The Target Factor of Heavy Metal Toxicity and Its Molecular Mechanism

Review

Age-Dependent Modification of Intracellular Zn^{2+} Buffering in the Hippocampus and Its Impact

Haruna Tamano* and Atsushi Takeda

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

Received August 17, 2018

The basal concentrations of extracellular Zn^{2+} and intracellular Zn^{2+} , which are approximately 10 nM and 100 pM, respectively, in the brain, are markedly lower than those of extracellular Ca^{2+} (1.3 mM) and intracellular Ca^{2+} (100 nM), respectively, resulting in much less attention paid to Zn^{2+} than to Ca^{2+} . However, intracellular Zn^{2+} dysregulation, which is closely linked with glutamate- and amyloid β -mediated extracellular Zn^{2+} influx, is more critical for cognitive decline and neurodegeneration than intracellular Ca^{2+} dysregulation. It is estimated that the age-dependent increase in the basal concentration of extracellular Zn^{2+} in the hippocampus plays a key role in cognitive decline and neurodegeneration. The characteristics of extracellular Zn^{2+} influx in the hippocampus may be modified age-dependently, probably followed by modification of intracellular Zn^{2+} buffering that is closely linked with age-related cognitive decline and neurodegeneration. Reduction of intracellular Zn^{2+} -buffering capacity may be linked with the pathophysiology of progressive neurodegeneration such as Alzheimer's disease. This paper deals with age-dependent modification of intracellular Zn^{2+} buffering in the hippocampus and its impact. On the basis of the estimated impact, we propose a potential defense strategy against Zn^{2+} -mediated neurodegeneration, *i.e.*, metallothionein induction in the hippocampus.

Key words Zn^{2+} ; metallothionein; ZnAF-2DA; Zn^{2+} buffering; hippocampus; aging

1. INTRODUCTION

Zrt-Irt-like proteins (ZIPs), which are involved in the transport of Zn^{2+} into the cytoplasm, and the zinc transporter (ZnT) family, which is involved in the transport of Zn^{2+} out of the cytoplasm, serve to maintain Zn^{2+} homeostasis in the living body including the brain.^{1–3)} The zinc concentration in the brain increases in the process of development (infant brain, $8.2 \pm 0.8 \mu\text{g/g}$ wet weight) and reaches $13.3 \pm 0.3 \mu\text{g/g}$ wet weight in the human adult brain.⁴⁾ Zinc homeostasis in the brain is strictly regulated through the brain barrier system, *i.e.*, the blood–brain and blood–cerebrospinal fluid (CSF) barriers.^{5,6)} The cells constructing the brain barrier system, which express ZIPs and ZnT, regulate Zn^{2+} transport into the brain parenchyma, *i.e.*, neurons and glial cells.⁷⁾ Zn^{2+} is very slowly transported through the brain barrier system, especially the blood–CSF barrier.^{8,9)}

Approximately 80% of brain zinc is zinc metalloproteins, while approximately 20%, which is histochemically reactive as determined by Timm's sulfide-silver staining,^{10,11)} serves as a signal factor, free Zn^{2+} , in both the intracellular and extracellular compartments. Synaptic Zn^{2+} dynamically functions in conjunction with synaptic activity, especially glutamatergic synapse activity, and is involved in synaptic function.¹²⁾ Synaptic Zn^{2+} plays a key role in cognitive function, while it can also lead to cognitive decline.

Brain aging is characterized by cognitive decline and neuronal loss, while it elevates susceptibility to neurological disorders.¹³⁾ The changes emerging in the process of brain

aging are often linked with the modification of synaptic Zn^{2+} dynamics. Zinc transporter-3 (ZnT-3) protein, which is responsible for Zn^{2+} transport into presynaptic vesicles,¹⁴⁾ regulates the availability of Zn^{2+} at zincergic synapses that concentrate zinc in presynaptic vesicles and activity-dependently releases Zn^{2+} with glutamate. ZnT-3 is involved in hippocampus-dependent memory and its expression decreases with aging.¹⁵⁾ The decrease in ZnT-3 protein may be involved in age-related cognitive decline. Adlard *et al.* reported that clioquinol, a copper/zinc ionophore, and PBT2, a second-generation 8-hydroxyquinoline analogue, which serve as metal chaperones in the brain, increase the availability of Zn^{2+} and prevent normal age-related cognitive decline.^{16,17)} It is estimated that the decrease in the availability of Zn^{2+} , which originates in presynaptic vesicles, is a cause of normal age-related cognitive decline. On the other hand, intracellular Zn^{2+} dysregulation, which is linked with extracellular Zn^{2+} influx mediated by glutamate and amyloid β ($\text{A}\beta$), a key pathogenic peptide of Alzheimer's disease (AD), may readily emerge with aging and is also a cause of normal age-related cognitive decline, as described below.^{18,19)} Therefore, the regulation of intracellular Zn^{2+} dynamics is critical for cognitive activity under physiological and pathological conditions.

