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# High Manganese, A Risk for Alzheimer's Disease: High Manganese Induces Amyloid-β Related Cognitive Impairment

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**Abstract**. Excess manganese (Mn) in brain can be neurotoxic, implicated in several neurodegenerative disorders such as sporadic Alzheimer's disease (AD). However, little is known about the altered metal environment including elevated Mn in the progressive cognitive impairment of AD. Indeed, whether high Mn is associated with AD risk remains elusive. In the study, we recruited 40 Chinese elders with different cognitive statuses and investigated concentrations of Mn in whole blood and plasma amyloid-β (Aβ) peptides. Surprisingly, there were significant correlations of Mn with Mini-Mental State Examination score and Clinical Dementia Rating Scale score. In addition, plasma Aβ peptides increased with elevated Mn. Further studies both *in vitro* and *in vivo* demonstrated dose-related neurotoxicity and increase of Aβ by Mn treatment, which was probably caused by disrupted Aβ degradation. These data suggested that high Mn may be involved in the progress of AD as an essential pathogenic factor.

Keywords: High manganese (Mn), cognitive impairment, amyloid-beta (Abeta), Alzheimer's disease (AD), Aβ degradation

## INTRODUCTION

As an essential trace metal, manganese (Mn) is present in all tissues and is required for the maintenance of proper function and regulation of numerous biochemical and cellular reactions [1]. Mn is a cofactor or required metal ion for many enzymes such as superoxide dismutase [2] and polynucleotide phosphorylase [3], and has important physiological roles, involved in immune function, regulation of metabolism, reproduction, digestion, bone growth, blood clotting, and brain development [4, 5].

While Mn deficiency rarely occurs in humans, high Mn induced toxicity, in particular Mn neurotoxicity, is more prevalent [6, 7]. Cases of Mn neurotoxicity have been reported particularly in miners, smelters, and workers in the alloy industry where exposures occur predominantly via the inhalation of Mn fumes or Mn-containing dusts [8]. Chronic exposure to Mn leads to excessive Mn accumulation in the nervous system [9], and dose-related cognitive deficits have been reported [10, 11], which highlights Mn toxicity on cognitive function. Reports have shown that high Mn is implicated in several neurodegenerative disorders such as Parkinson's disease and Huntington's disease [12–14].

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What's more, Mn was shown significantly higher levels in the parietal cortex of the Alzheimer's disease (AD) brain [15], and there was a significant inverse correlation of cerebrospinal fluid (CSF) amyloid-β $(Aβ)_{42}$ with Mn [16]. Together, these studies suggest that high Mn could be a potential risk for AD.

Here in order to unfold the role of high Mn in the pathological process of AD, we measured concentrations of Mn in whole blood and neuropsychological tests in humans. We first found Mn in whole blood was inversely correlated with Mini-Mental State Examination (MMSE) score and positively correlated with plasma Aβ peptides, which drove our interest to Mn's role in Aβ related cognitive impairment. Then both *in vivo* and *in vitro* studies revealed dose dependent Mn neurotoxicity with Aβ level via disrupted Aβ degradation. What's more, with Mn chelation therapy, cognitive impairment was ameliorated in the AD model mice, which indicated a novel strategy for the intervention of AD pathogenesis.

## MATERIALS AND METHODS

### *Subjects*

All patients and age-matched controls from the Beijing Geriatric Hospital (Beijing, China) provided informed consent for study participation. For assessment of global cognitive impairment, all subjects received the standard neuropsychological tests, including the MMSE scores [17] and the Clinical Dementia Rating Scale (CDR) scores [18], and divided into four groups accordingly [50, 51] as shown in Table 1. Whole blood samples of 10 ml were obtained from veins and stored into standard commercial evacuated tubes containing sodium heparin at −80°C until analysis.

### *Assay of metal ions*

The total concentrations in human whole blood or the rodent brain of Mn, copper, mercury, and lead were determined by inductively coupled plasma-mass spectrometry (ICP-MS; Thermo X7, Thermo Elemental, Winsford, UK) in accordance with Gerhardsson and colleagues [20]. To ensure the accuracy of the analytical methods and results, samples with 2% $HNO_3$ and quality control (QC) samples were analyzed along with the collected samples (Seronorm Trace Elements Serum Lot MI0181; SERO AS, Billingstad). The background contamination of the collection vessels was below the detection level of the analytical method used, and the obtained values for the QC samples showed good agreement with the recommended concentrations.

### *Animals, housing and treatment*

Male AβPPswe/PS1dE9 transgenic mice (bought from Institute of Laboratory Animal Science, Chinese Academy of Medical Science, abbreviated as AβPP mice for convenience), as an AD mice model, and age-matched wild-type mice were used. All work was approved by the Peking University Biomedical Ethics Committee Experimental Animal Ethics Branch. The animals were housed under controlled conditions of temperature and photoperiod, with free access to water and conventional mice chow. For Mn toxicity treatment, 27 mice of $12 \pm 1$ weeks old were used as follow: mice of low Mn group ($n = 9$) were treated with 15 mg/kg body weight, and mice of high Mn group ($n = 9$) with 60 mg/kg body weight, inorganic Mn ($MnCl_2 \cdot 4H_2O$, 99% purity; Sigma-Aldrich, St. Louis, MO, USA) dissolved in distilled water to 1 ml/kg administration volume; mice of the control group ($n = 9$) received distilled water, which naturally contains Mn about 5 ng/ml detected by ICP-MS. Administration was done by gavage, 5 days a week, and for 8 continuous weeks in total. For Mn chelation treatment, 16 mice of 6 months old were divided into two groups: one group of 8 mice was treated intraperitoneally four times weekly with 200 μL 0.2 M $Na_2CaCDTA$ (Strem Chemical, Inc., MA01950) for 3 months, and the other received normal saline intraperitoneally.

### *Cell culture and treatments*

Mouse N2a neuroblastoma cells stably expressing both wild type presenilin 1 (PS1wt) and Swedish mutant AβPP (AβPPsw) (abbreviated as AβPPsw-N2a cells for convenience) were kindly provided by Drs. Sangram S. Sisodia, and SeongHun Kim (University of Chicago) [21], and were maintained in normal DMEM free of Mn (Hyclone, South Logan, UT), and supplemented with 10% FBS. Cells were passaged at a ratio of 1:6 when 90% confluence had been reached and discarded after 20 passages. 24 h after splitting, the conditioned DMEM medium containing 10% FBS was replaced with fresh serum-free Mn-free DMEM (Hyclone). All experiments were performed in the absence of serum in order to exclude additional $Mn^{2+}$ from FBS. Manganese chloride ($MnCl_2$) was obtained from Sigma-Aldrich (St. Louis, MO). Cells were treated with different concentrations of

Table 1
Demographic data. For age and MMSE score, values represent mean ± SD

| | CDR 0 (Control) | CDR 0.5 (MCI) | CDR 1 (Mild dementia) | CDR ≥2 (Dementia) |
|---|---|---|---|---|
| Subjects | 10 | 10 | 4 | 16 |
| Age, year | 75.0 ± 5.2 | 73.2 ± 4.1 | 75.2 ± 2.7 | 75.9 ± 7.7 |
| Gender (male/female) | 5/5 | 4/6 | 2/2 | 7/9 |
| CDR | 0 | 0.5 | 1 | 2-3 |
| MMSE | 27.8 ± 0.6 | 10.8 ± 0.5 | 11.8 ± 1.7 | 5.2 ± 5.9 |

MMSE, Mini Mental Status Examination; CDR, Clinical Dementia Rating Scale; MCI, mild cognitive impairment.

extracellular manganese ([$Mn^{2+}$]o) for the time periods of 0, 12, 24, or 48 h at 37°C.

