Golgi Phosphoprotein 4 (Gpp130) Is A Sensitive And Selective Cellular Target Of Manganese Exposure

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UNIVERSITY OF CALIFORNIA
SANTA CRUZ

GOLGI PHOSPHOPROTEIN 4 (GPP130) IS A SENSITIVE AND SELECTIVE CELLULAR TARGET OF MANGANESE EXPOSURE

A thesis submitted in partial satisfaction of the requirements for the degree of

MASTER OF SCIENCE

in

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by

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ABSTRACT

GOLGI PHOSPHOPROTEIN 4 (GPP130) IS A SENSITIVE AND SELECTIVE CELLULAR TARGET OF MANGANESE EXPOSURE

by

MELISA MASUDA

While chronic environmental exposure to manganese (Mn) is associated with neurocognitive and fine motor deficits in children, relatively little is understood about cellular responses to Mn spanning the transition between physiologic to toxic levels of exposure. It was recently reported that elevated (500 µM) exposure and uptake of Mn into the Golgi of HeLa cells led to the lysosomal degradation of Golgi Phosphoprotein 4 (GPP130), and that blocking Mn uptake into the Golgi protected against GPP130 degradation, suggesting GPP130 may also play a role in cellular Mn homeostasis (Mukhopadhyay et al., 2010). Here, we investigated the specific, sensitive, and temporal response of GPP130 to Mn in AF5 GABAergic neuronal cells, and whether GPP130 degradation occurs in brain cells in vivo in rats subchronically exposed to Mn. Results show that GPP130 degradation is specific to Mn in AF5 cells, and does not occur following exposure to cobalt, copper, iron, nickel, or zinc. GPP130 degradation occurs without measurable increases in intracellular Mn levels and at Mn exposures as low as 0.54 µM. Furthermore, GPP130 protein was detected in only ~15 - 30 % of striatal and cortical brain cells in control animals, and Mn-exposed animals exhibited a significant reduction in both the number of GPP130-postive cells, and the overall cellular levels of GPP130 protein, demonstrating the in vivo relevance of this Mn-specific response within the primary target organ of Mn toxicity. These results may provide insight into important specific mechanism(s) of cellular Mn regulation and toxicity within the brain, including the selective susceptibility of cells to Mn cytotoxicity.

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DEDICATION

To my family, for their love and support each step of the way.
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Introduction

Manganese (Mn) is a transition metal that serves as a cofactor for a number of enzymes, and is essential for many biological processes, including brain development (Keen, 1984; Prohaska, 1987). At elevated exposures, however, Mn can accumulate widely throughout the brain and act as a neurotoxin (Reaney et al., 2006; Criswell et al., 2012), leading to deficits in executive and motor function (Aschner et al., 2005; Kern et al., 2010; Lucchini et al., 2011). The specific mechanisms leading to these functional deficits are not well-understood, though Mn has been shown to target dopaminergic and GABAergic neurons in the basal ganglia and elsewhere (Stanwood et al., 2009; Gwiazda et al., 2002; Crooks et al. 2007a, b). For example, Stanwood et al. (2009) reported Mn cytotoxicity in dopaminergic and GABAergic neurons exposed in vitro to 10 to 800 µM Mn, with levels of 100 µM Mn leading to changes in neurite length and integrity, and increased cytoskeletal abnormalities. Using a GABAergic AF5 neuronal cell model, Crooks et al. (2007a, b) reported altered cellular metabolism, including increased intracellular GABA and disrupted cellular iron homeostasis at exposure levels of 25 to 300 µM Mn. However, while much is known about the pathophysiology of Mn neurotoxicity at elevated exposure levels (Racette et al., 2012), relatively little is understood about cellular responses to lower exposures that may only slightly exceed physiologic levels of Mn.

The transition from physiologic to toxicologic cellular Mn likely occurs when homeostatic influx/efflux processes become imbalanced. Cellular Mn uptake/influx into brain cells occurs via divalent metal transporter-1 (DMT1), transferrin receptor (TfR), and voltage regulated and store-operated Ca2+ channel mechanisms (Gunshin et. al., 1997; Davidsson et. al., 1989; Lucaciu et. al., 1997; Riccio et. al., 2002). However, comparatively little is known about the mechanisms of cellular Mn efflux from cells in the brain, even though systemic Mn is regulated largely through hepatic efflux of excess Mn into the bile (Bertinchamps et al.,
Emerging evidence suggests that cellular Mn, like iron, may be effluxed by ferroportin, and that elevated exposures to Mn may induce ferroportin expression in brain (Madejczyk and Ballatori, 2012; Yin Z et al., 2010.). Other cellular proteins, including secretory pathway Ca2+ Mn2+ ATPases (SPCA) (Leitch et al., 2011) and ATP13A2 (Tan et al., 2011), have also been suggested to play a role in cellular Mn efflux. ATP13A2 may localize Mn to the lysosome and may possibly mediate Mn transportation in the neuron (Tan et al., 2011).

SPCA1 is a Golgi transmembrane protein in the brain capable of transporting Mn into the Golgi lumen with high affinity (Sepulveda et. al., 2009). Studies by Leitch et al. (2011) showed that SPCA1 knock down in hepatocyte derived (WIF-B) cells led to an increase in Mn specific cell death, whereas over expression of SPCA1 in human embryonic kidney cells (HEK-293T) protected cells against Mn toxicity. Similarly, Mukhopadhyay et al. (2011) reported that increased activity of SPCA1 led to increased Mn transport into the Golgi and decreased Mn cytotoxicity in HeLa cells, while blocking Mn transport into or out of the Golgi increased cytotoxicity, suggesting that the Golgi may play an important role in Mn homeostasis and detoxification in HeLa cells.

