanding of mitochondrial ion transport and its response to fractionation. Mitochondrial sequestration of Ca^{2+} or Mn^{2+} does not represent simple binding but weak binding within a steady state controlled by both influx and efflux of these ions[26]. Mn^{2+} is sequestered by the mitochondrial Ca^{2+} uniporter, primarily energized by the internally negative membrane potential ($\Delta\psi$), and effluxed by the Na^{+}-independent mechanism, primarily energized by the pH gradient[16, 26, 27]. Both of these gradients are maintained by energy-dependent proton pumping across an intact inner membrane. If ($\Delta\psi$) falls, uptake velocity decreases precipitously - by over 83% as $\Delta\psi$ falls from 180 to 160 mV, for example[16]. If $\Delta\psi$ falls near zero, the weakly bound ions rush out by reverse uniport[28, 29]. Furthermore, the "isolated mitochondria" produced by fractionation, whether by mechanical action or by detergents, represent resealed fragments of the original cellular mitochondrial network[16, 30]. In these resealed mitochondria, $\Delta\psi$ is dissipated during fractionation, then rebuilt by proton pumping energized by endogenous substrate --

e.g., pyruvate, a product of glycolysis that in the intact cell is transported continually into mitochondria for use in the TCA cycle. However, the fractionation procedure greatly dilutes glycolytic enzymes and substrates, and the amount of endogenous substrate within isolated, resealed mitochondria is greatly reduced and no longer replenished. It has been well known since the 1960's that appreciable Ca^{2+} or Mn^{2+} uptake by these resealed mitochondria requires addition of mitochondrial substrate; however, none was added in the fractionation studies cited above. Why, then, did all except Kalia et al find Mn in the mitochondria? Maynard and Cotzias[1] stressed that they treated animals with less Mn than that present in the food. Other *ex vivo* Mn distribution studies[5, 6] examined Mn concentrations in brain fractions; since brain Mn uptake is limited both by the blood-brain barrier and by rigorous homeostatic control of absorption and excretion, the amounts reaching mitochondria were probably not large. In contrast, Kalia et al exposed their cells, including PC12 cells, for 24 hours to 100 µM Mn^{2+}. Based on the uptake that we measured in PC12 cells at 10 µM for 24 hours (11.7 nmoles/mg cell protein)[31], we estimate that the mitochondria of Kalia et al were exposed to [Mn^{2+}]s over 100 times higher than those of Maynard and Cotzias and much higher than in the other fractionation studies. Following fractionation, the resealed mitochondria would begin to resequester and cycle the surrounding Mn^{2+}, using energy from endogenous substrate. However, in the presence of large amounts of Mn^{2+}, as in the experiments of Kalia et al, Mn cycling would quickly dissipate the endogenous substrate, $\Delta\psi$ would fall, and the Mn^{2+} would be released again from the mitochondria to bind to available sites such as nuclei.

5. We also showed data on the amount of free [Mn^{2+}] outside a suspension of energized mitochondria when the amounts of added Mn^{2+} were varied over the range between 3.7 µM and 147 µM and found that this free manganese varied from 80 nM following addition of 3.7 µM total Mn^{2+} to around 3.4 µM following addition of 147 µM total Mn^{2+}. The reason for the increase in external Mn^{2+} as added Mn^{2+} is increased is that the increased total Mn^{2+} causes more cycling of Mn^{2+} into and out of the mitochondria and this process dissipates energy shifting the steady state toward less uptake. The data also showed single points of data from heart and brain mitochondria and the effects of added Ca^{2+} on this Mn^{2+} steady state. Since Ca^{2+} activates the uniporter, it shifts the Mn^{2+} steady state toward more uptake and therefore less external free Mn^{2+} at a given amount of total Mn^{2+}. What this suggests is that a significant amount of mitochondrial substrate should be added to the media before any fractionation to minimize redistribution of Mn^{2+}.

Key Research Accomplishments:

1. Have set up the best experimental approach for determining whether Mn^{2+} can be transported via the RaM mechanism.
2. Have made Mn^{3+} transferrin and set up the EPR and visible/uv techniques necessary for identifying possible Mn^{3+} complexes with citrate and other TCA cycle intermediates.
3. Have identified the problems involving redistribution of Mn^{2+} during and following fractionation in the paper by Kalia et al (2008) which claimed to show that intracellular mitochondria didn't sequester Mn^{2+}.
4. With original data have shown that energized mitochondria exposed to Mn^{2+} over the range of total [Mn^{2+}] between 3.7 and 147 µM will sequester the Mn^{2+} and pump the external [Mn^{2+}] to levels between 80 nM and 3.4 µM depending on the total Mn^{2+} added. This range of concentrations then represent the levels at which mitochondrial sequestration of Mn^{2+} competes with other cellular binding for Mn^{2+} in the cytosol.

5. Have identified the problems in the Nature paper by Trenker et al (2007) to show that the uncoupling proteins 2 and 3 do not represent a component of the mitochondrial Ca^{2+} uniporter or RaM.

Reportable Outcomes

Manuscript: Supported by this project (last 2 years)

- Gunter, T.E., and Sheu, S.-S. Characteristics and possible functions of mitochondrial Ca^{2+} transport mechanisms. Biochem. *Biophys. Acta Bioenergetics* **Jan. 6 [Epub ahead of print]:** 2009
- Brookes, P.S., Parker, N., Buckingham, J.A.,Vidal-Puig, A., Halestrap, A.P., Gunter, T.E., Nicholls, D.G., Bernardi, P., LeMasters, J.J., and Brand, M.D. UCPs - Unlikely calcium porters. *Nature Cell Biology* **11:** 1235 - 1237, 2008.

Current faculty receiving support from the grant

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- Karlene Gunter PhD

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14. ABSTRACT

A case–control study of a small group of welders with typical idiopathic PD (Racette et al. 2001) showed no clinical differences between them and the typical PD population, but their disease was distinguished by a statistically significant younger age of onset (46 years) for welders compared with 63 years for the controls. This promoted the hypothesis that employment as a welder may be a risk factor for PD, accelerating or triggering the onset of the disease. This remains controversial but impossible to dismiss. A case–referent study represents the most appropriate means of investigating this theory. The objectives of the study are to test the following hypotheses:
I. A significantly higher proportion of those diagnosed as having PD have been welders of steel, or otherwise exposed to manganese–containing metal fumes, than those in a matched control group (age and sex) who do not have that diagnosis.
II. Within those diagnosed with PD, the age of onset is lower among those who have been occupationally exposed to manganese than those who have not.
Federal Wide assurance has been obtained by the researchers and ethical approval for the study has been granted both locally (Solihull LREC) and by the USAMRMC. Weekly data collection commenced in Jan 2008 with PD patients in a single hospital clinic, with data collection in a second clinic coming on-line in late 2008, and this is due to continue for the next 6 months.