*Assessment of cell survival by 3-(4,5-Dimethyl thiazol- 2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay*

Cells were seeded in a 96-well plate at a density of $1 \times 10^4$ cells/100 μl. After being cultured in normal DMEM containing 10% FBS for 24 h, the cells were treated with varying [$Mn^{2+}$]o (0.0–400 μM) for 12 h, 24 h, and 48 h. For MTT-reducing activity, the cells were then incubated with 0.5 mg/ml MTT (Sigma-Aldrich) at 37°C for 4 h. Media were removed, and dimethyl sulfoxide (100 μl) was added to each well to solubilize the formazan crystals generated by viable mitochondrial succinate dehydrogenase from MTT. The absorbance at 570 nm was measured using a VERSA MAX enzyme-linked immunosorbent assay reader (Molecular Devices, Sunnyvale, CA) as the MTT reducing activity of the cells. The resultant data were expressed as the percentage of viable cells relative to controls.

*Lactate dehydrogenase (LDH) assay*

LDH release is an indicator of the integrity of cell membrane in that LDH releases from cells after cells were injured. After Mn treatments, LDH releases in the medium were measured as previously described [49]. The values of absorbance were read at 440 nm by the use of the microplate reader (Molecular Devices, Sunnyvale, CA). The resultant data were expressed as the percentage of viable cells relative to controls.

*Quantification of plasma, medium and brain Aβ peptide levels by sandwich enzyme-linked immunosorbent assay (ELISA)*

For plasma Aβ, a 2-ml sample of venous blood was split and centrifuged at 1,000 *g* for 10 min at 4°C. For medium Aβ, following the 24 h treatment with varying [$Mn^{2+}$], cells and conditioned media were harvested. Complete Protease Inhibitor cocktail was added to the medium to prevent degradation of Aβ protein and the cell debris was removed by centrifugation at 3,000 *g* for 10 min at 4°C. For brain Aβ, half of fresh-frozen mouse brain was serially homogenized into detergent-soluble and guanidine HCl-soluble fractions as described [22]. All samples were assayed for $Aβ_{40}$ and $Aβ_{42}$ by sandwich ELISA according to the manufacturer's instructions (Biosource International, Camarillo, CA). The detection limit for this assay was 5 pg/ml for $Aβ_{40}$ and 10 pg/ml for $Aβ_{42}$. The Aβ concentration in the medium was normalized based on the amount of cells in each culture (as determined by protein content in the cell lysates). All measurements were performed in duplicate.

*Western blot analysis*

Brains and cells were harvested and lysed on ice in western blot lysis buffer containing 50 mM Tris-HCl, pH 6.8, 8 M urea, 5% β-mercaptoethanol, 2% SDS, and protease inhibitors. The lysates were collected, centrifuged at 12,000 *g* 4°C for 5 min, and quantified for total proteins by the BCA protein assay kit. For western blot analysis, total proteins were separated on 10% T 5% C Bicine/Tris, 8 M urea, SDS-PAGE or 10–18% regular SDS-PAGE system [23, 24]. Protein was transferred to 0.45 μm polyvinylidene difluoride membranes (Immobilon-P; Millipore, Bedford, MA), blocked for 1 h in 5% (m/v) nonfat milk in Tris-buffered saline (TBS, pH 7.5), supplemented with 0.1% Tween 20. Antibodies and their dilutions used in this study include 6E10 (1:1000, COVANCE) or A8717 (1:10000, Sigma) for AβPP derivatives, NEP-specific mAb (1:1000, R&D), IDE pAb (1:5000, EMD Millipore) and anti-β-actin mouse mAb (1:5000, Sigma) as an internal reference control. Following incubation with the appropriate horseradish peroxidase-conjugated secondary antibody for 1 h at ambient temperature, the immunoblots were developed using the ECL system. Quantitative densitometric

analyses were performed on digitized images of immunoblots with Quantity One software (Bio-Rad, Hercules, CA). The γ-secretase inhibitor DAPT (500 nM, 8 h treatment) was employed in the experiments as a positive control for CTFα and CTFβ. Representative blots from at least three independent experiments are shown.

*RT-PCR analysis*

Total RNA was extracted with TRIZOL (Invitrogen) and converted to cDNA by reverse transcriptase using random hexamers to prime superscript III RNasefree reverse transcriptase (Invitrogen) according to the manufacturer's instructions. Each RT-PCR primer used in this study was as follows; neprilysin sense primer, 5′-AGCCTCTCTGTGCTTGTCTTGC-3′; neprilysin antisense primer, 5′-CACTCATAGTAGCCTCTG GAAGGG-3′; β-actin sense primer, 5′-TGTACG CCTCTGGCCGTAC-3′; β-actin antisense primer, 5′-CCACGTCACACTTCATGATGG-3′; insulin degrading enzyme (IDE) sense primer, 5′-CCCACC ACAGACAAGTCCTC-3′; IDE antisense primer, 5′-AAAACTGCGCAAACCTGTCC-3′. PCR reactions were performed at 94° for 30 s, 55° for 1 min, and 68° for 2 min during 40 cycles, followed by a final extension of 7 min at 68° [25]. Additionally, the resulting PCR products were analyzed on a 1% agarose gel stained with ethidium bromide.

*Morris water maze test*

Behavior was performed with a modified version of the Morris water maze used to assess spatial navigation learning and memory retention as before [26, 27] with minor modifications.

Initially, animals received a habituation trial during which the animals were handled for several minutes by the experimenter and allowed to explore the pool of water (diameter 150 cm, height 40 cm, temperature 23 ± 1°C) without the platform present. Following habituation, visible platform training was performed for 2 consecutive days to measure motivation of the mice to find a platform, visual acuity of the mice, and the ability of mice to use local cues. In the acquisition phase, we measured the ability of mice to form a representation of the spatial relationship between a safe, but invisible (submerged 1 cm below the water level), platform (10 cm in diameter) and visual cues surrounding the maze. Animals were allowed 60 s to locate the platform and 20 s to rest on it. Mice that failed to find the platform were led there by the experimenter and allowed to rest there for 20 s. Twenty-four hours following the last acquisition trial; a single 60 s probe trial was administered to assess spatial memory retention. For the probe trial, animals were returned to the maze as during training, but with no platform present, and parameters were recorded to assess the ability of the mouse to remember the previous location of the platform.

During the acquisition phase, escape latency (acquisition time to reach the platform) and path length (distance swam to the platform) were used to analyze and compare the performance between different treatment groups. The time spent in each of the four quadrants, the number of crossings of the former platform location, time to the former platform location, time to the platform quadrant, and the number of entries into the target quadrant were recorded and analyzed during the probe trials.

*Statistical analysis*

Analyses were conducted using the GraphPad Prism 5 for Windows (GraphPad Software, San Diego, CA). As most of the variables showed a skewed distribution (checked by normal probability plots), nonparametric statistical processing was applied (Kruskal-Wallis one-way analysis of variance, Mann-Whitney U test, Spearman's correlation coefficients). Comparisons between multiple groups were made by one-way ANOVA followed by Dunnett's Multiple Comparison Test, and the Student's t test was used for comparisons between two groups. $P$ values <0.05 were regarded as statistically significant (two-tailed tests).

## RESULTS

*Manganese in whole blood with different cognitive statuses*

Demographic data for the four groups with different cognitive statuses are shown in Table 1. Concentrations of Mn in whole blood of all subjects were measured and analyzed: CDR 0 (age-matched healthy control, $n = 10$) $11.20 \pm 0.95$ ng/ml; CDR 0.5 (Mild Cognitive Impairment, $n = 10$) $12.37 \pm 1.08$ ng/ml; CDR 1 (Mild dementia, $n = 4$) $9.63 \pm 1.11$ ng/ml; CDR $\geqq 2$ (dementia, $n = 16$) $13.98 \pm 0.88$ ng/ml (Fig. 1A). A significant change in Mn concentration was observed among four groups ($F_{(3,36)} = 2.925$, $p = 0.0469$), and there were significant changes between CDR $\geqq 2$ and CDR 0 ($p = 0.0293$) and between CDR $\geqq 2$ and CDR 1 ($p = 0.0309$) (Fig. 1A). After the correlative analysis

