In vivo synaptic activity-independent co-uptakes of amyloid β₁–42 and Zn²⁺ into dentate granule cells in the normal brain

Haruna Tamano¹, Naoya Oneta², Aoi Shioya¹, Paul A. Adlard², Ashley I. Bush² & Atsushi Takeda¹

Neuronal amyloid β₁–42 (Aβ₁–42) accumulation is considered an upstream event in Alzheimer’s disease pathogenesis. Here we report the mechanism on synaptic activity-independent Aβ₁–42 uptake in vivo. When Aβ₁–42 uptake was compared in hippocampal slices after incubating with Aβ₁–42, in vitro Aβ₁–42 uptake was preferentially high in the dentate granule cell layer in the hippocampus. Because the rapid uptake of Aβ₁–42 with extracellular Zn²⁺ is essential for Aβ₁–42-induced cognitive decline in vivo, the uptake mechanism was tested in dentate granule cells in association with synaptic activity. In vivo rapid uptake of Aβ₁–42 was not modified in the dentate granule cell layer after co-injection of Aβ₁–42 and tetrodotoxin, a Na⁺ channel blocker, into the dentate gyrus. Both the rapid uptake of Aβ₁–42 and Zn²⁺ into the dentate granule cell layer was not modified after co-injection of CNQX, an AMPA receptor antagonist, which blocks extracellular Zn²⁺ influx. Both the rapid uptake of Aβ₁–42 and Zn²⁺ into the dentate granule cell layer was not also modified after either co-injection of chlorpromazine or genistein, an endocytic repressor. The present study suggests that Aβ₁–42 and Zn²⁺ are synaptic activity-independently co-taken up into dentate granule cells in the normal brain and the co-uptake is preferential in dentate granule cells in the hippocampus. We propose a hypothesis that Zn-Aβ₁–42 oligomers formed in the extracellular compartment are directly incorporated into neuronal plasma membranes and form Zn²⁺-permeable ion channels.

Alzheimer’s disease (AD) has a preclinical phase of 20–30 years prior to clinical onset and is the most common cause of dementia¹². Amyloid-β (Aβ) peptides are produced by processing of amyloid precursor protein (APP)³⁴ and have a characteristic of self-assembly into oligomers in the extracellular compartment⁵. Aβ₁–40 and Aβ₁–42 are the two major isoforms⁶. Aβ₁–42 more readily forms oligomers than Aβ₁–40 and is more neurotoxic⁷. Formation and propagation of misfolded aggregates of Aβ₁–42 may contribute to AD pathogenesis rather than those of Aβ₁–40. C-Terminal carboxylate anion of Aβ₁–42, which forms the C-terminal hydrophobic core, leads to accelerate neurotoxic oligomerization⁸. C-Terminal Ala42 that is absent in Aβ₁–40 forms a salt bridge with Lys28 and constructs a self-recognition molecular switch. The switch is the Aβ₁–42-mediated self-replicating amyloid-propagation machinery⁹.

In AD pathogenesis, Zn²⁺ has been implicated by inducing rapid Aβ oligomerization¹⁰–¹². The aggregation property of Aβ₁–42 is accelerated with Zn²⁺, followed by much higher affinity of Aβ₁–42 (Kₐ = 3–30 nM) to Zn²⁺ than Aβ₁–40 (Kₐ = 0.1–60 μM), which leads to neuronal Zn-Aβ₁–42 uptake and then synaptic dysfunction. In the hippocampus, Aβ₁–42 released into the extracellular compartment can capture Zn²⁺, which is estimated to be approximately 10 nM in the extracellular fluid¹³, even at 500 pM Aβ₁–42 in vivo. We have reported that Aβ₁–42 takes Zn²⁺ as a cargo into dentate granule cells in the normal brain, followed by cognitive decline¹⁴–¹⁶. Although the mechanism of Zn-Aβ₁–42 uptake remains to be solved, extracellular Zn²⁺ is essential for Aβ₁–42 uptake into dentate granule cells¹⁷.

¹Department of Neurophysiology, School of Pharmaceutical Sciences, University of Shizuoka, 52-1 Yada, Suruga-ku, Shizuoka, 422-8526, Japan. ²The Florey Institute of Neuroscience and Mental Health, The University of Melbourne, Parkville, VIC, 3052, Australia. Correspondence and requests for materials should be addressed to A.T. (email: takedaa@u-shizuoka-ken.ac.jp)
It had been reported that Aβ oligomers form Ca\(^{2+}\)-permeable plasma membrane pores that form Aβ channels, leading to a disruption of neuronal Ca\(^{2+}\) homeostasis\(^{17,18}\), which may be linked with synaptic dysfunction and neurodegeneration\(^{19}\). Aβ-mediated Ca\(^{2+}\) channels interact with Zn\(^{2+}\) and Zn\(^{2+}\) blocks extracellular Ca\(^{2+}\) influx in the range of high micromolar concentrations (>250 μM)\(^{20}\), although the gating kinetics and Ca\(^{2+}\) permeability of Aβ pores are not well understood\(^{21}\).