15. SUBJECT TERMS
Manganese, neurotoxicology, welding, occupational, Parkinson's disease, foundry, retrospective

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Introduction

Parkinson's Disease (PD) is a common neurological disorder affecting one in 500 of the general population and 1% of those over the age of 65. The first observable symptom of Parkinson's Disease is often tremor (trembling or shaking) of a limb, especially when the body is at rest. The tremor is initially unilateral, frequently in one hand. Other common symptoms include slow movement (bradykinesia), an inability to move (akinesia), rigid limbs, a shuffling gait, and a stooped posture. People with Parkinson's Disease often show reduced facial expressions and may speak quietly. Occasionally, the disease also causes depression, personality changes, dementia, sleep disturbances, speech impairments, or sexual difficulties. The severity of Parkinson's symptoms tends to worsen over time. This has led to the identification of idiopathic Parkinson's Disease, although other types of PD exist with better understood causes, although this only accounts for as small number of cases, and these are brought together under the term Parkinsonism. Research currently indicates that genetic factors are most likely to predispose patients to develop PD if combined with other gene mutations or environmental toxins.

A similar collection of symptoms to those displayed in idiopathic PD is a specific neurological disorder that may be attributed to excess absorption of manganese is manganism, (known as chronic managanese intoxication) typified by slowly progressive deterioration of well-being coupled with specific disturbances of mood and muscle function. Manganese is recommended in dietary intake (2 to 5 mg/day), and manganese is included in parenteral nutrition. The toxic effects in manganism are considered to result from interference by manganese in the metabolism of biogenic amines such as dopamine, and *in vitro* welding fume enhances dopamine oxidation, raising the possibility that manganese in welding fume may contribute to dopamine deficiencies in those who develop PD. Manganese-induced neurotoxicity has been reported to occur only after chronic exposure to high levels of manganese, usually above the permissible exposure limits. The disorder bears a marked similarity to Parkinson's Disease (PD) and has been referred to as manganese-induced Parkinsonism or secondary Parkinsonism. Exposure to high airborne levels of manganese in foundry and mine workers has historically been associated with a neurotoxicity that resembles Parkinson's Disease.

The greatest use of manganese ores is in the production of iron, steel, and manganese alloys, and when welding such metals and flame cutting, fumes containing several respirable substances including manganese are produced. If the suggested link between manganese exposure and PD-like symptoms is correct, it would be expected that that low grade exposure to manganese fumes experienced by metal welders, may increase the risk for the development of Parkinson's Disease and other basal ganglia and movement disorders. Compounds released during welding and flame cutting may accelerate the onset of Parkinson's disease. The number of well designed epidemiological studies and cohort studies which have evaluated these occupational issues is small. While the link between manganese and the development of PD remains controversial it is currently impossible to dismiss the possibility that occupational exposure to manganese compounds in welding fume may enhance genetic susceptibility to or interact with other environmental agents that may cause or expedite the onset of PD. A larger-scale study may be required.

Body

Manganese oxides are a constituent of the fume emitted from cutting and arc welding of steel and applying hard facing to tools. Steel welders probably constitute the largest occupational group exposed to manganese and its compounds. Some research has suggested that welders may be at a higher risk for developing Parkinsonism, and some have proposed that welding is a risk factor for PD. Such claims have entered the legal system and lawsuits have been filed on behalf of welders alleging that toxic fumes generated by welding rods (containing manganese) have caused not just Parkinsonism, but also Parkinson's Disease. However, several mortality surveys of large populations of welders found elevated rates of certain cancers, accidents strokes, cirrhosis, heart disease and even suicide, but not of PD or any other neurological diseases (Coggon *et al.* 1995). Conversely, other research has shown welders to have lower levels of PD than control occupations (Kirkey *et al.* 2001).

A case-control study of a small group of welders with typical idiopathic PD (Racette *et al.* 2001) showed there were no clinical differences between them and the typical PD population, but their disease was solely distinguished by the younger age of onset for welders (46 years) compared with the control group (63 years). Despite the significant and important results of the Racette *et al.* study, the study did not provide any evidence that welders were more likely to develop PD than the general population; the authors suggested that welding acted to accelerate the onset of PD. There were some important methodological limitations to the study that must be considered, mostly concerning the appropriateness of the welders involved: especially as patients were not randomly selected and there was no evidence that the welders in the study were representative of all welders. The data was collected from a center specializing in Parkinsonism disorders, (rather than a general movement disorder clinic) which may have raised the possibility of referral bias, as patients referred to specialty clinics may often be more atypical than general PD patients. Additionally, the method of referral to the specialty clinic was not specified, and 53% of the welders in the study had a family history of PD - higher than the 15% reported in unselected PD patients. Importantly there is a known link between family-history and younger-onset PD, possibly complicated by genetic factors. It has been concluded that genetics rather than welding was the major risk factor in these relatively young patients with PD who happened to be welders (Jankovic 2005).

Given the contradictory nature of the research body, this case-referent study represents the most appropriate means of investigating this hypothesis. The feasibility study involves the identification of a population of males newly-diagnosed with PD and a population of controls matched for age, gender and socio-economic status. The study population is restricted to males only, since the majority of welders are male. Detailed assessment of occupational histories, working practices for key / high exposure occupations, clinical histories, smoking and dietary intake are carried out for each case and control, via a clinical interview with a consultant neurologist and an occupational psychologist. The objectives of such a study would be to test the following two hypotheses:

H_i 1:

A significantly higher proportion of those diagnosed as having PD have been electric arc welders of steel, or otherwise exposed to manganese - containing metal fumes, than those in a matched control group (age, sex, education) who do not have that diagnosis.

H_i 2:

Within those diagnosed as having PD, the age of onset will be lower among those who have been occupationally exposed to manganese than those who have not.

Aims

Primary: If any manganese-effects are detected or nearly detected, the aim of this study is to suggest the methodological and statistical feasibility of a larger-scale study.

Secondary. To identify if occupational exposure to manganese (mainly derived from welding of steel) is associated with the development of Parkinson's Disease among males, in terms of a younger age of onset among exposed PD patients than non-exposed PD patients. The age of onset of PD may also be related to time as a welder and the estimated dose of welding fumes and metal fumes.

Key Research Accomplishments

a) Applied for and gained Local Research Ethical Committee approval to conduct a feasibility study.

b) Applied for and gained USAMRMC approval to conduct a feasibility study.

c) Developed patient / control participant (i) info sheets (ii) consent forms (iii) data collection sheets (iv) occupational history questionnaires.

d) Secured access to clinical populations at two movement disorder clinics in Birmingham.

e) Commencement of data collection from PD patients in movement disorder clinic #1 commenced in January 2008. Due to delay in gaining access to movement disorder clinic #2 (obtained in Nov 2008) PD patient data collection needs to continue until Spring 2009.

f) Identification of the control participants (age-sex-socioeconomic matched) with a non PD diagnosis has begun, and will utilize mild stroke and cardiovascular patients in Spring 2009.

g) Following the departure of the Principal Investigator (Prof Jouni Jaakkola) from the Institute of Occupational & Environmental Medicine, the new Director of the IOEM, Prof John Ayres has acted as Principal Investigator on the study for the remainder of the data collection period.

Reportable Outcomes

Some brief outcomes are presented below but this is to be viewed as a work in progress with data collection still ongoing and expected to accelerate with the recent addition of movement disorder clinic #2 in the study.

1. Participation rates in the study have been high, with 90% of newly-diagnosed PD cases who were consecutively asked to participate, agreeing to.

2. Ethnicity of PD cases includes 90% Caucasian, with 10% from India / Pakistan origin. Of the Caucasian PD cases, approximately 40% are from Irish immigrant roots (Birmingham has a large Irish community) and they have often confirmed childhood / adolescent diets “back home” that were high in cabbage content.

