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Author/s:

Venkataramani, V;Doeppner, TR;Willkommen, D;Cahill, CM;Xin, Y;Ye, G;Liu, Y;Southon, A;Aron, A;Au-Yeung, HY;Huang, X;Lahiri, DK;Wang, F;Bush, AI;Wulf, GG;Ströbel, P;Michalke, B;Rogers, JT

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**Manganese causes neurotoxic iron accumulation via translational repression of
Amyloid Precursor Protein (APP) and H-Ferritin**

**Vivek Venkataramani^{1,2*}, Thorsten R. Doeppner³, Desiree Willkommen¹⁰, Catherine M.
Cahill⁶, Yong-juan Xin^{4,5}, Guilin Ye^{4,5}, Yanyan Liu⁶, Adam Southon⁷, Allegra Aron⁹, Ho
Yu Au-Yeung¹¹, Xudong Huang⁶, Debomoy K. Lahiri⁸, Fudi Wang^{4,5}, Ashley I. Bush⁷,
Gerald G. Wulf¹, Philipp Ströbel², Bernhard Michalke¹⁰, Jack T. Rogers⁶**

¹Department of Hematology and Medical Oncology, ²Institute of Pathology, ³Department of
Neurology, University Medical Center Göttingen (UMG), Robert-Koch-Strasse 40, 37075
Göttingen, Germany; ⁴Department of Nutrition, Precision Nutrition Innovation Center, School
of Public Health, Zhengzhou University, Zhengzhou 450001; ⁵Department of Nutrition,
Nutrition Discovery Innovation Center, Institute of Nutrition and Food Safety, School of Public
Health, The First Affiliated Hospital, Zhejiang University School of Medicine, Hangzhou
310058, China; ⁶Neurochemistry Laboratory, Department of Psychiatry, Massachusetts
General Hospital and Harvard Medical School, Charlestown, MA 02129, USA; ⁷Melbourne
Dementia Research Centre, Florey Institute of Neuroscience and Mental Health, University of
Melbourne, 30 Royal Parade, Parkville Vic, Australia 3052; ⁸Department of Psychiatry,
Indiana Alzheimer Disease Center, Stark Neurosciences Research Institute, Indiana
University School of Medicine; ⁹Department of Chemistry, University of California, Berkeley,
CA 94720, USA; ¹⁰Helmholtz Zentrum München, Deutsches Forschungszentrum für
Gesundheit und Umwelt (GmbH), Research Unit Analytical BioGeoChemistry, Ingolstädter
Landstr. 1, 85764 Neuherberg; ¹¹Department of Chemistry, The University of Hong Kong,
Pokfulam Road, Hong Kong, P. R. China

***Corresponding author:**

Address correspondence and reprint requests to Vivek Venkataramani, MD; Department of
Hematology and Oncology and Department of Pathology; University Medical Center

Göttingen (UMG), Robert-Koch-Strasse 40, 37075 Göttingen, Germany; mail:
ramani@med.uni-goettingen.de

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Abbreviations used

amyloid precursor protein (APP)

Deferoxamine mesylate salt (DFO)

Ferritin heavy-chain (H-Ferritin)

Iron responsive protein (IRP)

Iron responsive element (IRE)

Ferric ammonium citrate (FAC)

Manganese (Mn)

Mn superoxide dismutase (MnSOD)

Part per million (p.p.m)

Reactive oxygen species (ROS)

Untranslated region (UTR)

ABSTRACT

For more than 150 years it is known that occupational overexposure of manganese (Mn) causes movement disorders resembling Parkinson's disease (PD) and PD-like syndromes. However, the mechanisms of Mn toxicity are still poorly understood. Here, we demonstrated that Mn dose- and time-dependently blocks the protein translation of amyloid precursor protein (APP) and heavy-chain Ferritin (H-Ferritin), both iron homeostatic proteins with neuroprotective features. APP and H-Ferritin are post-transcriptionally regulated by iron responsive proteins (IRPs), which bind to homologous iron responsive elements (IREs) located in the 5'-untranslated regions (5'-UTRs) within their mRNA transcripts. Mn exposure repressed the 5'-UTR-activity of APP and H-Ferritin, presumably via increased IRP-IRE binding, ultimately blocking their protein translation. Loss of the protective axis of APP and H-Ferritin resulted in unchecked accumulation of redox-active ferrous iron (Fe^{2+}) fueling neurotoxic oxidative stress. Enforced APP expression partially attenuated Mn-induced generation of cellular and lipid reactive oxygen species (ROS) and neurotoxicity. Lastly, we could validate the Mn-mediated suppression of APP and H-Ferritin in two rodent *in vivo* models mimicking acute and chronic Mn exposure. Together, these results suggest that Mn-induced neurotoxicity is partly attributable to the translational inhibition of APP and H-Ferritin resulting in impaired iron metabolism and exacerbated neurotoxic oxidative stress.

INTRODUCTION

The essential trace element and transition metal manganese (Mn) displays an important role as vital co-factor in receptors, transporters and many enzymatic reactions, including the anti-oxidant enzyme Mn-superoxide dismutase (MnSOD). At physiological levels, Mn is required for wound-healing, bone and cartilage formation, mitochondrial function and energy production. Thus, a balanced Mn homeostasis is crucial for cell maintenance and preservation of life (Peres *et al.* 2016, Farina *et al.* 2013).

However, Mn has also been documented as a potent neurotoxic agent for about 150 years (Dobson *et al.* 2004, Farina *et al.* 2013). Excessive or prolonged exposures cause

irreversible damage to the nervous system, in particular to the iron-rich basal ganglia resulting in the development of “*manganism*”, a neurological disorder with symptoms such as bradykinesia and rigidity resembling Parkinson’s disease (PD) and PD-like disturbances (Pal *et al.* 1999, Chia *et al.* 1993, Guilarte 2013, Gorell *et al.* 1997). Furthermore, the PD-linked gene ATP13A2 (also known as PARK9) is strikingly implicated in neuronal detoxification of Mn as well as suppressing α -synuclein toxicity (Gitler *et al.* 2009, Tan *et al.* 2011) and newly identified loss-of-function mutations in the Mn efflux transporter gene SLC30A10 are linked to PD-like symptoms (Quadri *et al.* 2012, Tuschl *et al.* 2012), altogether underscoring the biological complexity of these multifactorial neurodegenerative disorders.

