Novel defense by metallothionein induction against cognitive decline:
from amyloid β_{1-42} -induced excess Zn^{2+} to functional Zn^{2+} deficiency

Abbreviated title: Metallothionein & $\text{A}\beta_{1-42}$ -induced cognitive decline

Atsushi Takeda^{1*}, Haruna Tamano¹, Wakana Hashimoto¹, Shuhei Kobuchi¹, Hiroki Suzuki¹, Taku Murakami¹, Munekazu Tempaku¹, Yuta Koike¹, Paul A. Adlard²,
Ashley I. Bush²

¹ Department of Neurophysiology, School of Pharmaceutical Sciences, University
of Shizuoka, 52-1 Yada, Suruga-ku, Shizuoka 422-8526, Japan

² The Florey Institute of Neuroscience and Mental Health, The University of
Melbourne, Parkville, VIC., 3052, Australia

*To whom correspondence should be addressed.

TEL: 81-54-264-5733

FAX: 81-54-264-5909

E-mail: takedaa@u-shizuoka-ken.ac.jp

Keywords: metallothionein, extracellular Zn^{2+} , amyloid β_{1-42} , dentate gyrus, cognitive decline, Alzheimer's disease

Abstract

The role of metallothioneins (MTs) in cognitive decline associated with intracellular Zn^{2+} dysregulation remains unclear. Here we report that hippocampal MT induction defends cognitive decline, which was induced by amyloid β_{1-42} ($\text{A}\beta_{1-42}$)-mediated excess Zn^{2+} and functional Zn^{2+} deficiency. Excess increase in intracellular Zn^{2+} , which was induced by local injection of $\text{A}\beta_{1-42}$ into the dentate granule cell layer, attenuated in vivo perforant pathway LTP, while the attenuation was rescued by preinjection of MT inducers into the same region. Intraperitoneal injection of dexamethasone, which increased hippocampal MT proteins and blocked $\text{A}\beta_{1-42}$ -mediated Zn^{2+} uptake, but not $\text{A}\beta_{1-42}$ uptake, into dentate granule cells, also rescued $\text{A}\beta_{1-42}$ -induced impairment of memory via attenuated LTP. The present study indicates that hippocampal MT induction blocks rapid excess increase in intracellular Zn^{2+} in dentate granule cells, which originates in Zn^{2+} released from $\text{A}\beta_{1-42}$, followed by rescuing $\text{A}\beta_{1-42}$ -induced cognitive decline. Furthermore, LTP was vulnerable to $\text{A}\beta_{1-42}$ in the aged dentate gyrus, consistent with enhanced $\text{A}\beta_{1-42}$ -mediated Zn^{2+} uptake into aged dentate granule cells, suggesting that $\text{A}\beta_{1-42}$ -induced cognitive decline, which is caused by excess intracellular Zn^{2+} , can more frequently occur along with aging. On the other hand, attenuated LTP under functional Zn^{2+} deficiency in dentate granule cells was also rescued by MT induction. Hippocampal MT induction may rescue cognitive decline under lack of cellular transient changes in functional Zn^{2+} concentration, while its induction is an attractive defense strategy against $\text{A}\beta_{1-42}$ -induced cognitive decline.

Introduction

Zinc is essential for brain functions and most of zinc is tightly bound to metalloproteins, which require it for catalytic activity or structural stability. Zinc is concentrated in presynaptic vesicles of a subclass of glutamatergic neurons, i.e., zincergic neurons and is released along with synaptic activity [1,2]. Extracellular Zn^{2+} dynamics, which is often linked with Zn^{2+} release from zincergic terminals, is critical for not only synaptic plasticity such as long-term potentiation (LTP) but also synaptic dysfunction associated with cognitive decline [3,4]. Metallothioneins (MTs) are small, cysteine-rich proteins and bind zinc and copper. They function as metal ion regulation and detoxification [5]. MT-I and MT-II are expressed in all tissues, while MT-III and MT-IV are expressed specifically in the brain [6,7] and stratified squamous epithelia [8], respectively. MT-III is highly expressed in zincergic neurons and is most abundantly observed in dentate granule cells [9].

It is well known that the reactivity and dynamic coordination of MTs with Zn^{2+} and Cu^{+} are linked with the biological requirements for controlling these cellular metal ions [10]. MTs are unique biological molecules for buffering Zn^{2+} as well as zinc transporters, exporters and importers. Transient increase in intracellular (cytosolic) Zn^{2+} concentration functions as intracellular signaling and is involved in LTP, a cellular mechanism of memory [11]. The expression of MTs is involved in modulating and controlling zinc-dependent cellular signaling [12]. However, MT-III-deficient mice exhibit normal spatial learning and memory [13]. MT-I, II-deficient mice exhibit an impaired rate of spatial learning but once learned retain the information as well as the wild-type mice [14]. Therefore, physiological role of MTs in cognitive function remains unclear.

The basal levels of extracellular Zn^{2+} are in the range of low nanomolar concentrations (~ 10 nM) in the hippocampus and probably increased age-dependently [15,16]. In the dentate gyrus, non-zincergic perforant pathway innervates dentate granule cells. Therefore, it is estimated that extracellular Zn^{2+} concentration is not increased at the perforant pathway synapses even after synaptic excitation. Nonetheless, the rapid excess influx of extracellular Zn^{2+} into dentate granule cells, which more readily occurs in aged rats after local injection of 100 mM K^+ into the dentate molecular layer, is a cause of aged-related cognitive decline [17,18]. Interestingly, extracellular Zn^{2+} influx into dentate granule cells is increased by not only glutamatergic synapse excitation with high K^+ [17-19] but also amyloid- β ($\text{A}\beta$), a causative candidate for the pathogenesis of Alzheimer's disease (AD) [20]. The formation of $\text{Zn-A}\beta_{1-42}$ in the extracellular compartment rapidly increases both intracellular Zn^{2+} and $\text{A}\beta_{1-42}$ in dentate granule cells of normal young rats, followed by $\text{A}\beta_{1-42}$ -induced cognitive decline that is due to increase in intracellular Zn^{2+} released from $\text{A}\beta_{1-42}$ [21]. $\text{A}\beta_{1-42}$ -induced cognitive decline is rescued in the presence of not only an extracellular Zn^{2+} chelator, CaEDTA but also an intracellular Zn^{2+} chelator, ZnAF-2DA [20]. It is possible that the $\text{A}\beta_{1-42}$ -induced cognitive decline is increased along with normal aging and is a cause of short-term memory loss in the pre-dementia stage of AD. Therefore, defense strategy against the $\text{A}\beta_{1-42}$ -induced cognitive decline may lead the elderly to preventing and/or delaying AD pathogenesis.