3. Currently, detailed occupational history and exposure data has been collected from 20 newly-diagnosed PD patients, and this is expected to be completed and analyzed by Spring 2009.

4. Three PD cases have reported occupational exposure to manganese, not through welding or cutting of steel, but from foundry working. These individuals spent the majority of their working lives in those foundry jobs.

5. Approximately 60% of the PD cases interviewed were from manual jobs, with the remainder from professional or white collar working backgrounds.

6. Further statistical breakdown and analysis will be performed when the PD participant numbers achieve a more sizable sample. This will be provided to the funders in anticipation of the Washington DC meeting in 2009.

Conclusions

This case-control study has the potential to show interesting differences within a unique sample based upon consecutively diagnosed new PD cases in a heavily industrialized part of the UK. With more time to collect data from the PD cases and the control group, this study may provide the basis to consider a future larger-scale retrospective study of the PD population. It is anticipated that completion of this feasibility study can be secured by Autumn 2009.

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AWARD NUMBER: W81XWH-05-1-0239

TITLE: Longitudinal Study of Health Effects Over 3 Years in Mn Exposed Bridge Welders

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Not applicable

14. ABSTRACT

Manganese (Mn) exposure among welders has been shown in some reports to have adverse health consequences. A 2005 study of 43 bridge welders showed that an average of 18 months confined space welding, without adequate ventilation and protection, was associated with neuropsychological health effects. Accounting for potential confounders, increased Mn in blood was significantly related to lower full scale and verbal IQ, the VCI and PSI, cognitive flexibility, executive function, learning and memory. In 2008, 26 welders returned for a follow-up. Preliminary matched pair analyses indicate improvement from 2005 in PIQ and VIQ, VCI, PSI. Additionally, significant improvement was observed for: Auditory Consonant Trigrams (ACT) 18 seconds, Rey-Osterrieth Complex Figure, Fingertapping, and Grooved Pegboard. Prior elevated SCL90-R scores showed a trend towards improvement. For certain tests, age influenced improvement, with the younger welders showing improvement, and the older workers deteriorating further (ACT 18 second, Days of Physical and Mental Health per month). Evidence suggests that cessation of confined space Mn exposure contributed to significant functional improvements.

15. SUBJECT TERMS
Manganese, neurotoxicology, welding, follow-up study, neurological, neuropsychological

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Standard Form 298 (Rev. 8-98)
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Introduction

A group of 43 Mn-exposed bridge welders who welded 1.5 years in confined spaces without adequate protection and with poor ventilation were evaluated in January 2005 (T1, baseline) and briefly in 2006 (T2). At baseline, in 2005, their results showed dose-effect relations of Mn in blood and air with decrements in both full and verbal IQ. This project re-evaluated these welders 3¼ years later at (T3). Neuropsychological function, sensory/autonomic nervous system function, respiratory function, and blood Mn were re-assessed. The goal of the study was to : 1) compare functional outcomes at baseline and at the present follow-up in 2008; and because very few tests in comparison to baseline were evaluated at T2, the decision was made to primarily compare exposure to Mn between T1 and T3, and 2) correlate health outcomes with Mn exposure and describe the association of Mn and function between the T1 and T3 assessments. The welders were removed from unprotected exposure within 6 months of the T1 assessment in 2005, i.e. after a period (2002-04) of sub-acute exposure to Mn from welding fumes (1.5 yrs on average). This study evaluates if functional health status improved (reversibility) over time and if blood Mn has decreased. It was hypothesized that neurotoxic and pneumotoxic outcomes will be better compared to baseline; that decreased exposure to Mn will be associated with improved function; and that special characteristics of welders, such as age and use of personal protective equipment (PPE), may modify the functional outcomes.

There are still large gaps in the knowledge about the fate of adverse health effects in welders after a decrease or cessation of Mn exposure. This follow-up study addresses this gap of information in the scientific literature in that it seeks to investigate whether improvement or reversibility of neuropsychological, sensory/autonomic nervous system, and respiratory functions may occur in a cohort of bridge welders after no longer being exposed without protection to Mn-containing fumes.

Body

Longitudinal Study of Health Effects Over 3 Yrs in Mn Exposed Bridge Welders

Design

This study used a **longitudinal design** to re-evaluate the cohort of 43 welders 3¼ yrs (T3) after baseline (T1). An attempt was made to contact all 43 welders to be re-invited to participate. Although it was expected that most of the welders still resided locally, this was not entirely the case.

Recruitment procedure

The participants were contacted by letter and telephone calls. They were offered $50 gift cards as compensation for their participation. A mutually convenient appointment time during a 3-day weekend between August 22, 23 and 24, 2008 was offered and it was anticipated that 32 participants would agree to return. Six welders had relocated for work out of California and could not come, six welders were not reachable and/or did not return telephone calls, and one person was unwilling to return due to his own litigation issues. After mailing recruitment letters and contacting the welders by phone, appointments were made with 30 participants. Despite reminder telephone calls made 2 days prior to the appointment, four welders did not appear on the weekend of testing and could not be reached. This left in total 26 welders to whom all relevant tests could be administered, with the exception of 1 welder who could not come but was rescheduled within the following week. Consequently, this welder could not give blood as the phlebotomist was no longer available and 1 welder, similar to 2005, could not give blood because he had a needle phobia.

Testing procedure

Dr. Rosemarie Bowler was successful in obtaining the collaboration and participation of Dr. Harry Roels (neurotoxicology), Mr. Robert Park (work histories), Dr. Jayne Wilkinson (neurology and UPDRS) and Dr. Nadia Abdelouahab (CATSYS). Both the phlebotomist and the respiratory technician were the same persons used in 2005; in order to maximize comparison with 2005. Most of the same neuropsychologists returned to perform the testing in addition to a trained team of graduate students who administered the simpler tests. The order of test administration was the same for all participants. The battery included tests that were shown to be sensitive to Mn exposure in the first study, including measures of IQ (WAIS III), Cognitive flexibility (ROCF, D-KEFS), Information Processing (Stroop), Working Memory and Attention (ACT), Memory (NAB Memory

Module), Visuomotor tracking speed (Digit Symbol Coding), Verbal kills (WAIS III VCI), Motor dexterity and strength (Fingertapping, Grooved Pegboard, Dynamometer), and tremor (CATSYS, UPDRS). In addition, work histories and health questionnaires covering the time period since the first study were administered, and blood was drawn and analyzed for levels of Mn and lead. Currently, feedback letters are being drafted for each welder to include both their data for 2005 and the comparison of their performance with 2008, in addition to the values of blood Mn for both dates.

Work history

Detailed work and Mn exposure histories were asked at baseline testing in the clinical interview and a follow-up work interview administered by Mr.Robert Park from NIOSH, which is enclosed. Employment dates, shifts worked, average hours per day, days per week, and type of welding used were recorded, analyzed and described in manuscripts following the baseline testing in 2005 (Bowler RM and RP 2006; Park, Bowler et al. 2006). The supplemental work history included what job activities they have had, what type of welding was performed and under what conditions (outdoors/open construction, indoors/welding shop, in confined spaces, etc.), and use of protective equipment and ventilation, including the duration of use and the type of respiratory protection. Of the welders who returned in 2008, only 50% were still welding, 27 % were disabled, 12% were working in another field, 8% were injured, and 4% were laid off.