In the present study, we postulate that Zn-Aβ\(^{1–42}\) oligomers formed in the extracellular compartment form Zn\(^{2+}\)-permeable plasma membrane pores, based on the evidence of permeation of Zn\(^{2+}\) through Ca\(^{2+}\) channels\(^{22,23}\). Here we report the mechanism on synaptic activity-independent Aβ\(^{1–42}\) uptake in vivo.

**Results**

When Aβ\(^{1–42}\) is bound to extracellular Zn\(^{2+}\) in vivo, Aβ\(^{1–42}\) is rapidly taken up into dentate granule cells and affects memory via attenuated LTP\(^{14–16}\). Both uptake of Aβ\(^{1–42}\) and Zn\(^{2+}\) are observed 5 min after Aβ\(^{1–42}\) injection into the dentate gyrus, followed by Aβ toxicity and are blocked by coinjection of CaEDTA, an extracellular Zn\(^{2+}\) chelator, followed by blockade of Aβ toxicity. On the other hand, endogenous Zn\(^{2+}\) released from the hippocampal slices promotes Aβ\(^{1–42}\) uptake (retention), which is determined by in vitro Aβ immunohistochemistry (monoclonal antibody 4G8), in the absence of additional extracellular Zn\(^{2+}\)\(^{15}\).

In the present study, we postulate that Zn-Aβ\(^{1–42}\) oligomers formed in the extracellular compartment form Zn\(^{2+}\)-permeable plasma membrane pores, based on the evidence of permeation of Zn\(^{2+}\) through Ca\(^{2+}\) channels\(^{22,23}\). Here we report the mechanism on synaptic activity-independent Aβ\(^{1–42}\) uptake in vivo.

**Figure 1.** In vitro Aβ\(^{1–42}\) uptake in the hippocampus. Aβ immunostaining was determined in the dentate gyrus (DG), the CA3, and the CA1 15 min after incubation with 50μM Aβ\(^{1–42}\) in ACSF. GCL, dentate granule cell layer; PCL, pyramidal cell layer. Aβ uptake in the GCL (n = 32), CA3 PCL (n = 22), and CA1 PCL (n = 22) was determined with Alexa 633 intensity, which was represented by the ratio to Alexa 633 intensity in the control GCL without 50μM Aβ\(^{1–42}\) in ACSF expressed as 100% (lower-right). Alexa 633 intensity in the control CA3 PCL and the control CA1 PCL was also represented by the ratio to Alexa 633 intensity in the control GCL.

**Results**

When Aβ\(^{1–42}\) is bound to extracellular Zn\(^{2+}\) in vivo, Aβ\(^{1–42}\) is rapidly taken up into dentate granule cells and affects memory via attenuated LTP\(^{14–16}\). Both uptake of Aβ\(^{1–42}\) and Zn\(^{2+}\) are observed 5 min after Aβ\(^{1–42}\) injection into the dentate gyrus, followed by Aβ toxicity and are blocked by coinjection of CaEDTA, an extracellular Zn\(^{2+}\) chelator, followed by blockade of Aβ toxicity. On the other hand, endogenous Zn\(^{2+}\) released from the hippocampal slices promotes Aβ\(^{1–42}\) uptake (retention), which is determined by in vitro Aβ immunohistochemistry (monoclonal antibody 4G8), in the absence of additional extracellular Zn\(^{2+}\)\(^{15}\).

In the present study, Aβ\(^{1–42}\) uptake was determined in rat hippocampal slices 15 min after incubation with Aβ\(^{1–42}\). Aβ\(^{1–42}\) uptake was preferentially high in the dentate granule cell layer in the hippocampus, compared with the CA3 and CA1 pyramidal cell layer (Fig. 1).

To examine the involvement of neuronal activity in Aβ\(^{1–42}\) uptake, Aβ\(^{1–42}\) and tetrodotoxin, a Na\(^{+}\) channel blocker, was co-injected into the dentate gyrus of rats. The present dose (15μM) of tetrodotoxin was determined based on the in vivo action as a Na\(^{+}\) channel blocker (10μM)\(^{24}\). Aβ\(^{1–42}\) uptake was determined by ex vivo Aβ immunohistochemistry 5 min after the co-injection. The rapid uptake of Aβ\(^{1–42}\) was not modified in the dentate granule cell layer even by co-injection of tetrodotoxin (Fig. 2).

