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Pathophysiology of manganese-associated neurotoxicity

Brad A. Racette [a,*], Michael Aschner [b], Tomas R. Guilarte [c], Ulrike Dydak [d], Susan R. Criswell [a], Wei Zheng [d]

[a] Department of Neurology, Washington University School of Medicine, 660 South Euclid Avenue, St. Louis, MO 63131, USA
[b] Departments of Pediatrics, Pharmacology and The Kennedy Center for Research on Human Development, Vanderbilt University Medical Center, 110 Magnolia Circle, Nashville, TN 37203, USA
[c] Department of Environmental Health Sciences, Mailman School of Public Health, Columbia University, 722 W. 168th St., New York, NY 10032, USA
[d] School of Health Sciences, Purdue University, 550 Stadium Mall Dr., West Lafayette, IN 47907, USA

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CONFERENCE SUMMARY

Manganese (Mn) is a well established neurotoxin associated with specific damage to the basal ganglia in humans. The phenotype associated with Mn neurotoxicity was first described in two workers with occupational exposure to Mn oxide (Couper, 1837). Although the description did not use modern clinical terminology, a parkinsonian illness characterized by slowness of movement (bradykinesia), masked facies, and gait impairment (postural instability) appears to have predominated. Nearly 100 years later an outbreak of an atypical parkinsonian illness in a Chilean Mn mine provided a phenotypic description of a fulminant neurologic disorder with parkinsonism, dystonia, and neuropsychiatric symptoms (Rodier, 1955). Exposures associated with this syndrome were massive and an order of magnitude greater than modern exposures (Rodier, 1955; Hobson et al., 2011). The clinical syndrome associated with Mn neurotoxicity has been called manganism.

Modern exposures to Mn occur primarily through occupations in the steel industry and welding. These exposures are often chronic and varied, occurring over decades in the healthy workforce. Although the severe neurologic disorder described by Rodier and Couper are no longer seen, several reports have suggested a possible increased risk of neurotoxicity in these workers (Racette et al., 2005b; Bowler et al., 2007; Harris et al., 2011). Based upon limited prior imaging and pathologic investigations into the pathophysiology of neurotoxicity in Mn exposed workers (Huang et al., 2003), many investigators have concluded that the syndrome spares the dopamine system distinguishing manganism from Parkinson disease (PD), the most common cause of parkinsonism in the general population, and a disease with characteristic degenerative changes in the dopaminergic system (Jankovic, 2005).

The purpose of this symposium was to highlight recent advances in the understanding of the pathophysiology of Mn associated neurotoxicity from *Caenorhabditis elegans* to humans. Dr. Aschner's presentation discussed mechanisms of dopaminergic neuronal toxicity in *C. elegans* and demonstrates a compelling potential role of Mn in dopaminergic degeneration. Dr. Guilarte's experimental, non-human primate model of Mn neurotoxicity suggests that Mn decreases dopamine release in the brain without loss of neuronal integrity markers, including dopamine. Dr. Racette's presentation demonstrates a unique pattern of dopaminergic dysfunction in active welders with chronic exposure to Mn containing welding fumes. Finally, Dr. Dydak presented novel magnetic resonance (MR) spectroscopy data in Mn exposed smelter workers and demonstrated abnormalities in the thalamus and frontal cortex for those workers. This symposium provided some converging evidence of the potential neurotoxic impact of Mn on the dopaminergic system and challenged existing paradigms on the pathophysiology of Mn in the central nervous system.

* Corresponding author at: Department of Neurology, Washington University School of Medicine, 660 South Euclid Avenue, Campus Box 8111, St. Louis, MO 63110, USA. Tel.: +1 314 747 0531; fax: +1 314 747 3258.
E-mail addresses: racetteb@neuro.wustl.edu (B.A. Racette), Michael.aschner@vanderbilt.edu (M. Aschner), trguilarte@columbia.edu (T.R. Guilarte), wzheng@purdue.edu (W. Zheng).