On the basis of the data that in vivo K_d value of Zn^{2+} to $\text{A}\beta_{1-42}$ may be in the range of ~ 3 – 30 nM [21], which is higher than that of MTs (~ 1 pM) [22], here, we tested the defensive effect of hippocampal MT induction on cognitive decline, which was induced by $\text{A}\beta_{1-42}$ -mediated excess Zn^{2+} and functional Zn^{2+} deficiency. Hippocampal MT induction is of benefit to defend $\text{A}\beta_{1-42}$ -induced cognitive decline of normal rats. The present study also discusses the physiological role of hippocampal

MTs on cellular transient changes in Zn^{2+} concentration required for cognitive function.

Materials and methods

Animals and chemicals

Male Wistar rats (young, <20 weeks of age; aged, >60 weeks of age) were purchased from Japan SLC (Hamamatsu, Japan). All the experiments were performed in accordance with the Guidelines for the Care and Use of Laboratory Animals of the University of Shizuoka that refer to the American Association for Laboratory Animals Science and the guidelines laid down by the NIH (NIH Guide for the Care and Use of Laboratory Animals) in the USA. The Ethics Committee for Experimental Animals in the University of Shizuoka has approved this work (approval numbers: 136043).

Synthetic human $\text{A}\beta_{1-42}$ was purchased from ChinaPeptides (Shanghai, China). ZnAF-2DA, a membrane-permeable zinc indicator was kindly supplied from Sekisui Medical Co., LTD (Hachimantai, Japan). ZnAF-2DA is taken up into the cells through the cell membrane and is hydrolyzed by esterase in the cytosol to yield ZnAF-2 ($K_d = 2.7 \times 10^{-9}$ M for Zn^{2+}), which cannot permeate the cell membrane [23,24]. The fluorescence indicators were dissolved in dimethyl sulfoxide (DMSO) and then diluted to artificial cerebrospinal fluid (ACSF) containing 119 mM NaCl, 2.5 mM KCl, 1.3 mM MgSO_4 , 1.0 mM NaH_2PO_4 , 2.5 mM CaCl_2 , 26.2 mM NaHCO_3 , and 11 mM D-glucose (pH 7.3).

Most elements in vehicle and ACSF used in the present study are at or below detection limits (<1 ppb=15 nM): Li, B, Al, Ti, Cr, Mn, Fe, Co, Ni, Cu, Zn, Se, Rb, Sr, Mo, Ru & Cd (Agilent 7700 series ICPMS instrument).

***In vivo* LTP**

Male rats were anesthetized with chloral hydrate (400 mg/kg) and placed in a stereotaxic apparatus. A bipolar stimulating electrode and a monopolar recording electrode made of tungsten wire attached to an injection cannula (internal diameter, 0.15 mm; outer diameter, 0.35 mm) were positioned stereotaxically so as to selectively stimulate the perforant pathway while recording in the dentate gyrus. The electrode stimulating the perforant pathway was positioned 8.0 mm posterior to the bregma, 4.5 mm lateral, 3.0-3.5 mm inferior to the dura. A recording electrode was implanted ipsilaterally 4.0 mm posterior to the bregma, 2.5 mm lateral and 3.0-3.5 mm inferior to the dura. Rats were allowed to recover for one week in individual home cages. All the stimuli were biphasic square wave pulses (200 μ s width) and their intensities were set at the current that evoked 40% of the maximum population spike (PS) amplitude. Test stimuli (0.05 Hz) were delivered at 20 s intervals to monitor PS amplitude and PS amplitudes were measured for 20 min for the baseline recording in freely moving and unanesthetized rats. One day later, LTP was induced by delivery of high-frequency stimulation (HFS; 10 trains of 20 pulses at 200 Hz separated by 1 s) and under the same condition. PS amplitudes, which were measured for 10 min, were recorded 1 h and immediately (0 h) before HFS stimulation and 1 h after HFS stimulation, averaged, and expressed as percentages of the mean PS amplitude measured during the 20-min baseline period prior to LTP induction, which was expressed as 100%.

In another experiments, LTP recording was performed 1 h after implantation of the electrodes under anesthetized condition (Fig. 4 and 6). After stable baseline recording for at least 30 min, LTP was induced by delivery of high-frequency stimulation (HFS; 10 trains of 20 pulses at 200 Hz separated by 1 s) and recorded for 60 min under the same condition. PS amplitudes (test frequency: 0.05 Hz) were

averaged over 120-second intervals and expressed as percentages of the mean PS amplitude measured during the 10-min baseline period, which was expressed as 100%. PS amplitudes for the last 10 min were also averaged and represented as the magnitude of LTP.

Slice preparation

Wistar rats were anesthetized with ether and decapitated in accordance with the Japanese Pharmacological Society guide for the care and use of laboratory animals. The brain was quickly removed and immersed in ice-cold choline-ACSF containing 124 mM choline chloride, 2.5 mM KCl, 2.5 mM MgCl₂, 1.25 mM NaH₂PO₄, 0.5 mM CaCl₂, 26 mM NaHCO₃, and 10 mM glucose (pH 7.3) to suppress excessive neuronal excitation. Coronal brain slices or horizontal hippocampal slices (400 μ m) were prepared in ice-cold choline-ACSF using a vibratome ZERO-1 (Dosaka Kyoto, Japan) in an ice-cold choline-ACSF. Slices were then maintained in ACSF at 25°C for at least 30 minutes. All solutions used in the experiments were continuously bubbled with 95% O₂ and 5% CO₂.