Additionally, Mukhopadhyay et al. (2010) reported that elevated (500 µM) exposure and uptake of Mn into the Golgi of HeLa cells led to the lysosomal degradation of Golgi Phosphoprotein 4 (GPP130), and that blocking Mn uptake into the Golgi protected against GPP130 degradation, suggesting GPP130 may also play a role in cellular Mn homeostasis. The cellular function(s) of GPP130, a cis-Golgi associated transmembrane protein, is not fully understood, but it has been shown to mediate the cellular trafficking of protein cargo from the endosome to the Golgi via the bypass pathway. By utilizing this bypass pathway, proteins and toxins are able to avoid lysosomal degradation and are able to exhibit their effects by trafficking to the Golgi (Mukhopadhyay et al., 2010). Knockdown of GPP130 leads to increased cycling of endosomal proteins between the cell surface and endosomes (Linstedt et al., 1997; Natarajan and Linstedt, 2004). However, the relationship between Mn and
GPP130 within neuronal cells, and the extent that Mn, versus other divalent cations, specifically elicits GPP130 degradation within brain cells in vivo is not known.

The objectives of this study were to demonstrate the specific, sensitive, and temporal response of GPP130 to Mn exposure in AF5 GABAergic neuronal cells, and to determine the extent to which GPP130 degradation occurs in brain cells in vivo in rats subchronically exposed to Mn. Our results show that GPP130 degradation is specific to Mn in AF5 cells, and does not occur following exposure to cobalt, copper, iron, nickel, or zinc. GPP130 degradation occurs rapidly (<1 hr post Mn exposure) and at Mn exposures as low as 0.54 µM, which are nearly 100-times lower than previously reported exposures leading to GPP130 degradation (Mukhopadhyay et al., 2010; 2011). Furthermore, GPP130 protein was detected in only ~15 - 30 % of striatal and cortical brain cells in control animals, and Mn-exposed animals exhibited a significant reduction in both the number of GPP130-positive cells, and the overall cellular levels of GPP130 protein, demonstrating the in vivo relevance of this Mn-specific response within the predominant target organ of Mn toxicity. These results may provide insight into important specific mechanism(s) of cellular Mn regulation and toxicity within the brain.

Materials and Methods

Cell Culture

The immortalized mesencephalic-derived AF5 cell line was a generous gift provided by Dr. W.J. Freed of NIH/NIDA. For all experiments utilizing the AF5 cell line, cells were grown to confluence in T75 flasks in Dulbecco’s Modified Eagle Medium (DMEM; Gibco Life Technologies, Gaithersburg, Md.) containing 10% fetal bovine serum (FBS; Gibco Life Technologies, Gaithersburg, Md.) and 100 µg/mL streptomycin (Bio-Whittaker, Walkersville, Md.), and maintained in a 37°C humidified environment in a 5% CO₂ incubator. Cells were split into either 6-well plates or T25 flasks and grown to 80% confluence, then differentiated
for 4 days post 80% confluence in Neurobasal-A medium with 10% FBS, 2% B-27 serum-free
growth supplement (B-27, Gibco Life Technologies, Gaithersburg, Md.) and 1.25% 200mM L-
Glutamine (Gibco Life Technologies, Gaithersburg, Md.). For metal treatments, Neurobasal
medium was removed and replaced with Neurobasal medium spiked with the indicated metal
concentrations for exposure durations ranging from 1 – 24 hr, depending on the experiment.
The actual metal concentrations in control and exposure medium were determined using a
Finnigan MAT Element high resolution inductively coupled plasma – mass spectrometer
(ICP-MS), as described below. Following treatment, cells were harvested by trypsinization
and collected for analysis by centrifugation at 10,000 x g for 10 minutes; cell pellets were
frozen at -80°C until further analysis. Lysate protein concentrations were determined using
the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific Inc., Rockford, IL), following the
manufacturers instructions.

Immunoblot Analysis

AF5 cell pellets were lysed in RIPA buffer and sonication, and lysates were adjusted
to identical total protein concentrations following measurement of total lysate protein levels
using the BCA assay. Cell lysate protein (20 µg per lane) and the molecular weight marker
(10 µg) were separated by SDS-PAGE on a 4-12% Bis-Tris gel (Novex; Invitrogen Life
Technologies, Gaithersburg, Md.) and transferred to a PVDF membrane. Membranes were
blocked in 5% non-fat dry milk tris-buffered saline and Tween (PlusOne Tween 20; GE
Healthcare Life Sciences, Pittsburgh, PA) (TBST) overnight at 4°C. Membranes were
incubated with primary antibody (Anti-GOLPH4, ab28049; Abcam, Cambridge, UK) (1:1000)
for 1 hour, washed in TBST, and then incubated with secondary antibody (bovine anti-rabbit
IgG-HRP, sc-2370; Santa Cruz Biotech, Santa Cruz, CA) (1:1000) for 1 hour. The
membranes were visualized using ECL Plus (GE Healthcare Life Sciences, Pittsburgh, PA)
and imaged using a Typhoon Fluorescent Scanner. The protein bands were analyzed using ImageQuant.