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C. elegans and the role of dopamine in manganese-induced neurodegeneration

Michael Aschner, PhD

Departments of Pediatrics, Pharmacology and The Kennedy Center for Research on Human Development, Vanderbilt University Medical Center, Nashville, TN, USA

Manganese homeostasis is crucial given the delicate relationship between its essentiality and toxicity. Control over Mn concentrations in all tissues, including the central nervous system (CNS) is maintained by several mechanisms including the divalent metal transporter (DMT1) (Andrews, 1999). The latter belongs to the family of natural resistance-associated macrophage protein (NRAMP) (Garrick et al., 2003; Gruenheid et al., 1995; Gunshin et al., 1997). In the basal ganglia, an area known to accumulate the highest Mn concentrations, DMT1 levels are also the highest in the CNS (Huang and Lin, 2004). The existence of multiple DMT proteins which are differentially regulated at the transcriptional and post-translational levels and within specific tissues (intestine, liver, kidney, brain) and under various conditions [Mn or iron (Fe) levels, infection], has been a challenge in elucidating their specific functions. To better characterize DMT1 function, studies in our lab have utilized a *C. elegans* model.

C. elegans offers many distinct advantages. Its small size (~1.5 mm adult), short lifespan (~3 weeks) and rapid lifecycle (~3 days) (Leung et al., 2008; Sulston et al., 1983) permit rapid analysis. A *C. elegans* hermaphrodite produces ~300 progeny thus providing ample number of animals for analysis. *C. elegans* with <1000 cells is transparent allowing for direct visualization of cells. Furthermore, RNA interference (RNAi) and chromosomal deletion can be achieved with relative ease and provide critical information on mutant strains and sensitivity to environmental insults (Jiang and Zheng, 2005; Lund et al., 2002; Reichert and Menzel, 2005). Strains can also be frozen and thawed, and proteins can be labeled with green fluorescence (GFP) and easily visualized.

In recent studies in our lab we identified and cloned three functional *C. elegans* DMT1 orthologues SMF-1, SMF-2 and SMF-3 (Au et al., 2009). These proteins were shown to play distinct roles in Mn transport and support an evolutionary conserved function for DMT1 isoforms in the regulation of Mn uptake. Specifically, our results have documented that SMF-3 is the major Mn transporter in the worm. *smf-3(ok1035)* was shown to be the mutant most resistant to acute Mn exposure and the only mutant that displayed a significant decrease in Mn content upon Mn treatment suggesting that SMF-3 depletion may not be compensated for by SMF-1 or SMF-2. *smf-3* was also shown to be down-regulated both transcriptionally and post-translationally upon exposure to Mn thus limiting the toxic accumulation of Mn in the worm. In contrast, SMF-2 was shown to partially protect against Mn exposure, most likely by allowing: Mn excretion, its sequestration, or the modulation of Mn uptake *via* the other DMT1-like isoforms, or a combination of the above. We posited that SMF-2 most likely functions as a sensor of environmental Mn levels, and a downstream signaling pathway would either impact Mn uptake *via* SMF-3 and SMF-1 or Mn excretion *via* as of yet uncharacterized transporters. Finally, SMF-1's contribution to Mn uptake was minor compared to SMF-3 and we posited that it is a counterpart of NRAMP1 in the worm, and is predominantly required for Fe uptake.

These studies were conducted as a first step in establishing that both Mn uptake and toxicity mechanisms involving DMTs are conserved from nematodes to man. Given the evolutionary conserved function for DMT1 isoforms in *C. elegans*, we then used this platform as a tool to dissect out the role of various neurotransmitters in mediating Mn neurotoxicity. As such, additional studies were conducted in which it was demonstrated that dysfunction in the dopamine re-uptake transporter, DAT-1, sensitizes the worm to Mn neurotoxicity (Benedetto et al., 2010). Analogous to observations in mammalian studies, Mn toxicity in the worm was also associated with elevated formation of reactive oxygen species (ROS) and reduced longevity. Consistent with the oxidative stress associated with Mn exposure, SKN-1 (the worm's NRF-2 homolog) overexpression protected from Mn-induced toxicity. Furthermore, increased extracellular, but not intracellular dopamine (DA) concentrations due to direct DA exposure or genetic manipulation potentiated the Mn-induced lethality, oxidative-stress, and the worms' reduction in lifespan (Benedetto et al., 2010). Finally, we have also shown that the dual-oxidase, BLI-3, is involved in the DA-dependent Mn-induced toxicity and that *bli-3* loss-of-function attenuates the DA-dependency of Mn toxicity.