2. Zn^{2+} PHYSIOLOGY IN THE BRAIN

Divalent cations such as Ca^{2+} and Mg^{2+} are required for neuronal activity, *i.e.*, synaptic neurotransmission.²⁰⁾ The Ca^{2+} concentration is the highest among divalent cations in

*To whom correspondence should be addressed. e-mail: tamano@u-shizuoka-ken.ac.jp

Fig. 1. Intracellular Zn^{2+} -Buffering Capacity as Assessed with Intracellular ZnAF-2

MTs function in intracellular Zn^{2+} buffering and are mainly in the form of Zn_5MT under basal (static) conditions. Extracellular glutamate accumulation induces Zn^{2+} influx through Ca^{2+} -permeable AMPA receptors (Ca^{2+} -AMPA). Extracellular $\text{A}\beta_{1-42}$ also induces Zn^{2+} influx via the formation of $\text{Zn-A}\beta_{1-42}$ complexes, which is independent of AMPA receptor activation.⁶²⁾ Increased Zn^{2+} is captured with MTs. When MTs are saturated with Zn^{2+} via the formation of Zn_5MT , intracellular free Zn^{2+} is neurotoxic. Induced MTs increase the intracellular Zn^{2+} -buffering capacity, as assessed based on intracellular ZnAF-2 fluorescence. $[\text{Zn}^{2+}]_i$, basal concentration of intracellular Zn^{2+} ; $[\text{Zn}^{2+}]_o$, basal concentration of extracellular Zn^{2+} . (Color figure can be accessed in the online version.)

the brain and is approximately 1.3 mM in the CSF and brain extracellular fluid in adult rats.²¹⁾ Artificial cerebrospinal fluid (ACSF) used for *in vivo* and *in vitro* experiments contains approximately 2 mM Ca^{2+} , based on the essentiality of intracellular Ca^{2+} signaling for neurons and glial cells.^{22,23)} However, excess influx of extracellular Ca^{2+} into neurons, which is induced by glutamate excitotoxicity, is involved in the pathological process of neuronal death.²⁴⁻²⁶⁾

The basal concentrations of extracellular Zn^{2+} and intracellular Zn^{2+} , which are approximately 10 nM²⁷⁾ and 100 pM,^{28,29)} respectively, in the brain, are markedly lower than those of extracellular Ca^{2+} (1.3 mM) and intracellular Ca^{2+} (100 nM), respectively, resulting in much less attention paid to Zn^{2+} than to Ca^{2+} . For studying synaptic function, no attention has been paid to Zn^{2+} ; Zn^{2+} is not included in ACSF, *i.e.*, the brain extracellular medium widely used for *in vitro* and *in vivo* experiments. Spontaneous presynaptic activity in the stratum lucidum where mossy fibers are contained, which is determined with FM4-64, an indicator of exocytosis (presynaptic activity), is suppressed in brain slices from young rats bathed in ACSF containing 10 nM Zn^{2+} , indicating that glutamatergic presynaptic activity is enhanced in brain slices bathed in ACSF without Zn^{2+} .³⁰⁾ We reported that not only neuronal excitation but also synaptic plasticity such as long-term potentiation (LTP) are modified in brain slices bathed in ACSF without Zn^{2+} where the original neurophysiology may be modified.^{30,31)}

The action of extracellular Zn^{2+} at physiological concentrations is important to understand synaptic function precisely as well as to understand bidirectional Zn^{2+} actions under physiological and pathological conditions. It has been recognized that low nanomolar concentrations of Zn^{2+} are more physiologically relevant than its micromolar concentrations, which have been widely used and are often neurotoxic. On the other hand, the role of endogenous Zn^{2+} released from zincergic neurons has been studied in acute brain slice preparations using ACSF without Zn^{2+} . Because zinc concentrations in the presynaptic vesicles are reduced in the process of slice preparation, it is estimated that *in vitro* Zn^{2+} release is reduced to approximately 25% as compared with *in vivo* Zn^{2+} release.³²⁾ Interestingly, the extracellular zinc concentration, which was determined by *in vivo* microdialysis, increases age-dependent-

ly in the hippocampus, suggesting that the extracellular Zn^{2+} concentration is also physiologically increased in the hippocampus with aging.³³⁾

3. MODIFICATION OF INTRACELLULAR Zn^{2+} BUFFERING WITH AGING

It was reported that vulnerability to Ca^{2+} dysregulation is facilitated in the process of brain aging.³⁴⁻³⁶⁾ Ca^{2+} dysregulation is not ubiquitous in the brain but has been observed in specific cell populations and areas. For example, the expression of L-type Ca^{2+} channels is age-dependently elevated in hippocampal pyramidal cells.³⁷⁾ *N*-Methyl-D-aspartate (NMDA) receptor function is age-dependently reduced in the frontal cortex and the hippocampus,³⁸⁾ suggesting that a compensatory mechanism is induced in the process of brain aging to maintain the availability of intracellular Ca^{2+} signaling. On the other hand, intracellular Ca^{2+} buffering, which is involved not only in cognitive function but also in neurodegeneration, is weakened during brain aging.³⁵⁾