AMPA receptor activation induces extracellular Zn\(^{2+}\) influx in vivo, which is blocked in the presence of 2 mM CNQX, and the excess influx leads to cognitive decline via attenuated LTP\(^{25,26}\). The present dose of CNQX (2 mM) was used according to the previous papers. Both the rapid uptake of Aβ\(^{1–42}\) and Zn\(^{2+}\) into the dentate granule cell layer was not modified by co-injection of CNQX (Fig. 3), suggesting Aβ\(^{1–42}\)-mediated extracellular Zn\(^{2+}\) influx.

We examined whether endocytosis is involved in the rapid uptake of Aβ\(^{1–42}\) and Zn\(^{2+}\). Chlorpromazine (500μM), a clathrin/caveolae-mediated endocytosis blocker, was used based on the in vivo action as a blocker.
In vivo uptake of Aβ1-42 in the dentate gyrus in the presence of tetrodotoxin. Aβ1-42 immunostaining was determined in the dentate gyrus 5 min after injection of 50μM Aβ1-42 (n = 6), and 50μM Aβ1-42 + 15μM tetrodotoxin (n = 8) in ACSF into the dentate gyrus of anesthetized rats (left). GCL, dentate granule cell layer. Bar, 50μm. Aβ1-42 uptake in the dentate granule cell layer determined with Alexa 633 intensity, which is represented by the ratio to the control (n = 12) without 50μM Aβ1-42 in ACSF expressed as 100%. *p < 0.05, vs. control.

Figure 2.

Discussion

In vivo LTP at medial perforant pathway-dentate granule cell synapses, which is closely linked to object recognition memory, is not affected even under perfusion with 1,000nM Aβ1-42 in ACSF without Zn2+, but attenuated under pre-perfusion with 500pm Aβ1-42 in ACSF containing 10nM Zn2+. The attenuation is rescued by extracellular Zn2+ chelation with CaEDTA. These data indicate that high picomolar Aβ1-42 captures extracellular Zn2+ and subsequently attenuates LTP15. The evidence is consistent with Aβ1-42-induced object recognition memory deficit, which is also rescued by CaEDTA and ZnAF-2DA, an intracellular Zn2+ chelator13. Thus, extracellular Zn2+ is essential for Aβ1-42-induced cognitive decline via attenuated LTP in the normal brain, consistent with in vivo complete blocking rapid uptake of Aβ1-42 and Zn2+ into dentate granule cells after co-injection of Aβ1-42 and CaEDTA into the dentate gyrus15. In the present study, we tested the uptake mechanism of Aβ1-42 oligomers in vitro and in vivo.

When Aβ1-42 uptake was determined in hippocampal slices after incubation with Aβ1-42, Aβ1-42 uptake was preferentially high in the dentate granule cell layer in the hippocampus. Aβ1-42 uptake was also considerably high in the CA3 pyramidal cell layer, while Aβ1-42 uptake was not observed in the CA1 pyramidal cell layer. Extracellular Zn2+ interaction with Aβ1-42 is essential for Aβ1-42 uptake into dentate granule cells in vivo and its essentiality is the same even under in vitro hippocampal slice condition13. Endogenous Zn2+ release from the hippocampal slices is required for the Aβ1-42 uptake. In hippocampal slices bathed in ACSF without Zn2+, extracellular Zn2+ level determined with ZnAF-2 is the highest in the hilar close to the dentate granule cell layer and the second highest in the stratum lucidum close to the CA3 pyramidal cell layer14. Endogenous Zn2+, which is released from mossy fibers, may be closely linked with the Aβ1-42 uptake. Although the Schaffer collaterals also release Zn2+, it is estimated that its release is not enough for the Aβ1-42 uptake into CA1 pyramidal cells in vitro. In contrast, it is estimated that Aβ1-42 captures extracellular Zn2+ and is taken up into CA1 pyramidal cell in vivo. In vivo CA1 LTP is impaired after intracerebroventricular injections of Aβ peptide fragments31, implying that extracellular Zn2+ is potentially involved in the impairment. Intracellular infusion of oligomerised Aβ1-42 via passive diffusion from the patch pipette induces the rapid synaptic insertion of Ca2+-permeable AMPA receptors in CA1 pyramidal cells8. When oligomer Aβ1-42 captures extracellular Zn2+ in vivo, it is also possible that intracellular oligomeric Aβ1-42 affect neuronal functions. Postmortem studies suggest that the hippocampus and entorhinal cortex are the first brain regions to be affected in Alzheimer's disease30,34. The perforant pathway-dentate granule cell synapses are vulnerable to Aβ synapse toxicity34, which may be linked with extracellular Zn2+-mediated Aβ1-42 uptake into dentate granule cells. This uptake may be associated with the high level of extracellular Zn2+ in the hilar that may be due to Zn2+ release form mossy fibers.