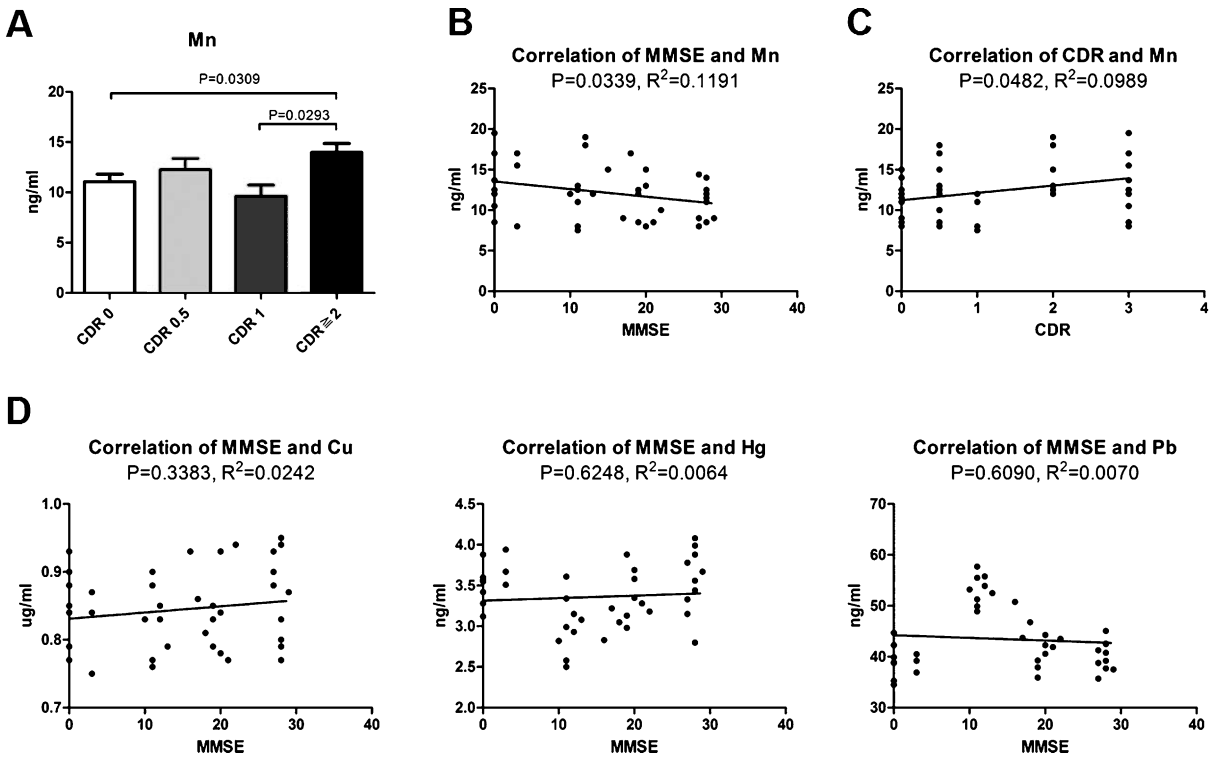


Fig. 1. Manganese (Mn) in whole blood is inversely correlated with MMSE scores. A) Mn in whole blood of different cognitive statuses: CDR 0 (age matched healthy control, $n=10$) $11.20 \pm 0.95$ ng/ml; CDR 0.5 (Mild Cognitive Impairment, $n=10$) $12.37 \pm 1.08$ ng/ml; CDR 1 (Mild dementia, $n=4$) $9.63 \pm 1.11$ ng/ml; CDR $\geq 2$ (dementia, $n=16$) $13.98 \pm 0.88$ ng/ml. B, C) Graphic representation of partial regression graph for Mn in whole blood with MMSE and CDR scores: $p<0.05$, $n=40$. D) Graphic representation of partial regression graph for copper (Cu), mercury (Hg), and lead (Pb) in whole blood with MMSE scores: $p>0.05$. MMSE, Mini Mental Status Examination; CDR, Clinical Dementia Rating Scale.

with MMSE and CDR scores, regardless of the grouping factor, we surprisingly discovered that there was a significant inverse correlation between MMSE score and Mn level ($p=0.0339$, $R^2=0.1191$) (Fig. 1B), and a positive correlation between CDR score and Mn level ($p=0.0482$, $R^2=0.0989$) (Fig. 1C). Concentrations of other neurotoxic metals (copper, mercury, and lead) [28, 29] had also been measured, but none had a significant association with MMSE scores (Fig. 1D). These results suggested that Mn might be strongly related to the progressive cognitive impairment. Indeed, more cases and follow-up evaluations are needed.

### *Manganese in whole blood with plasma Aβ peptides*

Plasma Aβ peptides, especially $A\beta_{42}$, were potential risk biomarkers of AD [30, 31]. Here we demonstrated that in all subjects plasma $A\beta_{42}$ was inversely correlated with MMSE ($p<0.01$, Fig. 2A), but plasma $A\beta_{40}$ was not ($p>0.05$, Fig. 2C). Interestingly, when analyzed with concentrations of Mn, both plasma $A\beta_{42}$ and $A\beta_{40}$ were increased with elevated Mn ($p<0.01$ for $A\beta_{42}$, Fig. 2B; $p<0.05$ for $A\beta_{40}$, Fig. 2D), which suggested that Mn might play a causal role in Aβ metabolism.

### *Exposure to high manganese induced Aβ related neurotoxicity and cognitive impairment*

We investigated the effects of high Mn on Aβ release in cultured neuronal cells for its neurotoxicity. MTT assay for cell viability demonstrated dose and time dependent toxicity of exposure to high Mn (Fig. 3A). LDH assay was used to assess the cell membrane integrity, and we found that after Mn treatment, LDH release was significantly increased only at 200 μM for 48 h and 400 μM for 12–48 h (Fig. 3B). These data revealed that after 200 μM Mn treated for 24 h, cell viability was decreased but cells did not undergo necrosis. Further, levels of $A\beta_{40}$ and $A\beta_{42}$ in the medium were measured (Fig. 3C, D), and both were found to sig-

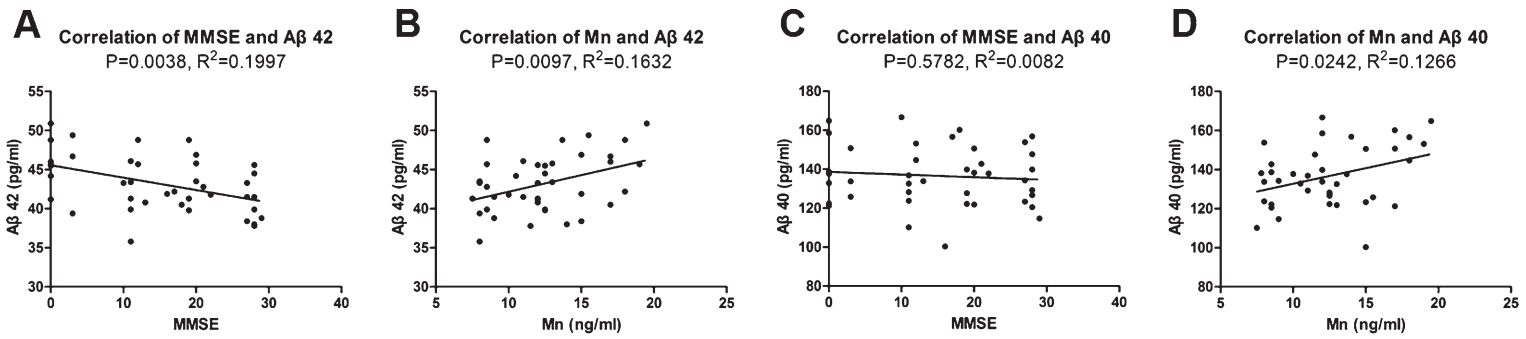


Fig. 2. Elevated manganese (Mn) with increased plasma $A\beta_{42}$ and $A\beta_{40}$. A, C) Graphic representation of partial regression graph for plasma $A\beta_{42}$ and $A\beta_{40}$ with MMSE scores ($p < 0.01$ for $A\beta_{42}$ and $p > 0.05$ for $A\beta_{40}$, $n = 40$). Only plasma $A\beta_{42}$ showed negative correlation with MMSE scores. B, D) Graphic representation of partial regression graph for Mn in whole blood with plasma $A\beta_{42}$ and $A\beta_{40}$ ($p < 0.01$ for $A\beta_{42}$ and $p < 0.05$ for $A\beta_{40}$, $n = 40$). Mn was positively correlated with both plasma $A\beta_{42}$ and $A\beta_{40}$. MMSE, Mini Mental Status Examination.