***In vivo* Zn²⁺ imaging**

Saline and ZnCl₂ in saline (100 μ M) (1 μ l) were bilaterally injected via injection cannulas (internal diameter, 0.15 mm; outer diameter, 0.35 mm) as described above. Twenty-three hours later, saline and 25 μ M A β ₁₋₄₂ in saline containing 100 μ M ZnAF-2DA (2 μ l) were injected in the same manner. Five minutes later, the brain slices were prepared, transferred to a chamber filled with ACSF, loaded with 2 μ M Calcium Orange AM in ACSF for 30 min to identify hippocampal regions, and then rinsed in ACSF for at least 15 min. The brain slices were transferred to a recording chamber filled with ACSF. The fluorescence of ZnAF-2 (laser, 488 nm; emission,

505–530 nm) and Calcium Orange (laser, 543 nm; emission, above 560 nm) was captured with a confocal laser-scanning microscopic system. Intracellular Zn^{2+} levels in the dentate granule cell layer, which were determined with intracellular ZnAF-2, were represented by the ratio to the control without $\text{A}\beta_{1-42}$ in saline expressed as 100%.

In vivo immunostaining

Male rats were anesthetized with chloral hydrate (400 mg/kg) and placed in a stereotaxic apparatus. The skull was exposed, two burr holes were drilled, and injection cannulae (internal diameter, 0.15 mm; outer diameter, 0.35 mm) were bilaterally inserted into the dentate granule cell layer (4.0 mm posterior to the bregma, 2.3 mm lateral, 2.9 mm inferior to the dura). Thirty minutes later following recovery from the insertion damage, 50 μM $\text{A}\beta_{1-42}$ in ACSF, or 50 μM $\text{A}\beta_{1-42}$ with 500 μM CaEDTA in ACSF were bilaterally injected via the injection cannulae into the dentate granule cell layer of anesthetized rats at the rate of 0.25 $\mu\text{l}/\text{min}$ for 8 min. Five minutes later, the brain was quickly removed and hippocampal slices were prepared as described above. Slices were then fixed with paraformaldehyde (4% in 0.01 M PBS) for 15 min. Slices were rinsed in 0.01 M PBS three times. Tissues were then blocked in 5% normal goat serum for 30 min, rinsed in 0.01M PBS three times, incubated with 70% formic acid for 5 min, rinsed with 0.01 M PBS three times, and incubated at 4°C with $\text{A}\beta$ monoclonal antibody, 4G8 (COVANCE, 1:500 dilution in 0.01M PBS) for 48 h. Slices were then rinsed with 0.01 M PBS three times, incubated with Alexa Fluor 633 goat anti-mouse IgG secondary antibody (1: 200 dilution in 0.01M PBS) for 1 h, rinsed in 0.01 M PBS three times, incubated with 4',6-diamidino-2-phenylindole (DAPI) for 10 min and then rinsed again with 0.01 M PBS three times prior to mounting on glass slides. Images for immunostaining were captured using a confocal laser-scanning microscopic system LSM 510 META (Carl Zeiss), equipped with an

inverted microscope (Axiovert 200M, Carl Zeiss) through a 10× objective. Florescence intensity was analyzed using NIH Image J. The region of interest was set in the dentate granule cell layer in the dentate gyrus.

MT measurement

The hippocampus excised was homogenized by sonication in 4 volume of ice-cold 0.03 M PBS (pH 7.4) and the homogenates (300 µl) were incubated with silver nitrate solution (20 mg/l, 250 µl) and 0.5 M glycine buffer (pH 8.5, 400 µl) for 5 min. Rat hemolyzed erythrocytes (100 µl) were added to the samples, boiled for 2 min to precipitate excess silver, centrifuged at 4,000 g for 5 min at 4°C. This process was repeated three times to obtain the supernatant containing only silver bound to MT proteins. The concentration of MT proteins was calculated from silver concentration in the supernatant as determined by inductively coupled plasma atomic emission spectroscopy (ICP-AES).

Object recognition memory

The object recognition tests were performed in a separate cohort of animals. Briefly, injection cannulae (internal diameter, 0.15 mm; outer diameter, 0.35 mm) were bilaterally implanted into the dentate granule cell layer of rats as described above. Five days later, saline or dexamethasone in saline (10 mg/kg) was intraperitoneally (i.p.) injected into the rats once a day for two days. One hour after the last injection, rats were allowed to explore an open field (70 × 60 cm arena surrounded by 70 cm high walls, made of a black-colored plastic) for 10 min. Twenty-three hours later, agents in saline (1 µl) were bilaterally injected via injection cannulae into the dentate granule cell layer of unanesthetized rats at the rate of 0.25 µl/min for 4 min. One hour after injection, rats were trained and tested in a novel object recognition task. Training in

the object recognition task took place in the same area used for the open field exploration. Thus the open field exploration was used as a context habituation trial for the recognition memory task. The object recognition test requires that the rats recall which of two earthenware objects they had been previously familiarized with. Training was conducted by placing individual rats in the field, in which two identical objects (objects A1 and A2; sake bottle) were positioned in two adjacent corners, 15 cm from the walls. Rats were left to explore the objects for 5 min. Rats were not used for the test when the total of the object exploration time was less than 20 s. In the test given 1 h after training, the rats explored the open field for 3 min in the presence of one familiar (A) and one novel (B; cup) object. All objects were of similar texture, color and size, but were a distinctive shape (we confirmed that there was no preference for the objects used). All objects were washed with 70% ethanol between trials. The behavior of the rats was recorded with a video camera during the training and the test phases of the experiment, and then two people independently measured exploratory time and the averaged time was used. Exploration was defined as sniffing or touching the object with the nose and/or forepaws. A recognition index calculated for each rat was expressed by the ratio $T_B/(T_A + T_B)$ [T_A =time spent to explore the familiar object A; T_B =time spent to explore the novel object B].

Data analysis

Student's paired *t*-test was used for comparison of the means of paired data. For multiple comparisons, differences between treatments were assessed by one-way ANOVA followed by post hoc testing using the Tukey's test (the statistical software, GraphPad Prism 5). A value of $p < 0.05$ was considered significant. Data were expressed as means $\pm$ standard error. The results of statistical analysis are described in each figure legend.

Results

MT induction rescues excess Zn^{2+} -induced attenuation of LTP

In vivo LTP at medial perforant pathway-dentate granule cell synapses underlies the formation and retention of object and space recognition memory [19,21]. The effect of MT inducers on excess Zn^{2+} -induced cognitive decline was assessed by recording the in vivo LTP. LTP was attenuated 1 h after local injection of ZnCl_2 into the dentate granule cell layer of control young rats, which were subjected to preinjection of vehicle into the same region. When ZnCl_2 or corticosterone was preinjected into the dentate granule cell layer to induce MT-I and MT-2 [7], ZnCl_2 -induced attenuation of LTP was rescued (Fig. 1A and 1B).