The mechanism of Aβ1-42 uptake was tested in the dentate granule cell layer in vivo. When Aβ1-42 was co-injected with tetrodotoxin, a Na+ channel blocker, into the dentate gyrus, the rapid uptake of Aβ1-42 was not modified in the dentate granule cell layer. AMPA receptor activation induces extracellular Zn2+ influx and the
excess influx leads to cognitive decline via attenuated LTP\(^\text{25,26}\). In contrast, both the rapid uptake of A\(\beta\)\(^{1–42}\) and Zn\(^{2+}\) into the dentate granule cell layer was not modified after co-injection of CNQX, an AMPA receptor antagonist, suggesting that Zn-A\(\beta\)\(^{1–42}\) oligomers are ionotropic glutamate receptor activation-independently taken up into dentate granule cells. Both the rapid uptake of A\(\beta\)\(^{1–42}\) and Zn\(^{2+}\) into the dentate granule cell layer was not also modified after either co-injection of chlorpromazine or genistein, an endocytic repressor. It is likely that Zn-A\(\beta\)\(^{1–42}\) oligomers are rapidly taken up into dentate granule cells without interaction with plasma membrane receptor proteins.

In human SH-SY5Y neuroblastoma, monomeric A\(\beta\)\(^{1–42}\) is selectively internalized via clathrin- and dynamin-independent endocytosis compared to monomeric A\(\beta\)\(^{1–40}\)\(^\text{36}\). Genistein, a major phytoestrogen in soybean, may reduce the A\(\beta\)\(^{1–42}\)-induced cell toxicity by suppressing the formation of toxic, cell membrane penetrating A\(\beta\)\(^{1–40}\) oligomers in human SH-SY5Y neuroblastoma\(^\text{37}\). Zn\(^{2+}\) interaction with A\(\beta\)\(^{1–42}\) has not been taken into account in these in vitro cell culture systems, suggesting that Zn-A\(\beta\)\(^{1–42}\) oligomers formed in vivo are taken up into dentate granule cells via a novel mechanism and causes cytotoxicity. On the other hand, A\(\beta\) production occurs in acidic vesicular organelles and contributes both to A\(\beta\) secretion and to the direct accumulation of A\(\beta\) within neurons\(^\text{37}\). Intracellular A\(\beta\) produced by the direct accumulation can exist as a monomeric form that further aggregates into oligomers and it may mediate pathological events\(^\text{38}\), although it is unknown whether Zn\(^{2+}\) is involved in the pathological effects.

The A\(\beta\) ion channel hypothesis has been reported in AD pathogenesis. These authors have reported that A\(\beta\) peptides disrupt Ca\(^{2+}\) homeostasis in neurons and increase intracellular Ca\(^{2+}\) level, resulting in synaptic

**Figure 3.** *In vivo* uptake of A\(\beta\)\(^{1–42}\) and Zn\(^{2+}\) in the dentate gyrus in the presence of CNQX. A\(\beta\)\(^{1–42}\) immunostaining (upper-left) and Zn\(^{2+}\) imaging (upper-right) were determined in the dentate gyrus 5 min after injection of 50\(\mu\)M A\(\beta\)\(^{1–42}\) and 50\(\mu\)M A\(\beta\)\(^{1–42}\) + 2 mM CNQX in ACSF into the dentate gyrus of anesthetized rats. GCL, dentate granule cell layer. Bar, 50 \(\mu\)m. A\(\beta\)\(^{1–42}\) uptake in the dentate granule cell layer determined with Alexa 633 intensity, which is represented by the ratio to the control (control, \(n=6\); A\(\beta\), \(n=5\); A\(\beta\)/CNQX, \(n=6\)) without 50\(\mu\)M A\(\beta\)\(^{1–42}\) in ACSF expressed as 100% (lower-left). Zn\(^{2+}\) uptake in the dentate granule cell layer determined with ZnAF-2 intensity, which is represented by the ratio to the control (control, \(n=11\); A\(\beta\), \(n=10\); A\(\beta\)/CNQX, \(n=6\)) without 50\(\mu\)M A\(\beta\)\(^{1–42}\) in ACSF expressed as 100% (lower-right). *p < 0.05, **p < 0.01, vs. control.
dysfunction and neurodegeneration. The hypothesis is based on the results from in vitro experimental systems such as artificial membranes and neuronal culture\(^{18,39}\). Judging from capturing extracellular Zn\(^{2+}\) with high picomolar (100–500 pM) A\(^{1–42}\) in the hippocampal extracellular compartment, which disrupts Zn\(^{2+}\) homeostasis in neurons and increases intracellular Zn\(^{2+}\) level\(^{14}\), the A\(^{1–42}\)-mediated Ca\(^{2+}\) channel hypothesis might be changed into the A\(^{1–42}\)-mediated Zn\(^{2+}\) channel hypothesis in AD pathogenesis. Because A\(^{1–42}\) stain is observed around the nuclei in dentate granule cells\(^{15}\), a portion of A\(^{1–42}\) is co-taken up with Zn\(^{2+}\) into dentate granule cells even if A\(^{1–42}\)-mediated Zn\(^{2+}\) channels (pores) is formed in the plasma membrane.