**Intracellular Mn Concentration Measurement**

Cellular Mn levels were measured using trace metal clean methods as previously described (Crooks et al., 2007; Smith et al., 2000; Kwik-Uribe et al., 2003). Briefly, AF5 cells were harvested by trypsinization, and the pellets were washed once with phosphate buffered saline (PBS) supplemented with 10 mM ethylenediaminetetraacetic acid (EDTA; Gibco Life Technologies, Gaithersburg, Md.), followed by a second wash with PBS alone to remove surface-associated Mn from the cells. Cell pellets were digested using 100 µL 1N nitric acid and heated on a heat block at 80 °C for 30 minutes. The digestate was diluted using Milli-Q water for analyses of total intracellular Mn levels using a Thermo XR-ICP-MS, measuring masses $^{55}$Mn (medium resolution) and $^{103}$Rh, the latter as an internal standard. Manganese concentrations were determined by external standardization using certified standards (Inorganic Ventures, Christiansburg, VA). The analytical detection limit for Mn analyses was 0.01 ng/mL.

**Animals and Mn Treatment**

Adult female Long Evans (*Rattus Norvegicus*) rats were dosed with either control vehicle (n=3) or 9.6 mg Mn/kg (n=3) by intraperitoneal (i.p.) injection, once a day, 3 days a week, for a duration of 4 weeks. A Mn stock solution of 49.6 mg/mL was prepared using MnCl$_2$-hexahydrate diluted in Milli-Q water, and subsequently diluted to 6.7 mg/mL and filter sterilized for delivery to the animals. Manganese concentrations in the dosing solutions were routinely verified by atomic absorption spectrometry. This Mn exposure regimen was selected based on prior studies in our lab showing it was well-tolerated but produced subtle neurochemical and neuromotor deficits (Gwiazda et al., 2007).
**Perfusion and Blood and Tissue Collection**

Twenty-four hours after the final dose was administered, the rats were sacrificed by i.p. injection with 75 mg/kg pentobarbital, followed immediately by whole blood collected via cardiac puncture, and *in situ* brain fixation via upper body perfusion through the heart with ice cold 4% paraformaldehyde (PFA). The brain was removed and immediately immersed in 4% PFA and fixed for 12 hours at 4°C. The solution was changed to a 10% sucrose solution and fixed for 24 hours at 4°C, and then the solution was changed again to a 30% sucrose solution for 48 hours at 4°C. Whole brains were then embedded in freezing medium and stored at -70°C.

**Immunohistochemistry**

Immunohistochemical (IHC) analysis was performed in cortical and striatal brain regions, as previously described (Kern et al., 2010). Briefly, PFA-fixed brains were sectioned coronally in 20 µm slices at -20°C using a cryotome (Leica Microsystems, Inc. model CM30505). Slices containing dorsal striatum and S1 dysgranular zone cortex (Bregma 0.48 mm) (Paxinos, 1998) were mounted on Superfrost/Plus slides, with three slices per animal per treatment on each slide (i.e., six brain slices per slide balanced by treatment) and stored at -20°C. Six brain slices per animal per treatment group for the cortex and one representative brain slice per animal per treatment for the striatum were analyzed for GPP130 by IHC.

For immunostaining, mounted brain slices were blocked with 4% normal goat serum and permeabлизed with 0.1% Triton (Triton X-100; Sigma-Aldrich) for 1 hour. Tissues were then washed three times with PBS, and incubated with primary antibody (Anti-GOLPH4, ab28049; Abcam, Cambridge, UK) (1:1000) overnight at 4°C. Tissues were then washed with PBS, phosphate buffered saline Tween (PBST), and incubated with secondary antibody (goat
anti-rabbit IgG, Alexa Fluor 488; Molecular Probes). Slides were washed again with PBST and stained for 10 min with Draq5 (4084; Cell Signaling Technology, Beverly, MA), followed by a final washing with PBS. Slides were then loaded with Fluoromount GTM (Southern Biotech) and cover-slipped prior to analyses by confocal microscopy.

Confocal Microscopy

Immunostained brain slices were analyzed using a Zeiss LSM 5 Pascal Laser Scanning Microscope. All images on each slide were taken with constant settings at either x20 or x63 magnification using the same detector gain and amplifier offset settings within each magnification for fluorescent image comparison. The x20 images were taken from two separate fields per brain region per brain slice, while the x63 images were taken from 10 separate fields per brain region per slice.

Image Analysis and Quantification

Brain slices per region per animal were qualitatively scored for protein fluorescence as previously described (Kern et al. 2010). A total of six (x20 cortex) or one (x63 cortex and x63 striatum) immunostained brain slice(s) per brain region per animal per treatment were analyzed for GPP130. For the x20 images, a total of 36 fields/treatment for the cortex were qualitatively scored for protein (based on two fields per brain region x six brain slices per animal x three animals per treatment). For the x63 images a total of 30 fields/treatment for the striatum (based on 10 fields per brain region x one representative brain slice per animal x one representative animal per treatment) were quantified and analyzed for treatment-based comparisons of fluorescent density within each slide using Metamorph software (MetaXpress, multiwavelength cell scoring and count nuclei module; Molecular Devices Corporation). For these analyses total grayscale values (pixel brightness) were obtained by summing all of the grayscale values for all objects detected above the defined threshold for each slide.
Fluorescence density in the Mn-treated animals was compared with that of control animals within each slide to determine Mn effects. Threshold limits were set by analyzing three fields/brain over three brain slices/animal and identifying the cells that were considered to be positive. From this, the Approximate Minimum Width, Approximate Maximum Width, and Intensity Above Local Background settings were adjusted and set to capture and identify all cells that were determined to be positive within a given field; these settings were 3 µm, 15 µm, and 80 gray/level, respectively.

**Statistical Analysis**

Treatment comparisons were made using analysis of variance (ANOVA) and Tukey’s post hoc tests. P-Values of <0.05 were considered statistically significant. All analyses were conducted using JMP software (Version 9.0; SAS Institute).