When $\text{A}\beta_{1-42}$ was injected into the dentate granule cell layer, intracellular Zn^{2+} determined with intracellular ZnAF-2 was markedly increased in the dentate granule cell layer 5 min after $\text{A}\beta_{1-42}$ injection (Fig. 2D) and was not increased in the CA1 and CA3 (data not shown). LTP was attenuated 1 h after $\text{A}\beta_{1-42}$ injection, probably due to the rapid increase in intracellular Zn^{2+} [20]. $\text{A}\beta_{1-42}$ -induced attenuation of LTP was rescued by the preinjection of ZnCl_2 , CdCl_2 , or corticosterone into the same region, which was performed 24 h before the $\text{A}\beta_{1-42}$ injection (Fig. 2A, 2B and 2C), consistent with blocking the increase in intracellular Zn^{2+} in the dentate granule cell layer by preinjection of ZnCl_2 (Fig. 2D). Even in the case of ZnCl_2 preinjection into young rats, it is estimated that induced MTs bind Zn^{2+} released from $\text{Zn-A}\beta_{1-42}$ in dentate granule cells.

Dexamethasone-induced MTs rescue $\text{A}\beta_{1-42}$ -induced cognitive decline

Intraperitoneal (i.p.) injection of dexamethasone increases MT-I mRNA in the brain, but not MT-III mRNA [6]. On the basis of the data that MT proteins were maximally increased in the hippocampus 24 h after i.p. injection of dexamethasone into young rats (Fig. 3A), the effect of the increase was examined in ZnCl₂-induced and A β ₁₋₄₂-induced attenuation of LTP. Both attenuations were rescued by the preinjection of dexamethasone prior to injection of ZnCl₂ or A β ₁₋₄₂ (Fig. 4A and 4B). The preinjection of dexamethasone blocked A β ₁₋₄₂-induced increase in intracellular Zn²⁺ in the dentate granule cell layer (Fig. 3C). On the other hand, the preinjection did not modify A β ₁₋₄₂ stain in the dentate granule cell layer (Fig. 3D). Simultaneously A β ₁₋₄₂-induced object recognition memory deficit was rescued in young rats, which were subjected to preinjection of dexamethasone in the same manner (Fig.4D). The preinjection of dexamethasone had no effect on object recognition memory.

To assess the effect of induced MTs on Zn²⁺-buffering, high K⁺-induced increase in intracellular Zn²⁺ was compared between brain slices from the control and dexamethasone-pretreated rats. The increase in intracellular Zn²⁺ was suppressed in the dentate granule cell layer by the preinjection of dexamethasone (Fig. 3E), suggesting that dexamethasone-induced MTs buffer the rapid excess increase in intracellular Zn²⁺ in dentate granule cells after exposure to high K⁺.

A β ₁₋₄₂-induced increase in intracellular Zn²⁺ is enhanced age-dependently

On the basis of the rapid uptakes of A β ₁₋₄₂ and Zn²⁺ into young dentate granule cells [21], it is possible that both uptakes are modified in aged dentate granule cells, consistent with age-related increase in extracellular zinc in the hippocampus [18]. To test age-related A β ₁₋₄₂ retention in vivo, A β ₁₋₄₂ immunostaining was performed in the dentate gyrus captured 5 min after local injection of A β ₁₋₄₂ into the dentate granule cell layer of young and aged rats. A β ₁₋₄₂ staining was more increased in the aged dentate

granule cell layer, although it was not significant increase (Fig. 5A and 5B). On the other hand, A β_{1-42} -induced increase in intracellular Zn²⁺, which was measured with intracellular ZnAF-2, was significantly more in the aged dentate granule cell layer (Fig. 5C and 5D). Both A β_{1-42} staining and A β_{1-42} -induced increase in intracellular Zn²⁺ were blocked in both the young and aged dentate granule cell layers by coinjection of CaEDTA, an extracellular Zn²⁺ chelator, suggesting that A β_{1-42} uptake and A β_{1-42} -induced increase in intracellular Zn²⁺ require extracellular Zn²⁺ even in aged rats.

The rapid Zn²⁺ influx via A β_{1-42} in aged rats implies age-related vulnerability to A β_{1-42} -induced synapse dysfunction. A β_{1-42} injection (25 pmol) into the dentate granule cell layer, which rapidly increases intracellular Zn²⁺ in the dentate granule cell layer [20], attenuated LTP in young rats. However, the half dose of A β_{1-42} (12.5 pmol) did not attenuate LTP (Fig. 6A) and did not increase intracellular Zn²⁺ in the dentate granule cell layer (Fig. 6C). In contrast, LTP was attenuated 1 h after A β_{1-42} injection (12.5 pmol) into the aged dentate granule cell layer (Fig. 6B), which rapidly increased intracellular Zn²⁺ in the aged dentate granule cell layer (Fig. 6D). In aged rats, A β_{1-42} -induced attenuation of LTP was rescued by coinjection of CaEDTA (Fig. 6B).

The basal level of MT proteins in the hippocampus, which was higher in aged rats than young rats, was also increased 24 h after i.p. injection of dexamethasone into aged rats (Fig. 3B). High K⁺-induced increase in intracellular Zn²⁺ was suppressed in the aged dentate granule cell layer, as well as the young dentate granule cell layer (Fig. 3E), by the preinjection of dexamethasone (Fig. 3F), suggesting that dexamethasone-induced MTs can buffer Zn²⁺ released from A β_{1-42} in dentate granule cells of aged rats and rescue A β_{1-42} -induced cognitive decline. Actually, A β_{1-42} -induced attenuation of LTP was rescued in aged rats, as well as young rats (Fig. 4B), by the preinjection of dexamethasone prior to A β_{1-42} injection (Fig. 4C).