To maintain the availability of intracellular Zn^{2+} signaling, it is likely that a compensatory mechanism is also induced in the process of brain aging. The ZnT-3-dependent zinc concentration in presynaptic vesicles is decreased with aging,^{15,39)} while the extracellular zinc concentration is age-dependently increased in the hippocampus.³³⁾ Intracellular Zn^{2+} buffering is critical not only for cognitive function but also for neurodegeneration via regulating the availability of intracellular Zn^{2+} signaling. However, the Zn^{2+} -buffering system is more poorly understood than the Ca^{2+} -buffering system. It was reported that weakened intracellular Ca^{2+} buffering, with a net decrease in the Ca^{2+} -buffering capacity, is linked with both normal aging³⁵⁾ and neurological disorders such as AD.⁴⁰⁾

The Zn^{2+} -buffering system is composed of Zn^{2+} transporters (ZIPs and ZnT), Zn^{2+} -binding proteins such as metallothioneins (MTs), internal stores containing Zn^{2+} , and Ca^{2+} -permeable channels, which is dynamically linked with synaptic activity. Judging from the increased extracellular Zn^{2+} concentration and extracellular Zn^{2+} influx in the aged rat hippocampus,³³⁾ it is estimated that intracellular Zn^{2+} buffering is modified in the dentate gyrus of aged rats.⁴¹⁾ ZnAF-

Fig. 2. Proposed Defense Strategy against Neurotoxic Zn^{2+} Released from $\text{Zn-A}\beta_{1-42}$ Complexes

When $\text{Zn-A}\beta_{1-42}$ complexes are taken up into dentate granule cells, neurotoxic Zn^{2+} is released from the complexes. Neurotoxic Zn^{2+} can be captured with MTs. Thus, the induction of MTs, which increases the intracellular Zn^{2+} -buffering capacity, is a promising defense strategy against neurotoxic Zn^{2+} . (Color figure can be accessed in the online version.)

2DA, a membrane-permeable Zn^{2+} indicator, is taken up into the cells through the plasma membrane and is hydrolyzed by esterase in the cytosol, resulting in the production of ZnAF-2 (dissociation constant $[K_d] = 2.7 \times 10^{-9} \text{ M}$ for Zn^{2+}). Intracellular ZnAF-2 cannot permeate the plasma membrane.^{42,43} Thus we used ZnAF-2DA to determine the changes in the Zn^{2+} -binding capacity, which was assessed by the changes in intracellular ZnAF-2 fluorescence (Fig. 1). The *in vivo* increase in intracellular ZnAF-2 fluorescence is more rapidly lost in the aged dentate molecular layer where medial perforant pathway-dentate granule cell synapses are present than in the young dentate molecular layer, suggesting that intracellular Zn^{2+} -buffering capacity is reduced in the aged dentate gyrus.⁴¹ The characteristics (easiness) of extracellular Zn^{2+} influx may lead to reduced intracellular Zn^{2+} -buffering capacity in the aged dentate gyrus, which represents weakened intracellular Zn^{2+} buffering. It was reported that the dentate gyrus is vulnerable to aging and AD in the hippocampal formation.^{44,45} The vulnerability seems to be linked with age-related modification of extracellular Zn^{2+} influx, which is induced by glutamate and $\text{A}\beta_{1-42}$ in the extracellular compartment.^{33,41,46,47}

4. IMPACT OF MODIFIED INTRACELLULAR Zn^{2+} BUFFERING ON NEURODEGENERATION

Glutamate excitotoxicity is a common pathway to neurodegeneration, and extracellular glutamate accumulation causes neurodegeneration *via* Ca^{2+} influx through calcium channels such as NMDA receptors. On the other hand, Zn^{2+} can pass through calcium channels and preferentially passes through GluR2-lacking Ca^{2+} -permeable α -amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA) receptors.^{48,49} It was reported that glutamate excitotoxicity, which is involved in the pathology of most neurodegenerative disorders including ischemia, epilepsy, and AD, is due to Zn^{2+} neurotoxicity rather than to Ca^{2+} neurotoxicity.⁵⁰⁻⁵² This may be due to the extremely low concentration (approx. 100 pM) of basal intracellular Zn^{2+} ,^{28,29} which reflects the necessity of strict Zn^{2+} regulation in the intracellular (cytosolic) compartment to maintain the intracellular environment.