**Results**

1. **GPP130 degradation in AF5 cells is Mn-specific.**

   In order to provide insight into the cellular regulation of Mn and/or the mechanism of cellular Mn toxicity, we investigated whether GPP130 degradation in AF5 neuronal-like cells was Mn-specific, or if GPP130 degradation also occurred with other divalent metal treatments. Results show that Mn exposure (150 µM) led to >80% reduction in cellular GPP130 protein levels, while exposure to Ni, Zn, Co (all 150 µM), and Fe (300 µM) had no measurable effect (Figure 1). Interestingly, treatment with 150 µM Cu led to a small (~17%) but statistically significant increase in GPP130 protein levels, compared to control. These results demonstrate that the effect of metal exposure on GPP130 degradation, at metal levels that do not cause measurable overt cytotoxicity (Crooks et al., 2007a), is highly Mn-specific.
2. **GPP130 degradation in AF5 cells is sensitive to unmeasureable changes in intracellular Mn concentration.**

To elucidate the sensitivity of the GPP130 response to Mn over the transition from physiologic to supra-physiologic intracellular Mn levels, AF5 cells were treated with a range of physiologically relevant and sub-toxic Mn concentrations. Results show that significant cellular GPP130 degradation (~50% reduction) can be observed at the lowest Mn exposure level explored here (0.54 µM) (Figure 2a.), even though total intracellular Mn concentrations did not measurably increase until Mn exposure levels in the medium reached 140 µM (Figure 2b). Note, however, that there were trending but non-significant (p=~0.1) increases in intracellular Mn levels at the 5.3 and 27 µM exposure levels. This indicates that GPP130 degradation is highly sensitive to Mn exposure, occurring at exposure levels below those that produce measureable increases in intracellular Mn.

3. **GPP130 degrades rapidly over time in parallel with a rapid increase, then decrease in intracellular Mn concentrations.**

In order to evaluate the rapid temporality of the GPP130 response to Mn exposure, AF5 cells were treated with sub-toxic Mn concentrations for durations of 1, 2, 4, 8, or 24 hours. Significant GPP130 degradation can be seen in cells treated with 5.4 µM Mn (~15% reduction) and 140 µM Mn (~25% reduction) as early as 1 hour post Mn exposure, the earliest time point evaluated here (Figure 3a). Notably, intracellular Mn levels significantly increased over the first 2 hours of exposure in both the 5.4 and 140 µM treatments, and then significantly decreased over the subsequent 22 hours (hours 2-24 of exposure) even in the presence of continued Mn exposure (Figure 3b). The close temporal association between changes in intracellular Mn levels (rapid increase, then decrease) with GPP130 degradation suggests a possible role for GPP130 in cellular Mn homeostasis (e.g., loss of GPP130 favors cellular Mn efflux).
4. **GPP130 rate of recovery is slower than the rate of disappearance following Mn exposure.**

   Cellular GPP130 degradation occurs rapidly (see above) via the lysosome (Mukhopadhyay et al., 2010), though the rate of recovery of cellular GPP130 levels following cessation of Mn exposure is not known. Here, AF5 cells were exposed to control (0.09 µM), 5.4 µM, or 140 µM Mn for 8 hours, and then allowed to recover in control medium for the subsequent 16 hours. Results show significant degradation of GPP130 by 8 hours in the 5.4 µM and 140 µM treatments (to ~40% and 25% of control, respectively), with small but significant increases (recovery) in cellular GPP130 levels by 24 hours (to ~55% and 45% of control, respectively) (Figure 4A). Notably, the apparent rate of GPP130 recovery after the cessation of Mn exposure was nearly identical in both the 5.4 µM and 140 µM treatment groups. In parallel, intracellular Mn levels increased significantly after 8 hours exposure to 140 µM Mn, which then rapidly and significantly declined after cessation of Mn exposure over the subsequent 16 hours; in contrast, intracellular Mn levels were not measurably increased after 8 hours exposure to 5 µM Mn, nor did levels measurably change after cessation of exposure (Figure 4B). These data indicate that recovery of cellular GPP130 levels following cessation of Mn exposure is incomplete and temporally much slower compared to the initial degradation response to Mn.

5. **GPP130 degradation occurs in vivo in response to Mn and may be cell specific.**

   We explored whether sub-chronic Mn exposure in rats resulted in reductions in brain GPP130 protein levels in order to validate the relevance of our in vitro findings in AF5 neuronal-like cells in intact organisms. For this, rats were exposed to Mn for 4 weeks using an exposure regimen (9.6 mg/kg/d x 3 days/week x 4 weeks via i.p. injection) shown to produce subtle asymptomatic neurotoxic effects (Gwiazda et. al., 2005). Results show that there was no significant difference in the total number of cells detected via Draq5 staining in
either the cortex or dorsal striatum of control versus Mn treated animals (Table I). However, there was a significant reduction in the percentage of cells that were identified as GPP130-positive in Mn-treated animals, with ~20 – 30% of cells identified as GPP130-positive in the S1 dysgranular zone of the cortex of control animals compared to <10% in Mn-treated animals (p<0.001), based on Metamorph analysis of images at x20 and x63 magnification. Similar differences between control and Mn-exposed animals occurred in the dorsal striatum (i.e., 10 - 20% GPP130-positive cells in controls versus 4 – 5% in Mn-treated animals, p<0.05) (Table I).