A body of evidence suggests that Mn-driven neurotoxicity is associated with altered iron metabolism, both systemically and at the cellular level due to similar physiochemical properties and shared absorptive pathways, such as the divalent metal transporter (DMT1) that competitively regulates the uptake Mn and iron (Chua & Morgan 1996, Kwik-Urbe *et al.* 2003, Gunshin *et al.* 1997). Despite these similarities there are also some essential differences between both transition metals. Common oxidation states of Mn are 2+, 3+, 4+, 6+ and 7+, with Mn^{2+} (later referred to as Mn) being the most stable oxidation state (Farina *et al.* 2013). Within biological systems, iron occurs in one of two oxidation states known as ferrous (Fe^{2+}) and ferric iron (Fe^{3+}). While Fe^{3+} is redox-inactive, Fe^{2+} is an intrinsic generator of reactive oxygen species (ROS) by catalyzing the decomposition of H_2O_2 producing highly toxic hydroxyl radicals and membrane lipidperoxidation via Haber-Weiss and Fenton reactions (Kehrer 2000, Gaschler & Stockwell 2017). Both, overproduction of cellular ROS and peroxidized phospholipids corrupt the integrity of proteins, lipids and DNA which can result in detrimental consequences on core cellular functions (Sies 2015) and even trigger a programmed necrotic cell death, known as “ferroptosis” (Dixon *et al.* 2012, Stockwell *et al.* 2017). In contrast, Mn is not capable to generate such hydroxyl radicals via Haber-Weiss or Fenton reaction like Fe^{2+} (Archibald & Tyree 1987). However, it was also reported that Mn-catalyzed auto-oxidation of dopamine involves redox-cycling of Mn^{3+} and Mn^{2+} in a reaction that can result in ROS and dopamine-o-quinone generation, both fueling oxidative stress

(Segura-Aguilar & Lind 1989). We and others previously demonstrated that Mn exposure results in a shift of $\text{Fe}^{2+}/\text{Fe}^{3+}$ ratio towards redox active Fe^{2+} accompanied with depleted levels of the antioxidant glutathione and increased oxidative stress markers *in vitro* and *in vivo* (Fernsebner *et al.* 2014, Kwik-Urbe *et al.* 2003, Neth *et al.* 2015). However the molecular basis of Mn-induced iron-shift and redox-alteration remains to be elucidated.

To avoid cellular accumulation of free toxic Fe^{2+} , iron metabolism requires the tight coordination of a wide variety of genes that are primarily controlled through post-transcriptional regulatory mechanisms, including the iron storage protein ferritin (light-chain (L-Ferritin) and the heavy-chain type (H-Ferritin)), iron importer transferrin receptor 1 (TFR1) and iron exporter ferroportin (FPN) (Bogdan *et al.* 2016). All such iron homeostasis proteins contain iron-responsive elements (IRE) on their respective mRNAs located within the 5'- or 3'-untranslated regions (UTRs) flanking their coding sequences. Binding of iron regulatory proteins (IRP1 and IRP2) to IRE-sequences have opposite effects on target mRNA expression (Anderson *et al.* 2012, Hentze *et al.* 2010): While IRP binding to 3'-UTR IREs enhances mRNA stability that results in increased protein expression, IRP binding to 5'-UTR IREs blocks the protein translation of iron-responsive target genes. In this context, we previously identified a fully functional IRE-like RNA stem loop in the 5'-UTR of the amyloid precursor protein (APP) (Rogers *et al.* 2002). Consistently, we showed that genetic removal of IRP1 facilitates translational activation of APP mRNA ultimately increasing its protein expression (Cho *et al.* 2010).

APP is a ubiquitously expressed type-I-transmembrane protein that is known to contain copper and zinc-binding domains (Bayer *et al.* 1999). Pathological APP processing by β - and γ -secretases generates neurotoxic $\text{A}\beta$ -peptides which is believed to be the key event in the pathogenic cascade in Alzheimer's disease (AD) (Selkoe 2001). However, under physiological conditions, APP is predominantly processed in a non-amyloidogenic manner via α -secretase that precludes $\text{A}\beta$ production and releases the large N-terminal fragment $\text{sAPP}\alpha$ (Sisodia 1992). Compared to the neurotoxic properties of $\text{A}\beta$ peptides (Haass & Selkoe 2007), $\text{sAPP}\alpha$ possesses cell protective and growth-promoting features that have

been validated both *in vitro* and *in vivo* , including neuronal and multiple cancer cell lines as well as several animal models (Venkataramani *et al.* 2010, Venkataramani *et al.* 2012, Zheng & Koo 2011, Müller *et al.* 2017). Intriguingly, we and others recently elucidated that APP and sAPP α indirectly facilitate iron efflux via stabilizing the key Fe²⁺-exporter FPN (Duce *et al.* 2010, Wong *et al.* 2014, McCarthy *et al.* 2014). In line, we showed in APP^{-/-} mice a significant accumulation of total and labile iron pool accompanied with a marked induction of oxidative stress levels in their brain and other organs compared to wild-type mice (Duce *et al.* 2010, Ayton *et al.* 2015).

Given the role and functions of APP and H-Ferritin in orchestrating iron homeostasis and responses, we here investigated if Mn interferes with the translational control of both proteins resulting in embargoed iron efflux and depleted iron storage in neuronal-like SH-SY5Y cells and in two different rodent *in vivo* models.

MATERIALS AND METHODS

Reagents and antibodies:

Manganese-(II)-chloride hydrate and Manganese-(II)-acetate (both referred as Mn), ferric ammonium citrate (FAC), desferrioxamine mesylate salt (DFO) and 3-Hydroxy-1,2-dimethyl-4(1H)-pyridone (Deferiprone, DEF) were purchased from Sigma-Aldrich (St. Louis, MO). Calcein-AM (calcein-acetoxymethyl ester, C3100MP) was purchased from Thermo Fisher Scientific. Stocks of FAC (10 mM), Mn (100 mM), DFO (10 mM) and DEF (100 mM) were prepared in DMEM and purified water, respectively. The following antibodies were used: IRP1 (ab126595, abcam), IRP2 (PA1-16544, Thermo Fisher Scientific), β -actin (C-11, Sigma-Aldrich and AC-15, abcam), anti-FTH1 (H-Ferritin) antibodies (D1D4, Cell Signaling and ab137758, abcam), LC3B (D11) and Beclin-1 (D40C5) (both Cell signaling). For APP detection we used the c-terminal APP antibody (A8717; Sigma-Aldrich) and the N-terminal APP antibody (22C11; Millipore).