Aging is a major risk factor for the onset of AD. The first

step in pathological alteration in the AD brain is the abnormal processing of $\text{A}\beta$ peptides and their accumulation, which is initiated more than two decades before the onset of AD.^{53,54} $\text{A}\beta$ is secreted into the extracellular space after sequential cleavage of the amyloid precursor protein (APP).⁵⁵ $\text{A}\beta$ has self-aggregation ability and $\text{A}\beta$ fibrils are the main components of $\text{A}\beta$ plaques, a pathological hallmark of AD. $\text{A}\beta$ is bound to Zn^{2+} *via* histidine residues, and the K_d values of Zn^{2+} to $\text{A}\beta_{1-40}$ are in the range of 0.1–60 μM .⁵⁶ On the other hand, it was reported that the formation and propagation of misfolded aggregates of $\text{A}\beta_{1-42}$ rather than of $\text{A}\beta_{1-40}$ contribute to AD pathogenesis. However, the structural details of misfolded $\text{A}\beta_{1-42}$ are poorly understood.⁵⁷ The C-terminal carboxylate anion of $\text{A}\beta_{1-42}$ constructs the C-terminal hydrophobic core, which accelerates neurotoxic oligomerization.⁵⁸ C-terminal Ala42 that is absent in $\text{A}\beta_{1-40}$ constructs a salt bridge with Lys28 to create a self-recognition molecular switch, which is the $\text{A}\beta_{1-42}$ -selective self-replicating amyloid-propagation machinery.⁵⁹ The aggregating property of $\text{A}\beta_{1-42}$ is rapidly promoted with Zn^{2+} , resulting in much higher affinity of $\text{A}\beta_{1-42}$ to Zn^{2+} than that of $\text{A}\beta_{1-40}$, and the K_d values of Zn^{2+} to $\text{A}\beta_{1-42}$ are in the range of 3–30 nM.⁴⁶ $\text{A}\beta_{1-42}$, unlike $\text{A}\beta_{1-40}$, captures extracellular Zn^{2+} at high picomolar levels, and the formation of $\text{Zn-A}\beta_{1-42}$ in the extracellular compartment is essential for $\text{A}\beta_{1-42}$ uptake into dentate granule cells, followed by cognitive decline (Fig. 2). Soluble $\text{A}\beta_{1-42}$ oligomers that are strong synaptotoxic molecules in AD induce synaptic dysfunction and cognitive decline.^{60,61}

The cytosolic Zn^{2+} concentration in dentate granule cells is much lower than the extracellular Zn^{2+} concentration. When $\text{Zn-A}\beta_{1-42}$ formed is taken up into dentate granule cells, Zn^{2+} can be released from $\text{A}\beta_{1-42}$, resulting in an increase in neurotoxic Zn^{2+} (Fig. 2), which is assessed based on the increase in intracellular ZnAF-2 fluorescence^{46,62} (Fig. 1). $\text{Zn-A}\beta_{1-42}$ uptake is increased in the aged dentate gyrus compared with the young dentate gyrus⁴⁷, consistent with the estimated increase in extracellular Zn^{2+} in the hippocampus with aging.³³ It is likely that Zn^{2+} neurotoxicity, which originates in $\text{A}\beta_{1-42}$, is readily induced with aging. On the other hand, weakened intracellular Zn^{2+} buffering is observed in the aged dentate gyrus.⁴¹ Although aging is a major risk factor for AD, the

characteristic age-related Zn- $A\beta_{1-42}$ formation in the extracellular compartment and weakened intracellular Zn²⁺ buffering may be linked with the major risk.

It is possible that the $A\beta_{1-42}$ -mediated increase in intracellular Zn²⁺ facilitates hyperphosphorylation of the microtubule-associated protein tau, which is a main pathological hallmark as well as $A\beta$ deposits and affects axonal transport, subsequently leading to neurodegeneration. Neuronal loss, tau hyperphosphorylation, and hippocampus-dependent cognitive decline are observed in mice subjected to repeated injection of $A\beta_{1-42}$ oligomers into the hippocampus.⁶³⁾ Tau hyperphosphorylation in AD is linked with a reduction in protein phosphatase 2A (PP2A) activity.^{64,65)} PP2A regulates tau phosphorylation at multiple sites in the normal human brain. Zn²⁺ promotes tau hyperphosphorylation by inactivating PP2A.^{66,67)} Zn²⁺ binds PP2A and directly inhibits its activity at a low micromolar concentration of Zn²⁺ (10 μ M) *in vitro*.⁶⁸⁾ Furthermore, Zn²⁺ 0.25 μ M changes the conformation of tau, and Zn²⁺ 5 μ M promotes tau aggregation *in vitro*.⁶⁹⁾ Although the Zn²⁺ concentrations used in *in vitro* experiments is extremely high compared with physiologically estimated intracellular Zn²⁺ in the *in vivo* picomolar range, it is possible that a local increase in intracellular Zn²⁺ induced by $A\beta_{1-42}$ is linked with tau hyperphosphorylation *via* PP2A inactivation and tau aggregation (Fig. 2).

5. PERSPECTIVE ON A DEFENSE STRATEGY AGAINST NEURODEGENERATION INDUCED BY Zn- $A\beta_{1-42}$

The blockage of $A\beta_{1-42}$ binding to extracellular Zn²⁺ and capturing Zn²⁺ from Zn- $A\beta_{1-42}$ in the intracellular compartment, which are performed using extracellular Zn²⁺ and intracellular Zn²⁺ chelators, respectively, may be an effective strategy for preventing $A\beta_{1-42}$ -mediated cognitive decline and neurodegeneration⁶²⁾ (Fig. 2). *In vivo* local co-injection of $A\beta_{1-42}$ and Ca ethylenediaminetetraacetic acid (EDTA), an extracellular Zn²⁺ chelator, or CdCl₂, a zinc-replacing metal, into the rat dentate gyrus prevents $A\beta_{1-42}$ -mediated impairments of LTP induced at perforant path-dentate granule cell synapses and of recognition memory.⁴⁶⁾ However, CaEDTA and CdCl₂ are not adaptable for clinical use because they cannot permeate the blood-brain barrier.