Taken together these studies show that in Mn-treated *C. elegans*, physiological aspects of parkinsonism are recapitulated. Specifically, we corroborate the importance of NRAMP/DMT orthologues in the toxicity process (Salazar et al., 2008; Song et al., 2007; Zhang et al., 2009), the specificity and dose-dependency of the DAergic neurodegeneration (Barzilai and Melamed, 2003), the involvement of the dopamine transporter (DAT) (Afonso-Oramas et al., 2009), the synergy between DA and Mn (Prabhakaran et al., 2008) as well as the role of Mn-induced oxidative stress in the neurodegenerative process (Milatovic et al., 2007, 2009). Our finding on the role of extracellular DA in mediating Mn neurotoxicity also have important implications for contemporary treatment modalities of Parkinson disease (PD) since different strategies are necessary for controlling extracellular *vs.* intracellular DA levels. The most commonly used treatment for PD, L-DOPA, could accelerate or exacerbate DAergic neurodegeneration over a protracted time period. However, only one clinical trial has investigated the impact of L-DOPA on disease progression and no worsening in PD was found (Fahn, 1999). If the dual-oxidases also oxidize DA in vertebrates, the ideal pharmacologic treatment for PD would maintain high affinity for DA receptors and DAT, but serve as poor substrates for the dual-oxidases (Benedetto et al., 2010). Alternatively, one could limit the extent of the DAergic neurodegeneration by directly inhibiting the dual-oxidases. Promoting Nrf2 activity could also offer a beneficial strategy in limiting Mn-induced toxicity. Importantly, the series of studies described herein establishes the critical benefit in conducting biochemical and genetic investigations in the *C. elegans* model. Clearly, studies on environment–gene interactions in age-related DAergic neurodegenerative disorders are highly amenable in the nematode, providing crucial information on mechanisms associated with Mn-induced neurotoxicity.

Dysregulation of in vivo dopamine release in the striatum of manganese-exposed non-human primates measured by PET

Tomas Guilarte, PhD

Department of Environmental Health Sciences, Mailman School of Public Health, Columbia University, New York, NY, USA

Accumulation of Mn in the CNS can result in a parkinsonian syndrome with clinical features that may overlap with idiopathic PD (Perl and Olanow, 2007; Guilarte, 2010). The neuropathology in PD is well characterized and comprises the progressive loss of dopamine-containing neurons in the substantia nigra pars compacta. The loss of dopamine neurons leads to a dramatic decrease in dopamine concentrations and the structural loss of dopamine terminal markers in the caudate and putamen, the brain regions that are primarily affected and account for the movement

abnormalities in PD. Unlike PD, the underlying neurobiology of Mn-induced parkinsonism has been a subject of great debate in the scientific literature (Guilarte, 2010). Historically, there has been a paucity of data and the available evidence has been contradictory on the mechanism(s) by which Mn induces movement abnormalities. Accordingly, our laboratory undertook a multidisciplinary study using non-human primates to address the early neurological effects of exposure to moderate levels of Mn on behavior and motor function. These longitudinal studies comprise assessment of cognitive and motor function in conjunction with neuroimaging studies that interrogate structural and functional aspects of the dopaminergic system using Positron Emission Tomography (PET). Finally, verification of *in vivo* PET findings was performed using a variety of neuropathological endpoints.

The results of these studies indicate that moderate levels of Mn exposure produce subtle changes in fine motor control and small decrements in motor function (Guilarte et al., 2006b, 2008a; Schneider et al., 2006) These motor function changes were associated with a marked decrease of *in vivo* dopamine release in the absence of a change in dopamine transporter levels in the caudate/putamen (Guilarte et al., 2006a, 2008a) measured by PET in the same animals. We also found a small but significant decrease in D2-dopamine receptor levels in the caudate and putamen measured by PET. The neuropathological studies confirmed that Mn exposure did not alter levels of tyrosine hydroxylase or other structural markers of dopamine terminals in the caudate and putamen, or dopamine concentrations (Guilarte et al., 2006b, 2008a). Thus, the findings from our studies clearly indicate that the movement abnormalities in non-human primates associated with exposure to moderate levels of Mn were not the result of dopamine neuron loss; but in fact, they are associated with the inability of dopaminergic neurons to release dopamine.

One important aspect of our comprehensive non-human primate studies is that there are a number of cognitive tasks that are assessed along with motor function tests. We have found that in the same Mn-exposed animals that express subtle deficits in motor function, they also express working memory deficits (Schneider et al., 2009). Since dopamine plays an important role in working memory in the frontal cortex (Aalto et al., 2005; Landau et al., 2009), we are currently investigating whether the same level of Mn exposure that result in impairments of *in vivo* dopamine release in the caudate/putamen, also affects dopamine release in the frontal cortex. We have previously shown that Mn exposure in these non-human primates results in significant neurodegenerative changes in the frontal cortex (Guilarte et al., 2008b), a brain region that has not been previously associated with Mn neurotoxicity. The *in vivo* dopamine release studies in the frontal cortex using PET are being performed in parallel with assessment of working memory in the same animals. The results from these studies should provide valuable information that may help explain the role of the dopamine system in various aspects of Mn-induced neurological dysfunction.