Cell culture and transfection:

The human neuroblastoma cell line SH-SY5Y was initially purchased from ATCC. We have confirmed in this cell line the heterozygous ALK F1174L mutation by Sanger sequencing. Stably expressing cell lines overexpressing human wildtype APP₆₉₅ isoform (SH-SY5Y APP₆₉₅) or empty vector (pCEP4) were previously established (Venkataramani et al. 2010). The stable SH-SY5Y cell line transfected with the APP 5'-UTR luciferase or HIRE_CAT constructs were described previously (Rogers et al. 2016, Thomson et al. 2005). Immortalized APP^{-/-} MEFs and corresponding wild-type MEFs (APP^{+/+}) were provided by Ulrike Müller (University Heidelberg) and described previously (Li et al. 1996). All cell lines were cultured in DMEM (Dulbecco's modified Eagle's medium) supplemented with 10% (v/v) fetal calf serum, 1% L-glutamine and 1% penicillin/streptomycin. Complete medium were changed every 2-3 days.

Protein extracts and Western blot analyses:

Cells at 60-70% confluency were treated with indicated conditions and time points. After subsequent washing in PBS, cells were scraped into modified radioimmunoprecipitation assay (RIPA) lysis buffer on ice for 30 min with gentle agitation. Cell lysates were centrifuged at 10,000 x g for 10 min. Mice and rat whole brains were collected in cold PBS on ice. For all mice experiments cortex tissue (frontal cortex) were isolated, fully homogenized in modified RIPA buffer. For all shown rat experiments, whole brain tissues were lysed in ice cold extraction buffer (10 mM Tris-HCl, pH 7.4, previously purged with helium for 3 h) as previously published (Fernsebner et al. 2014). Total protein concentrations were analyzed using Bio-Rad protein assay. Unless stated otherwise, cell extracts (15-20 µg/well) were separated by SDS-PAGE at 100V constant using Mini-PROTEAN TGX precast 4-15% gradient gels (Bio-Rad, Germany) and transferred using the Bio-Rad nitrocellulose and Trans-Blot Turbo System. Unless stated otherwise, western blots from three independent experiments were quantified using ImageJ software (version 1.41o, National Institutes of Health). Images were captures using a CCD camera. To enhance visibility of bands, images

were equally adjusted for contrast in Adobe Photoshop. If comparisons are to be made between images, the blots were taken with identical conditions and manipulated equally.

Quantitative Real-Time-PCR analysis:

Total RNA was isolated using TRI-reagent (Sigma-Aldrich) according to the manufacturer's instructions. Assays were performed using an ABI Prism 7000 system (Applied Biosystems, Foster City, CA). APP primers (forward, 5-GCCCTGCGGAATTGACAAG-3; reverse, 5-CCATCTGCATAGTCTGTGTCTG-3), ferroportin primers (forward, 5-CTACTTGGGGAGATCGGATGT-3; reverse, 5-CTGGGCCCACTTTAAGTCTAGC-3), ferritin primers (forward, 5-CCCCCATTTGTGTGACTTCAT-3; reverse, 5-GCCCGAGGCTTAGCTTTCATT-3); transferrin receptor primers (forward, 5-GGCTACTTGGGCTATTGTAAAGG-3; reverse, 5-CAGTTTCTCCGACAACTTTCTCT-3); and β -actin primers (forward, 5-CATGTACGTTGCTATCCAGGC-3; reverse, 5-CTCCTTAATGTACGCACGAT-3) were purchased from Life Technology.

Quantification of luciferase (luc) and chloramphenicol acetyltransferase (CAT) activity:

Firefly Luciferase Assay Kit (Hayward, CA) was used according to manufacturer's directions to measure the luciferase activity. Reporter assays for balanced luciferase assays using the transfectants encoding the APP 5'-UTR luciferase (luc) reporter construct pGAL were as described previously (Rogers et al. 2016). The activity of the Ferritin-H chain specific iron-responsive element (H-Ferritin IRE) was assessed using the plasmid construct, designated as HIRE_CAT, in which the H-Ferritin IRE had been ligated in front of a chloramphenicol acetyltransferase (CAT) reporter gene. CAT enzyme-linked immunosorbent assays were performed according to manufacturer's instructions (Roche Applied Science) as previously described (Thomson et al. 2005).

Cell Viability and cell number quantification:

Cell viability was measured by a colorimetric assay either by using the MTT (thiazolyl blue tetrazolium, Sigma-Aldrich) viability assay or the MTS-Assay (CellTiter96 AQassay, Promega) according to the protocol of the supplier (Venkataramani et al. 2010). Cell viability data are presented as relative changes in % compared to untreated controls. In parallel, cells were grown in 12-well plates for cell number analysis. Repetitive aliquots were taken and counted using a hemocytometer with trypan blue exclusion assay. For each treatment condition, triplicate wells were counted, and values were averaged. Data were presented as relative values in % compared to untreated controls.

Quantification of the labile iron pool (LIP):

Increasing Mn concentrations were incubated with SH-SY5Y cells or complete DMEM media alone using isoplate 96 well plates (Perkin Elmer). Cells or media alone were loaded with calcein-AM at a final concentration of 0.25 μ M. Fluorescence (Excitation/Emission: 485/520 nm) was assessed using the FlexStation3 multi-mode microplate reader (Molecular Devices). Background fluorescence was subtracted and data was expressed as mean $\pm$ SEM from 6 replicates.

Fe²⁺-specific probes Rho-Nox1 and IP-1 were prepared by Allegra Aron, Ho Yu Au-Yeung and Christopher J. Chang (Department of Chemistry, University of California, Berkeley) and freshly dissolved in DMSO at a stock concentration of 2 mM (Niwa *et al.* 2014, Au-Yeung *et al.* 2013). Cells were incubated with DMEM media alone or increasing Mn doses for 24 h, washed with PBS and loaded with 5 μ M RhoNox-1 dissolved in OptiMEM media for 60 min. After incubation, cells were washed once with PBS. As positive control, cells treated for 24 h with Mn were washed with PBS and incubated with 100 μ M DEF for 1 h and washed again with PBS. Fluorescence (Excitation/Emission: 540/575 nm) was analyzed using a TECAN reader. In a similar fashion, cells were incubated with a 20 μ M final concentration of IP-1 dissolved in OptiMEM media for 2 h. Cells were washed with PBS and fluorescence (Excitation/Emission: 488/550 nm) was analyzed using a TECAN reader.