On the other hand, increasing the intracellular Zn²⁺-buffering capacity may be also an effective strategy for preventing intracellular Zn²⁺ toxicity induced by $A\beta_{1-42}$ and glutamate⁴⁷⁾ (Fig. 1). MTs are major Zn²⁺-binding proteins in the cytosol and play a critical role in intracellular Zn²⁺ buffering. Thus, the induction of MT synthesis can increase the capacity for intracellular Zn²⁺ buffering. Our data show that the systemic administration of dexamethasone, an inducer of MT synthesis, prevents the $A\beta_{1-42}$ -mediated increase in intracellular Zn²⁺ and $A\beta_{1-42}$ -mediated impairments of LTP and recognition memory in young rats⁴⁷⁾ (Fig. 2). The basal level of MTs in the hippocampus is approximately 65 nM in young rats, as determined in the silver-binding assay.⁴⁷⁾ MTs can bind seven Zn²⁺ in the form of Zn₇MT with varying affinity (K_d : approx. 10⁻⁸ to 10⁻¹² M), and cytosolic MTs exist mostly in the form of Zn₅MT under physiological (static) conditions, which has the capacity to capture two more free Zn²⁺.^{70,71)} Thus, it is estimated that hippocampal MTs can buffer 130 nM of

free Zn²⁺ in the cytosol of young rats. Even if the cytosolic Zn²⁺ concentration reaches the extracellular concentration (approx. 10 nM) *via* $A\beta_{1-42}$ -mediated Zn²⁺ influx, induced MTs capture Zn²⁺ released from Zn- $A\beta_{1-42}$. On the other hand, the basal level of MTs in the hippocampus is slightly higher in aged than in young rats. When the increase in intracellular Zn²⁺-buffering capacity is compared between the young and aged hippocampus, as determined based on the reduction in intracellular ZnAF-2 fluorescence, after the systemic administration of dexamethasone, there is no appreciable difference between them.⁴⁷⁾ Therefore, a blood-brain barrier-permeable MT-inducing agent may be promising for preventing neurodegenerative disorders such as AD linked with intracellular Zn²⁺ toxicity⁷²⁾ (Fig. 2).

Conflict of Interest The authors declare no conflict of interest.

REFERENCES

- 1) Dufner-Beattie J, Kuo YM, Gitschier J, Andrews GK. The adaptive response to dietary zinc in mice involves the differential cellular localization and zinc regulation of the zinc transporters ZIP4 and ZIP5. *J. Biol. Chem.*, **279**, 49082–49090 (2004).
- 2) Cousins RJ, Liuzzi JP, Lichten LA. Mammalian zinc transport, trafficking, and signals. *J. Biol. Chem.*, **281**, 24085–24089 (2006).
- 3) Fukada T, Kambe T. Molecular and genetic features of zinc transporters in physiology and pathogenesis. *Metallomics*, **3**, 662–674 (2011).
- 4) Markesbery WR, Ehmann WD, Alauddin M, Hossain TIM. Brain trace element concentrations in aging. *Neurobiol. Aging*, **5**, 19–28 (1984).
- 5) Takeda A. Movement of zinc and its functional significance in the brain. *Brain Res. Brain Res. Rev.*, **34**, 137–148 (2000).
- 6) Takeda A. Zinc homeostasis and functions of zinc in the brain. *Bio-metals*, **14**, 343–351 (2001).
- 7) Belloni-Olivi L, Marshall C, Laal B, Andrews GK, Bressler J. Localization of zip1 and zip4 mRNA in the adult rat brain. *J. Neurosci. Res.*, **87**, 3221–3230 (2009).
- 8) Takeda A, Akiyama T, Sawashita J, Okada S. Brain uptake of trace metals, zinc and manganese, in rats. *Brain Res.*, **640**, 341–344 (1994).
- 9) Takeda A, Sawashita J, Okada S. Localization in rat brain of the trace metals, zinc and manganese, after intracerebroventricular injection. *Brain Res.*, **658**, 252–254 (1994).
- 10) Frederickson CJ. Neurobiology of zinc and zinc-containing neurons. *Int. Rev. Neurobiol.*, **31**, 145–238 (1989).
- 11) Frederickson CJ, Danscher G. Zinc-containing neurons in hippocampus and related CNS structures. *Prog. Brain Res.*, **83**, 71–84 (1990).
- 12) Takeda A, Nakamura M, Fujii H, Tamano H. Synaptic Zn²⁺ homeostasis and its significance. *Metallomics*, **5**, 417–423 (2013).
- 13) Mocchegiani E, Bertoni-Freddari C, Marcellini F, Malavolta M. Brain, aging and neurodegeneration: role of zinc ion availability. *Prog. Neurobiol.*, **75**, 367–390 (2005).
- 14) Cole TB, Wenzel HJ, Kafer KE, Schwartzkroin PA, Palmiter RD. Elimination of zinc from synaptic vesicles in the intact mouse brain by disruption of the ZnT3 gene. *Proc. Natl. Acad. Sci. U.S.A.*, **96**, 1716–1721 (1999).
- 15) Adlard PA, Parncutt JM, Finkelstein DI, Bush AI. Cognitive loss in zinc transporter-3 knock-out mice: a phenocopy for the synaptic and memory deficits of Alzheimer's disease? *J. Neurosci.*, **30**, 1631–1636 (2010).
- 16) Adlard PA, Sedjahtera A, Gunawan L, Bray L, Hare D, Lear J,