In conclusion, the present study suggests that amyloid A\(^{1–42}\) and Zn\(^{2+}\) are synaptic activity-independently co-taken up into dentate granule cells in the normal brain and the co-uptake is preferential in dentate granule cells. We propose a hypothesis that Zn-A\(^{1–42}\) oligomers formed in the extracellular compartment are directly incorporated into neuronal membranes and form Zn\(^{2+}\)-permeable ion channels. Because extracellular Zn\(^{2+}\) is age-relatedly increased in the rat hippocampus\(^{30}\), Zn-A\(^{1–42}\) oligomers are more readily produced in the extracellular compartment of the aged hippocampus, followed by vulnerability to Zn-A\(^{1–42}\) oligomers in aging\(^{36,41}\). Therefore, controlling intracellular Zn\(^{2+}\) dysregulation may be an effective strategy for overcoming AD pathogenesis.

**Experimental Procedures**

**Animals and chemicals.** Wistar rats (male, 7–9 weeks of age) were obtained from Japan SLC (Hamamatsu, Japan). Rats were housed under the standard laboratory conditions (23 ± 1°C, 55 ± 5% humidity) and had access
to water and food ad libitum. Rats were used for experiments approximately 1 week after housing. All the experiments were performed according to the Guidelines for the Care and Use of Laboratory Animals in the University of Shizuoka, which 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 present study has been approved by the Ethics Committee for Experimental Animals in the University of Shizuoka.

Synthetic human Aβ_{1-42} was purchased from ChinaPeptides (Shanghai, China). Aβ was dissolved in saline at the time of need. Aβ_{1-42} prepared in saline was mainly monomers with a small fraction of low order oligomers in SDS-PAGE. ZnAF-2DA (K_d = 2.7 \times 10^{-9} M for Zn^{2+}) is a membrane-permeable Zn^{2+} indicator and was kindly supplied from Sekisui Medical Co., LTD (Hachimantai, Japan). This indicator is taken up into the cells through the plasma membrane and is hydrolyzed by esterase in the cytosol to produce ZnAF-2, which does not permeate the plasma membrane. ZnAF-2 is selectively bound to Zn^{2+}, but not bound to other divalent cations such as Ca^{2+}, Mg^{2+}, and Cu^{2+}. Calcium Orange AM is a membrane-permeable Ca^{2+} indicator and was purchased from Molecular Probes, Inc. (Eugene, OR). The fluorescence indicators were dissolved in dimethyl sulfoxide and diluted to artificial cerebrospinal fluid (ACSF) containing 119 mM NaCl, 2.5 mM KCl, 1.3 mM NaH_2PO_4, 2.5 mM CaCl_2, 26.2 mM NaHCO_3, and 11 mM D-glucose (pH 7.3).

**Hippocampal slice preparation.** Rats were anesthetized with ether and decapitated under anesthesia. The brain was quickly removed from rats and bathed in ice-cold choline-ACSF, which consists of 124 mM choline chloride, 2.5 mM KCl, 2.5 mM MgCl_2, 1.25 mM NaH_2PO_4, 0.5 mM CaCl_2, 26 mM NaHCO_3, and 10 mM glucose (pH 7.3) to block excessive neuronal excitation. Horizontal hippocampal slices (400 μm) were prepared in

**Figure 5.** In vivo uptake of Aβ_{1-42} and Zn^{2+} in the dentate gyrus in the presence of genistein. Aβ_{1-42} immunostaining (upper-left) and Zn^{2+} imaging (upper-right) were determined in the dentate gyrus 5 min after injection of 50 μM Aβ_{1-42}, and 50 μM Aβ_{1-42} + 250 μM genistein in ACSF into the dentate gyrus of anesthetized rats. GCL, dentate granule cell layer. Bar, 50 μm. Aβ_{1-42} uptake in the dentate granule cell layer determined with Alexa 633 intensity, which is represented by the ratio to the control (control, n = 5; Aβ, n = 4; Aβ/genistein, n = 6) without 50 μM Aβ_{1-42} in ACSF expressed as 100% (lower-left). Zn^{2+} uptake in the dentate granule cell layer determined with ZnAF-2 intensity, which is represented by the ratio to the control (control, n = 8; Aβ, n = 5; Aβ/genistein, n = 5) without 50 μM Aβ_{1-42} in ACSF expressed as 100% (lower-right). *p < 0.05, vs. control.
ice-cold choline-ACSF by using a vibratome ZERO-1 (Dosaka Kyoto, Japan). Hippocampal slices were placed in ACSF at 25 °C for at least 30 minutes. All solutions were continuously bubbled with 95% O₂ and 5% CO₂.