Quantification of Fe²⁺, Fe³⁺ and Mn²⁺ using IC-ICP-sf-MS and CE-ICP-qMS analysis:

Speciation and quantification of Fe²⁺, Fe³⁺ and Mn²⁺ was performed by IC-ICP-MS as described previously (Fernsebner et al. 2014, Solovyev et al. 2017) and in addition with CE-ICP-MS. In short terms the IC-ICP-MS methods used a Thermo- Dionex IonPac™ CS5A RFIC 4*250 mm column and a guard column IonPac™ CG5A column hyphenated to high-resolution sectorfield ICP-sf-MS (Element II, Thermo Scientific). Detailed experimental conditions were given in (Solovyev et al. 2017). For cross-validation a coupling of capillary electrophoresis (PrinCe 760, Prince Technologies) with ICP-qMS (Nexlon 360 M, Perkin Elmer) was employed. The CE-ICP-MS method operated in positive electrophoretic mode at acidic pH (background electrolyte: 20 mM HCl, +25 kV). Monitored isotopes were ⁵⁵Mn, ⁵⁶Fe and ⁵⁷Fe, using DRC cell technology with ammonia as DRC gas in accordance to (Quintana et al. 2006) for removing of interferences at respective isotopes.

Determination of cytoplasmic and lipid reactive oxygen species (ROS):

For detection of cytoplasmic ROS, cells were seeded in 6-well plates at a density of 1x10⁵ cells in full media. After 24 h Mn treatment, cells were washed twice in PBS and subsequently incubated with a 25 µM working solution of H2DCFDA (Molecular Probes, Stock 20 mM dissolved in methanol) for 10 min light-protected at 37°C. After brief washing with PBS, fluorescence was analyzed via FACS (FL-1 channel). For detection of lipid peroxidation (Lipid-ROS), cells were incubated with a 10 µM working solution of BODIPY 581/591 C11 (Molecular Probes, Stock 2 mM in DMSO) and incubated for 45 min light-protected at 37°C. Lipid-ROS was analyzed via flow cytometry (excitation at 488 nm and emission at 530 (FL-1)) and (excitation at 488 nm and emission at 585 nm (FL-2)). Data were analyzed by FlowJo software and presented as relative induced ROS compared to untreated controls. For Lipid-ROS the fluorescence ratio FL1/FL2 reflecting the oxidation of the probe were presented either in arbitrary units or as relative induced lipid ROS compared to untreated controls.

Rodent experiments and treatments:

All mice were housed in a specific pathogen-free facility and maintained on a purified AIN-76A diet (Research Diets, New Brunswick, NJ, USA). All mice were maintained under a 12-hour light/dark cycle, controlled temperature ($22 \pm 2^{\circ}\text{C}$) and humidity ($65 \pm 5\%$). Eight weeks male mice with C57BL6/J background were housed in cages for 5 days before the experiment. The mice (20-25g) were randomly divided into four groups: Mice were either feed with standard diet (control group: Mn 10 p.p.m, diet D08080401) or high Mn diet (Mn group: 2400 p.p.m. diet D17020702) for 91 days. Furthermore, mice were intraperitoneally (i.p.) injected with either PBS (control group) or MnCl_2 (Mn group: 40 mg/kg body weight/day, Sigma) for 7 days. At the end of each experiment, all mice were sacrificed by anesthesia with chloral hydrate. All mice experiments were approved by the Institutional Animal Care and Use Committee of Zhengzhou University.

All showed rat experiments were previously published by Fernsebner et al. (2014). In brief, 12 male Sprague-Dawley rats (RjHan:SD) were purchased from Janvier (Janvier S.A.S., France). For acclimatization, rats were maintained in standard cages with air filter hoods and free access to food and water. Subsequently, animals were divided in two groups: control group (n=6) received a standard diet with 23 mg/kg fodder without Mn whereas the treated group received Mn-enriched fodder (500mg/kg body weight). The whole experiment lasted 53 days, thereafter euthanasia was performed by perforating the aorta abdominals suprarenalis after deep narcotization. The brains were snap frozen immediately in liquid nitrogen until sample preparation and stored at -80°C . Exactly the same animal brains were used for western blot experiments in this current study. Levels of redox-active Fe^{2+} and Mn from each brain were previously published by Fernsebner et al. (2014) using ion chromatography hyphenated to ICP-OES (IC-ICP-OES). All performed rat experiments were in accordance with the institutional Animal Welfare Committee as well as approved by the Bavarian federal state government under the file number 55.2-1-54-2531-180-12.

Statistical analysis:

Differences between treatment groups were either evaluated using unpaired t-test, one- or two-way ANOVA followed by Bonferroni post-hoc analysis. The correlation between Fe²⁺/total iron ratio and relative APP protein expression was analyzed by Spearman correlation and linear regression methods. All statistical analyses were performed using GraphPad Prism Software (GraphPad Software Inc.).

RESULTS

Manganese reduced cell viability in a delayed manner.

The dopaminergic neuroblastoma cell line SH-SY5Y was exposed to increasing concentrations of Mn for up to 48 h. Cell viability analysis after 24 h incubation revealed an IC₅₀ of ~175 µM, while prolonged treatment after 48 h showed a more pronounced decrease in cellular viability with an IC₅₀ of less than ~10 µM Mn. When administering 100 µM of Mn for 24 h, we detected in SH-SY5Y cells a viability drop of 36.6 ± 3.5% for 24 h, and 82.3 ± 6.8 % for 48 h that even further decreased to 89.0 ± 2.5% for 72 h (data not shown) (**Fig. 1a**). In a similar fashion, cell counting data via hemocytometer revealed that cells stopped growing, closely reflecting cell viability measurements (**Fig. 1b**).

Manganese acutely reduced protein levels of APP and H-Ferritin, known as neuroprotective iron homeostatic gene products.

We previously reported that the xenobiotic metal lead caused a translational repression of APP and H-Ferritin in SH-SY5Y cells (Rogers et al. 2016). Based on this, we evaluated Mn-dose responsive effects in the range of 10-100 µM and analyzed protein expression of both iron homeostatic gene products APP and H-Ferritin. Indeed, Mn led to a significant and abrupt decreased expression of both proteins after 24 h exposure (**Fig. 1c**). At 100 µM of Mn, where cell viability was decreased by only ~37%, we observed a marked reduction of H-Ferritin (~92.3 ± 10.9%) and APP levels (~82.6 ± 10.8 %) compared to untreated controls, supporting that the effects of Mn on these proteins was not an artifact of

immediate cell toxicity. Diametrically opposite, FAC treatment strongly increased the protein expression of APP and H-Ferritin, underlining the iron-responsive nature of both proteins (Cho et al. 2010, Rogers et al. 2002) (**Fig. 1d**). In agreement, time course experiment revealed that Mn limited steady state APP and H-Ferritin protein levels beginning at 6 h after metal-exposure in a directly opposing pattern to the inductive response of after FAC exposure (**Fig. 2a,b**).