- Doble P, Bush AI, Finkelstein DI, Cherny RA. A novel approach to rapidly prevent age-related cognitive decline. *Aging Cell*, **13**, 351–359 (2014).
- 17) Adlard PA, Parncutt J, Lal V, James S, Hare D, Doble P, Finkelstein DI, Bush AI. Metal chaperones prevent zinc-mediated cognitive decline. *Neurobiol. Dis.*, **81**, 196–202 (2015).
 - 18) Takeda A, Tamano H. Impact of synaptic Zn^{2+} dynamics on cognition and its decline. *Int. J. Mol. Sci.*, **18**, 2411 (2017).
 - 19) Takeda A, Tamano H. Is vulnerability of the dentate gyrus to aging and amyloid- β_{1-42} neurotoxicity linked with modified extracellular Zn^{2+} dynamics? *Biol. Pharm. Bull.*, **41**, 995–1000 (2018).
 - 20) Kim NK, Robinson HP. Effects of divalent cations on slow unblock of native NMDA receptors in mouse neocortical pyramidal neurons. *Eur. J. Neurosci.*, **34**, 199–212 (2011).
 - 21) Jones HC, Keep RF. Brain fluid calcium concentration and response to acute hypercalcaemia during development in the rat. *J. Physiol.*, **402**, 579–593 (1988).
 - 22) Neves G, Cooke SF, Bliss TVP. Synaptic plasticity, memory and the hippocampus: A neural network approach to causality. *Nat. Rev. Neurosci.*, **9**, 65–75 (2008).
 - 23) Lisman J, Yasuda R, Raghavachari S. Mechanisms of CaMKII action in long-term potentiation. *Nat. Rev. Neurosci.*, **13**, 169–182 (2012).
 - 24) Alberdi E, Sánchez-Gómez MV, Cavaliere F, Pérez-Samartín A, Zugaza JL, Trullas R, Domercq M, Matute C. Amyloid β oligomers induce Ca^{2+} dysregulation and neuronal death through activation of ionotropic glutamate receptors. *Cell Calcium*, **47**, 264–272 (2010).
 - 25) Bickler PE, Warren DE, Clark JP, Gabatto P, Gregersen M, Brosnan H. Anesthetic protection of neurons injured by hypothermia and rewarming: Roles of intracellular Ca^{2+} and excitotoxicity. *Anesthesiology*, **117**, 280–292 (2012).
 - 26) Abushik PA, Sibarov DA, Eaton MJ, Skatchkov SN, Antonov SM. Kainate-induced calcium overload of cortical neurons *in vitro*: Dependence on expression of AMPAR GluA2-subunit and down-regulation by subnanomolar ouabain. *Cell Calcium*, **54**, 95–104 (2013).
 - 27) Frederickson CJ, Giblin LJ, Krezel A, McAdoo DJ, Muelle RN, Zeng Y, Balaji RV, Masalha R, Thompson RB, Fierke CA, Sarvey JM, de Valdenegro M, Prough DS, Zornow MH. Concentrations of extracellular free zinc (pZn) in the central nervous system during simple anesthetization, ischemia and reperfusion. *Exp. Neurol.*, **198**, 285–293 (2006).
 - 28) Sensi SL, Canzoniero LMT, Yu SP, Ying HS, Koh JY, Kerchner GA, Choi DW. Measurement of intracellular free zinc in living cortical neurons: routes of entry. *J. Neurosci.*, **17**, 9554–9564 (1997).
 - 29) Colvin RA, Bush AI, Volitakis I, Fontaine CP, Thomas D, Kikuchi K, Holmes WR. Insights into Zn^{2+} homeostasis in neurons from experimental and modeling studies. *Am. J. Physiol. Cell Physiol.*, **294**, C726–C742 (2008).
 - 30) Tamano H, Nishio R, Shakushi Y, Sasaki M, Koike Y, Osawa M, Takeda A. *In vitro* and *in vivo* physiology of low nanomolar concentrations of Zn^{2+} in artificial cerebrospinal fluid. *Sci. Rep.*, **7**, 42897 (2017).
 - 31) Takeda A, Tamano H. Significance of low nanomolar concentration of Zn^{2+} in artificial cerebrospinal fluid. *Mol. Neurobiol.*, **54**, 2477–2482 (2017).
 - 32) Suh SW, Danscher G, Jensen MS, Thompson R, Motamedi M, Frederickson CJ. Release of synaptic zinc is substantially depressed by conventional brain slice preparations. *Brain Res.*, **879**, 7–12 (2000).
 - 33) Takeda A, Koike Y, Osawa M, Tamano H. Characteristic of extracellular Zn^{2+} influx in the middle-aged dentate gyrus and its involvement in attenuation of LTP. *Mol. Neurobiol.*, **55**, 2185–2195 (2018).
 - 34) Foster TC. Calcium homeostasis and modulation of synaptic plasticity in the aged brain. *Aging Cell*, **6**, 319–325 (2007).
 - 35) Kumar A, Bodhinathan K, Foster TC. Susceptibility to calcium dysregulation during brain aging. *Front. Aging Neurosci.*, **1**, 2 (2009).