MT induction rescues attenuated LTP under functional intracellular Zn^{2+} deficiency

The lack of intracellular Zn^{2+} in dentate granule cells induced by perfusion with ZnAF-2DA attenuates LTP [25]. We tested whether the lack of transient changes in intracellular Zn^{2+} concentration, which is required for LTP, could be rescued by MT induction. LTP was attenuated 1 h after local injection of ZnAF-2DA into the dentate granule cell layer. ZnAF-2DA-induced attenuation of LTP was rescued by the preinjection of ZnCl_2 or CdCl_2 into the same region, which was performed 24 h before the ZnAF-2DA injection (Fig. 7A and 7B). ZnAF-2DA-induced attenuation of LTP was also rescued by the preinjection of dexamethasone prior to the injection (Fig. 7C). On the other hand, the preinjection of dexamethasone had no effect on LTP induction (Fig. 7C), suggesting that dexamethasone-induced MTs do not affect LTP, as well as the cases of MT induction with ZnCl_2 , CdCl_2 , and corticosterone (Fig. 1 and 2).

Discussion

MT-I and MT-II expressions are regulated by a wide variety of metals, hormones, and xenobiotics, while MT-III expression hardly responds to these stimuli [6,26]. MT-III is upregulated by brain ischemia and plays an important role in protecting neurons from ischemic insult by decreasing neurotoxic Zn^{2+} [27]. In contrast, MT-I and MT-II protect neuroglial cells from oxidative damage [28]. The evidence indicates that MT-III is hardly induced by increase in intracellular Zn^{2+} in hippocampal neurons, while MT-I and MT-II expressions are responsive to it [29]. The basal concentration of intracellular (cytosolic) Zn^{2+} is estimated to be less than 1 nM (~0.1 nM) [30,31]. The rapid increase in free Zn^{2+} in the intracellular compartment is a cause of age-related

and A β_{1-42} -induced cognitive decline in addition to neuronal death [18,32]. Intracellular MTs can buffer free Zn²⁺ based on its varying affinities for Zn²⁺ [33,34], which physiologically overlap with cellular transient changes in Zn²⁺ concentration, i.e., intracellular Zn²⁺ signaling. However, it is estimated that the binding capacity is saturated by the rapid increase in intracellular Zn²⁺, which is induced by glutamatergic synapse excitation with high K⁺ [18,19] and extracellular A β_{1-42} [20]. If intracellular Zn²⁺ concentration reaches 10 nM, which is the estimated concentration of extracellular Zn²⁺ [35], MTs bind up to 7 equivalents of Zn²⁺ and become the holo-MTs, Zn₇MTs [36], resulting in loss of the Zn²⁺-buffering ability. Therefore, it is thought that an increase in intracellular MTs is effective for buffering the rapid excess increase in intracellular Zn²⁺ associated with cognitive decline, while an increase in extracellular MTs might not be effective for blocking extracellular Zn²⁺-dependent Zn-A β_{1-42} uptake [21].

To examine our idea, the rescuing effect of MT induction on excess Zn²⁺-induced attenuation of LTP was examined in young rats 24 h after local injection of three MT inducers. Excess Zn²⁺-induced attenuation of LTP, which was induced by A β_{1-42} injection into dentate granule cell layer, was rescued by the preinjection of ZnCl₂, CdCl₂, or corticosterone into the same region, consistent with blocking the rapid excess increase in intracellular Zn²⁺ in dentate granule cells by preinjection of ZnCl₂, suggesting that induced MT-I and MT-II proteins rescue A β_{1-42} -induced attenuation of LTP.

MT proteins in the hippocampus were maximally increased 24 h after i.p. injection of dexamethasone. This increase subsequently rescued A β_{1-42} -induced attenuation of LTP and A β_{1-42} -induced memory deficit. Dexamethasone-induced MTs blocked A β_{1-42} -induced increase in intracellular Zn²⁺ in dentate granule cells, while did not modify A β_{1-42} uptake into dentate granule cells, in agreement with our idea. On the

other hand, high K^+ -induced increase in intracellular Zn^{2+} , which induces cognitive decline [17-19], was suppressed in dentate granule cells of brain slices from rats pretreated with dexamethasone in the same manner, suggesting that dexamethasone-induced MTs capture free Zn^{2+} taken up into dentate granule cells after exposure to high K^+ . High K^+ -induced increase in intracellular Zn^{2+} is due to Zn^{2+} influx through Ca^{2+} -permeable α -amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA) receptors [17,18], while Ca^{2+} -permeable AMPA receptors may not be involved in $A\beta_{1-42}$ -induced increase in intracellular Zn^{2+} [20]. The present study indicates that hippocampal MTs induced with dexamethasone block rapid increase in intracellular Zn^{2+} in dentate granule cells, which originates in Zn^{2+} released from $A\beta_{1-42}$, followed by rescuing $A\beta_{1-42}$ -induced cognitive decline.

When the basal level of intracellular Zn^{2+} is ~ 0.1 nM in dentate granule cells, MTs have Zn^{2+} -binding sites [37] and can capture Zn^{2+} released from $A\beta_{1-42}$ in the cells. MTs bind four Zn^{2+} with high affinity (K_d , ~ 1 pM) [37] and bind another two Zn^{2+} with lower affinity. MTs bind the seventh Zn^{2+} with nanomolar affinity (K_d , ~ 1 nM) [36]. Although the mechanism of cellular Zn - $A\beta_{1-42}$ uptake is unknown, it is estimated that induced MTs do not affect extracellular Zn^{2+} -dependent Zn - $A\beta_{1-42}$ uptake into dentate granule cells. Even if induced MTs are secreted from the cells [38,39], extracellular MTs readily become hono-MTs, Zn_7 MTs by binding to extracellular Zn^{2+} under the condition of extracellular Zn^{2+} concentration (10 nM) [36], resulting in no effect on Zn - $A\beta_{1-42}$ uptake into dentate granule cells.