Manganese-dependent transcriptional response of APP and H-Ferritin does not reflect protein changes.

To determine if differential regulation of iron homeostasis genes occurs at the transcriptional level, we performed quantitative real-time PCR (qRT-PCR) targeting the mRNA levels of APP, H-Ferritin, transferrin receptor 1 (TFR1) and ferroportin (FPN1). Interestingly, 24 h treatment with Mn resulted in a more than 3-fold increase of APP mRNA, while mRNA levels of H-Ferritin remained nearly unaltered. Moreover, Mn caused a strong induction of TFR1 mRNA without affecting FPN1 mRNA levels. In contrast, FAC administration did not result in marked changes of any mRNA levels (**Fig. 2c**). As a positive control, we treated SH-SY5Y cells with the iron chelator deferoxamine (DFO), resulting in a more than eight-fold increment of TFR1 mRNA levels and a suppression of FPN mRNA levels underlining a cellular iron-deficient state (**Supplemental Fig. 1**).

These results demonstrated a clear discrepancy between protein and mRNA after Mn and FAC exposure, suggesting the involvement of post-transcriptional gene regulatory mechanisms of APP and H-Ferritin.

Manganese post-transcriptionally blocks 5'-UTR-mediated translation of APP and H-Ferritin

We first conducted alignments of the equivalent 5'-untranslated regions (5'-UTRs) in the transcripts for the human, mouse and rat APP transcripts, the Ferritin-L and -H chains mRNAs and also the putative 5'-UTR specific iron specific element (IRE) in the human FPN

mRNA (**Supplemental Fig. 2**). Here we could confirmed that there is a IRE stem loop in the 5'-UTR of the APP mRNA, which is similar to the canonical IRE loops found in H- and L-chain Ferritin (Rogers et al. 2002, Thomson et al. 2005). Application of NCBI align showed that these 5'-UTR structures align in such a way as to generate characteristic AGA or AGU tri-loops that can bind to the iron regulatory proteins (IRP1 and IRP2) (Cho et al. 2010, Goforth et al. 2010, Ke et al. 1998).

Based on this finding, we next used SH-SY5Y cells expressing a luciferase (luc) reporter fused to the 146-nucleotide 5'-UTR sequence of the APP mRNA (APP 5'-UTR-luc) that we previously established to discover APP 5'-UTR-directed compounds (Bandyopadhyay et al. 2006) and iron responsiveness (Rogers et al. 2002). Compared to pGL3-luc empty plasmid, 50 μ M FAC resulted in a significant increase of APP 5'-UTR-luc activity. By contrast, Mn treatment suppressed APP 5'-UTR specific activity by >50% at 50 μ M, while not significantly affecting pGL3-luc activity (**Fig. 3a**). In a second series of experiments we utilized a H-Ferritin IRE-CAT reporter system to monitor the 5'-UTR activity of H-Ferritin mRNA. In a similar fashion, 24 h FAC treatment significantly induced CAT reporter activity, while Mn exposure resulted in a significant suppression of H-Ferritin 5'-UTR activity (**Fig. 3b**). Since, IRPs are central post-transcriptional regulators that bind to the 5'-UTR-IRE regions of both proteins blocking their ribosomal protein translation, we observed that Mn promoted the proteolysis of IRP2 while leaving IRP1 protein levels unchanged (**Fig. 3c**).

In conclusion, our data show that Mn exposure left IRP1 unchanged and presumably still bound to the 5'-UTR-IREs, providing an explanation for suppressed 5'-UTR activities of APP and H-Ferritin and subsequent blockage of their endogenous protein translation.

Manganese increases intracellular labile iron pool (LIP) and oxidative stress levels.

APP and H-Ferritin can be readily modeled to play key roles in export and storage of excess toxic redox active ferrous iron (Fe^{2+}). Therefore, we asked if Mn treatment causes changes in Fe^{2+} levels that represent the cellular labile iron pool (LIP). In our first attempt, we

used the established calcein-AM assay that has been benchmarked in many reports as convenient assay to measure levels of cytosolic redox-active Fe^{2+} in the labile iron pool (LIP). Calcein is a fluorochrome that chelates free Fe^{2+} under quenching its green calcein fluorescence (Tenopoulou *et al.* 2007). Mn exposure to SH-SY5Y cells resulted in a concentration- and time-dependent decrease of calcein fluorescence. Surprisingly, cell-free analysis using DMEM media with increasing Mn doses resulted even in a stronger concentration-dependent shutdown of calcein fluorescence indicating the limitation of this assay to specifically determine LIP alterations (**Supplemental Fig. 3a**). We therefore utilized two first-generation activity-based “turn-on” probes that are capable to selectively detect Fe^{2+} over other biologically relevant metals including Mn (Aron *et al.* 2018). RhoNox-1 interacts with Fe^{2+} resulting in the release of fluorescent rhodamine and was recently reported to selectively detect endogenous changes in LIP of lung carcinoma cells (Adachi *et al.* 2016, Niwa *et al.* 2014), while IP-1 relies on an oxygen-dependent, three-component sensing mechanism to detect Fe^{2+} (Au-Yeung *et al.* 2013). Indeed, we could detect a significant concentration-dependent increase in RhoNox-1 and IP-1 fluorescence in SH-SY5Y cells (**Fig. 4 a**), while no significant changes could be detected with both dyes in a cell-free environment (**Supplemental Fig. 3b**). Moreover, subsequent 1-h incubation with 100 μM of the iron chelator deferiprone (DEF) completely rescued RhoNox-1 and IP-1 fluorescence, confirming Mn-mediated intracellular Fe^{2+} accumulation in SH-SY5Y cells (**Fig. 4 a**). We finally validated the Mn-induced shift in $\text{Fe}^{2+}/\text{Fe}^{3+}$ ratio unequivocally reflecting increased LIP using IC-ICP-MS and CE-ICP-MS that can clearly distinguish and quantify iron in both redox-states (**Fig. 4b and Supplemental Fig. 3c**).