It is noteworthy that only ~20 – 30% of Draq5-identified cells in the S1 dysgranular zone of the cortex and 10 – 20% in the dorsal striatum of control animals were identified as GPP130 positive using the defined threshold criteria (see Materials and Methods) (Table I, Figure 5). Metamorph analysis of x20 images from the cortex, which captured ~600 Draq-5 positive cells/field, contained ~23% GPP130-positive cells, while analysis of higher resolution x63 images, which captured an average of 12 Draq-5 positive cells/field, showed that ~34% of cells were GPP130 positive. Metamorph analysis was conducted on images at x20 and x63 magnification because the x20 images provided analysis of a large number of cells/field, while the x63 images provided improved fluorescence background and increased sensitivity of fluorescence detection. For the x20 images ~10,800 total cells/treatment were available for analysis (based on ~600 cells/field x two fields/brain x three brain slices/animal x three animals/treatment). By comparison for the x63 images, there were ~120 total cells/treatment analyzed (based on ~12 cells/field x 10 fields/brain x one brain/animal x one animal/treatment).

Further Metamorph analysis of GPP130 protein levels in the cortex at x20 and x63 magnification shows that Mn treatment reduced GPP130 protein levels to ~42% and ~15% of controls, respectively (p<0.005), based on total cellular fluorescence, with a parallel reduction in the percent of GPP130-positive cells to ~40% and ~23% of control (p<0.001) (see Figure
6a and b, ‘Total Fluorescence, All Cells’). However, in cells identified as GPP130-positive, GPP130 protein levels were only slightly reduced in Mn-treated animals to ~90% of controls (see Figure 6a and b, ‘Total Fluorescence in GPP-Positive Cells’), suggesting there exists a population of brain cells that do not exhibit a GPP130 degradation response to Mn.

GPP130 fluorescence threshold detection limits were conservatively set to optimize detection of GPP130 protein above background fluorescence, which was higher in the x20 versus the x63 images. However, it is possible that some cells contained very low levels of GPP130 fluorescence staining below the defined fluorescence threshold detection limits, and therefore were incorrectly classified as non-positive GPP130 cells. In light of this, we analyzed GP130 protein fluorescence in cells identified as non-positive for GPP130. The results show that Mn treatment significantly reduced levels of GP130 fluorescence levels to 14% (x63 images) and 48% of controls in cells initially identified as non-positive for GPP130 (Figure 6a and b). These results parallel the Mn effect on GPP130 levels in GPP130 positive cells, and indicate cells that weakly stain for GPP130 are equally as responsive to Mn.

We also evaluated whether dorsal striatal cells similarly responded to Mn exposure using the x63 images, since the striatum is a well-recognized target region for Mn neurotoxicity (Gwiazda et al., 2002). Results show that GPP130 protein levels in striatal cells were decreased to ~50% of controls based on total fluorescence across all cells, and that Mn treatment reduced the number of cells identified as GPP130-positive to ~20% of controls (Table II) (i.e., 17% of cells identified as GPP130 positive in controls versus 4% GPP130 positive in Mn-treated animals, Table I). Thus, the Mn effect on GPP130 protein levels in the dorsal striatum is very comparable to results in the cortex brain region (Figure 6b, Table I and II). It is noteworthy, however, that the GPP130 staining pattern in the striatum appeared different than in the cortex. In the striatum, GPP130 staining appeared primarily on the surface of the cells, and was typically localized to cell processes (Figure 5), compared to the
cortex, in which GPP130 staining appeared within the cell in a pattern suggesting Golgi localization (Figure 5).

Discussion

While cellular mechanisms of Mn neurotoxicity at elevated exposures are well understood, relatively little is known about cellular responses to exposures that transition from physiologic to toxic levels of Mn. Here, we show that GPP130 protein degradation is a specific and highly sensitive cellular response to Mn spanning the transition from physiologic to supra-physiologic/subtoxic exposures in GABAergic neuronal-like AF5 cells. Moreover, we show in a rodent model that GPP130 protein was detected in only ~15 - 30 % of striatal and cortical brain cells in control animals, and that Mn exposure produced a significant reduction in the number of GPP130-positive cells, demonstrating the in vivo relevance of this Mn-specific response within the brain, the predominant target organ of Mn toxicity.

Our results show that GPP130 degradation was specific to Mn exposure, and not to other cation metals such as Co, Ni, Zn, Cu, or Fe (Figure 1). Since Co(II) is a biologic analog to Mn(II), while Fe(III) is an analog to Mn(III) (Silva and Williams, 1991), this specificity suggests that the GPP130 degradation in response to Mn is a physiological response, as opposed to toxicological. However, the molecular basis for this specificity to Mn is not known. Studies in HeLa cells mapped the Mn-responsive region of GPP130 to its Golgi luminal stem domain; deletion of this stem domain led to a loss of GPP130 sensitivity to Mn and the displacement of GPP130 from the cis-Golgi toward the trans-Golgi network (Mukhopadhyay et al., 2010). Moreover, when this stem domain construct was placed in a related cis-Golgi protein (GP73), it led to the Mn-sensitive targeting of GP73 to multi-vesicular bodies (MVBs) (Mukhopadhyay et al., 2010). Thus, while there is no evidence of direct Mn binding or interaction with the Mn-sensitive luminal stem domain of GPP130, it is clear that this domain
confers transferable, Mn-sensitive responsiveness to the protein and mediates trafficking from the cis-Golgi to MVBs (Mukhopadhyay et al., 2010).