**In vitro immunostaining.** Hippocampal slices were bathed in 50μM Aβ₁–42 in ACSF for 15 min. Slices were then rinsed twice with ACSF for 5 min to remove extracellular agents, and fixed with paraformaldehyde (4% in 0.01 M PBS) for 15 min. Slices were rinsed in 0.01 M PBS three times. Slice tissues were blocked in 10% normal goat serum for 30 min, followed by rinse in 0.01 M PBS three times, incubated with 0.1% Triton X-100 for 5 min, rinsed with 0.01 M PBS three times, and bathed at 4 °C in Aβ monoclonal antibody, 4G8 (COVANCE, 1:500 dilution in 0.01 M PBS) for 48 h. Slices were then rinsed with 0.01 M PBS three times, bathed in Alexa Fluor 633 goat anti-mouse IgG secondary antibody (1: 200 dilution in 0.01 M PBS) for 1 h, rinsed with 0.01 M PBS three times, bathed in 4′,6-diamidino-2-phenylindole (DAPI) for 10 min, rinsed again with 0.01 M PBS three times, and mounted on glass slides. Immunostaining images were obtained by using a confocal laser-scanning microscopic system (Nikon A1 confocal microscopes, Nikon Corp.) through a 10× objective. Fluorescence intensity was analyzed by the NIH Image J.

**Ex vivo immunostaining.** Rats anesthetized with chloral hydrate (400 mg/kg) were placed in a stereotoxic apparatus. The skull was exposed and two burr holes were drilled. 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) of the anesthetized rats. Thirty minutes later following recovery from the insertion damage, 50μM Aβ₁–42 in ACSF, or 50μM Aβ₁–42 with either 15μM tetrodotoxin, a Na+ channel blocker, 2μM 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), an α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA) receptor antagonist, 500μM chlorpromazine, a clathrin/caveolae-mediated endocytosis blocker, or 250μM genistein, a caveolae/raft-mediated endocytosis blocker, in ACSF were bilaterally injected into the dentate granule cell layer at the rate of 0.25μl/min for 8 min via the injection cannulae. Five minutes later, the brain was quickly removed from the rats and immunostaining images in hippocampal slices were obtained in the same manner except for changing the 10% goat serum with 5% goat serum. The positions of the injection cannulae were confirmed in the slice preparation.

**In vivo intracellular Zn²⁺ imaging.** Fifty μM Aβ₁–42 in ACSF containing 100μM ZnAF-2DA, or 50μM Aβ₁–42 with either 15μM tetrodotoxin, 2μM CNQX, 500μM chlorpromazine, or 250μM genistein in ACSF containing 100μM ZnAF-2DA was bilaterally injected via injection cannulae into the dentate granule cell layer of anesthetized rats at the rate of 0.25μl/min for 8 min. Five minutes later, the hippocampal slices were prepared in the same manner. Slices were transferred to a chamber filled with ACSF, loaded with 2μM Calcium Orange AM in ACSF for 30 min, and then rinsed in ACSF for 30 min. The hippocampal slices were transferred to a recording chamber filled with ACSF. The fluorescence of ZnAF-2 (laser, 488.4 nm; emission, 500–550 nm), and calcium orange (laser, 561.4 nm; emission, 570–620 nm) was measured with a confocal laser-scanning microscopic system (Nikon A1 confocal microscopes, Nikon Corp.). Calcium Orange AM was used to identify hippocampal regions in slices. The positions of the injection cannulae were confirmed in the slice preparation.

**Data analysis.** For multiple comparisons, differences between treated groups were analyzed by one-way ANOVA followed by post hoc testing using the Tukey’s test. A value of p < 0.05 was considered significant (the statistical software, GraphPad Prism 5). Data were expressed as means ± standard error. The control group with vehicle was compared with the treated groups in all figures for the statistical analysis. In Fig. 1, Aβ group in the DG was also compared with those in the CA3 and the CA1.

**Ethics statement.** All experiments were done according to the Guidelines for the Care and Use of Laboratory Animals of the University of Shizuoka, which refer to 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. All experimental protocols were approved by the ethics committee of the University of Shizuoka.