Molecular imaging of Mn exposed humans

Brad A. Racette, M.D.

Department of Neurology, Washington University School of Medicine, St. Louis, MO, USA

Several single-photon emission computed tomography (SPECT) or PET-based approaches use different radiotracers to assess various aspects of presynaptic dopaminergic nigrostriatal neurons (Brooks et al., 2003). PET has the advantage of substantially greater resolution. We have recently used FDOPA PET to investigate the pathophysiology of Mn associated neurotoxicity in welders. FDOPA PET primarily reflects neuronal decarboxylase activity that converts FDOPA into the charged molecule [^{18}F] dopamine that is subsequently trapped in the brain (Martin and Perlmutter, 1994). Multiple studies in humans and nonhuman primates suggest that FDOPA uptake reflects nigrostriatal function. FDOPA uptake is decreased in PD patients compared to normal subjects (Martin et al., 1989; Heiss and Hilker, 2004) and striatal uptake modestly correlates with parkinsonian signs (Ishikawa et al., 1996). Several studies have attempted to correlate striatal uptake of FDOPA with postmortem measures of striatal dopamine or numbers of substantia nigra pars compacta (SNpc) cell bodies of nigrostriatal neurons but FDOPA probably best correlates with dopamine content (Snow et al., 1993; Pate et al., 1993; Yee et al., 2001). FDOPA PET has greater sensitivity to detect dysfunction of the nigrostriatal system than clinical examination and identifies dopaminergic dysfunction in pre-symptomatic relatives several years prior to symptoms in genetic studies for PD (Brooks, 1991; Doudet et al., 1998; Shinotoh et al., 1996) and other genetic parkinsonisms (Kishore et al., 1996).

FDOPA PET studies in patients with Mn toxicity provide conflicting results. Wolters et al. (1989) examined four workers with parkinsonism due to ventilatory malfunction in a ferromanganese smelter. All four subjects had normal FDOPA PET and T1 MRI scans despite blood Mn levels 7–700 times the normal value. Repeat FDOPA PET and MRI scans in four of these patients, four years later were reportedly normal (Shinotoh et al., 1997). Kim et al. (1999) performed FDOPA PET on a welder with Mn exposure and clinically typical idiopathic PD and found reduced FDOPA uptake in the left putamen. These same authors later described two additional cases of patients with parkinsonism and Mn exposure with dopaminergic imaging using [123_I]-(1r)-2b-carboxymethoxy-3b-(4-iodophenyl)tropane ([123_I]-β-CIT) SPECT (Kim et al., 2002). One subject was a welder and the other worked in a foundry. Both had reduced striatal uptake of [123_I]-β-CIT suggesting dysfunction of the nigrostriatal pathway. Unfortunately, coincident PD cannot be excluded in those subjects with dopaminergic dysfunction, especially in older subjects.

To investigate the integrity of the dopamine system in Mn exposed humans, we imaged 20 asymptomatic welders exposed to Mn containing fumes, 20 subjects with PD, and 20 non-exposed reference subjects using FDOPA PET (Criswell et al., 2011). The mean Unified Parkinson Disease Rating Scale motor subsection part 3 (UPDRS3) was 8.3 (±3.82) as compared to 1.08 (±1.32) (ANOVA; $p \leq 0.001$) demonstrating mild parkinsonian signs. Despite having mild parkinsonian signs, these subjects had no neurologic symptoms or neurologic diagnoses. For each subject we identified basal ganglia volumes of interest and generated the specific uptake of FDOPA, Ki, for each region. Repeated measures general linear model analysis demonstrated a significant interaction between diagnostic group and region $F(4, 112) = 15.36$, $p < 0.001$. Caudate Ki's were significantly lower in asymptomatic welders ($0.0098 \pm 0.0013\ \text{min}^{-1}$) compared to non-exposed reference subjects ($0.0111 \pm 0.0012\ \text{min}^{-1}$, $p = 0.002$). The regional pattern of uptake in welders was most affected in the caudate > anterior putamen > posterior putamen. PD patients demonstrated the typical regional pattern of uptake with most severe reductions in the posterior putamen > anterior putamen > caudate. This study demonstrates dysfunction in the nigrostriatal dopamine system in active, Mn exposed welders without neurologic symptoms. We did not find a dose–response relationship between FDOPA Ki and cumulative welding exposure. However, the upregulation of dopa decarboxylase in dopamine deficiency states may obscure a dose–response relationship. The caudate Ki reduction in welders may represent an early (asymptomatic) marker of Mn neurotoxicity and may be distinct from the pattern of dysfunction found in symptomatic PD. Our data cannot exclude a specific regulatory effect of welding fume and Mn on