We characterized both extracellular (exposure medium) and AF5 intracellular Mn concentrations so as to elucidate the sensitivity of the GPP130 response to Mn over the transition from physiologic to supra-physiologic intracellular Mn levels. The ~50% reduction in cellular GPP130 levels following 24 hr exposure to 0.54 µM Mn, the lowest Mn exposure level explored here, and the ~80% reduction following exposure to up through 27 µM Mn occurred without measurable increases in total intracellular Mn concentrations (Figure 2). A more detailed assessment of the temporal relationship between intracellular Mn concentrations and cellular GPP130 protein levels over the 24 hr exposure period showed that intracellular Mn levels actually increased and peaked over the first 2 hrs of exposure to 5.4 or 140 µM Mn in association with a rapid significant decrease in cellular GPP130 protein levels (Figure 3). However, over the subsequent 22 hrs of exposure, intracellular Mn levels declined even in the presence of continued Mn exposure, while GPP130 protein levels continued to significantly decline (Figure 3). This close temporal association between changes in intracellular Mn levels (rapid increase, then decrease) with GPP130 degradation suggests a possible role for GPP130 in cellular Mn homeostasis, i.e., loss of GPP130 favors cellular Mn efflux.

The suggestion that loss of GPP130 favors cellular Mn efflux is consistent with a role for GPP130 protein in the transition of cellular Mn exposure from physiologic to supra-physiologic. While systemic Mn is regulated largely through hepatic efflux of excess Mn into the bile (Bertinchamps et al., 1966), comparatively little is known about the mechanisms of cellular Mn efflux from cells in the brain. Recent studies suggest that cellular Mn, like iron, may be effluxed by ferroportin, and that elevated exposures to Mn may induce ferroportin expression in brain (Madejczyk and Ballatori, 2012; Yin et al., 2010). Other cellular proteins, including secretory pathway Ca2+ Mn2+ ATPases (SPCA) (Leitch et al., 2011) and ATP13A2
(Tan et al., 2011), have also been suggested to play a role in cellular Mn efflux. In particular, SPCA1 was identified to facilitate transport of intracellular Mn into the Golgi, and protected cells against Mn cytotoxicity (Mukhopadhyay et al., 2011). In contrast, blocking Mn transport either into or out of the Golgi led to increased cytotoxicity, supporting an important role of the Golgi in cellular Mn detoxification (Mukhopadhyay et al. 2011). Overall, these data indicate that the Golgi, and SPCA1 and GPP130 in particular, plays an important role in cellular Mn homeostasis and resistance to elevated Mn exposures, including possibly cellular Mn efflux (Figure 7).

Our results in rodents address a significant knowledge gap on GPP130 expression and response to Mn in vivo by demonstrating that GPP130 protein appears to be expressed selectively in brain cells, and that Mn exposure produces significant reductions in cellular GPP130 protein levels in a subset of these brain cells. In control animals, only ~20 – 30% of Draq5-identified cells in the S1 dysgranular zone of the cortex and 10 – 20% in the dorsal striatum were identified as GPP130 positive. Moreover, the GPP130 staining pattern in the striatum appeared different than in the cortex. In the striatum, GPP130 was largely localized to the cell surface with evidence of staining of cell processes, while in the cortex, GPP130 protein appeared largely within the cell body in a pattern suggesting localization to the Golgi (Figure 5). The basis for this apparent cell-selective expression and intracellular localization of GPP130 protein is not known.

Manganese-exposed rats exhibited a ~50% to ~80% decrease in both total GPP130 protein levels and the number of GPP130 positive cells in the cortex and striatum, compared to controls. The relative effect size of Mn exposure on GPP130 protein levels in the cortex and dorsal striatum is comparable to decreases in cellular GPP130 protein observed in the AF5 cell studies noted above. However, results from the animal studies suggest that the decrease in brain GPP130 protein levels with Mn exposure is due largely to a reduction in the number of GPP130-positive cells, rather than a reduction in GPP130 across all cells. This
suggestion is further supported by the specific analyses of cells identified as GPP130-positive in both the control and Mn-treated animals; i.e., in the GPP130-positive cells, GPP130 protein levels were only slightly reduced by ~10% in Mn-treated animals compared to controls.

Previous studies have suggested that although Mn is taken up by different types of brain cells via DMT1, the highest levels of Mn accumulation primarily occurs in the dopaminergic and GABAergic-rich basal ganglia region of the brain, which includes the striatum (Garrick et al., 2003; Gunter et al., 2006; Stanwood et al., 2009). While it is recognized that cells and regions within the brain vary in susceptibility to elevated Mn exposure (Garrick et al. 2003; Gunter et al., 2006; Stanwood et al. 2009), the basis for these cell to cell or region to region differences are not well understood. It is notable that here only a subset of cells in the cortex (~20 – 30%) and dorsal striatum (~10 – 20%) were identified as GPP130-positive in control animals, and that a large subset of these cells appeared responsive to Mn exposure, based on reductions in GPP130. Depending on the physiological consequence of the drastic reductions in cellular GPP130 in response to Mn exposure, which presently remains unclear, these results may suggest that GPP130 plays a role in cell-specificity of susceptibility/resistance to elevated Mn exposure.

The very low Mn exposure levels that elicited the degradation of GPP130 protein are noteworthy because they span the physiologic to supra-physiologic range. The lowest level of Mn exposure explored here that caused a significant reduction in cellular GPP130 protein levels, 0.54 µM Mn, was only ~6-fold higher than background Mn levels in the culture medium (0.09 µM Mn), and represents a relative increase that is well within the range of differences in blood Mn concentrations in humans. For instance, normal adult blood Mn levels range from ~7 – 14 ng/mL (i.e., 0.14 – 0.28 µM Mn), though levels in women during late term pregnancy and in neonates range from 40 – 70 ng/mL (i.e., 0.8 – 1.4 µM Mn) (Zota et al., 2009; Montes et al., 2007). Notably, however, the lower Mn exposures in this study (e.g., 0.54 -27 µM Mn for 24 hrs) led to intracellular Mn concentrations of 4.7 – 8.1 ng Mn/mg protein, which were
not significantly different from controls (i.e., 3.6 ng Mn/mg protein in controls exposed to 0.09 µM Mn).