Exposure assessment

The goal of the exposure re-assessment at T3 was to re-compute Mn exposure in blood with the outcome variables and to develop a second cumulative exposure index (CEI2) for each worker. While the first goal was met to have blood Mn and Pb available for further analyses, the second goal for recomputing a CEI2 could not be accomplished; since TI the welders had worked in varied work environments, their self-reports of exposure were judged as insufficient to accurately compute this CEI2 without air measurements. Therefore, it was judged that analyses of total time welding will be a better surrogate of continued exposure for the lack of more detailed exposure information.

Preliminary Results

Preliminary matched-pair analyses showed significant improvement for: Performance and Verbal IQ, Cognitive Flexibility (Stroop Color Word), delayed memory with distraction (ACT 18 second delay), Executive function (ROCF), tactile manipulative ability (Grooved Pegboard), as well as summary index of processing speed (WAIS-III PSI), verbal comprehension (VCI) and 2 out of 12 subtests of the WAIS-III: a test of word knowledge (Vocabulary) and knowledge of conventional standards of behavior (Comprehension). Additionally, there appeared to be a significant decline for grip strength (dynamometer). Analysis of the CATSYS and Unified Parkinson's Disease Rating Scale (UPDRS) revealed a decline in function as well. Matched pair analyses indicated increased right hand tremor compared to 2005, for tremor intensity, frequency dispersion, and Harmonic Index. Postural sway intensity also increased. Non-parametric tests for 2005-2008 UPDRS scales revealed significant increases in rigidity and motor-postural instability, but no difference in resting tremor. Matched paired analysis also indicated a significant decrease in Mn levels in blood from an average of 9.9 µg/L in 2005 to 8.6 µg/L in 2008.

Those participants from T1 who did not return in 2008 were compared to the cohort returning in 2008. Demographic results indicate no differences between the groups, with the exception of ethnicity (fewer whites returned in 2008). There were also no differences on welding status or total number of years welding. Results of the neuropsychological outcomes indicate no differences in verbal skills, motor dexterity and strength, tremor and postural sway, and mood. However, IQ results indicate slightly lower IQ for those who returned. Working and delayed memory scores in those who returned in 2008 were also lower, as was one test of executive function.

Also as part of the preliminary analysis, a Mixed Effects regression model was used in comparing blood Mn levels with mean differences in outcome variables, as well as matched-pair comparisons of those variables. The results and implications of these analyses are still being interpreted. With the preliminary findings, the PI and collaborators have submitted abstracts for a symposium to be given at the 2009 APA conference in Toronto, Canada. The titles and authors of those presentations are listed below.

Conclusion:

Overall, the findings suggest that 3.5 years after cessation of exposure, improvement is possible in some neuropsychological domains. However, the data from the CATSYS and UPDRS suggest that tremor and sway,

probably mediated by other CNS pathways, continue to deteriorate even when Mn exposure ceases or is reduced. The findings described in this interim report are based on preliminary analyses, and further interpretations will be drawn as analysis continues. Further results and implications will be included in the final report, which will be submitted within the next six months.

Key Research Accomplishments – preliminary data

1. A symposium, prepared with preliminary data analyses of the welders will be presented at the American Psychologial Association Meeting in Toronto, August 2009 entitled **Manganese health effects in welding: Scientific investigation addressing the controversy:**

   Follow-up study of SF Welders: Evolution of dose-effect manganese relationships, Bowler, R.M., Roels, H.

   Follow-up of CATSYS and neurological results of manganese-exposed welders, Abdelouahab.N, Wilkinson.J, Mergler.D, Bouchard, M., Roels.H., Bowler.R.

   Health Effects of Manganese Exposure in Welders: Methods for Study, Gysens, S.

   Follow-up Study of Psychological Effects of Manganese-Exposed SF Bridge Welders, Wecker, N.

   NAB Memory and ACT, Rey-O, WAIS-III performance in Mn-exposed welders, Gocheva, V., Hubbard, J.

   A Follow-Up Study of ROCF Scores among Manganese-Exposed Welders, Tara Dennehy

2. A manuscript is currently in preparation entitled: **Follow-up of dose-effect relationships and neurological, neuropsychological, and pulmonary function in manganese exposed bridge welders.**

3. Dr. Bowler will also give a presentation at the MHRP Showcase Conference, June 24-25 2009 in Washington, DC entitled: **Health Effects in Mn Exposed Bridge Welders: A 3 ½ Year Follow-up Study.**

Reportable Outcomes

Manuscript in preparation and Presentations: Supported by this project (last 2 years)

- Bowler, R.M.,Roels, H.,

Current faculty receiving support from the grant:

- Rosemarie M. Bowler, PhD

References

Not applicable

AD_____
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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 2009

TYPE OF REPORT: Final report

PREPARED FOR: U.S. Army Medical Research and Materiel Command
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Costa, Lucio G., PhD

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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. 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

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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 uM 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.
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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 (Table 1). When astrocytes were incubated for 24 h with different concentrations of $MnCl_2$ (50, 100, 200, 500 uM), 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. At the concentration of 100 uM and above, $MnCl_2$ decreased the average axon length and the average neurite length, without affecting the number of neurite 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 uM. 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. Based on these studies, a concentration of $MnCl_2$ of 200 uM was chosen for further experiments.

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
Manganese 500 uM	59.3 ± 3.8 ****	6.9 ± 1.5****	4.6 ± 0.5*****
Manganese 200 uM	91.2 ± 6.2 ****	11.8 ± 2.2***	6.4 ± 1.1****
Manganese 100 uM	115.2 ± 11.6***	15.3 ± 2.0***	7.3 ± 0.9
Manganese 50 uM	153.0 ± 10.0	18.0 ± 5.1	7.7 ± 0.6

Rat astrocytes were incubated in the presence or absence of different concentrations of $MnCl_2$ 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 um. Results represent the mean (± 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 $MnCl_2$ (200 uM) in the presence of the antioxidants melatonin (200 uM) or N-t-butyl-alpha-phenylnitrone (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) (Table 2). In contrast, depletion of astrocytic GSH with buthionine sulfoxime (BSO, 25 uM for 24 h), 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.

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	8.1 ± 1.4
$MnCl_2$ + 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-phenylnitrone (PBN). After 24 hr, astrocytes were washed out and incubated with freshly prepared rat hippocampal neurons for 48 hr. Length of axon and neurites is expressed in um. Results represent the mean (± SD) of three separate experiments. *Significantly different from control (untreated astrocytes), $p<0.01$.

Table3. 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 ± 2.3	9.1 ± 1.5
$MnCl_2$ 200 uM	104.9 ± 22.1*	8.7 ± 1.4*	6.2 ± 1.5*
BSO 25uM	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 um. Results represent the mean (± SD) of three separate experiments. *Significantly different from control, $p<0.05$; #Significantly different from $MnCl_2$-treated astrocytes, $p< 0.05$.

Mn decreases the expression of fibronectin protein and mRNA in astrocytes, and this effect is antagonized by antioxidants. The ability of astrocytes to induce neuronal differentiation is most likely mediated by the release of neurite-promoting molecules. A proteomics analysis of astrocyte secreta 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 a extracellular matrix glycoprotein, fibronectin, because of its reported permissive role in neurite outgrowth. 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 (Guizzetti et al. 2008). Fig. 1 shows that $MnCl_2$ caused a concentration-dependent decrease in the expression of intracellular fibronectin protein in astrocytes. A time-course study indicated that $MnCl_2$ (200 uM) decreased fibronectin mRNA after 8-24h of incubation. Both the decrease in protein and mRNA caused by 200 uM $MnCl_2$ were antagonized by increasing intracellular GSH with GSHee (2.5 mM), and by the antioxidant melatonin (200 uM). These two compounds also antagonized the decrease in fibronectin protein caused by 200 uM $MnCl_2$ in the astrocyte medium.