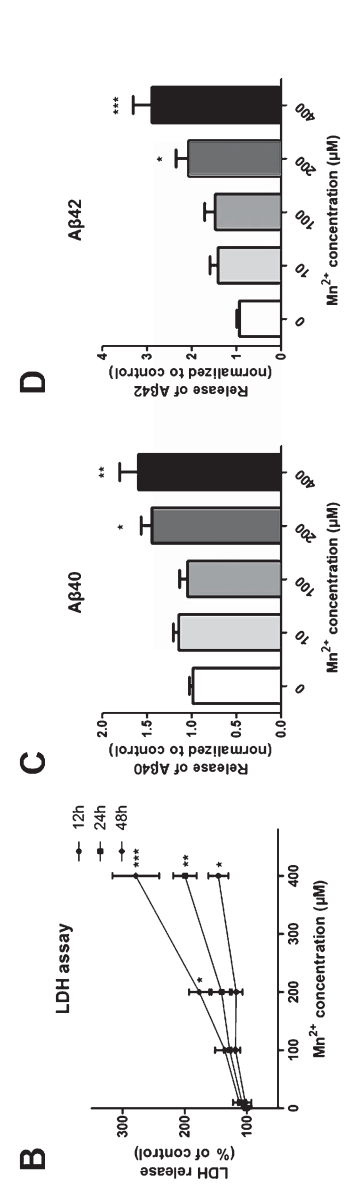


Fig. 3. High Mn-induced neurotoxicity in cultured AβPPsw-N2a cells. A) MTT assay for cell viability and (B) LDH assay were carried out on AβPPsw-N2a cells treated with various Mn concentrations (0–100 μM) for different time period (12, 24, and 48 h). Data are represented as % change relative to control (cells treated with Mn-free DMEM). C, D) ELISA for $A\beta_{40}$ and $A\beta_{42}$ in the medium was carried out on AβPPsw-N2a cells treated with various Mn concentrations (0–100 μM) for 24 h. The Aβ concentration was normalized based on the amount of cells in each culture, and then to the Mn-free control. Data are mean ± SEM (error bars) from 6–10 different preparations; $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$.

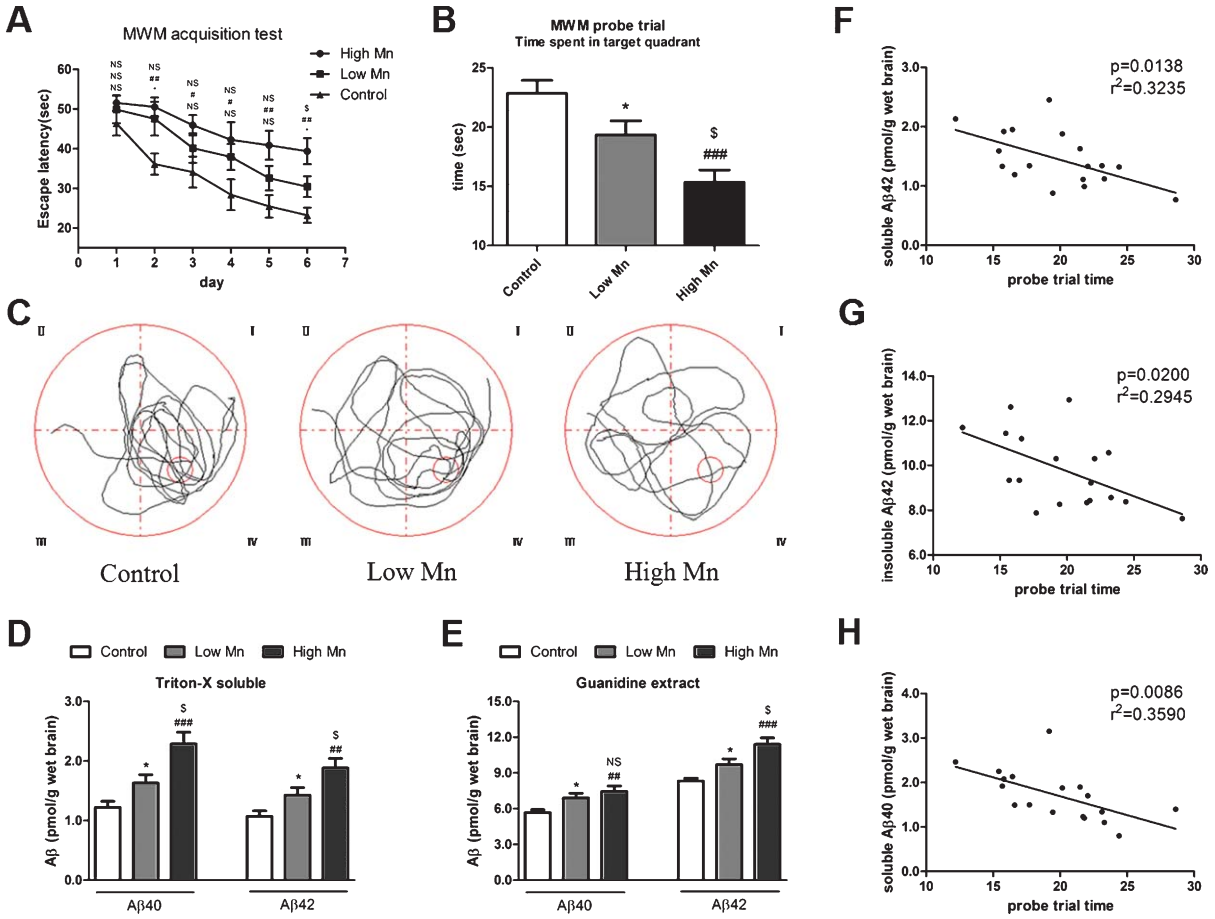


Fig. 4. Mn-induced Aβ related cognitive impairment in AβPPsw/PS1dE9 transgenic mice. A–C) Morris water maze test at 20 weeks. Escape latencies in hidden-platform of acquisition test (A), times spent in target quadrant (B), and representative swim paths (C) during the probe trial test are shown ($n = 9$ per group). There was a significant treatment effect on escape latency ($F_{(2,144)} = 23.62$, $p < 0.0001$). D, E) Quantification of brain $A\beta_{40}$ and $A\beta_{42}$ concentrations. Both Triton-X–soluble (D) and -insoluble (guanidine extract) (E) Aβ were measured ($n = 6$). $^{*}p < 0.05$ for Low Mn group versus Control group; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, and $^{\#\#\#}p < 0.001$ for High Mn group versus Control group; $\$p < 0.05$ for High Mn group versus Low Mn group. F–H) Graphic representation of partial regression graph for soluble $A\beta_{42}$ (F), insoluble $A\beta_{42}$ (G), and soluble $A\beta_{40}$ (H) with the corresponding probe trial time ($n = 18$).

nificantly increase in response to high Mn for 24 h at 200 and 400 μM. These data revealed that Mn induced neurotoxicity was, at least partially, due to the elevated Aβ level.

Evidence had shown significant higher Mn levels in the parietal cortex of the AD brain [15], then based on our *in vivo* study, we demonstrated Mn neurotoxicity in AβPPsw/PS1dE9 transgenic mice (AβPP mice), a mouse model for AD. Male AβPP mice at age 12 weeks were fed low dose of Mn (Low Mn) or high dose of Mn (High Mn) for 8 weeks, and compared to age-matched AβPP mice on a normal diet (Control). During the Morris water maze acquisition test (Fig. 4A), Low Mn group performed worse at day 2 and day 6, High Mn group performed worse from day 2 to 6 compared to Control group, and High Mn group performed even worse than Low Mn group at day 6. Indeed, there was a significant treatment effect on escape latency ($F_{(2,144)} = 23.62$, $p < 0.0001$) (Fig. 4A), accompanied by dose dependent decrease of time spent in target quadrant during the probe trial test (Fig. 4B, C), which revealed a dose related Mn toxicity on cognitive function.