dopa decarboxylase. Use of a radioligand that binds to VMAT2 may provide a better dose–response relationship since VMAT2 is not upregulated in dopamine deficiency states like the dopamine transporter and dopa decarboxylase.

We have also reported FDOPA PET in two subjects with Mn exposure due to end stage liver disease (ESLD). Patients with ESLD accumulate Mn in the brain due to impaired hepatic excretion (Burkhard et al., 2003). This provides a potentially informative model to study the neurotoxic effects of chronic Mn exposure since these patients presumably have consistent elevations in blood Mn. The first subject presented with a severe parkinsonian illness with symmetric signs and severe gait impairment and had response to levodopa with improvement in her UPDRS3 from 45 to 33.5 (Racette et al., 2005a). MRI demonstrated increased T1 signal in the globus pallidum and FDOPA PET revealed marked reduction in FDOPA uptake with a caudate: posterior putamen ratio of 1.43. More recently, we described another ESLD subject who had mild parkinsonism with a UDPRS3 of 12 who also had an elevated pallidal index, indicating Mn deposition in the brain. This subject had reduced FDOPA uptake in the caudate (24.7% reduction), anterior putamen (28.0% reduction), and posterior putamen (29.3% reduction) (Criswell et al., in press). The caudate: posterior putamen ratio was 0.99.

These human studies suggest that early Mn neurotoxicity results in dopaminergic dysfunction that primarily affects the caudate and may be associated with cognitive and behavioral symptoms (Bowler et al., 2007). However, as the syndrome progresses to a more severe motor phenotype, the neurotoxicity affects the posterior putamen. This latter pattern is similar to that seen in PD patients and provides a possible pathophysiologic link between Mn neurotoxicity and the neurodegeneration seen in PD.

Altered brain GABA and NAA concentrations in occupational manganese exposure measured by in vivo MRS

Ulrike Dydak, PhD

School of Health Sciences, Purdue University, West Lafayette, IN, USA

While Mn neurotoxicity is clearly linked to a dysfunctional dopaminergic system (Burton and Guilarte, 2009; Criswell et al., 2011), the exact roles of the neurotransmitters glutamate and γ-aminobutyric acid (GABA) are less well understood. GABA is an inhibitory neurotransmitter, involved in projecting neuronal signals in the basal ganglia and thalamic regions and coordinating the body's fine movement. Thus, GABA plays an important role in many movement disorders (Galvan and Wichmann, 2007). Several animal studies have suggested increased striatal GABA levels following Mn exposure (Anderson et al., 2008; Bonilla, 1978; Gianutsos and Murray, 1982; Gwiazda et al., 2002). Due to novel developments in the non-invasive imaging technique of magnetic resonance spectroscopy (MRS) over the past decade, it has become feasible to measure *in vivo* brain GABA levels in addition to many other brain metabolites in subjects exposed to Mn. Furthermore, Mn deposition in their brains may be visualized by T1-weighted MRI.

A recent study of 10 Mn-exposed smelters and 10 matched controls was conducted using the MEGA-PRESS MRS sequence (Mescher et al., 1998) to determine GABA concentrations (Edden and Barker, 2007) in a mainly thalamic brain region and a standard short echo time MRS sequence to measure six more brain chemicals in several brain regions, including the frontal cortex, globus pallidus, thalamus and putamen (Dydak et al., 2011). A 3D high-resolution T1-weighted MR imaging sequence was used to investigate differences in Mn-deposition in the brain as determined by the Pallidal Index (PI), a ratio of the T1-weighted signal intensities referenced to a white matter region in the brain with no to little Mn deposition.

While a clear group difference was found between exposed and non-exposed subjects using the PI ($p = 0.007$), only seven out of the ten exposed subjects did show a hyper-intense signal in the T1-weighted images, revealing Mn deposition. Thus, no clear cutoff value for the PI could be determined to discriminate between the two groups. The question as to why a small percentage of exposed workers did not show brain Mn deposition could not be explained by the duration of exposure, different work practices, age, blood Mn levels or any other factors studied in this group. It is possible that the size of samples in this study may not be sufficient to reveal the difference; however, it is also possible that a protective mechanism may exist among these subjects.

