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Metallothionein synthesis increased by Ninjin-yoei-to, a Kampo medicine protects neuronal death and memory loss after exposure to amyloid $\beta_{1\text{-}42}$

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Abstract

Background: It is possible that increased synthesis of metallothioneins (MTs), Zn^{2+}-binding proteins is linked with the protective effect of Ninjin-yoei-to (NYT) on Zn^{2+} toxicity ferried by amyloid $\beta_{1\text{-}42}$ ($A\beta_{1\text{-}42}$).

Methods: Judging from the biological half-life (18-20 h) of MTs,the effective period of newly synthesized MT on capturing Zn^{2+} isestimated to be approximately 2 days. In the present paper, a diet containing3% NYT was administered to mice for 2 days and then $A\beta_{1\text{-}42}$ was injectedinto the lateral ventricle of mice.

Results: MT level in the dentate granule cell layer was elevated 2 days after administration of NYT diet, while the administration reduced intracellular Zn^{2+} level increased 1 h after $A\beta_{1\text{-}42}$ injection, resulting in rescuing neuronal death in the dentate granule cell layer, which was observed 14 days after $A\beta_{1\text{-}42}$ injection. Furthermore, Pre-administration of NYT diet rescued object recognition memory loss via affected perforant pathway long-term potentiation after local injection of $A\beta_{1\text{-}42}$ into the dentate granule cell layer of rats.

Conclusion: The present study indicates that pre-administration of NYT diet for 2 days increases synthesis of MTs, which reduces intracellular Zn^{2+} toxicity ferried by extracellular $A\beta_{1\text{-}42}$, resulting in protecting neuronal death in the dentate gyrus and memory loss after exposure to $A\beta_{1\text{-}42}$.

Keywords: Metallothionein, Amyloid $\beta_{1\text{-}42}$, Alzheimer's disease, Zn^{2+} dysregulation, Ninjin-yoei-to, Kampo medicine

Background

In the Alzheimer's disease (AD) pathogenesis, neuronal accumulation of amyloid $\beta_{1\text{-}42}$ ($A\beta_{1\text{-}42}$), a causative peptide causes synaptic and neuronal losses, which affect hippocampus-dependent memory [1, 2]. In persons with mild cognitive impairment prior to the AD pathogenesis, approximately 30% neurons are lost in the entorhinal cortex and induce synaptic loss to the dentate gyrus. The loss is correlated with cognitive impairment [1, 3] and the perforant pathway-dentate granule cell synapse is an earliest site affected in $A\beta_{1\text{-}42}$-mediated pathogenesis [4]. $A\beta_{1\text{-}42}$ readily captures Zn^{2+} in the extracellular fluid and Zn-$A\beta_{1\text{-}42}$ complexes are preferentially taken up into dentate gyrus neurons, resulting in cognitive impairment and neuronal death, which are linked with intracellular Zn^{2+} toxicity ferried by $A\beta_{1\text{-}42}$ [5–7]. The protection of dentate gyrus neurons against Zn^{2+} toxicity is a potential target to protect the $A\beta_{1\text{-}42}$-mediated pathogenesis [8, 9].

Cholinergic degeneration in the brain is associated with AD pathophysiology and maintenance of choline

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acetyltransferase activity is benefit to the patients with AD [10–12]. It has been reported that donepezil a cholinesterase inhibitor, is effective for the symptom alleviation of the patients with AD [13, 14]. Furthermore, the treatment with both donepezil and Ninjin-yoei-to (NYT), a Kampo medicine more than 2 years ameliorates cognitive performance and alleviates AD-associated depression [15]. However, there is no report that NYT itself is effective on the AD pathophysiology. We have reported that neuronal death in the dentate gyrus induced by $A\beta_{1\text{-}42}$ is protected by pre-administration of NYT for 14 days [16]. In the present study, we presumed that NYT-induced synthesis of metallothioneins (MTs), Zn^{2+}-binding proteins, which may reduce intracellular Zn^{2+} toxicity by $A\beta_{1\text{-}42}$, contributes to the protective effect. On the basis of the data on the biological half-life (18–20 h) of MTs [17], we orally administered NYT diet to mice for 2 days and tested the protective effect on neuronal death in the dentate gyrus. Because intracellular Zn^{2+} toxicity by $A\beta_{1\text{-}42}$ in the dentate gyrus also affects object recognition memory [5], we also checked the effect of NYT diet on memory loss.