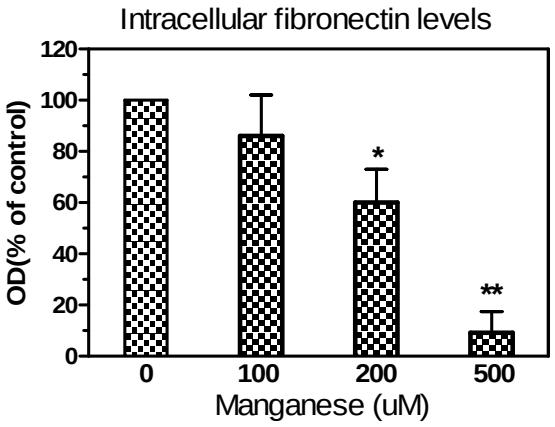

Fig. 1. Manganese decreases fibronectin protein levels in astrocytes. Astrocytes were exposed to MnCl2 at the indicated concentrations for 24h. Cells were then washed out and collected for Western blot analyis, as described by Guizzetti et al. (2008). Beta-actin was used as a loading control. Results are expressed as mean (± SD) of six separate experiments performed in duplicate. *p<0.01; **p<0.001 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. 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.
- 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, Guizzetti M, Costa LG. Manganese inhibits the ability of astrocytes to promote neuronal differentiation (in preparation).

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 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. 2009 (in press).

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AWARD NUMBER: W81XWH-05-1-0239

TITLE: Effects of manganese in welding fumes on cognitive function

PRINCIPAL INVESTIGATOR: Tomás R. Guilarte, PhD and Alison Geyh, PhD

CONTRACTING ORGANIZATION: Vanderbilt University Medical Center
Nashville, TN 37203

REPORT DATE: February 2009

TYPE OF REPORT: First report

PREPARED FOR: U.S. Army Medical Research and Materiel Command
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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. The proposed studies will determine if exposure to Mn in welding fumes at levels that are relevant to occupational exposures have an effect on cognitive function. We will also obtain data on neurochemical changes associated with these exposure levels. Concentrations of Mn in the air, blood and brain will be obtained in order to determine the level of cumulative exposure that produces behavioral and neurochemical changes.

15. SUBJECT TERMS
Manganese, welding, cognitive function, mice

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Introduction

Due to contract negotiations between Vanderbilt University and Johns Hopkins University, the start of this project was delayed for several months. We did not have a signed contract until late 2008. Since then, we have tested the welding fume chambers where the mice are going to be exposed. We are waiting to receive mice this coming week and hope to start animal exposure by February 24, 2009.

Body

N/A

Conclusion:

N/A

Key Research Accomplishments

N/A

Reportable Outcomes

N/A

Current faculty receiving support from the grant:

- Tomas Guilarte, PhD
- Alyson Geyh, PhD

References

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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 2009

TYPE OF REPORT: Progress report

PREPARED FOR: U.S. Army Medical Research and Materiel Command
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Wasserman, Gail, PhD
Liu, Xinhua, PhD

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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, 2nd Edition.

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**
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**			Joseph Graziano, PhD
					19b. TELEPHONE NUMBER *(include area code)* (212) 305-1678

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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, we will carry out the following objectives:

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 Araihazar, Bangladesh. The study participants will be selected from the Northwestern region of Araihazar, 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, 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).

Progress for this study has been severely encumbered because of slow IRB approval by the Bangladesh Medical Research Council (BMRC).

The award was approved by Vanderbilt University in January 2008. An application for human subjects research approval was submitted to the Columbia University (CU) IRB at that time and Columbia IRB approval was obtained on May 12, 2008. The approved protocol and consent form was forwarded to Dr. Michael Aschner on that day. Also at that time, the approved Columbia protocol was submitted to the BMRC for their approval; unfortunately that approval did not occur until November 23, 2008. Nevertheless, the good news is that we now have that approval in hand.

In December 2008, the BMRC approval, along with the requested modifications to the CU protocol was then forwarded to Kelly Dustin, RN, MS, CCRC, of the Human Research Protection Office (HRPO), U.S. Army Medical Research & Materiel Command. On January 15, 2009, we were informed by Kelly Dustin, RN, MS, CCRC, that all modifications were accepted and that we should now resubmit to the CU IRB for re-approval. At this time, the modified protocol has been submitted and is under review in the CU IRB. We anticipate that the CU IRB will approve this modification quickly, at which point we will submit the modification to the BMRC.

Recruitment of study participants will not begin until the CU IRB and BMRC have approved these minor modifications.

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: W81XWH-05-1-0239

TITLE: Oxidative damage and neurodegeneration in manganese-induced neurotoxicity

PRINCIPAL INVESTIGATOR: Dejan Milatovic, PhD and Michael Aschner, PhD

CONTRACTING ORGANIZATION: Vanderbilt University Medical Center
Nashville, TN 37203

REPORT DATE: February 2009

TYPE OF REPORT: Progress report

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

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Milatovic, Dejan, PhD
Aschner, Michael, PhD

5d. PROJECT NUMBER

5e. TASK NUMBER

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14. ABSTRACT Manganese (Mn) neurotoxicity in multiple animal models is associated with elevated levels of Mn in the brain, depletion of dopamine in the striatum, damage to neurons in the basal ganglia and/or the development of movement disorders. Since factors, such as oxidative stress and inflammatory activation within basal ganglia are strongly implicated in the selective degeneration of dopaminergic neurons, we proposed to use pharmacologic and morphologic approaches to test the hypothesis that suppression of oxidative damage and neuroinflammation prevent Mn-induced striatal neurodegeneration. To test this hypothesis, we (1) investigate novel markers of oxidative damage and neuroinflammation and quantify associated changes in dendritic degeneration of striatal medium spiny neurons (MSNs) in mice exposed to Mn, and (2) evaluate whether treatment with antioxidant or an anti-inflammatory agent suppress the development of oxidative injury and neurodegeneration following Mn exposure. Currently, we are analyzing data from the experiments related to specific aim I and progressing with experiments related to suppression of oxidative damage and neurodegeneration as scheduled.

15. SUBJECT TERMS
Manganese, neurotoxicology, oxidative stress, neuronal injury

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Introduction

Manganese (Mn) is an essential nutrient and it functions as a critical cofactor for many key enzymes involved in cellular metabolism. However, elevated occupational exposure to Mn pose an increased risk for Parkinsonian-like symptoms, but it is not known to what extent this may occur. Once established, symptoms associated with Mn intoxication or Parkinson's Disease (PD) usually become progressive and irreversible, reflecting upon damage to neuronal structures. The mechanisms that underlie motor complications of Mn intoxication and PD are not fully understood; however, oxidative stress and inflammatory processes play prominent roles in the degeneration of dopamine-containing neurons. Due to dopamine as well as dopamine receptor oxidation, initial degeneration of dopaminergic projections to neostriatal medium spiny neurons (MSNs), may lead to secondary late-stage degeneration, as well as loss of MSNs and their axonal projections in late stage of disease. Changes in dendritic length and spine density of MSNs following Mn intoxication have not been investigated, leaving open the questions of whether the changes in the spine density and dendritic length are associated with oxidative stress, and neuroinflammation following Mn exposure.