To examine whether the impaired cognitive function in Mn treated AβPP mice might be due to increased Aβ load in the brain, Aβ content in the brain-soluble and brain-insoluble fractions was quantified. The levels of soluble and insoluble $A\beta_{40}$ and $A\beta_{42}$ in Low Mn and High Mn mice brain were significantly higher than those in Control mice, and significant differences

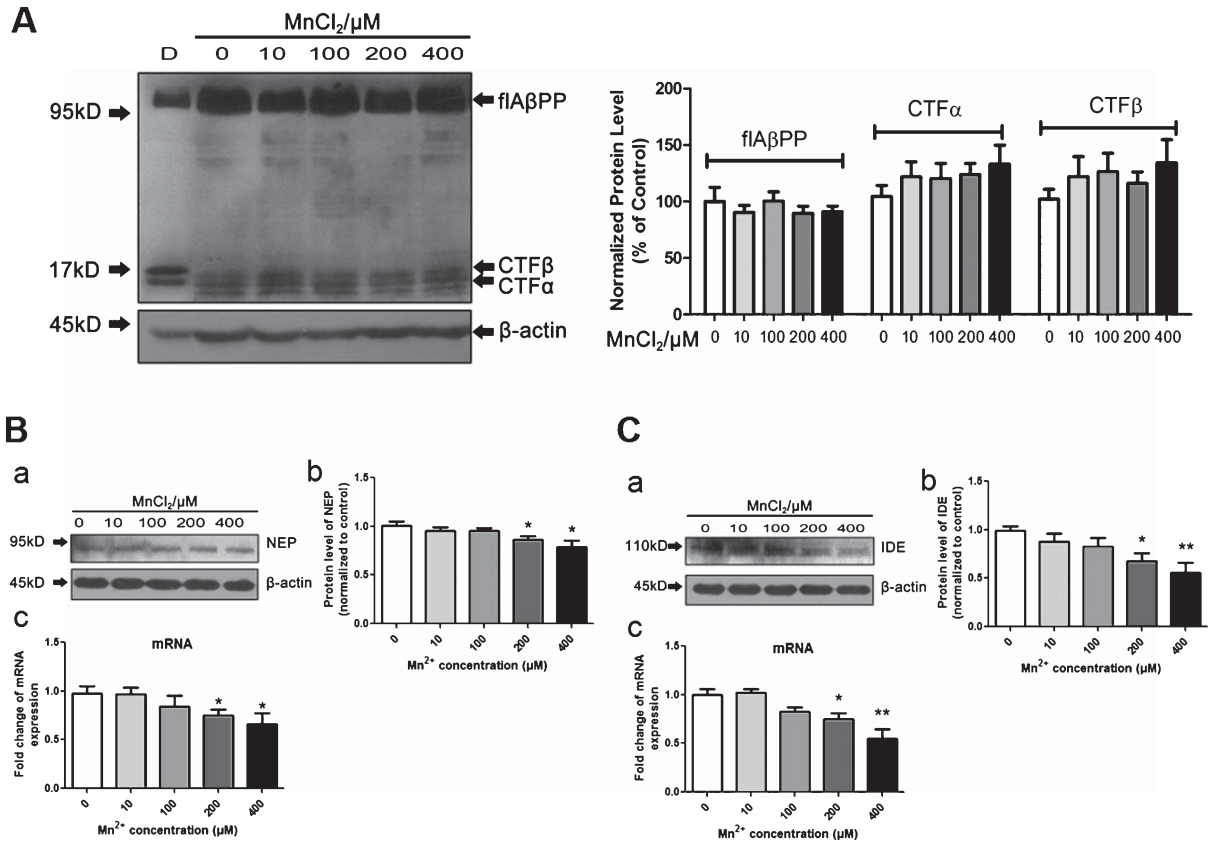


Fig. 5. Effects of varying [$Mn^{2+}$] on Aβ production and Aβ degradation in cultured AβPPsw-N2a cells. A) After treatment with varying [$Mn^{2+}$]o for 24 h, cell lysates were prepared, flAβPP and CTFs in cell lysates were analyzed by western blot analysis using antibody A8717. Note that exposure to varying [$Mn^{2+}$]o for 24 h did not alter the levels of flAβPP, CTFβ, and CTFα normalized to β-actin. As a positive control, the γ-secretase inhibitor DAPT (500 nM, for 8 h treatment) was employed to induce the accumulation of CTFβ and CTFα (far left lane). B, C) NEP and IDE protein and mRNA levels with varying [$Mn^{2+}$]o for 24 h. Cell lysates were immunoblotted for NEP or IDE and β-actin was used as a loading control. NEP and IDE mRNA levels were quantitated by RT-PCR and normalized to β-actin controls and expressed as ratios of control cell levels. Data are mean ± SEM (error bars) ($n = 4$); *$p < 0.05$; **$p < 0.01$. NEP, neprilysin; IDE, insulin degrading enzyme.

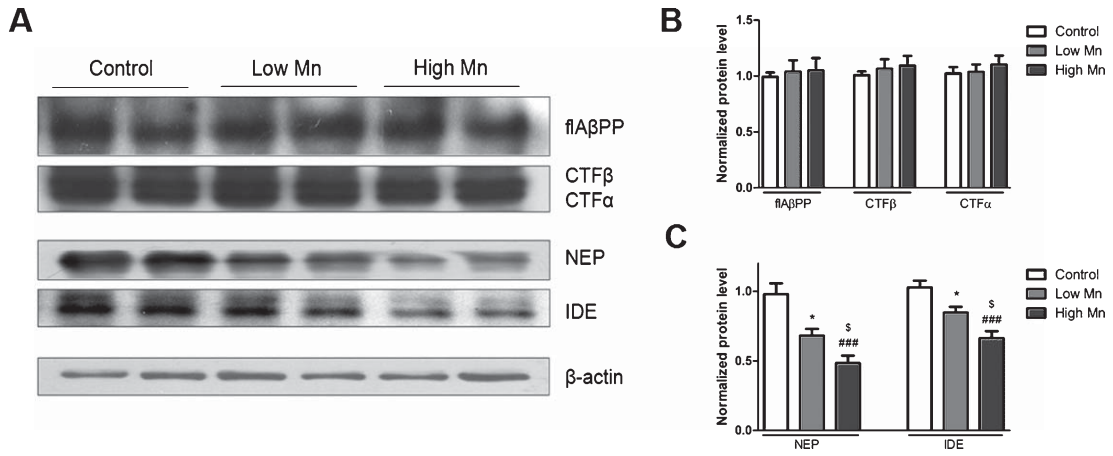


Fig. 6. Mn-induced decrease of Aβ degradation enzymes in AβPPsw/PS1dE9 transgenic mice. Representative western blots (A) and quantitative analysis (B, C) of flAβPP and CTFα/β for AβPP processing, and NEP and IDE for Aβ degradation, in brains of Control, Low Mn, and High Mn groups. Data are mean ± SEM (error bars) ($n = 6$ for each group). *$p < 0.05$ for Low Mn group versus Control group; ###$p < 0.001$ for High Mn group versus Control group; \$$p < 0.05$ for High Mn group versus Low Mn group. NEP, neprilysin; IDE, insulin degrading enzyme.