Moreover, the lower Mn exposure levels explored here, all of which produced a significant GPP130 degradation response, were generally much lower than levels used in other studies reporting sensitive cellular targets of Mn exposure. For example, Mukhopadhyay et al. (2010, 2011) showed that GPP130 degradation occurs in HeLa cells at Mn concentrations from 100 µM to 500 µM (cellular Mn concentrations were not reported), or ~200 – 1000-fold higher than the lowest exposure levels explored here. Prior studies in differentiated AF5 cells treated with 25 to 300 µM Mn for 24 hrs showed evidence of altered cellular metabolism, including increased intracellular GABA and disrupted cellular iron homeostasis at exposure levels as low as 25 - 50 µM Mn (with intracellular Mn levels of ~20 ng Mn/mg protein; Crooks et al. 2007a, b), or Mn levels ~5 - 10-fold higher than the lowest Mn levels causing a GPP130 effect in the present study. In undifferentiated PC-12 cells, Mn exposure levels as low as 1 µM for 24 hrs, which produced intracellular Mn levels of ~30 ng Mn/mg protein, were shown to disrupt cellular iron homeostasis, based on increased IRP-2 binding and protein levels and intracellular labile iron levels (Kwik-Uribe et al. 2003; 2006). More recently, Tamm et al. (2008) reported apoptotic cell death via a mitochondrial-mediated pathway in murine-derived multipotent neural stem cells exposed to 50 µM Mn; intracellular Mn levels were not reported in that study. Collectively, these Mn exposure levels and resultant intracellular Mn concentrations were ~5 to 1000 fold higher than exposure levels that generated a GPP130 degradation response in the present study, further underscoring the highly sensitive and specific nature of the GPP130 degradation response to Mn.

Finally, brain Mn levels were not determined in the animal studies reported here because brains were PFA-perfused in situ, thereby potentially altering inherent brain Mn levels. However, previous studies in our lab showed that a comparable Mn exposure regimen (i.e., 9.6 mg Mn/kg/day i.p. x 3 doses/wk x 5 wks) produced brain Mn levels of ~1.8 µg/g (wet
wt.) compared to 0.35 µg/g in controls (Lucchini et al., 2012). These brain Mn levels are equivalent to 16 and 3 ng Mn/mg brain protein for the Mn-treated and control animals, respectively, based on a brain tissue protein content of 115 mg protein/g brain (Banay-Schwartz et al., 1992). Notably, these brain Mn levels (i.e., ng Mn/mg brain protein) are highly comparable to levels in the Mn-treated and control AF5 GABAergic cells reported here, i.e., 22 and 3.6 ng Mn/mg protein for the 140 µM Mn-treated and control cells, respectively (Figure 2b), supporting both the relevance and translation of the AF5 cell study results to Mn exposures in intact organisms.

Currently, there is little known about the cellular responses and molecular mechanism(s) by which exposure to Mn over the transition between physiologic to supra-physiologic/toxic levels leads to cellular and neurological dysfunction. This study addressed this knowledge gap by characterizing a known Mn specific cellular target, GPP130. Collectively, the results indicate that GPP130 degradation is a very early and sensitive cellular response to even very low Mn exposures, and hence may play a role in cellular Mn homeostasis over the transition from physiologic to subtoxic levels of Mn. The full ramifications of the relationship between Mn and GPP130 are as yet unknown and await future study to help elucidate the effects of Mn at low exposure levels that are relevant to environmental exposures in humans. If the mechanism by which Mn leads to neurotoxicity can be elucidated, this could potentially contribute to the development of treatment regimens for Mn toxicity.
Figure 1. GPP130 degradation is Mn-specific. Cellular GPP130 levels by Western Blot in AF5 cells treated for 24 hrs with indicated metals (150 µM for all except Fe was 300 µM). Data are mean percent of control (±SD, n=3/treatment); data are from a representative experiment performed in triplicate. Asterisks indicate significantly different from control (* p<0.05; ** p<0.001). A representative Western Blot is shown above.

<table>
<thead>
<tr>
<th>Metal Treatment</th>
<th>Control</th>
<th>Mn</th>
<th>Ni</th>
<th>Zn</th>
<th>Co</th>
<th>Cu</th>
<th>Fe</th>
</tr>
</thead>
<tbody>
<tr>
<td>GPP130 Band Density (% of Control)</td>
<td>100</td>
<td>80</td>
<td>60</td>
<td>90</td>
<td>80</td>
<td>120</td>
<td>0</td>
</tr>
</tbody>
</table>