**References**

1. Nestor, P. J., Scheltens, P. & Hodges, J. R. Advances in the early detection of Alzheimer’s disease. *Nat. Med.* 10(Suppl 1), S34–S41 (2004).
2. Querfurth, H. W. & LaFerla, F. M. Alzheimer’s disease. *N. Engl. J. Med.* 362, 329–344 (2010).
3. Haass, C. et al. Amyloid beta-peptide is produced by cultured cells during normal metabolism. *Nature* 359, 322–325 (1992).
4. Hardy, J. A. & Higgins, G. A. Alzheimer’s disease: the amyloid cascade hypothesis. *Science* 256, 184–185 (1992).
5. Gu, L., Tran, J., Jiang, L. & Guo, Z. A new structural model of Alzheimer’s Aβ42 fibrils based on electron paramagnetic resonance data and Rosetta modeling. *J. Struct. Biol.* 194, 61–67 (2016).
6. Schoonenboom, N. S. et al. Amyloid beta 38, 40, and 42 species in cerebrospinal fluid: more of the same? *Annu. Neurol.* 58, 139–142 (2005).
7. Mucke, L. et al. High-level neuronal expression of abeta 1–42 in wild-type human amyloid protein precursor transgenic mice: synaptotoxicity without plaque formation. *J. Neurosci.* 20, 4050–4058 (2000).
8. Masuda, Y. et al. Identification of physiological and toxic conformations in Abeta42 aggregates. *Chembiochem.* 10, 287–295 (2009).
9. Xiao, Y. et al. Aβ(1–42) fibril structure illuminates self-recognition and replication of amyloid in Alzheimer’s disease. *Nat. Struct. Mol. Biol.* 22, 499–505 (2015).
10. Bush, A. I. The metal theory of Alzheimer’s disease. *J. Alzheimers. Dis.* 33(Suppl 1), S277–281 (2013).
11. Bush, A. I. et al. Rapid induction of Alzheimer A beta amyloid formation by zinc. *Science* 265, 1464–1467 (1994).
12. Adlard, P. A. & Bush, A. I. Metals and Alzheimer’s Disease: How Far Have We Come in the Clinic? *J. Alzheimers Dis.* 62, 1369–1379 (2018).
13. Frederickson, C. J. et al. Concentrations of extracellular free zinc (pZn) in the central nervous system during simple anesthetization, ischemia and reperfusion. *Exp. Neurol.* 198, 285–293 (2006).
14. Takeda, A. et al. Amyloid β-mediated Zn²⁺ influx into dentate granule cells transiently induces a short-term cognitive deficit. *PLoS One* 9, e115923 (2014).
15. Takeda, A. et al. Extracellular Zn\(^{2+}\) is essential for amyloid \(\beta_{1-42}\)-induced cognitive decline in the normal brain and its rescu. J. Neurosci. 37, 7253–7262 (2017).
16. Takeda, A. et al. Novel defense by metallothionein induction against cognitive decline: from amyloid \(\beta_{1-42}\)-induced excess Zn\(^{2+}\) to functional Zn\(^{2+}\) deficiency. Mol. Neurobiol. 55, 7775–7788 (2018).
17. Arispe, N., Rojas, E. & Pollard, H. B. Alzheimer disease amyloid \(\beta\) protein forms calcium channels in bilayer membranes: Blockade by tromethamine and aluminum. Proc. Natl. Acad. Sci. USA 99, 567–571 (1999).
18. Arispe, N., Rojas, E. & Pollard, H. B. Giant multilevel cation channels formed by Alzheimer disease amyloid \(\beta\) protein [A\(\beta\)-(1–40)] in bilayer membranes. Proc. Natl. Acad. Sci. USA 10, 10573–10577 (1993).
19. Kawahara, M. Neurotoxicity of \(\beta\)-amyloid protein: oligomerization, channel formation and calcium dyshomeostasis. Curr. Pharm. Des. 16, 2779–2789 (2010).
20. Arispe, N., Pollard, H. B. & Rojas, E. Zn\(^{2+}\) interactions with Alzheimer's amyloid \(\beta\) protein calcium channels. Proc. Natl. Acad. Sci. USA 93, 1710–1715 (1996).
21. Ullah, G., Demuro, A., Parker, I. & Pearson, J. E. Analyzing and Modeling the Kinetics of Amyloid Beta Pores Associated with Alzheimer's Disease Pathology. PLoS One 10, e0137357 (2015).
22. Kwak, S. & Weiss, J. H. Calcium-permeable AMPA channels in neurodegenerative disease and ischemia. Curr. Opin. Neurobiol. 16, 281–287 (2006).
23. Weiss, J. H. Ca permeable AMPA channels in diseases of the nervous system. Front. Mol. Neurosci. 4, 42 (2011).