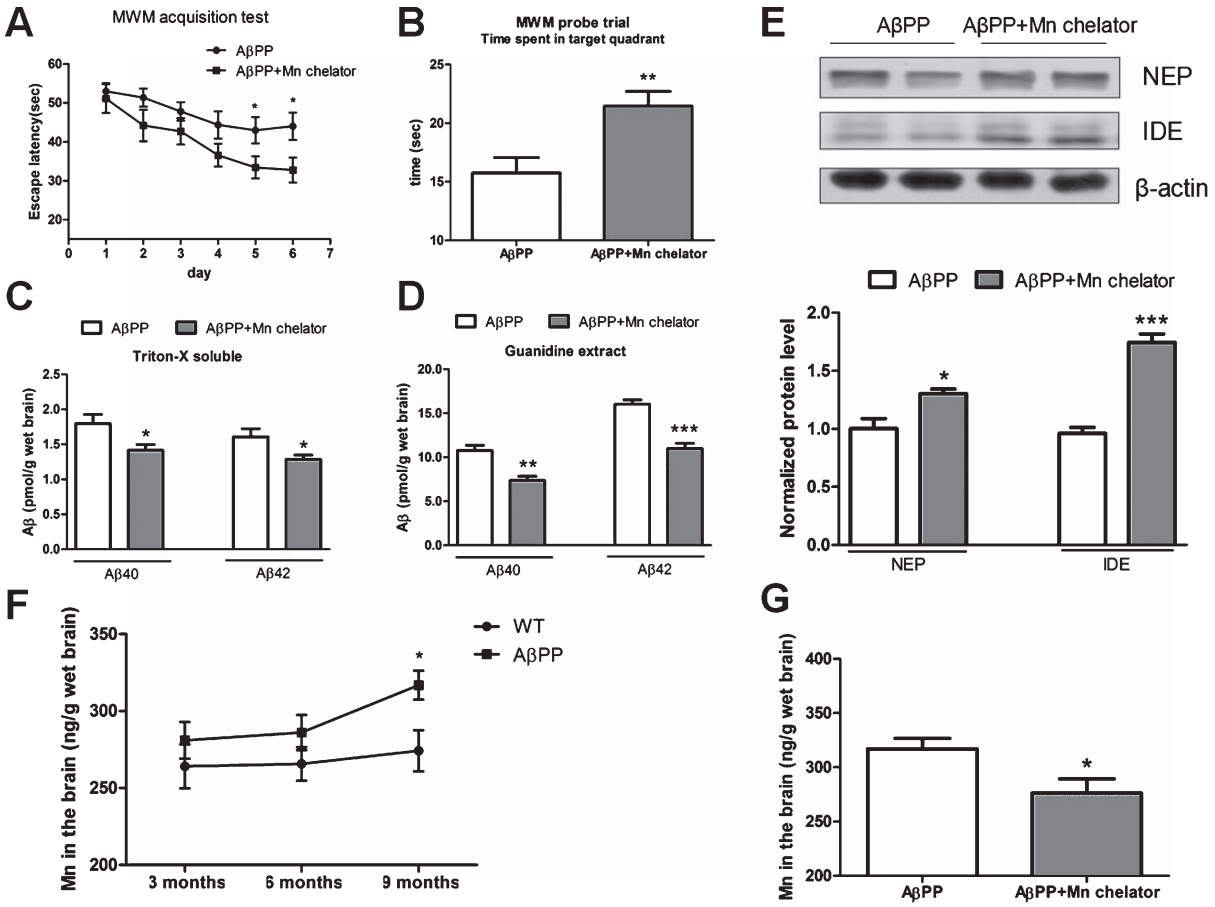


Fig. 7. Manganese chelator ameliorated cognitive impairment in the AβPPsw/PS1dE9 transgenic mice. A, B) Morris water maze test at 9 months. Escape latencies in hidden-platform of acquisition test (A) and times spent in target quadrant during the probe trial test (B) were shown ($n = 8$ per group). There was a significant treatment effect on escape latency ($F_{(1,84)} = 15.31$, $p = 0.0002$). C, D) Quantification of brain $A\beta_{40}$ and $A\beta_{42}$ concentrations. Both Triton-X–soluble (C) and -insoluble (guanidine extract) (D) Aβ were measured ($n = 6$). E) Representative western blots and quantitative analysis of NEP and IDE in brains of two groups ($n = 6$ for each group). F) Concentration of Mn in brains of WT and AβPP mice at the age of 3, 6 and 9 months ($n = 6$). G) Concentration of Mn in brains of AβPP and AβPP+Mn chelator mice ($n = 6$). Data are mean ± SEM (error bars). $^*p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$. AβPP, the AβPPsw/PS1dE9 transgenic mice; AβPP+Mn chelator, AβPP mice treated with $Na_2CaCDTA$; NEP, neprilysin; IDE, insulin degrading enzyme.

of soluble $A\beta_{40}$, soluble $A\beta_{42}$, and insoluble $A\beta_{42}$ were observed between Low Mn and High Mn mice (Fig. 4D, E). Furthermore, there was a negative correlation of the level of brain soluble $A\beta_{40}$, soluble $A\beta_{42}$, and insoluble $A\beta_{42}$ with the probe trial performance of mice of Mn exposure, suggesting a causative relation between the increased Aβ and the aggravated cognitive impairment (Fig. 4F–H).

*High manganese did not affect Aβ production, but impaired Aβ degradation*

To clarify how high Mn induced elevation of Aβ level, we compared amyloid-β precursor protein (AβPP) processing for Aβ production among differed concentrations of Mn. However, as shown in Fig. 5A, increased Mn changed neither the level of full-length AβPP (flAβPP) nor its processing production CTFα and even CTFβ in cultured cells, which suggested high Mn had no impact on Aβ production. Then we examined two major enzymes of Aβ degradation, namely neprilysin (NEP) and insulin degrading enzyme (IDE). Surprisingly, NEP protein level decreased slightly but significantly by 14% at 200 μM and 21% at 400 μM of Mn exposure (Fig. 5Ba, b), and IDE protein level decreased more significantly by 31% at 200 μM and further 43% at 400 μM of Mn exposure (Fig. 5Ca, b), which indicated disrupted Aβ

degradation metabolism induced by high Mn. Levels of mRNA showed consistent results (Fig. 5Bc, Cc), which suggested a novel role of Mn as a transcription regulator.

After Mn treatment, AβPP processing remained unchanged in brains of Low Mn and High Mn group mice, compared to Control group (Fig. 6, two upper panels). Nevertheless, there were dose dependent decreases of NEP and IDE (Fig. 6, two middle panels): NEP decreased by 31% in Low Mn group and 51% in High Mn group, and IDE decreased by 16% in Low Mn group and 34% in High Mn group. In addition, NEP and IDE were both reduced in High Mn group, compared to Low Mn group (Fig. 6). Combined with the *in vitro* study above, it could be concluded that Mn overload caused down-regulation of Aβ degradation enzymes.

### *Manganese chelation therapy ameliorated cognitive impairment in the AD model mice*

Data above made us wonder whether reducing brain Mn level can be a potential therapeutic strategy for AD. Compared to age-matched wild type mice, brain Mn level of AβPP mice stayed normally at the age of 3 and 6 months but increased significantly at the age of 9 months (Fig. 7F). Then we treated 6-month-old AβPP mice intraperitoneally with Mn chelator $Na_2CaCDTA$ for 3 months. Surprisingly, after Mn chelator treatment, AβPP mice performed better during the acquisition test (Fig. 7A) and the probe trial test (Fig. 7B) of Morris water maze. Further, soluble and insoluble $A\beta_{40}$ and $A\beta_{42}$ were reduced (Fig. 7C, D), along with elevated NEP protein level by 30% and IDE protein level by 74% (Fig. 7E), which revealed rescue of Mn chelation therapy on Aβ related cognitive impairment. Additionally, brain Mn was detected and also reduced after Mn chelation (Fig. 7G).

## DISCUSSION

### *Mn in the blood as a tracker for the progressive cognitive impairment*

An imbalance in Mn homeostasis is well known to result in severe CNS dysfunction [6]. Increased Mn concentrations in the brain can occur under a variety of conditions, such as occupational, iatrogenic, medical, and environmental exposures [32]. After oral Mn intake, the blood-CSF barrier might be the major route for Mn into the brain [6]. The homeostasis of Mn in the CSF appears to be directly influenced by plasma Mn concentrations [33]. When testing several possible Mn carriers, the most abundant Mn species was Mn-citrate [34]. Elevated Mn-Citrate in serum could be a valuable marker for increased total Mn in CSF (and brain), i.e., it could be a marker for elevated risk of Mn-dependent neurological disorders in occupational health [35]. Then the metal environment in the blood may be an efficient indicator for the CSF condition and the related pathological status.