Body

Specific Aim 1. To establish relationship between Mn-induced changes in cerebral biomarkers of oxidative damage, isoprostanes (F_2-IsoPs, F_4-NeuroPs and IsoFs), nitric oxide (citrulline), indicators of inflammatory response (PGE_2) and degeneration of the MSNs dendritic system in mice.

To investigate the mechanisms underlying this neurotoxicity, we studied the effects of Mn on reactive oxygen species (ROS) formation, neuroinflammation, changes in high-energy phosphates (HEP) and associated neuronal dysfunctions in mouse brain and neonatal rat primary neurons. Biomarkers of oxidative damage, F_2-isoprostanes (F_2-IsoPs) and pro-inflammatory prostaglandins E_2 (PGE_2) were significantly ($p<0.0.5$) increased (145% and125%, respectively) in brain of C57Bl6 female mice exposed to Mn (100 mg/kg, sc) for 24 hours. At the same time, quantitative morphometric analysis of striatal medium spiny neurons (MSNs) revealed significant decreases ($p<0.05$) in spine number and spine density to 50% and 59% of controls, respectively. Additionally, cultured neonatal rat primary neurons exposed to 500 μM $MnCl_2$ for 2 hours showed significant ($p<0.05$) increase (139%) in F_2-IsoPs and decrease (67%) in HEP (ATP), as determined by GC-MS and HPLC, respectively. These results are consistent with oxidative stress, neuroinflammation, mitochondrial dysfunction and consequent neurodegeneration as major mechanisms in Mn-induced neurotoxicity

Specific Aim 2. To investigate whether treatment with antioxidant or an anti-inflammatory agent attenuates biomarkers of oxidative damage and neuroinflammation associated with Mn exposure, and the extent to which such attenuation is accompanied by rescue from neurodegeneration.

Our first set of data indicate that increased Mn striatal concentration induce significant increases in biomarkers of oxidative damage and neuroinflammation even following single Mn treatment. Thus, we have initiated experiments to investigate if pretreatment with ibuprofen (non-steroidal anti-inflammatory drug) or vitamin E suppress these biomarkers of oxidative injury and neurodegeneration following Mn exposure.

Our previous studies have shown that oxidative damage and neurodegeneration induced by activation of glial innate immunity are suppressed (with varied efficacy) by NSAIDs, ibuprofen, aspirin and naproxen. Ibuprofen was the most efficient NSAID in suppressing oxidative injury. As with F_2-IsoPs, ibuprofen completely protected the dendritic system from the degenerative consequences of neuroinflammation. We have previously shown that α-tocopherol (vitamin E) pretreatment suppressed oxidative injury and neurodegeneration in the model of activated innate immunity. Thus, we are applying neuroprotective strategies in our study proven to be effective to neurodegenerative patients

Conclusion:

Our initial experiments show that manganese exposure induces significant increase in biomarkers of oxidative damage, F_2-isoprostanes (F_2-IsoPs) and pro-inflammatory prostaglandins E_2 (PGE_2) in brain of C57Bl6 female mice. It has been also shown that oxidative stress and inflammatory response is accompanied by changes in dendritic lengths and spine density of striatal medium spiny neurons (MSNs) in mice exposed to Mn. Experiments currently in progress are design to determine whether pathways that involve oxidative stress and inflammation contribute causally to Mn-mediated neurodegeneration or whether neurodegeneration occurs by other mechanisms with secondary changes in biochemical endpoints. Currently, no ideal therapies are available for slowing the progression of the degeneration process and at the same time relieving symptomatic abnormalities associated with manganism. Therefore, our studies investigate two pathways to attenuate biomarkers of oxidative damage, neuroinflammation and consequent neurodegeneration associated with Mn exposure.

Key Research Accomplishments

- Mice treated with one or three injections of $MnCl_2$ (100 mg/kg, sc) did not show signs of significant toxicity.
- Mn exposed mice showed significant increase in cerebral biomarkers of oxidative damage (F_2-IsoPs) and neuroiflammation (PGE_2).
- Mn exposed mice showed significant alteration in striatal medium spiny neurons morphology as evaluated by quantification of dendritic length and spine density in Golgi-impregnated tissue.
- A model was developed to assess the relationship between Mn-induced oxidative damage and neurodegeneration brain

Reportable Outcomes

Publications/abstracts supported by this project (last 8 months)

- Milatovic, D., Yu, Y., Zaja-Milatovic, S., Gupta, R.C., Aschner, M. (2009) Oxidative damage and neurodegeneration in manganese-induced neurotoxicity. Society of Toxicology, abstract.

- Milatovic, D., Aschner, M. (2009) Measurement of isoprostanes as markers of oxidative stress in neuronal tissue. Current Protocols in Toxicology, in press.

- Gupta, R.C., Milatovic, D. (2009) Oxidative injury and neurodegeneration by OPs: protection by NMDA receptor antagonists and antioxidants. In: The neurochemical consequences of organophosphate poisoning in the CNS, ed. Weissman, B.A. Research Signpost/Transworld Research Network, submitted.

- Milatovic, D., Zaja-Milatovic, S., Yu, Y., Aschner, M. (2009) Oxidative damage and neurodegeneration in manganese-induced neurotoxicity. In preparation.

Current faculty receiving support from the grant:

- Michael Aschner, PhD
- Dejan Milatovic, PhD

References

Not applicable

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AWARD NUMBER:

TITLE: Role of Toxins and Genetics in Manganese-Induced DA Neuron Degeneration

PRINCIPAL INVESTIGATOR: Richard Nass, Ph.D

CONTRACTING ORGANIZATION: Indiana University School of Medicine, Indianapolis, IN

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Role of Toxins and Genetics in Manganese-Induced DA Neuron Degeneration

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6. AUTHOR(S)
Richard Nass, Ph.D

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Not applicable

14. ABSTRACT

Manganese (Mn^{2+}) neurotoxicity resembles a number of aspects of the dopamine (DA) neuron degenerating disorder Parkinson's disease (PD). Both PD and Mn^{2+} toxicity is characterized by motor deficits and damage to substantia nigra and other basal ganglia nuclei, and dopamine or its metabolites are believed to contribute to the disorder. Furthermore, expression of the pre-synaptic protein a-synuclein, and the oxidative stress-induced protein parkin have been proposed to contribute to the pathogenesis of both disorders, and occupational exposure to Mn^{2+} has been invoked to predispose individuals to PD. Despite the initial characterization of the disorder over 150 years ago, and intensive research within the past several decades, the origin of the pathogenesis and the molecular determinants involved in Mn^{2+} neurotoxicity have yet to be fully elucidated. A significant hindrance in dissecting the molecular components of Mn^{2+}-induced neurotoxicity is the high complexity of the vertebrate brain and lack of facile *in vivo* genetic models to determine and explore the mechanisms involved in the cell death. **We have developed a novel pharmacogenetic model using the genetically tractable nematode *C. elegans* to dissect and characterize the molecular components involved in DA neuron degeneration (see Nass et al, PNAS, 2002; Nass and Blakely, Ann. Rev. Toxicol. Pharmacol., 2003).** At the molecular level, the *C. elegans* nervous system is highly conserved both genetically and functionally with mammals, and all the genes responsible for DA biosynthesis, packaging, and reuptake are present and functional in the worm. We have shown that the nematode *C. elegans* DA neurons can be selectively damaged by exposure of whole animals to the parkinsonian-inducing neurotoxin 6-hydroxydopamine (6-OHDA) **(see Nass et al, PNAS, 2002)**. We have also recently shown that a brief exposure to Mn^{2+} causes DA neuron cell death in the worm, and that prior exposure to Mn^{2+} amplifies the 6-OHDA-induced DA neurodegeneration. **In our model system, the expression of the green fluorescent protein (GFP) in DA neurons will allow us a facile and powerful test to examine the role that DA, its metabolites, endogenous proteins, and neurotoxins play in Mn^{2+} -induced degeneration of DA neurons *in vivo*.** These studies will also include a genome-wide screen to identify mediators and suppressors of Mn^{2+}-induced toxicity that will facilitate the identification of novel genes and molecular pathways involved in this highly relevant health and environmental concern.