Judging from estimated increase in extracellular Zn^{2+} and weakened intracellular Zn^{2+} -buffering in the process of aging [18,40], it is possible that extracellular Zn^{2+} more frequently captures $A\beta_{1-42}$ and that Zn - $A\beta_{1-42}$ formed is rapidly taken up into aged dentate granule cells, resulting in enhanced vulnerability of aged dentate granule cells to Zn^{2+} released from $A\beta_{1-42}$. This process leads the elderly to an increased risk of

Zn-A β_{1-42} -induced cognitive decline. Both uptakes of A β_{1-42} and Zn²⁺ into dentate granule cells were enhanced in aged rats, supporting age-related increase in the risk. Furthermore, LTP induction was more vulnerable to A β_{1-42} in aged dentate gyrus, consistent with enhanced A β_{1-42} -mediated Zn²⁺ uptake into aged dentate granule cells. Hippocampal MTs induced with dexamethasone also rescued the vulnerability of aged dentate granule cells to A β_{1-42} -induced attenuation of LTP. Therefore, once ferried by A β_{1-42} into dentate granule cells, the Zn²⁺ cargo is released in dentate granule cells. This encounter may be facilitated in the normal aging process. Synaptic activity increases both extracellular A β_{1-42} [41,42] and extracellular Zn²⁺ at zincergic synapses [4]. So we hypothesize that a decrease in clearance mechanisms in aging process may lead to the inappropriate combination of Zn²⁺ with A β_{1-42} , age-dependently facilitating the formation of a toxic aggregate that enters neurons.

Zn²⁺- or Cu²⁺-induced aggregates of A β , i.e., soluble oligomers, have been implicated as the neurotoxic form of the peptides against synapse function and structure [43]. The potential for metal chelation-based drug therapy has been reported for AD. Clioquinol, a copper/zinc ionophore reduces zinc accumulation in neuritic plaques and inhibits the amyloidogenic pathway in the brain of the A β PP/PS1 transgenic mouse [44]. It also promotes the degradation of metal-dependent A β oligomers to restore endocytosis and ameliorates A β toxicity [45]. PBT2, a second-generation 8-hydroxyquinoline analog, significantly lowers A β levels in the cerebrospinal fluid and improves cognitive performance over baseline in several key executive function tests [46,47]. In contrast, MTs are non-toxic and functional as endogenous metal chelators. MT induction is an attractive strategy for defending A β_{1-42} -induced cognitive decline and this novel defense strategy may lead to preventing and/or delaying AD pathogenesis. It needs to clarify an effective procedure

to induce hippocampal MTs that continuously defend A β_{1-42} -induced cognitive decline via free Zn²⁺.

Finally we tested whether MTs could deliver Zn²⁺ in LTP induction. The lack of intracellular Zn²⁺, which was induced by injection of ZnAF-2DA into the dentate granule cell layer, attenuated LTP and the attenuation was rescued by preinjection of Zn²⁺ and Cd²⁺ into the same region. It is possible that ZnAF-2DA captures extracellular Zn²⁺ and Zn-bound ZnAF-2DA is taken up into neurons. In the case of injection of 100 μ M ZnAF-2DA into the extracellular compartment (extracellular Zn²⁺:ZnAF-2DA=1:10,000), however, it is likely that a major portion of ZnAF-2DA taken up into neurons is the form without Zn²⁺, resulting in the lack of intracellular Zn²⁺. The occupation of Zn²⁺ in MTs is positively correlated with intracellular Zn²⁺ levels and its occupying rate is lowered under the lack of intracellular Zn²⁺ [36]. Nonetheless, the present data first indicate that induced MTs deliver Zn²⁺ to Zn²⁺-required proteins, which are required for LTP and plastic changes in synapse structure, under the acute lack of intracellular Zn²⁺. Induced apo-MTs increase the influx of extracellular Zn²⁺ for binding Zn²⁺, while they do not modify the basal level of intracellular Zn²⁺ required for synapse function such as LTP. Thus, MTs may serve as a member for functional Zn²⁺-buffering in cognitive activity. However, the Zn²⁺-buffering system may be weakened in the aged dentate gyrus, probably due to characteristics (easiness) of extracellular Zn²⁺ influx [18,40]. The characteristics might be associated with the high basal level of MT proteins in the aged hippocampus, perhaps a compensative mechanism. However, intracellular Zn²⁺ regulation via MTs remains unclear.

In summary, hippocampal MT induction may rescue cognitive decline under lack of cellular transient changes in functional Zn²⁺ concentration, while its induction

is an attractive defense strategy against A β ₁₋₄₂-induced cognitive decline, which is due to excess increase in intracellular Zn²⁺.

Conflict of interest

All authors do not have any known or potential conflict of interest including any financial, personal or other relationships with other people or organizations within three years of beginning the submitted work that could inappropriately influence, or be perceived to influence, their work.

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Figure legend

Figure 1. MT induction rescues attenuated LTP under excess intracellular Zn^{2+}

(A) Saline and $ZnCl_2$ in saline (100 μM) (1 μl) were injected into the dentate granule cell layer of freely moving young rats via an injection cannula at the rate of 0.25 $\mu l/min$ for 4 min. Twenty-three hours later, saline and $ZnCl_2$ in saline (100 μM) (1 μl) were injected in the same manner as shown by the arrow. One hour later, high-frequency stimulation (HFS) was delivered (left). Saline/saline, $n=17$; saline/ $ZnCl_2$, $n=4$; $ZnCl_2/ZnCl_2$, $n=5$; $ZnCl_2/saline$, $n=4$. The magnitude of LTP (middle). *, $p<0.05$, vs. saline; #, $p<0.05$, v.s., saline/Zn (Tukey's test). Representative fEPSP recordings at the time -24 h (black line), -20 min (grey line) and 50 min (red line) are shown (right). Scale bar, vertical axis (6 mV), cross axis (5 ms). (B) Saline and corticosterone (CORT) in saline (100 μM) (1 μl) were injected and treated in the same manner as A (left). Saline/saline, $n=17$; saline/ $ZnCl_2$, $n=4$; CORT/ $ZnCl_2$, $n=5$; CORT/saline, $n=7$. *, $p<0.05$, vs. saline; #, $p<0.05$, v.s., saline/Zn (Tukey's test) (middle).