In our study, the concentrations of Mn in whole blood of patients were significantly negatively related to the MMSE scores and positively related to the CDR scores of elder subjects with different cognitive statuses, which indicated that Mn in whole blood may track the progressive cognitive impairment of AD. Mn in whole blood was also positively correlated with plasma Aβ peptides, the potential risk biomarkers of AD, which suggested the possible role of Mn in Aβ hallmarked AD. Reports had demonstrated that Mn showed significantly higher levels in the parietal cortex and plasma of AD in humans [15, 20], and diffused Aβ plaques were also found in the frontal cortex from Mn-exposed non-human primates [36]. Besides, exposure to Mn can cause the hyperphosphorylation of tau [37], which may lead to the formation of neurofibrillary tangles, one of the key clinical structure changes of AD. In summary, Mn in whole blood may be a potential noninvasive biomarker for AD.

### *High Mn induced Aβ related neurotoxicity on cultured neurons and rodent brains*

Chronic occupational exposure to Mn mostly by inhalation raises its accumulation in the central nervous system, especially in the basal ganglia, leading to degeneration of neurons and a loss of dopamine [34, 38]. Several mechanisms underlie its neurotoxicity. For example, inhibitory effect of Mn on AChE activity promotes increased neuronal oxidative stress and neuroinflammatory biomarkers [39]. Also Mn-induced neurotoxicity is associated with disruption of the glutamine/glutamate-c-aminobutyric acid cycle between astrocytes and neurons [40]. Besides neurons [38, 41], increasing studies focus on its activity/toxicity on astrocytes [8, 42–44], the blood-brain barrier [45] and the blood-CSF barrier [6, 46].

Here we examined Mn neurotoxicity with MTT assay, and found a dose and time dependent manner. Also, the released cellular Aβ peptides, which were initially neurotoxic, were increased after high Mn exposure, indicating a non-ignorable role of elevated Aβ level in Mn induced neurotoxicity. Further our *in*

*vivo* study found dose related cognitive impairment in the AD model mice after Mn treatment. Both brain-soluble and brain-insoluble Aβ peptides were increased and had a strong relationship with the probe trial performance, which suggested that high Mn could induce Aβ related cognitive impairment. The Mn chelation therapy supported us a novel strategy to prevent and treat AD. After intraperitoneal injection, Mn chelator $Na_2CaCDTA$ reduced the concentration of Mn in the brain, and restored the cognitive function of AD model mice, along with decreased Aβ peptides. Indeed, the application dosage of $Na_2CaCDTA$ had no effects on wild type mice (not shown).

### *High Mn impaired Aβ degradation but not Aβ production*

Aβ is generated from sequential cleavages of the AβPP by the β- and γ-secretases [47], and is removed through various clearance pathways and by enzyme-mediated degradation, mainly including the metalloproteases NEP and IDE [48]. We probed into effects of high Mn on AβPP processing for Aβ generation, but it did not differ among all concentrations of Mn exposure, while NEP and IDE decreased after high Mn exposure in cultured neurons. Furthermore, NEP and IDE were dose dependently reduced in Mn-treated mice, indicating disrupted Aβ degradation metabolism induced by high Mn. Evidence has shown that Mn can act as the metal cofactor of polynucleotide phosphorylase and affect the discrimination of RNA from DNA [3], and then Mn homeostasis might regulate the related protein level at the transcriptional level.

In conclusion, we first found Mn in whole blood was correlated with MMSE/CDR scores and plasma Aβ peptides, and high Mn could induce Aβ related cognitive impairment via disrupted Aβ degradation. Our results suggest that high Mn may be a risk and an essential pathogenic factor for AD.

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## REFERENCES

[1] Keen CL, Ensunsa JL, Clegg MS (2000) Manganese metabolism in animals and humans including the toxicity of manganese. *Met Ions Biol Syst* **37**, 89-121.

[2] Wedler FC, Denman RB (1984) Glutamine synthetase: The major Mn(II) enzyme in mammalian brain. *Curr Top Cell Regul* **24**, 153-169.

[3] Unciuleac MC, Shuman S (2013) Discrimination of RNA from DNA by polynucleotide phosphorylase. *Biochemistry* **52**, 6702-6711.

[4] Aschner M, Erikson KM, Dorman DC (2005) Manganese dosimetry: Species differences and implications for neurotoxicity. *Crit Rev Toxicol* **35**, 1-32.

[5] Takeda A, Ishiwatari S, Okada S (1999) Manganese uptake into rat brain during development and aging. *J Neurosci Res* **56**, 93-98.

[6] Bornhorst J, Wehe CA, Hüwel S, Karst U, Galla HJ, Schwerdtle T (2012) Impact of manganese on and transfer across blood-brain and blood-cerebrospinal fluid barrier *in vitro*. *J Biol Chem* **287**, 17140-17151.

[7] Dobson AW, Erikson KM, Aschner M (2004) Manganese neurotoxicity. *Ann N Y Acad Sci* **1012**, 115-128.

[8] Aschner M, Guilarte TR, Schneider JS, Zheng W (2007) Manganese: Recent advances in understanding its transport and neurotoxicity. *Toxicol Appl Pharmacol* **221**, 131-147.

[9] Martins EN, Pessano NT, Leal L, Roos DH, Folmer V, Puntel GO, Rocha JB, Aschner M, Ávila DS, Puntel RL (2012) Protective effect of Melissa officinalis aqueous extract against Mn-induced oxidative stress in chronically exposed mice. *Brain Res Bull* **87**, 74-79.

[10] Bowler RM, Roels HA, Nakagawa S, Drezgic M, Diamond E, Park R, Koller W, Bowler RP, Mergler D, Bouchard M, Smith D, Gwiazda R, Doty RL (2007) Dose-effect relationships between manganese exposure and neurological, neuropsychological and pulmonary function in confined space bridge welders. *Occup Environ Med* **64**, 167-177.

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

[12] Sidoryk-Wegrzynowicz M, Aschner M (2013) Role of astrocytes in manganese mediated neurotoxicity. *BMC Pharmacol Toxicol* **14**, 23.

[13] Guilarte TR (2010) Manganese and Parkinson's disease: A critical review and new findings. *Environ Health Perspect* **118**, 1071-1080.

[14] Williams BB, Li D, Wegrzynowicz M, Vadodaria BK, Anderson JG, Kwakye GF, Aschner M, Erikson KM, Bowman AB (2010) Disease-toxicant screen reveals a neuroprotective interaction between Huntington's disease and manganese exposure. *J Neurochem* **112**, 227-237.

[15] Srivastava RA, Jain JC (2002) Scavenger receptor class B type I expression and elemental analysis in cerebellum and parietal cortex regions of the Alzheimer's disease brain. *J Neurol Sci* **196**, 45-52.

[16] Strozyk D, Launer LJ, Adlard PA, Cherny RA, Tsatsanis A, Volitakis I, Blennow K, Petrovitch H, White LR, Bush AI (2009) Zinc and copper modulate Alzheimer Abeta levels in human cerebrospinal fluid. *Neurobiol Aging* **30**, 1069-1077.

[17] Folstein MF, Folstein SE, McHugh PR (1975) "Mini-mental state". A practical method for grading the cognitive state of patients for the clinician. *J Psychiatr Res* **12**, 189-198.

[18] O'Bryant SE, Waring SC, Cullum CM, Hall J, Lacritz L, Massman PJ, Lupo PJ, Reisch JS, Doody R; Texas

Alzheimer's Research Consortium (2008) Staging dementia using Clinical Dementia Rating Scale Sum of Boxes scores: A Texas Alzheimer's research consortium study. *Arch Neurol* **65**, 1091-1095.

[19] Dubois B, Feldman HH, Jacova C, Dekosky ST, Barberger-Gateau P, Cummings J, Delacourte A, Galasko D, Gauthier S, Jicha G, Meguro K, O'brien J, Pasquier F, Robert P, Rossor M, Salloway S, Stern Y, Visser PJ, Scheltens P (2007) Research criteria for the diagnosis of Alzheimer's disease: Revising the NINCDS-ADRDA criteria. *Lancet Neurol* **6**, 734-746.

[20] Gerhardsson L, Lundh T, Minthon L, Londos E (2008) Metal concentrations in plasma and cerebrospinal fluid in patients with Alzheimer's disease. *Dement Geriatr Cogn Disord* **25**, 508-515.