15. SUBJECT TERMS

Role of Toxins and Genetics in Manganese-Induced DA Neuron Degeneration

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
					Richard Nass, Ph.D
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Introduction

The goals of the award are to identify and characterize the molecular pathways and mechanisms involved in manganese (Mn)-induced dopamine (DA) neuron cell death. Due to the many significant similarities between Parkinson's disease (PD) and manganese toxicity, it is likely that many of the key molecular players in both disorders interact in similar molecular pathways. The establishment of our *C. elegans* PD model will allow us to examine the role that DA, its metabolites, endogenous proteins and neurotoxins play in Mn induced cell death.

Body

STUDY 1 – Determine whether Mn^{2+}-induced DA neuron degeneration is dependent on DA or DA neuron-specific proteins, and the neurodegeneration can be amplified by exposure to 6-OHDA or expression of human α-synuclein animals in vivo.

A goal of our first aim is to determine the role that dopamine or dopamine-associated proteins play in Mn^{2+}- or toxin-induced cell death. Our studies suggest that dopamine and several DA associated proteins contribute to DA neuron vulnerability to Mn. Utilizing HPLC we have found that Mn increases DA levels in worms treated with Mn, underscoring the potential role that DA may contribute to the degeneration. We have though identified by HPLC a highly reactive compound that is likely generated by Mn exposure that is not found in vivo that may significantly contribute to the degeneration. We are currently utilizing HPLC and MS to characterize this compound and its putative role in the neurodegeneration. Consistent with the change that DA may play a role in VMAT levels are immediately increased following brief exposures to Mn, suggesting the necessity of sequestering the increase in cellular DA into synaptic vesicle. Furthermore, our microarray and qrt-PCR studies also strongly suggest the synergistic role of dopamine and Mn in inducing cellular damage.

In vitro and *in vivo* vertebrate studies suggest that Mn^{2+} causes an increase in α-synuclein-induced cell death. We also show that Mn confers increase sensitivity to α-synuclein induced cellular death and in *C. elegans*. Mn induced degeneration has been implicated in conferring cellular death through mitochondria disfunction. Mn attenuates membrane potential in *C. elegans* following Mn exposure, and compounds that have shown to increase membrane potential can largely protect against the neuronal death in *C. elegans*. These compounds are especially efficacious post exposures to Mn, suggesting a potential therapeutic benefit in vertebrates if Mn toxicity is suspected.

Our microarray studies, qrt-PCR and Western blot analysis have identified a number of genes and proteins in which expression levels are significantly modulated following expression Mn exposure. A number of these genes have also been identified in vertebrate PD-association studies. Significant changes occur in pathways identified with the proteosome, ubiquitn mediated proteolysis, mitochondria, and ROS production.

STUDY 2 – Establish and evaluate transgenic lines overexpressing edongenous parkin and normal and mutant parkin and determine whether these genes play a role in Mn^{2+}-induced neurodegeneration in both WT and cell death pathway deficient mutants.

A major goal of our second aim is to determine the role of parkin in Mn^{2+}-induced DA neuron cell death in *C. elegans*. Parkin has previously been shown to attenuate Mn^{2+} induced DA neuron cell death in other systems. Parkin is also one of at least 5 proteins that when mutated increases the probability of developing the disorder. We have generated 40 *C. elegans* antibodies to PD, manganism, and stress response proteins in *C. elegans*. Brief Mn exposures confers significant increases in parkin expression as determined qrt-PCR and Western Blot analysis. Parkin mutants have a significant decrease in DA levels, and in the presence of other PD-associated mutants, can have further reductions. Our protein expression studies indicate that Parkin is expressed in DA neurons in *C. elegans*.

We have recently finished developing a novel fluorescent dissecting scope that can perform high throughput, automated, 3-D imaging and fluorescent signal analysis of *C. elegans* on a variable temperature controlled platform. This instrument will allow us to perform whole genome reverse screens in as little time as a month or less. Our reverse genetic, protein expression, and immunofluorescent studies have identified a number of

proteins that protect against Mn induced toxicity. A C. elegans orthologue to DMT-1, the vertebrate Mn-transporter, is expressed in C. elegans DA neurons (as well as other cell types) and contributes to Mn induced DA neuron cell death. We have also identified other previously uncharacterized stress response proteins that when expression is decreased, confers sensitivity to Mn and/or 6-OHDA in the DA neurons. Worms containing mutations within these genes also shows increase vulnerability to Mn-induced DA neuron cell death. Furthermore, movement defects are also observed with protein knockdown with a PD associated neurotoxin.

Conclusions

Our studies are consistent with dopamine contributing to Mn induced DA neurodegeneration. PD associated proteins modulate vulnerability of DA neurons to Mn. Molecular transporters and stress response specifically confer DA neuronal cellular protection against Mn. Our ongoing genetic screens for Mn- and PD-associated DA neurodegeneration should provide further significant insight into the molecular basis DA neuron vulnernerability.

Key Research Accomplishments

- *C. elegans* strains with mutations within DA neuron associated proteins have altered sensitivity to Mn.
- α-synuclein neurotoxicity is amplified by Mn exposure
- Microarray and qrt-PCR studies indicate proteins involved in oxidative phosphorylation, proteosome and mitochondria function play a role in Mn induced cell death
- Mn exposure attenuates mitochondria potential in C. elegans
- Several stress response proteins have been identified to play a role in protecting DA neurons against Mn induced cell death

Reportable Outcomes

Peterson, RT., **Nass, R.**, Boyd, WA., Freedman, JH., Dong, K., Narahashi, T. (2008) Use of non-mammalian alternative models for neurotoxicological study. *Neurotoxicology* **29**:545-54

Nass, R., Settivari, R. (2008) *C. elegans* models of Parkinson's disease: a robust genetic system to identify and characterize endogenous and environmental components involved in dopamine neuron degeneration (2008). In: *Parkinson's disease: Molecular and Therapeutic Insights from Model Systems*, eds. R. Nass and S. Przedborski. Elsevier Academic Press. 347-56

Nass, R. (2008) *C. elegans* Genetic Strategies to Identify Novel Parkinson's disease-associated therapeutic targets and leads In: *Parkinson's disease: Molecular and Therapeutic Insights from Model Systems*, eds. R. Nass and S. Przedborski. Elsevier Academic Press. 361-367