 - 36) Toescu EC, Vreugdenhil M. Calcium and normal brain ageing. *Cell Calcium*, **47**, 158–164 (2010).
 - 37) Thibault O, Landfield PW. Increase in single L-type calcium channels in hippocampal neurons during aging. *Science*, **272**, 1017–1020 (1996).
 - 38) Zamzow DR, Elias V, Shumaker M, Larson C, Magnusson KR. An increase in the association of GluN2B containing NMDA receptors with membrane scaffolding proteins was related to memory declines during aging. *J. Neurosci.*, **33**, 12300–12305 (2013).
 - 39) Lee JY, Kim JS, Byun HR, Palmiter RD, Koh JY. Dependence of the histofluorescently reactive zinc pool on zinc transporter-3 in the normal brain. *Brain Res.*, **1418**, 12–22 (2011).
 - 40) Berridge MJ. Dysregulation of neural calcium signaling in Alzheimer disease, bipolar disorder and schizophrenia. *Prion*, **7**, 2–13 (2013).
 - 41) Takeda A, Tamano H, Murakami T, Nakada H, Minamino T, Koike Y. Weakened intracellular Zn^{2+} -buffering in the aged dentate gyrus and its involvement in erasure of maintained LTP. *Mol. Neurobiol.*, **55**, 3856–3865 (2018).
 - 42) Hirano T, Kikuchi K, Urano Y, Nagano T. Improvement and biological applications of fluorescent probes for zinc, ZnAFs. *J. Am. Chem. Soc.*, **124**, 6555–6562 (2002).
 - 43) Ueno S, Tsukamoto M, Hirano T, Kikuchi K, Yamada MK, Nishiyama N, Nagano T, Matsuki N, Ikegaya Y. Mossy fiber Zn^{2+} spill-over modulates heterosynaptic N-methyl-D-aspartate receptor activity in hippocampal CA3 circuits. *J. Cell Biol.*, **158**, 215–220 (2002).
 - 44) Small SA, Schobel SA, Buxton RB, Witter MP, Barnes CA. A pathophysiological framework of hippocampal dysfunction in ageing and disease. *Nat. Rev. Neurosci.*, **12**, 585–601 (2011).
 - 45) Brickman AM, Khan UA, Provenzano FA, Yeung LK, Suzuki W, Schroeter H, Wall M, Sloan RP, Small SA. Enhancing dentate gyrus function with dietary flavanols improves cognition in older adults. *Nat. Neurosci.*, **17**, 1798–1803 (2014).
 - 46) Takeda A, Tamano H, Tempaku M, Sasaki M, Uematsu C, Sato S, Kanazawa H, Datki ZL, Adlard PA, Bush AI. Extracellular Zn^{2+} is essential for amyloid β_{1-42} -induced cognitive decline in the normal brain and its rescue. *J. Neurosci.*, **37**, 7253–7262 (2017).
 - 47) Takeda A, Tamano H, Hashimoto W, Kobuchi S, Suzuki H, Murakami T, Tempaku M, Koike Y, Adlard PA, Bush AI. Novel defense by metallothionein induction against cognitive decline: from amyloid β_{1-42} -induced excess Zn^{2+} to functional Zn^{2+} deficiency. *Mol. Neurobiol.*, **55**, 7775–7788 (2018).
 - 48) Weiss JH, Sensi SL, Koh JY. Zn^{2+} : a novel ionic mediator of neural injury in brain disease. *Trends Pharmacol. Sci.*, **21**, 395–401 (2000).
 - 49) Frederickson CJ, Koh JY, Bush AI. The neurobiology of zinc in health and disease. *Nat. Rev. Neurosci.*, **6**, 449–462 (2005).
 - 50) Colbourne F, Grooms SY, Zukin RS, Buchan AM, Bennett MV. Hypothermia rescues hippocampal CA1 neurons and attenuates down-regulation of the AMPA receptor GluR2 subunit after forebrain ischemia. *Proc. Natl. Acad. Sci. U.S.A.*, **100**, 2906–2910 (2003).
 - 51) Stork CJ, Li YV. Rising zinc: a significant cause of ischemic neuronal death in the CA1 region of rat hippocampus. *J. Cereb. Blood Flow Metab.*, **29**, 1399–1408 (2009).
 - 52) Sensi SL, Paoletti P, Bush AI, Sekler I. Zinc in the physiology and pathology of the CNS. *Nat. Rev. Neurosci.*, **10**, 780–791 (2009).
 - 53) Jack CR Jr, Knopman DS, Jagust WJ, Shaw LM, Aisen PS, Weiner MW, Petersen RC, Trojanowski JQ. Hypothetical model of dynamic biomarkers of the Alzheimer's pathological cascade. *Lancet Neurol.*, **9**, 119–128 (2010).
 - 54) Selkoe DJ, Hardy J. The amyloid hypothesis of Alzheimer's disease at 25 years. *EMBO Mol. Med.*, **8**, 595–608 (2016).
 - 55) Querfurth HW, LaFerla FM. Alzheimer's disease. *N. Engl. J. Med.*, **362**, 329–344 (2010).
 - 56) Tōgu V, Karafin A, Palumaa P. Binding of zinc(II) and copper(II)