[21] Kim SH, Leem JY, Lah JJ, Slunt HH, Levey AI, Thinakaran G, Sisodia SS (2001) Multiple effects of aspartate mutant presenilin 1 on the processing and trafficking of amyloid precursor protein. *J Biol Chem* **276**, 43343-43350.

[22] Takeda S, Sato N, Uchio-Yamada K, Sawada K, Kunieda T, Takeuchi D, Kurinami H, Shinohara M, Rakugi H, Morishita R (2010) Diabetes-accelerated memory dysfunction via cerebrovascular inflammation and Abeta deposition in an Alzheimer mouse model with diabetes. *Proc Natl Acad Sci U S A* **107**, 7036-7041.

[23] Li M, Sun M, Liu Y, Yu J, Yang H, Fan D, Chui D (2010) Copper downregulates neprilysin activity through modulation of neprilysin degradation. *J Alzheimers Dis* **19**, 161-169.

[24] Zhao G, Mao G, Tan J, Dong Y, Cui MZ, Kim SH, Xu X (2004) Identification of a new presenilin-dependent zeta-cleavage site within the transmembrane domain of amyloid precursor protein. *J Biol Chem* **279**, 50647-50650.

[25] Pardossi-Piquard R, Petit A, Kawarai T, Sunyach C, Alves da Costa C, Vincent B, Ring S, D'Adamio L, Shen J, Müller U, St George Hyslop P, Checler F (2005) Presenilin-dependent transcriptional control of the Abeta-degrading enzyme neprilysin by intracellular domains of betaAPP and APLP. *Neuron* **46**, 541-554.

[26] Liu Y, Ye Z, Yang H, Zhou L, Fan D, He S, Chui D (2010) Disturbances of soluble N-ethylmaleimide-sensitive factor attachment proteins in hippocampal synaptosomes contribute to cognitive impairment after repetitive formaldehyde inhalation in male rats. *Neuroscience* **169**, 1248-1254.

[27] Fitz NF, Cronican A, Pham T, Fogg A, Fauq AH, Chapman R, Lefterov I, Koldamova R (2010) Liver X receptor agonist treatment ameliorates amyloid pathology and memory deficits caused by high-fat diet in APP23 mice. *J Neurosci* **30**, 6862-6872.

[28] Butterworth RF (2010) Metal toxicity, liver disease and neurodegeneration. *Neurotox Res* **18**, 100-105.

[29] Weiss B (2010) Lead, manganese, and methylmercury as risk factors for neurobehavioral impairment in advanced age. *Int J Alzheimers Di* **2011**, 607543.

[30] Schupf N, Tang MX, Fukuyama H, Manly J, Andrews H, Mehta P, Ravetch J, Mayeux R (2008) Peripheral Abeta subspecies as risk biomarkers of Alzheimer's disease. *Proc Natl Acad Sci U S A* **105**, 14052-14057.

[31] Mayeux R, Honig LS, Tang MX, Manly J, Stern Y, Schupf N, Mehta PD (2003) Plasma A[beta]40 and A[beta]42 and Alzheimer's disease: Relation to age, mortality, and risk. *Neurology* **61**, 1185-1190.

[32] Burton NC, Guilarte TR (2009) Manganese neurotoxicity: Lessons learned from longitudinal studies in nonhuman primates. *Environ Health Perspect* **117**, 325-332.

[33] Zheng W, Ren S, Graziano JH (1998) Manganese inhibits mitochondrial aconitase: A mechanism of manganese neurotoxicity. *Brain Res* **799**, 334-342.

[34] Michalke B, Berthele A, Mistriotis P, Ochsenkühn-Petropoulou M, Halbach S (2007) Manganese speciation in human cerebrospinal fluid using CZE coupled to inductively coupled plasma MS. *Electrophoresis* **28**, 1380-1386.

[35] Michalke B, Lucio M, Berthele A, Kanawati B (2013) Manganese speciation in paired serum and CSF samples using SEC-DRC-ICP-MS and CE-ICP-DRC-MS. *Anal Bioanal Chem* **405**, 2301-2309.

[36] Guilarte TR (2013) Manganese neurotoxicity: New perspectives from behavioral, neuroimaging, and neuropathological studies in humans and non-human primates. *Front Aging Neurosc* **5**, 23.

[37] Cai T, Che H, Yao T, Chen Y, Huang C, Zhang W, Du K, Zhang J, Cao Y, Chen J, Luo W (2010) Manganese induces tau hyperphosphorylation through the activation of ERK MAPK pathway in PC12 cells. *Toxicol Sci* **119**, 169-177.

[38] Dukhande VV, Malthankar-Phatak GH, Hugus JJ, Daniels CK, Lai JC (2006) Manganese-induced neurotoxicity is differentially enhanced by glutathione depletion in astrocytoma and neuroblastoma cells. *Neurochem Res* **31**, 1349-1357.

[39] Santos D, Milatovic D, Andrade V, Batoreu MC, Aschner M, Marreilha dos Santos AP (2012) The inhibitory effect of manganese on acetylcholinesterase activity enhances oxidative stress and neuroinflammation in the rat brain. *Toxicology* **292**, 90-98.

[40] Sidoryk-Wegrzynowicz M, Aschner M (2012) Manganese toxicity in the central nervous system: The glutamine /glutamate-γ-aminobutyric acid cycle. *J Intern Med* **273**, 466-477.

[41] Kalia K, Jiang W, Zheng W (2008) Manganese accumulates primarily in nuclei of cultured brain cells. *Neurotoxicology* **29**, 466-470.

[42] Latronico T, Branà MT, Merra E, Fasano A, Di Bari G, Casalino E, Liuzzi GM (2013) Impact of manganese neurotoxicity on mmp-9 production and superoxide dismutase activity in rat primary astrocytes. Effect of resveratrol and therapeutical implications for the treatment of CNS diseases. *Toxicol Sci* **135**, 218-228.

[43] Lee E, Sidoryk-Wegrzynowicz M, Farina M, Rocha JB, Aschner M (2013) Estrogen attenuates manganese-induced glutamate transporter impairment in rat primary astrocytes. *Neurotox Res* **23**, 124-130.

[44] Deng Y, Xu Z, Xu B, Xu D, Tian Y, Feng W (2012) The protective effects of riluzole on manganese-induced disruption of glutamate transporters and glutamine synthetase in the cultured astrocytes. *Biol Trace Elem Res* **148**, 242-249.

[45] dos Santos AP, Milatovic D, Au C, Yin Z, Batoreu MC, Aschner M (2010) Rat brain endothelial cells are a target of manganese toxicity. *Brain Res* **1326**, 152-161.

[46] Zheng G, Chen J, Zheng W (2012) Relative contribution of CTR1 and DMT1 in copper transport by the blood-CSF barrier: Implication in manganese-induced neurotoxicity. *Toxicol Appl Pharmacol* **260**, 285-293.

[47] Ly PT, Wu Y, Zou H, Wang R, Zhou W, Kinoshita A, Zhang M, Yang Y, Cai F, Woodgett J, Song W (2013) Inhibition of GSK3β-mediated BACE1 expression reduces Alzheimer-associated phenotypes. *J Clin Invest* **123**, 224-235.

[48] Miners JS, Baig S, Palmer J, Palmer LE, Kehoe PG, Love S (2008) Abeta-degrading enzymes in Alzheimer's disease. *Brain Pathol* **18**, 240-252.

[49] Koh JY, Choi DW (1987) Quantitative determination of glutamate mediated cortical neuronal injury in cell culture by

lactate dehydrogenase efflux assay. *J Neurosci Methods* **20**, 83-90.

[50] Maki Y1, Yamaguchi T, Yamaguchi H (2013) Symptoms of Early Dementia-11 Questionnaire (SED-11Q): A brief informant-operated screening for dementia. *Dement Geriatr Cogn Dis Extra* **3**, 131-142.

[51] Rolstad S1, Berg AI, Bjerke M, Blennow K, Johansson B, Zetterberg H, Wallin A (2011) Amyloid-β42 is associated with cognitive impairment in healthy elderly and subjective cognitive impairment. *J Alzheimers Dis* **26**, 135-142.