24. Yang, G. & Iadecola. Glutamate microinjections in cerebellar cortex reproduce cerebrovascular effects of parallel fiber stimulation. Am. J. Physiol. 271, R1568–1575 (1996).
25. Suzuki, M., Fujise, Y., Tsuchiya, Y., Tamano, H. & Takeda, A. Excess influx of Zn\(^{2+}\) into dentate granule cells affects object recognition memory via attenuated LTP. Neurochem. Int. 87, 60–65 (2015).
26. Takeda, A. et al. Maintained LTP and memory are lost by Zn\(^{2+}\) influx into dentate granule cells, but not Ca\(^{2+}\) influx. Mol. Neurobiol. 55, 1498–1508 (2018).
27. Marchiando, A. M. et al. Caveolin-1-dependent occludin endocytosis is required for TNF-induced tight junction regulation in vivo. J. Cell Biol. 189, 111–126 (2010).
28. Zhang, F., Guo, H., Zhang, J., Chen, Q. & Fang, Q. Identification of the caveolea/raft-mediated endocytosis as the primary entry pathway for aequorin. Virology 513, 193–207 (2018).
29. Takeda, A. et al. Intracellular Zn\(^{2+}\) signaling in the dentate gyrus is required for object recognition memory. Hippocampus 24, 1404–1412 (2014).
30. Minami, A. et al. Inhibition of presynaptic activity by zinc released from mossy fiber terminals during tetanic stimulation. J. Neurosci. Res. 83, 167–176 (2006).
31. Freir, D. B., Holscher, C. & Herron, C. E. Blockade of long-term potentiation by beta-amyloid peptides in the CA1 region of the rat hippocampus in vivo. J. Neurophysiol. 85, 708–713 (2001).
32. Whitcomb, D. J. et al. Intracellular oligomeric amyloid-beta rapidly regulates GluA1 subunit of AMPA receptor in the hippocampus. Sci. Rep. 5, 10934 (2015).
33. Hyman, B. T., Van Hoesen, G. W., Damasio, A. R. & Barnes, C. L. Alzheimer's disease: cell-specific pathology isolates the hippocampal formation. Science 225, 1168–1170 (1984).
34. Moryś., J., Sadowski, M., Barcikowska, M., Maciejewska, B. & Narkiewicz, O. The second layer neurones of the entorhinal cortex and the perforant path in physiological ageing and Alzheimer's disease. Acta Neurobiol. Exp. (Wars) 68, 1404–1412 (2018).
35. Harris, J. A. et al. Transsynaptic propagation of amyloid-\(\beta\)-induced neuronal dysfunction within the entorhinal-hippocampal network. Neuron 68, 428–441 (2010).
36. Wen, E., Jefferis, G. D. M., Drebo, M. M. & Eubjörner, E. K. Endocytic uptake of monomeric amyloid-\(\beta\) peptides is clathrin- and dynamin-independent and results in selective accumulation of A\(\beta\)-(1–42) compared to A\(\beta\)-(1–40). Sci. Rep. 7, 2021 (2017).
37. Ren, B. et al. Genistein: A Dual Inhibitor of Both Amyloid \(\beta\) and Human Islet Amylin Peptides. ACS Chem. Neurosci. 9, 1215–1224 (2018).
38. LaFerla, F. M., Green, K. N. & Oddo, S. Intracellular amyloid-\(\beta\) in Alzheimer's disease. Nat. Rev. Neurosci. 8, 499–509 (2007).
39. Shirwany, N. A., Payette, D., Xie, J. & Guo, Q. The amyloid beta ion channel hypothesis of Alzheimer's disease. Neuropsychiatr. Dis. Treat. 3, 597–612 (2007).
40. 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).
41. Takeda, A. & Tamano, H. Is vulnerability of the dentate gyrus to aging and amyloid-\(\beta\) neurotoxicity linked with modified extracellular Zn\(^{2+}\) dynamics? Biol. Pharm. Bull. 41, 995–1000 (2018).
42. Hirano, T., Kikuchi, K., Uran, Y. & Nagano, T. Improvement and biological applications of fluorescent probes for zinc, ZnAFs. J. Am. Chem. Soc. 124, 6555–6562 (2002).
43. Unen, S. et al. Mossy fiber Zn\(^{2+}\) spillover modulates heterosynaptic N-methyl-D-aspartate receptor activity in hippocampal CA3 circuits. J. Cell Biol. 158, 215–220 (2002).

Author Contributions
Planned experiments: H.T. and A.T. Performed the Experiments: N.O. and A.S. Analyzed data: P.A. and A.B. Wrote the paper: H.T. and A.T. All authors reviewed the manuscript.

Additional Information
Competing Interests: The authors declare no competing interests.

Publisher’s note: Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.