![Western Blot Image]
Figure 2. GPP130 degradation is sensitive to unmeasurable changes in intracellular Mn concentration. (A) Cellular GPP130 levels by Western Blot, as percent of control, in AF5 cells treated for 24 hrs with Mn at various concentrations (control (0.09), 0.54, 1.8, 5.3, 27, and 140 µM). (B) Intracellular Mn concentrations in AF5 cells measured by ICP-MS. All data are mean (±SD, n=3/treatment); data are from a representative experiment performed in triplicate. Letters denote significant differences between treatment groups (p<0.05), based on Tukey’s post hoc test. A representative Western Blot is shown above.
Figure 3. GPP130 degradation occurs rapidly with a parallel rapid increase then decrease in intracellular Mn concentrations over time. (A) Cellular GPP130 levels by Western Blot, a percent of control, in AF5 cells treated for 1, 2, 4, 8, or 24 hrs with control (0.09 µM), 5 µM, or 150 µM Mn. (B) Intracellular Mn concentrations measured by ICP-MS in AF5 cells under the same conditions. Data are mean (±SD, n=3/treatment); data are from a representative experiment performed in triplicate. Superscript letters denote significant differences between experimental groups (p < 0.05), based on Tukey’s post hoc test.
Figure 4. Recovery of cellular GPP130 levels is slower than the rate of disappearance. (A) Cellular GPP130 levels by Western Blot, as percent of control, in AF5 cells treated with control (0.09 µM), 5 µM, or 150 µM Mn for 8 hours, followed by change to control medium for the subsequent 16 hours. (B) Intracellular Mn concentrations measured by ICP-MS in AF5 cells treated as above. Data are mean (±SD, n=3/treatment); data are from a representative experiment performed in triplicate. Letters denote significant differences between experimental groups (p < 0.05), based on Tukey's post hoc test.
Figure 5. Mn exposure in rats reduces brain GPP130 levels \textit{in vivo}. Representative IHC photomicrographs labeled with GPP130 (green) and Draq5 (red) from cortex (S1 dysgranular zone, Bregma 0.48 mm) and dorsal striatum. Mn-exposed animals show an overall decrease in GPP130 levels in the cortex region of the brain compared to control animals. IHC slides were prepared and stained with three brain slices/animal/slide balanced by control and Mn treatment, and photographed at x20 or x63 magnification under defined illumination conditions (see text for details). Scale bar = 50 µm for x20, 10 µm for x63.
Figure 6. Mn exposure in rats reduces brain GPP130 levels *in vivo* primarily through reductions in GPP130-positive cells. Data quantified from fields at (A) x20 magnification and (B) x63 magnification, as follows: Total GPP130 fluorescence staining across all cells identified by the Draq5 staining in the cortex (S1 dysgranular zone, Bregma 0.48 mm) of control and Mn exposed rats; percent of all cells identified that were GPP130-positive (see text for defined positive fluorescence threshold limits); total GPP130 fluorescence of GPP130-positive cells; and total GPP130 fluorescence in cells not identified as GPP130 positive (i.e., below the fluorescence threshold for GPP130-positive identification). Data are mean (±SD) expressed as percent of control group animals (see text for details). Asterisks indicate significantly different from respective control group (* p<0.05; ** p<0.001).
Figure 7. Cellular model for GPP130 in the presence of normal (A) and elevated (B) cellular Mn. (A) GPP130 is localized to the cis-Golgi and participates in the bypass pathway by facilitating endosome to Golgi traffic. (B) Upon elevated Mn exposure, Mn is transported into the Golgi lumen via SPCA1, which causes Golgi-localized GPP130 to traffic to MVBs and then to the lysosome, where it is ultimately degraded. As GPP130 degradation increases, the amount of intracellular Mn decreases as well, suggesting that GPP130 may play a role in Mn homeostasis and susceptibility/resistance to elevated Mn exposure.
Table I. Total number of cells identified via Draq5 staining, and the percent of GPP130-positive cells in cortex (S1 dysgranular zone, Bregma 0.48 mm) and dorsal striatum of Mn-treated rats, assessed at x20 or x63 magnification.

<table>
<thead>
<tr>
<th>Brain Region</th>
<th>Treatment</th>
<th>Average Total # Cells/Field</th>
<th>% GPP Positive Cells</th>
<th>Average</th>
<th>x20</th>
<th>x63</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td>Total # Cells/Field</td>
<td>% GPP Positive Cells</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>x20</td>
<td>x63</td>
<td>x20</td>
<td>x63</td>
<td></td>
</tr>
<tr>
<td>Cortex</td>
<td>Control</td>
<td>602 ± 88</td>
<td>12 ± 2</td>
<td>23 ± 12</td>
<td>34 ± 11</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Mn</td>
<td>609 ± 54</td>
<td>12 ± 3</td>
<td>9 ± 6**</td>
<td>7 ± 7**</td>
<td></td>
</tr>
<tr>
<td>Striatum</td>
<td>Control</td>
<td>646 ± 106</td>
<td>11 ± 2</td>
<td>9 ± 10</td>
<td>17 ± 19</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Mn</td>
<td>672 ± 58</td>
<td>10 ± 2</td>
<td>5 ± 5*</td>
<td>4 ± 8*</td>
<td></td>
</tr>
</tbody>
</table>

a Data are mean ± SD; based on two microscopy fields per brain region (slice), six brain slices per animal, and three animals per treatment. (* p<0.05; ** p<0.001).
b Data are mean ± SD; based on 10 fields per brain region (slice) from one representative brain slice/animal/treatment.
Table II. Total GPP130 fluorescence across all cells identified via Draq5 staining in the dorsal striatum of control and Mn exposed rats, percent of all cells identified that were GPP130-positive (see text for defined positive fluorescence threshold limits), and total GPP130 fluorescence of GPP130-positive cells, all quantified at x63 magnification. Data expressed as percent of control group animals (see text for details).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Total Fluorescence, All Cells(^a)</th>
<th>% of GPP-Positive Cells</th>
<th>Total Fluorescence, GPP-Positive Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>100 ± 42</td>
<td>100 ± 88</td>
<td>100 ± 94</td>
</tr>
<tr>
<td>Mn</td>
<td>53 ± 31**</td>
<td>23 ± 55**</td>
<td>36 ± 69*</td>
</tr>
</tbody>
</table>

\(^a\) Data are mean ± SD, based on 10 fields per brain region (slice) from one representative brain slice/animal/treatment. (* p<0.05; ** p<0.001).
Bibliography


23. Madejczyk and Ballatori, (2012). The iron transporter ferroportin can also function as a manganese exporter. Biochim Biophys Acta.1818: 651-7