Figure 2. MT induction rescues attenuated LTP under $A\beta$ -induced increase in intracellular Zn^{2+}

(A) Saline and $ZnCl_2$ in saline (100 μM) (1 μl) were injected in the same manner as Fig. 1A. Twenty-three hours later, saline and $A\beta_{1-42}$ in saline (25 μM) (1 μl) were injected as shown by the arrow (left). Saline/saline, $n=17$; saline/ $A\beta$, $n=10$; $ZnCl_2/A\beta$, $n=5$; $ZnCl_2/saline$, $n=4$. The magnitude of LTP (middle). ***, $p<0.001$, vs. saline; ##, $p<0.01$, v.s., saline/ $A\beta$ (Tukey's test). Representative fEPSP recordings at the time -24 h (black line), -20 min (grey line) and 50 min (red line) are shown (right). Scale bar, vertical axis (6 mV), cross axis (5 ms). (B) Saline and $CdCl_2$ in saline (100 μM) (1 μl) were injected and treated in the same manner as Fig. 2A (left). ***, $p<0.001$, vs.

saline; #, $p < 0.05$, ##, $p < 0.01$, v.s., saline/A β (Tukey's test) (middle). (C) Saline and corticosterone in saline (100 μ M) (1 μ l) were injected and treated in the same manner as Fig. 2A (left). ***, $p < 0.001$, vs. saline; #, $p < 0.05$, ##, $p < 0.01$, v.s. saline/A β (Tukey's test) (middle). (D) Saline and ZnCl₂ in saline (100 μ M) (1 μ l) were bilaterally injected via injection cannulas in the same manner as Fig. 1A. Twenty-three hours later, saline and 25 μ M A β ₁₋₄₂ in saline containing 100 μ M ZnAF-2DA (2 μ l) were injected in the same manner. In vivo Zn²⁺ imaging were performed (left). Intracellular Zn²⁺ levels in the dentate granule cell layer (GCL) were represented by the ratio to the control without A β ₁₋₄₂ in saline expressed as 100% (right). Saline/saline, $n=7$; Saline/A β , $n=10$; ZnCl₂/A β , $n=11$. ***, $p < 0.001$, vs. saline (control). ###, $p < 0.001$, vs. saline/A β (Tukey's test). Bar, 50 μ m.

Figure 3. MT induction modifies intracellular Zn²⁺ levels, but not intracellular A β levels

(A) Saline and dexamethasone sodium phosphate (DEX, 10 mg/kg) in saline were i.p. injected into young rats once a day for two days. Hippocampal MT proteins were determined 6 h (saline, $n=6$; DEX, $n=4$), 12 h (DEX, $n=4$), 24 h (saline, $n=10$. DEX, $n=9$), 48 h (DEX, $n=5$), and 72 h (saline, $n=4$; DEX, $n=6$) after the last injection of DEX. (B) Hippocampal MT proteins 24 h after DEX (10 mg/kg x 2) injection into young (saline, $n=17$; DEX, $n=16$) and aged (saline, 8; DEX, $n=10$) rats. ***, $p < 0.001$ vs. saline; #, $p < 0.05$ vs. young saline. (C) Saline and DEX (10 mg/kg x 2) in saline were injected into young rats in the same manner as Fig. 3A. Twenty-four hours later, saline and 25 μ M A β ₁₋₄₂ in saline containing 100 μ M ZnAF-2DA (2 μ l) were bilaterally injected into the dentate granule cell layer. Five minutes later, intracellular ZnAF-2 fluorescence were captured in the same manner as Fig. 2D (left). Intracellular Zn²⁺ levels in the GCL (right). Saline/saline, $n=10$; Saline/A β , $n=7$; DEX/A β , $n=7$. ***,

$p < 0.001$, vs. saline (control); ###, $p < 0.001$, vs. saline/A β . (Tukey's test). (D) A β_{1-42} immunostaining was performed 5 min after bilateral injection of saline and 50 μ M A β_{1-42} in saline (2 μ l) into the dentate granule cell layer of young rats pretreated with DEX in the same manner as Fig. 3C. Saline/saline, $n=10$; Saline/A β , $n=8$; DEX/A β , $n=8$. *, $p < 0.05$, ***, $p < 0.001$, vs. saline (control). (Tukey's test). Brain slices were prepared from young (E) and aged (F) rats 24 h after DEX (10 mg/kg x 2) injection in the same manner as Fig. 3A, loaded with 10 μ M ZnAF-2DA in ACSF for 30 min, and rinsed in ACSF for 10 min. The brain slices were transferred to a recording chamber filled with ACSF containing 10 nM ZnCl₂ and stimulated with high K⁺ (final concentration of KCl, 50 mM) to measure the changes in intracellular Zn²⁺. Intracellular ZnAF-2 images before stimulation (0 sec) and 300 sec after stimulation (left). Changes in intracellular ZnAF-2 fluorescence in the GCL after stimulation (middle) and 300 sec after stimulation (right). Young saline, $n=13$; Young DEX, $n=18$; Aged saline, $n=13$, aged DEX $n=9$. *, $p < 0.05$, vs. saline (*t*-test).

Figure 4. DEX-induced MTs rescue memory deficit via attenuated LTP

(A) Saline and ZnCl₂ in saline (100 μ M) (1 μ l) were injected in the dentate granule cell layer of young rats under anesthetized condition 24 h after pretreatment with DEX in the same manner as Fig. 3A (arrow) and HFS was delivered 1 h after injection (left). Saline/saline, $n=8$; saline/ZnCl₂, $n=5$; DEX/ZnCl₂, $n=6$; DEX/saline, $n=6$. The magnitude of LTP (middle). ***, $p < 0.001$, vs. saline; #, $p < 0.05$, ###, $p < 0.001$, vs. saline/Zn (Tukey's test). Representative fEPSP recordings at the time -70 min (black line), -20 min (grey line) and 50 min (red line) are shown (right). Scale bar, vertical axis (5 mV), cross axis (5 ms). (B) Saline and A β_{1-42} in saline (25 μ M) (1 μ l) were injected into DEX-pretreated young rats in the same manner as Fig. 4A (arrow) (left). Saline/saline, $n=8$; saline/A β , $n=8$; DEX/A β , $n=5$; DEX/saline, $n=5$. ***, $p < 0.001$, vs.

saline; ^{###}, $p < 0.001$, vs. saline/A β (Tukey's test). (C) Saline and A β_{1-42} in saline (25 μ M) (1 μ l) were injected into DEX-pretreated aged rats in the same manner as Fig. 4A (arrow) (left). Saline/saline, $n=15$; saline/A β , $n=8$; DEX/A β , $n=4$; DEX/saline, $n=4$. *, $p < 0.05$, vs. saline; [#], $p < 0.05$, vs. saline/A β (Tukey's test). (D) One hour after pretreatment with DEX in the same manner as Fig. 3A, young rats were allowed to explore an open field for 10 min. Twenty-three hours later, saline or 25 μ M A β_{1-42} in saline (1 μ l) were bilaterally injected into the dentate granule cell layer of the unanesthetized young rats. One hour after injection, the rats were trained and tested in a novel object recognition task. Saine/saline, $n=11$; saline/A β , $n=11$; DEX/A β , $n=9$; DEX/saline, $n=8$. **, $p < 0.01$, vs. saline, [#], $p < 0.05$, vs. saline/A β_{1-42} (Tukey's test). Note that recognition indices in the test were significantly ($p < 0.01$, t -test) elevated in the saline, DEX/A β_{1-42} , and DEX/saline groups.