- to the full-length Alzheimer's amyloid-beta peptide. *J. Neurochem.*, **104**, 1249–1259 (2008).
- 57) Ahmed M, Davis J, Aucoin D, Sato T, Ahuja S, Aimoto S, Elliott JI, Van Nostrand WE, Smith SO. Structural conversion of neurotoxic amyloid-beta(1–42) oligomers to fibrils. *Nat. Struct. Mol. Biol.*, **17**, 561–567 (2010).
 - 58) Masuda Y, Uemura S, Ohashi R, Nakanishi A, Takegoshi K, Shimizu T, Shirasawa T, Irie K. Identification of physiological and toxic conformations in Aβ42 aggregates. *ChemBioChem*, **10**, 287–295 (2009).
 - 59) Xiao Y, Ma B, McElheny D, Parthasarathy S, Long F, Hoshi M, Nussinov R, Ishii Y. Aβ(1–42) fibril structure illuminates self-recognition and replication of amyloid in Alzheimer's disease. *Nat. Struct. Mol. Biol.*, **22**, 499–505 (2015).
 - 60) Walsh DM, Klyubin I, Fadeeva JV, Cullen WK, Anwyl R, Wolfe MS, Rowan MJ, Selkoe DJ. Naturally secreted oligomers of amyloid β protein potently inhibit hippocampal long-term potentiation *in vivo*. *Nature*, **416**, 535–539 (2002).
 - 61) Selkoe DJ. Soluble oligomers of the amyloid β-protein impair synaptic plasticity and behavior. *Behav. Brain Res.*, **192**, 106–113 (2008).
 - 62) Takeda A, Nakamura M, Fujii H, Uematsu C, Minamino T, Adlard PA, Bush AI, Tamano H. Amyloid β-mediated Zn²⁺ influx into dentate granule cells transiently induces a short-term cognitive deficit. *PLOS ONE*, **9**, e115923 (2014).
 - 63) Brouillette J, Caillierez R, Zommer N, Alves-Pires C, Benilova I, Blum D, De Strooper B, Buée L. Neurotoxicity and memory deficits induced by soluble low-molecular-weight amyloid-β_{1–42} oligomers are revealed *in vivo* by using a novel animal model. *J. Neurosci.*, **32**, 7852–7861 (2012).
 - 64) Gong CX, Shaikh S, Wang JZ, Zaidi T, Grundke-Iqbal, Iqbal K. Phosphatase activity toward abnormally phosphorylated tau: decrease in Alzheimer's disease brain. *J. Neurochem.*, **65**, 732–738 (1995).
 - 65) Iqbal K, Gong CX, Liu F. Hyperphosphorylation-induced tau oligomers. *Front. Neurol.*, **4**, 112 (2013).
 - 66) Sun XY, Wei YP, Xiong Y, Wang XC, Xie AJ, Wang XL, Yang Y, Wang Q, Lu YM, Liu R, Wang JZ. Synaptic released zinc promotes tau hyperphosphorylation by inhibition of protein phosphatase 2A (PP2A). *J. Biol. Chem.*, **287**, 11174–11182 (2012).
 - 67) Xiong Y, Jing XP, Zhou XW, Wang XL, Yang Y, Sun XY, Qiu M, Cao FY, Lu YM, Liu R, Wang JZ. Zinc induces protein phosphatase 2A inactivation and tau hyperphosphorylation through Src dependent PP2A (tyrosine 307) phosphorylation. *Neurobiol. Aging*, **34**, 745–756 (2013).
 - 68) Xiong Y, Luo DJ, Wang XL, Qiu M, Yang Y, Yan X, Wang JZ, Ye QF, Liu R. Zinc binds to and directly inhibits protein phosphatase 2A *in vitro*. *Neurosci. Bull.*, **31**, 331–337 (2015).
 - 69) Huang Y, Wu Z, Cao Y, Lang M, Lu B, Zhou B. Zinc binding directly regulates tau toxicity independent of tau hyperphosphorylation. *Cell Reports*, **8**, 831–842 (2014).
 - 70) Krężel A, Maret W. The functions of metamorphic metallothioneins in zinc and copper metabolism. *Int. J. Mol. Sci.*, **18**, 1237 (2017).
 - 71) Atrián-Blasco E, Santoro A, Pountney DL, Meloni G, Hureau C, Faller P. Chemistry of mammalian metallothioneins and their interaction with amyloidogenic peptides and proteins. *Chem. Soc. Rev.*, **46**, 7683–7693 (2017).
 - 72) Tamano H, Nishio R, Morioka H, Takeda A. Extracellular Zn²⁺ influx into nigral dopaminergic neurons plays a key role for pathogenesis of 6-hydroxydopamine-induced Parkinson's disease in rats. *Mol. Neurobiol.*, **56**, 435–443 (2019).