Material and methods

NYT diet

NYT obtained from Tsumura & Co. (Tokyo, Japan) was in the form of dried powder extract. NYT was prepared from a mixture of Angelicae radix (4.0 g, root of *Angelica acutiloba* Kitagawa), Hoelen (4.0 g, fungus of *Poria cocos* Wolf), Rehmanniae radix (4.0 g, root of *Rehmannia glutinosa* Lib., var. purpurea Mak), Atractylodis rhizoma (4.0 g, root of *Atractylodes japonica* Koidzumi), Ginseng radix (3.0 g, root of *Panax ginseng* C.A.Mey), Cinnamomi cortex (2.5 g, bark of *Cinnamomum cassia* Bl.), Aurantii nobilis pericarpium (2.0 g, peel of *Citrus unshiu* Markovich), Polygalae radix (2.0 g, root of *Polygala tenuifolia* Willd), Paeoniae radix (2.0 g, root of *Paeonia lactiflora* Pall), Astragali radix (1.5 g, root of *Astragalus membranaceus* Bge.), Glycyrrhizae radix (1.0 g, root of *Glycyrrhiza uralensis* Fisher) and Schisandrae fructus (1.0 g, fruit of *Schisandra chinensis* Baill). A diet containing 3% NYT was prepared by Oriental Yeast Co. Ltd. (Yokohama, Japan). A control diet without NYT was also administered to mice and rats in place of NYT diet. The direct administration via mouth is better for a more accurate dosage, while it was difficult to prepare such an aqueous solution of NYT for administration because of the solubility. The administration as a NYT diet was selected in the present study.

Animals

Male ddY mice (10 weeks of age) and Male Wistar rats (10 weeks of age), which were obtained from Japan SLC (Hamamatsu, Japan), freely access a control diet, a 3% NYT-containing diet, and water. NYT diet did not modify the body weight of mice 4 weeks after administration because of the almost the same intake between the control and NYT diets in the previous study [16]. Body weight of mice and rats was also almost the same between intakes of the control and NYT diets in the present experiments. All the experiments were performed in accordance with the Guidelines for the Care and Use of Laboratory Animals of the University of Shizuoka. The Ethics Committee for Experimental Animals has approved the present study in the University of Shizuoka.

Intracerebroventricular (ICV) injection of Aβ

Saline (vehicle) and $A\beta_{1\text{-}42}$ (ChinaPeptides, Shanghai, China) in saline (25 μM) was delivered into the lateral ventricle of mice at the rate of 0.5 μl/min for 40 min (500 pmol/mouse) via a microinjection canula as described previously [16].

MT immunostaining

A 3% NYT-containing diet was administered to mice for 2 days. The mice were anesthetized with chloral hydrate and perfused with ice-cold 4% paraformaldehyde in PBS, followed by removal of the brain and overnight fixation in 4% paraformaldehyde in PBS at 4 °C. Fixed brains were cryopreserved in 30% sucrose in PBS for 2 day and frozen in Tissue-Tek Optimal Cutting Temperature embedding medium. Coronal brain slices (30 μm) were prepared at -20 °C in a cryostat, picked up on slides, adhered at 50 °C for 60 min, and stored at -20 °C. For immunostaining, the slices were first immersed in PBS for washing, incubated in blocking solution (3% BSA, 0.1% Triton X-100 in PBS) for 1 h, and rinsed with PBS for 5 min followed by overnight incubation with anti-MT antibody [UC1MT] ab12228 (Abcam) in 0.1% Triton X-100 in PBS (1:200 dilution) at 4 °C. The slides were rinsed with PBS for 5 min three times and incubated in blocking buffer containing Alexa Fluor 488 goat anti-mouse secondary antibody (Thermo Fisher Scientific) in 3% BSA, 0.1% Triton X-100 in PBS (1:200 dilution) for 3 h at room temperature. Following three rinses in PBS for 5 min, the slides were bathed in 0.1% DAPI in PBS for 5 min, rinsed with PBS for 5 min three times, mounted with Prolong Gold antifade reagent, and placed at 4 °C for 24 h. Immunostaining images were measured in the dentate gyrus using a confocal laser-scanning microscopic system (Ex/Em: 495 nm/519 nm) (Nikon A1 confocal microscopes, Nikon Corp.) as described previously [16]. To obtain the best fluorescence images and measure the difference in fluorescence intensity among groups exactly, we first checked the relationship between the gain (fluorescence sensitivity) and fluorescence intensity and then carefully

decided the best gain for measuring the exact changes in fluorescence intensity. This decision was separately performed in all experiments (Figs. 1, 2, 3 and 4).

In vitro ZnAF-2 imaging

$A\beta_{1\text{-}42}$ (25 μM) in saline was intracerebroventricularly injected via a microinjection canula at the rate of 0.5 μL/min for 40 min (500 pmol/mouse) of anesthetized mice. One hour after the start of injection, coronal brain slices (400 μm) were prepared in ice-cold choline-Ringer solution containing 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 suppress excessive neuronal excitation. Brain slices were immersed in 2 μM ZnAF-2DA in Ringer solution 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) for 30 min, immersed in ice-cold choline-Ringer solution for 60 min, and transferred to a recording chamber filled with Ringer solution. The fluorescence of ZnAF-2 (Ex/Em: 488 nm/505–530 nm) was captured in the dentate gyrus with a confocal laser-scanning microscopic system.