Based on this finding, we next evaluated for Mn-mediated induction of oxidative stress and used DCF fluorescence as a measure of cellular ROS and BODIPY 581/591 C11 dye to assess lipidperoxidation (lipid ROS) induction. In line with a disturbed redox homeostasis, Mn significantly increased cellular and lipid-ROS levels in a concentration and time-dependent fashion (**Fig. 4c-f**).

Our results confirmed previous observations by demonstrating that Mn exposure

alters the $\text{Fe}^{3+}/\text{Fe}^{2+}$ towards redox-active Fe^{2+} enabling cytoplasmic and lipid ROS generation (Fernsebner et al. 2014, Neth et al. 2015, Kwik-Urbe et al. 2003).

Forced expression of APP confers cellular resistance to Mn-induced toxicity.

Since Mn represses endogenous APP translation, we next asked if forced expression of APP driven by a CMV (cytomegalovirus) promoter can modify Mn-induced neurotoxicity. We therefore utilized SH-SY5Y cells stably overexpressing the neuronal APP₆₉₅-isoform around 20 fold-higher compared to endogenous APP levels of empty vector (pCEP4) transfected controls (Venkataramani et al. 2010). In line with the iron-exporting function of APP via FPN stabilization (Duce et al. 2010, Wong et al. 2014, McCarthy et al. 2014), SH-SY5Y APP₆₉₅ cells presented markedly suppressed basal levels of H-Ferritin, cellular and lipid ROS compared to controls (**Fig. 5a,c**). As expected, Mn reduced endogenous APP levels in mock-transfected cells while not affecting APP expression level in constitutively overexpressing APP₆₉₅ cells. However, only 50 μM Mn resulted in a slight decrease of H-Ferritin levels in APP₆₉₅ cells, while a similar treatment caused a complete depletion of H-Ferritin expression in mock-transfected controls (**Fig. 5a**). Consistently, Mn-induced generation of cytoplasmic and lipid ROS could be dramatically attenuated in APP₆₉₅ overexpressing cells compared to control cells (**Fig. 5b,c**). Moreover, forced expression of APP₆₉₅ in SH-SY5Y cells partially promoted cell survival against dose-responsive Mn-toxicity relative to Mock-transfected counterparts (**Fig. 5d**). Vice versa, APP-wild type (APP^{+/+}) mouse embryonic fibroblasts (MEFs) exhibited a greater resistance to Mn toxicity compared to APP^{-/-} MEFs. Notably, FAC pre-treatment resulted in a significant protection from Mn toxicity in APP^{+/+} MEFs, while in Mn-sensitive APP^{-/-} MEFs FAC presented no protective effect (**Supplemental Fig. 4**).

In conclusion, forced APP expression abrogated Mn-induced oxidative stress and cell toxicity. Moreover, FAC treatment in MEFs could partially reverse Mn toxicity in an APP-dependent manner.

Mn treatment reduces brain cortex APP and H-Ferritin levels two *in vivo* models.

To demonstrate the *in vivo* relevance of Mn-mediated APP and H-Ferritin suppression we fed male C57BL/6J mice with either a normal (10 p.p.m Mn) or high Mn diet (2400 p.p.m Mn). This oral Mn dose was previously demonstrated not to induce toxicity in mice (Sato *et al.* 1996). After 91 days of chronic Mn exposure we observed a significant reduction of APP and H-Ferritin expression in brain tissue lysates of high Mn fed mice compared to standard dietary fed controls (**Fig. 6a,b**). To evaluate the effects of acute Mn exposure, we intraperitoneally (i.p.) administered male C57BL/6J mice with either Mn (40 mg/kg body weight/day) or PBS (control) for 7 days. Consistently, we observed suppressed levels of APP and H-Ferritin protein expression in Mn-treated mice compared to controls (**Fig. 6 c,d**). We validated these *in vivo* findings in brain lysates that we obtained from a previous published Mn study from Fernsebner *et al.* (2014). In this study, male Sprague-Dawley rats were either orally challenged with Mn (500mg/kg body weight) or standard diet (control) for 53 days. Each brain lysate was previously analyzed by ion chromatography hyphenated to ICP-OES (IC-ICP-OES) to separately quantify Fe^{2+} and Fe^{3+} amounts (Fernsebner *et al.* 2014). Re-evaluation of these total brain extracts clearly demonstrated that Mn treatment resulted in a marked suppression of APP and H-Ferritin protein levels compared to control treated rats (**Fig. 6e,f**). In agreement with our *in vitro* findings, we observed in Mn treated rat brains reduced protein levels of IPR2, while IRP1 expression levels were unaltered (**Supplemental Fig. 5 a,b**). Together with the obtained concentrations of Fe^{2+} , Fe^{3+} and Mn^{2+} concentrations from Fernsebner *et al.* and the calculated ratio of APP/ β -actin from all rat brain extracts we performed Spearman correlation and linear regression analyses (Fernsebner *et al.* 2014). We observed a significant negative correlation between the Fe^{2+} /total iron ratio and relative APP protein levels (**Fig. 6g**). Interestingly, we could not observe a significant relationship between Mn levels and APP protein expression (**Fig. 6h**).

In conclusion, we could validate our *in vitro* data in two independent *in vivo* rodent models. Chronic and acute administration of Mn clearly resulted in suppressed protein expression of APP and H-Ferritin compared to respective controls. Moreover, we could

demonstrate a significant inverse correlation between redox-active Fe^{2+} and APP protein levels underlining the previously reported iron-regulatory function of APP via stabilizing the mammalian iron exporter FPN (Duce et al. 2010, Wong et al. 2014, McCarthy et al. 2014).

DISCUSSION

Chronic environmental exposure to Mn results in its excessive accumulation in iron-rich brain regions such as the basal ganglia resulting in dystonic neurodegenerative movement disorders that resembles features of PD and PD-like syndromes (Pal et al. 1999, Chia et al. 1993, Guilarte 2013, Gorell et al. 1997). However, the primary mechanism(s) that drives and propagates Mn-induced neurotoxicity remains to be established.