MRS revealed two major findings: First, the concentration of N-acetylaspartate (NAA), a marker of neuronal integrity, was found to be decreased in the frontal cortex of Mn-exposed workers ($p = 0.042$). Further, this decrease showed a significant correlation with duration of exposure ($R = -0.93$, $p < 0.001$). Second, the neurotransmitter GABA was increased by 80% on average in the

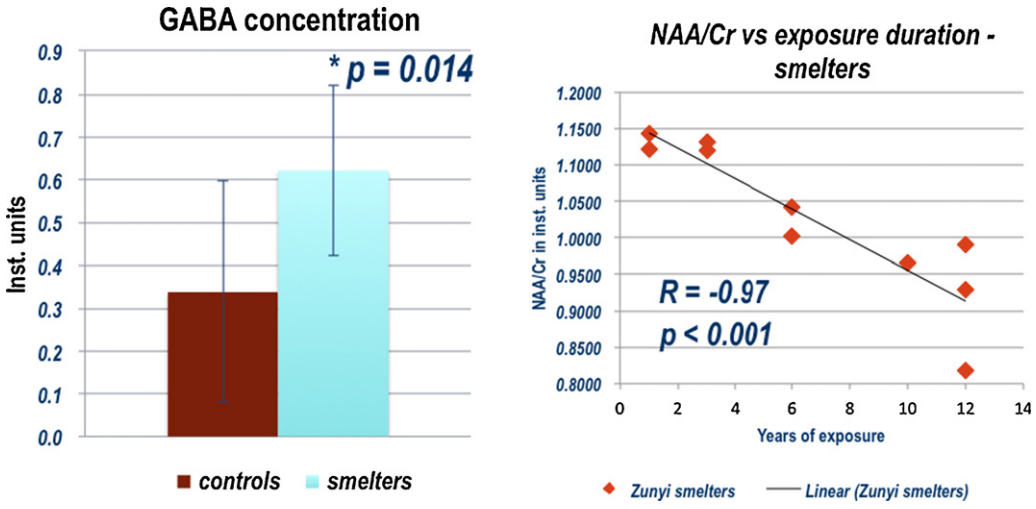

Fig. 1. GABA concentrations in the thalamus in Smelter workers and control subjects.

thalamus and adjacent brain regions in the Mn-exposed group (Fig. 1). The findings of increased GABA levels corroborate the findings in rodent studies mentioned above for the first time in humans. Since GABA levels did not correlate with the MRI signal hyperintensity indicative of high Mn deposition in the brain, and NAA was found to be decreased particularly in the frontal cortex, where less Mn is deposited than in the basal ganglia, Mn neurotoxicity may rather be defined by the intrinsic vulnerability of neuronal systems to injury caused by Mn than the amount of accumulated Mn, a notion already previously suggested.(Burton and Guilarte, 2009). Finally, with the GABA findings mirroring findings in movement disorders, and decreased NAA levels reflecting increased neuronal dysfunction, the MRS findings of this study may lead to new approaches identifying early presymptomatic effects of Mn neurotoxicity. Confirmation of these results in larger study cohorts is in progress.

Future directions

This symposium highlighted paradigm shifting research implicating Mn as a potential dopamine neuron toxin. The mechanisms of this toxicity remain unclear. Dr. Aschner provides compelling evidence of the mechanisms underlying Mn associated dopaminergic toxicity. Dr. Guilarte's work suggests that dopamine release is impaired but without neuronal degeneration. The human studies presented by Drs. Criswell and Racette demonstrate dopaminergic dysfunction in Mn exposed welders but the molecular imaging modalities do not necessarily indicate neurodegeneration. The human studies conducted by Drs. Dydak and Zheng broaden the spectrum of neurotransmitters impaired by Mn exposure and offers a potentially reliable new marker of Mn-associated early stage neuronal injury. Ultimately, human imaging-pathologic correlation will provide the unprecedented advantage to address lingering questions raised by the work presented at this symposium. The research progress presented by the speakers in this symposium also suggests that these imaging techniques may provide *in vivo* evidence to verify, compare, or discover protective or symptomatic treatments (Jiang et al., 2006; Zheng et al., 2009).

Conflict of interest

None declared.

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