Figure 5. In vivo A β uptake and A β -mediated Zn²⁺ uptake that require extracellular Zn²⁺ are enhanced in aged dentate granule cells

(A) Fifty micromolar A β_{1-42} in ACSF (young, $n=13$; aged, $n=11$) and 50 μ M A β_{1-42} + 500 μ M CaEDTA in ACSF (young, $n=11$; aged, $n=12$) were bilaterally injected into the dentate granule cell layer of anesthetized young and aged rats. A β_{1-42} immunostaining in the dentate gyrus were captured 5 min after injection. (B) A β_{1-42} uptake in the GCL determined with Alexa 633 fluorescence, which is represented by the ratio to the young control without A β_{1-42} in ACSF expressed as 100%. **, $p < 0.01$, ***, $p < 0.001$, vs. control (young, $n=15$; aged, 14), ^{##}, $p < 0.01$, ^{###}, $p < 0.001$, vs. A β (Tukey's test). (C) Fifty micromolar A β_{1-42} in ACSF (young, $n=8$; aged, $n=7$) and 50 μ M A β_{1-42} + 500 μ M CaEDTA in ACSF (young, $n=6$; aged, $n=4$) containing ZnAF-2DA (100 μ M) were bilaterally injected in the same manner as Fig. 5A. Intracellular Zn²⁺ fluorescence was captured 5 min after injection as described in Fig.

2D. (D) Intracellular Zn^{2+} level in the GCL determined with intracellular ZnAF-2 fluorescence, which is represented by the ratio to the young control without $A\beta_{1-42}$ in ACSF expressed as 100%. *, $p<0.05$, ***, $p<0.001$, vs. control (young, $n=6$; aged, $n=6$), #, $p<0.05$, ###, $p<0.001$, vs. $A\beta$. \$, $p<0.05$, young vs. aged $A\beta$ (Tukey's test).

Figure 6. Aged rats are vulnerable to $A\beta$ -induced increase in intracellular Zn^{2+}
Saline and $A\beta_{1-42}$ in saline (1 μ l) were injected in the dentate granule cell layer (arrow) of young (A) and aged (B) rats under anesthetized condition and HFS was delivered 1 h after injection (left). Saline, $n=7$; 12.5 μ M $A\beta_{1-42}$, $n=4$; 25 μ M $A\beta_{1-42}$, $n=4$. *, $p<0.05$, vs. saline (Tukey's test) (A, middle). Saline, $n=15$; 10 μ M $A\beta_{1-42}$, $n=4$; 12.5 μ M $A\beta_{1-42}$, $n=9$; 12.5 μ M $A\beta_{1-42}$ /500 μ M CaEDTA, $n=9$. *, $p<0.05$, vs. saline, #, $p<0.05$, vs. 12.5 μ M $A\beta_{1-42}$ (Tukey's test) (B, middle). Representative fEPSP recordings at the time -70 min (black line), -20 min (grey line) and 50 min (red line) are shown (right). Scale bar, vertical axis (5 mV), cross axis (5 ms). Saline and 12.5 μ M $A\beta_{1-42}$ in saline containing ZnAF-2DA (100 μ M) (1 μ l) were bilaterally injected into the dentate granule cell layer of anesthetized young (C) and aged (D) rats. Intracellular Zn^{2+} fluorescence was captured 5 min after injection as described in Fig. 2D (left). Intracellular Zn^{2+} level in the GCL determined in the same manner as Fig. 2D (right). Young saline, $n=14$; young $A\beta$, $n=16$; aged saline, $n=13$; aged $A\beta$, $n=10$. *, $p<0.05$, vs. saline (*t*-test).

Figure 7. MT induction rescues attenuated LTP under lack of intracellular Zn^{2+}

(A) Saline and $ZnCl_2$ in saline (100 μ M) (1 μ l) were injected into the dentate granule cell layer, and then saline and ZnAF-2DA in saline (100 μ M) (1 μ l) were injected 23 h after the injection as shown by the arrow in the same manner as Fig.1 (left). Saline/saline, $n=17$; saline/ZnAF-2DA, $n=10$; $ZnCl_2$ /ZnAF-2DA, $n=7$; $ZnCl_2$ /saline, $n=4$. *, $p<0.05$, vs. saline; #, $p<0.05$, ###, $p<0.001$, v.s. saline/ZnAF-2DA (Tukey's test)

(middle). Representative fEPSP recordings at the time -24 h (black line), -20 min (grey line) and 50 min (red line) are shown (right). Scale bar, vertical axis (6 mV), cross axis (5 ms). (B) Saline and CdCl₂ in saline (100 μM) (1 μl) were injected and treated in the same manner as A (left). Saline/saline, n=17; saline/ZnAF-2DA, n=10; CdCl₂/ZnAF-2DA, n=8; CdCl₂/saline, n=5. *, p<0.05, vs. saline; #, p<0.05, v.s. saline/ZnAF-2DA (Tukey's test) (middle). (C) Saline and ZnAF-2DA in saline (100 μM) (1 μl) were injected into DEX-pretreated young rats in the same manner as Fig. 4A (arrow) and HFS was delivered 1 h after injection (left). Saline/saline, n=8; saline/ZnAF-2DA, n=7; DEX/ZnAF-2DA, n=7; DEX/saline, n=5. ***, p<0.001, vs. saline; ###, p<0.001, vs. saline/ZnAF-2DA (Tukey's test). Representative fEPSP recordings at the time -70 min (black line), -20 min (grey line) and 50 min (red line) are shown (right). Scale bar, vertical axis (5 mV), cross axis (5 ms).