Our study demonstrates for the first time that Mn simultaneously suppresses protein expression of APP and H-Ferritin *in vitro* and two independent *in vivo* settings. Mechanistically, the repression of APP and H-Ferritin is in part mediated post-transcriptionally via blocking the conserved 5'-UTR-dependent regions of their mRNA transcripts. We propose that Mn-induced translational block is achieved by an increased binding of IRP1 to the IRE sequences within the 5'-UTR of APP and H-Ferritin (**see schematic model in Figure 7**). Indeed, under iron-depleted conditions when aconitase loses its labile Fe^{2+} and thus converts to IRP1, the coordination chemistry of Mn closely resembles that of Fe^{2+} , possibly allowing Mn to interact with Fe^{2+} in aconitase/IRP1, thus altering its correct function (Zheng et al. 1998). Moreover recently, comparative analysis of kinetic and thermodynamic properties of IRP1 showed that Mn increases 12-fold the IRE-mRNA/IRP1 binding, while Fe^{2+} decreases the interaction between IRP1 and IRE sequences (Khan et al. 2017). As part of a physiological reaction, iron treatment (in the form of FAC) is tightly regulated to avoid free generation of toxic Fe^{2+} . Iron exposure results in the release of IRPs from IRE-mRNAs enhancing the coordinated translation of iron-related genes (Bogdan et al. 2016). Consistently, we observed that FAC treatment in neuronal cells resulted in a concentration and time-dependent induction of the iron storage protein H-Ferritin and APP that facilitates the stability of membrane-bound FPN, each capable in reducing the amount of

intracellular toxic redox-active Fe^{2+} (Duce et al. 2010, Wong et al. 2014, McCarthy et al. 2014). *Vice versa*, we could demonstrate that Mn-mediated suppression of APP and H-Ferritin resulted in an increment of free Fe^{2+} accelerating toxic cytoplasmic and lipid ROS accumulation (**Figure 7**). Recently, Tai et al. demonstrated that low millimolar doses of Mn resulted in increased autophagic ferritin degradation also known as ferritinophagy (Tai et al. 2016). In this regard, we tested alteration of autophagy markers LC3B and Beclin-1 and observed no marked induction of both autophagy markers in Mn-treated rat brains compared to controls, thus excluding the significant involvement of autophagic ferritin degradation in our *in vivo* setting (**Supplemental Fig. 5 b,c**).

We furthermore demonstrated that forced expression of APP suppressed Mn-mediated toxic cytoplasmic ROS and lipidperoxidation generation and even partially rescued cellular toxicity. Moreover, FAC pretreatment could partially rescue increased Mn toxicity in wildtype $\text{APP}^{+/+}$ MEFs, while a similar FAC pretreatment presented no such significant protective effects altogether underlining the integral involvement of APP as neuroprotective and iron homeostatic gene product capable in attenuating Mn toxicity.

In summary, Mn altered IRP1/IRE-binding affinity coordinate the translational suppression of neuroprotective APP and H-Ferritin that in turn leads to a profound increase of redox-active iron, providing an extended explanation of Mn-induced shift in $\text{Fe}^{2+}/\text{Fe}^{3+}$ ratio and neurotoxic oxidative stress accumulation that we and others previously observed *in vitro* and *in vivo* (Fernsebner et al. 2014, Kwik-Urbe et al. 2003, Neth et al. 2015).

Acknowledgments and conflict of interest disclosure

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685

FIGURE LEGENDS

Figure 1. Opposing effects of Mn and FAC exposure to cell viability and protein expression of APP and H-Ferritin in human neuroblastoma cells. Human SH-SY5Y cells were incubated with increasing Mn concentrations for 24 h and 48 h and analyzed for **(a)** cell viability (n=2) and **(b)** cell number (24 h, n=6; 48 h, n=4). **(c)** Cell lysates of control and Mn treated SH-SY5Y cells were separated on 4-12% Bis-Tris gels. Western blot and densitometric quantification of APP (clone A8717) and H-Ferritin (clone D1D4) (n=3). **(d)** SH-SY5Y cells treated with increasing Mn FAC concentrations for 24 h, immunoblotted for APP (clone A8717) and H-Ferritin (clone D1D4). Densitometric quantification of both proteins were adjusted to loading control β -actin (n=3). Data shown represent mean $\pm$ SD. Differences were calculated using either one-way ANOVA followed by Bonferroni post hoc analyses **(a, b)** or unpaired t-test **(c, d)** (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$).

Figure 2. Acute exposures of Mn and FAC differentially impacts protein and mRNA levels of APP and H-Ferritin. **(a)** SH-SY5Y cells treated with 100 μ M Mn for indicated time points and immunoblotted for APP (clone 22C11) and H-Ferritin (clone D1D4). All densitometric analyses were standardized to corresponding β -actin levels (n=3). **(b)** SH-SY5Y cells were incubated with 100 μ M FAC for indicated time points and immunoblotted for APP (clone 22C11) and H-Ferritin (clone D1D4). All densitometric analyses were standardized to corresponding β -actin levels (n=3). **(c)** RT-PCR analysis of indicated iron responsive target genes (APP, H-Ferritin, TFR1 and FPN) from SH-SY5Y cells incubated for 24 h with 100 μ M FAC or 100 μ M Mn. All data are normalized to housekeeping gene β -actin and presented as relative fold-induction compared to untreated controls (n=4). RT-PCR analysis of DFO-treated SH-SY5Y cells were shown in **Supplemental Fig 1**. Data shown represent mean $\pm$ SD. Differences were calculated using unpaired t-test (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$).

Figure 3: The effect of Mn and FAC on IRE/IRP-dependent expression of APP and H-

Ferritin. (a) SH-SY5Y cells transfected with the pGL3 construct expressing the 146-nucleotide APP 5'UTR sequence (APP 5'-UTR) or empty plasmid (pGL3) were left untreated (Ctrl) or exposed to either 50 μ M Mn (Ctrl n=7; Mn n=8) or 50 μ M FAC (Ctrl n=8; FAC n=4) for 24 h and analyzed for luciferase activity. Data are depicted as relative luciferase activity compared to untreated controls. **(b)** In a similar fashion, SH-SY5Y cells were transfected with the HIRE-CAT construct (H-Ferritin 5'-UTR) and treated with either 50 μ M Mn or 50 μ M FAC for 24 h or left untreated (Ctrl) and analyzed for CAT (chloramphenicol acetyltransferase) reporter activity using an enzyme-linked immunosorbent assay (Ctrl n=4, FAC n=5; Mn n=4). **(c)** Immunoblot analysis of Iron-regulatory protein-1 (IRP1) and IRP2 protein expression in response to increasing Mn concentrations. β -actin served as loading control (n=3). Data shown represent mean $\pm$ SD. Differences were calculated using two-way ANOVA **(a)** or unpaired t-test **(b, c)** (*, $p < 0.05$; **, $p < 0.01$).