Nass, R. and Przedborski, S., (2008) eds. *Parkinson's disease: Molecular and Therapeutic Insights from Model System.* Elsevier Academic Press

Nass, R., Merchant, KM., Ryan, T., (2008) *Caenohabditis elegans* in Parkinson's disease drug discovery: addressing an unmet medical need. Molecular Interventions. 8:284-93

Current faculty receiving support from the grant:

- Richard Nass, Ph.D

References

Not applicable

Award Number: W81XWH-05-1-0239

Title: Proposal to Conduct a GLP-compliant *In Vivo* Micronucleus Assay According to OECD 474 Guideline and Critical Review of the Genetic Toxicology of Manganese

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6. AUTHOR(S) Leslie Recio, PhD DABT Michael Streicker		**5d. PROJECT NUMBER** **5e. TASK NUMBER** **5f. WORK UNIT NUMBER**
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Integrated Laboratory Systems, Inc. P.O. Box 13501 RTP, NC 27709		**8. PERFORMING ORGANIZATION REPORT NUMBER**
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12. DISTRIBUTION / AVAILABILITY STATEMENT		
13. SUPPLEMENTARY NOTES Not applicable		
14. ASTRACT Manganese is an essential trace nutrient in all forms of life. Manganese is also a crucial metal for industry and has many applications. However, there is research needed to assess the potential adverse health effects that may arise from high level exposure to manganese from industrial and other sources. The purpose of these studies is to assess the genotoxic potential of manganese in mice using the in vivo micronucleus assay. Preliminary studies have shown that female mice are more sensitive than male mice to the acute toxicity of manganese and a top dose level of 200 mg/kg will be uses to assess the in vivo genotoxic potential of manganese using a flow cytometry-based micronucleus assay.		
15. SUBJECT TERMS Manganese, genetic toxicology, micronucleus assay, genotoxicity		

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Table of Contents

Introduction

Manganese is an essential trace nutrient in all forms of life. Manganese is also a crucial metal for industry and has many applications. However, there is research needed to assess the potential adverse health effects that may arise from high level exposure to manganese from industrial and other sources. The purpose of these studies is to assess the genotoxic potential of manganese in mice using the in vivo micronucleus assay.

TASK 1 - CONDUCT A GLP-COMPLIANT *IN VIVO* MICRONUCLEUS ASSAY ACCORDING TO OECD 474 GUIDELINE

Test System

Micronuclei (MN) are well-characterized biomarkers of chromosomal damage that are formed from either chromosome fragments or from lagging, intact chromosomes (aneuploidy). The determination of MN frequencies is currently the most reliable method for evaluating the potential for a chemical to induce both structural and numerical chromosomal alterations (OECD 474). Of the variety of *in vivo* assays that are used to detect genotoxic chemicals, the most common is the *in vivo* rodent erythrocyte MN assay. This assay has been used routinely for decades to evaluate genotoxicity and is typically conducted by evaluating the frequency of micronucleated erythrocytes in bone marrow or peripheral blood slide preparations. ILS uses using flow cytometry (FCM) to measure MN frequencies in rodent peripheral blood that was validated compared to traditional microscopy methods used to enumerate MN in blood smears prepared on slides.

Objective

The objective of this task is to conduct of a GLP compliant micronucleus assay in mice according to the OECD Guideline for the Testing of Chemicals, OECD 474: Mammalian Erythrocyte Micronucleus Test.

Experimental Design

The basic experimental design used at ILS and proposed for the definitive in vivo micronucleus assay in manganese exposed mice is based on the OECD 474 Guideline and includes:

- Exposure to the test substance by an appropriate route
- Five animals per dose group, mice, one sex
- Vehicle control, positive control, 3 test substance dose groups
- Limit dose (2000 mg/kg) – dose range finder needed to identify top dose
- 2 administrations of test article 24 hrs apart, blood collection at 36 hours
- 20,000 cells scored per mouse for micronuclei
- 21 CFR 58 - GLP compliance and oversight by ILS QAU

Progress: Dose-range finder to determine top-dose for definitive in vivo micronucleus assay for Manganese

The purpose of the study was to determine the toxicity of Manganese Chloride tetrahydrate ($MnCl_2$); CAS No. 13446-34-9 in B6C3F1 mice treated by gavage to set dose levels for an *in vivo* micronucleus assay. For compliance to OECD 474 (No. 22), the top dose for the micronucleus assay is defined as the "dose producing signs of toxicity such that higher dose levels, based on the same dosing regimen, would be expected to produce lethality". The limit dose for the in vivo micronucleus assay based on OECD 474 is 2000 mg/kg and testing in a single sex is sufficient if there are no sex differences in response to manganese. For this study animals were handled strictly in accordance with Integrated Laboratory Systems, Inc. institutional guidelines and NIH regulations for humane treatment of research animals.

Literature reviews indicate that the oral LD_{50} for $MnCl_2$ in mice is 1330 mg/kg (equivalent to 581 mg Mn^{+2}/kg). To minimize the use of mice, the toxicity test used at ILS to set dose levels for the micronucleus assay uses 2 male and 2 female mice per dose group and signs of toxicity in a single animal per dose group is sufficient for dose-setting. Due to the many uncertainties associated with literature derived values for LD_{50} the first study was designed to comply with OECD 474 using dose levels of 1000, 500, 250 and 125 and 0 mg/kg MnCl2, 2 doses administered 24 hrs apart and euthanasia at 36 hrs after the second dose. Animals male and female in the 1000, 500, and 250 mg/kg showed clinical signs of toxicity and these dose levels were therefore too high for use in the definitive micronucleus assay. All animals in the 125 mg/kg treatment group and the vehicle control group did not show clinical signs of toxicity and survived to the terminal sacrifice. Therefore, a second study using dose levels of 225, 200 and 175 mg/kg with 2 male and 2 female mice per dose group was conducted to set the dose range and animal sex for the definitive micronucleus study. In this study, female mice but not male mice at the 225 dose group exhibited clinical signs on Day 1 of treatment eliminating this does level and males mice as the sex to be used for the definitive micronucleus assay. Blood was taken from animals demonstrating no clinical signs in the 200 and 175 mg/kg dose group for females and analyzed for percent reticulocytes (%RET), an indicator of bone marrow toxicity. The %RET with respect to the control in this study for females was 100% for the 175 mg/kg dose-group and 68.2% for the 200 mg/kg dose group.

The data from the range finder indicate that: 1. under the conditions used in this study, females are more sensitive than male mice to the acute effects of $MnCl_2$ and 2. a top dose of 200 mg/kg will be used in the definitive micronucleus assay.

TASK 2 – CRITICAL REVIEW OF THE GENETIC TOXICOLOGY OF MANGANESE

ILS will also propose to provide a critical review of the manganese genotoxicity that will provide a:

- comprehensive review of all genotoxicity testing

- critically review of testing data based on current testing guidelines
- identify data gaps
- recommend further testing if required under federal guidelines
- recommend further studies to clarify mode-of-action if needed

Progress report:
No progress on this task

Key Research Accomplishments

- Female mice are more sensitive than male mice to the acute effects of $MnCl_2$

- A top dose of 200 mg/kg will be used to conduct a micronucleus assay in female mice in compliance with OECD 474

Reportable Outcomes
None