Figure 4. Mn treatment increases labile iron pool (LIP), cytoplasmic and lipid ROS levels in a concentration and time-dependent manner. (a) SH-SY5Y cells were incubated for 24 h with indicated Mn concentrations and incubated with either 5 μ M RhoNox-1 dye for 1 h (left panel) or 20 μ M IP-1dye for 2h (right panel). As additional control Mn treated wells were incubated with 100 μ M of the iron chelator Deferiprone (DEF) for 1 h and incubated with both dyes. Fluorescence was presented as absolute fluorescence units (F.U.) (n=6). Cell-free fluorescence intensities of both Fe^{2+} -selective dyes $\pm$ 100 μ M Mn in OpiMEM were shown in **Supplemental Fig. 3b. (b)** IC-ICP-MS and CE-ICP-MS analysis were performed to determine Fe^{3+} and Fe^{2+} levels in RIPA lysates from SH-SY5Y incubated with DMEM media alone or 100 μ M Mn for 24 h. Data are presented as Fe^{3+} and Fe^{2+} to total iron (Fe^{3+} and Fe^{2+}) ratio (n=3). Absolute Fe^{3+} and Fe^{2+} values are presented in **Supplemental Fig. 3c. (c)** SH-SY5Y cells treated with increasing Mn concentrations for 24 h and analyzed for cellular oxidative stress levels via DCF fluorescence using flow cytometry. Mean relative fluorescence intensity (RFI) was presented as % increase to untreated controls (n=3). **(d)** SH-SY5Y cells treated with 100 μ M Mn for indicated time points and analyzed for DCF

fluorescence (n=3). **(e)** In a similar fashion lipid ROS was determined using the fluorescence dye BODIPY-C11 and flow cytometry after Mn treatment with increasing concentrations for 24 h **(f)** or 100 μ M Mn for indicated time points. Mean relative fluorescence intensity of DCF and FL1/FL2 ratio of BODIPY-C11 were presented as % increase to untreated controls (n=3). Data shown represent mean $\pm$ SD. Differences were calculated using unpaired t-test (*, $p < 0.05$; **, $p < 0.01$)

Figure 5: APP over-expression protects against Mn-induced toxicity via attenuating oxidative stress. **(a)** Representative histogram of cellular ROS (left panel) and lipid ROS (right panel) levels of SH-SY5Y cells, either stably overexpressing the APP₆₉₅-isoform (APP₆₉₅) or with the empty vector pCEP4 (Mock). **(b)** SH-SY5Y Mock and APP₆₉₅ cells were exposed to Mn for 24 h and analyzed for cellular oxidative cells via DCF dye (left panel) or lipid-ROS using BODIPY-C11 (right panel). DCF fluorescence is presented as arbitrary fluorescence units (F.U) (n=3). Lipid-ROS data are presented as FL1/FL2 ratio (n=3). **(c)** Mock and APP₆₉₅-transfected SH-SY5Y cells were incubated with increasing Mn concentrations for 24 h and analyzed for APP (clone 22C11) and H-Ferritin (clone D1D4) protein expression. **(d)** Cell viability was analyzed in Mock and APP₆₉₅-expressing SH-SY5Y cells after Mn exposure for 24 h (n=2-3). All other data are presented as mean $\pm$ SD. Differences were calculated using two-way ANOVA (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$).

Figure 6: Dietary Mn suppresses APP and H-Ferritin protein expression *in vivo* and inversely correlates with redox-active Fe²⁺ levels. **(a)** Mice were orally treated with standard diet (control group: 10 p.p.m Mn) or high Mn diet (Mn group: 2400 p.p.m Mn) for 91 days. Explanted cortices were analyzed for APP (clone A8717) and H-Ferritin (ab137758). β -actin (clone AC-15) served as loading control. **(b)** Relative protein levels were measured by densitometry and normalized to β -actin (control group: n=6 and Mn group: n=7). **(c)** Mice were either i.p. injected with PBS (control group) or with MnCl₂ (40 mg/kg/day) for 7 days. Cortices were blotted for APP (clone A8717) and H-Ferritin (ab137758). β -actin served as

loading control. **(d)** Relative protein levels were measured by densitometry and normalized to β -actin (control group: n=6 and Mn group: n=5). **(e)** Sprague-Dawley rats were orally treatment with Mn (500 mg/kg) or with standard diet for 53 days. Harvested whole brain lysates were blotted for rat APP (clone 22C11) and H-Ferritin (clone D1D4). β -actin served as loading control. **(f)** Densitometric quantification of APP and H-Ferritin were normalized to β -actin (each group: n=6). Representative western blots of IRP1, IRP2, Beclin-1 and LC3 are shown in **Supplemental Fig. 5**. **(g)** Redox-active Fe^{2+} levels (Fe^{2+} /total iron ($\text{Fe}^{2+}+\text{Fe}^{3+}$)) of individual brain samples were analyzed via IC-ICP-MS and previously published by Fernsebner et al (2014). Pearson correlation analysis between redox-active Fe^{2+} levels (mean value of two technical replicates) or **(h)** Mn and corresponding relative APP protein levels (mean value of two technical replicates) of control (red) and Mn treated (blue) rat brain samples showed significant relationship ($p=0.0065$, $r=-0.7063$). A linear regression line is shown when the correlation reaches statistical significance. All other data are presented as mean $\pm$ SD. *, $p<0.05$; **, $p<0.01$ by unpaired t-test.

Figure 7: Proposed model for Mn-induced neurotoxicity. **(a)** Under physiological conditions, free ferrous iron (Fe^{2+}) causes IRP1 release from iron responsive elements (IRE) that are located within the 5'-untranslated regions (5'-UTRs) of APP and H-Ferritin mRNA inducing enhanced ribosomal protein translation. To counteract toxic redox-active Fe^{2+} levels, APP facilitates the stabilization of membrane-bound Fe^{2+} -exporter ferroportin (FPN) while H-Ferritin safely sequesters Fe^{2+} via conversion to redox-inactive Fe^{3+} . **(b)** Manganese (Mn) decreases APP and H-Ferritin protein translation by increasing the binding of IRP1 to the IRE on the 5'-UTR of APP and H-Ferritin mRNA. Translational blockage of APP and H-Ferritin results in accumulation of toxic Fe^{2+} that fuels the generation of reactive oxygen species (ROS), ultimately resulting in neurotoxicity.