Propidium iodide (PI) staining

Fourteen days after ICV injection of $A\beta_{1\text{-}42}$, the brain was quickly removed from the mice under anesthesia and immersed in ice-cold choline-Ringer. Coronal brain slices (400 μm) were prepared using a vibratome ZERO-1 (Dosaka Kyoto, Japan) in ice-cold choline-Ringer, which were continuously bubbled with 95% O_2 and 5% CO_2. The brain slices were bathed in PI in Ringer solution (7 μg/ml) for 30 min, bathed in Ringer solution for 30 min and transferred to a recording chamber filled with Ringer solution. PI fluorescence (Ex/Em: 535 nm/617 nm) was captured in the dentate gyrus with a confocal laser-scanning microscopic system.

Fluoro-Jade B (FJB) staining

Fourteen days after ICV injection of $A\beta_{1\text{-}42}$, the mice were anesthetized with chloral hydrate and perfused with ice-cold 4% paraformaldehyde in PBS, followed by removal of the brain and overnight fixation in 4% paraformaldehyde in PBS at 4 °C. Fixed brains were cryopreserved in 30% sucrose in PBS for 2 day and frozen in Tissue-Tek Optimal Cutting Temperature embedding medium. Coronal brain slices (30 μm) were prepared at -20 °C in a cryostat, picked up on slides, adhered at 50 °C for 60 min, and stored at -20 °C. The slides were first immersed in a solution containing 1% sodium hydroxide in 80% alcohol (20 ml of 5% NaOH added to 80 ml ethanol) for 5 min. This was followed by 2 min in 70% ethanol and 2 min in distilled water. The slides were then transferred to a solution of 0.06% potassium permanganate for 15 min on a shaker table to insure consistent background suppression between slices. The slides were then rinsed in distilled

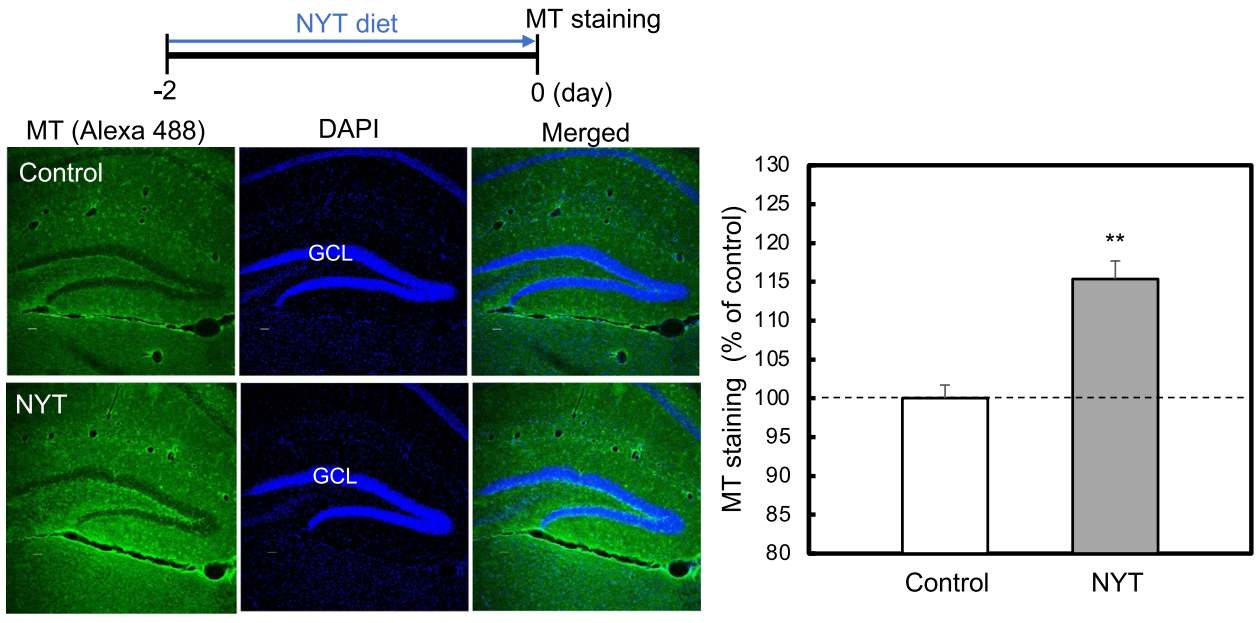

Fig. 1 MT level is elevated 2 days after administration of NYT diet. MT immunostaining was determined in the dentate granule cell layer (GCL) 2 days after administration of NYT diet to mice (left). The data (mean ± SEM) indicate the rate (%) of MT staining after NYT administration to that after the control diet administration that was indicated as 100% (right). **, $p < 0.01$, vs. control (*t*-test). Control, 16 slices from 4 mice; NYT, 20 slices from 5 mice

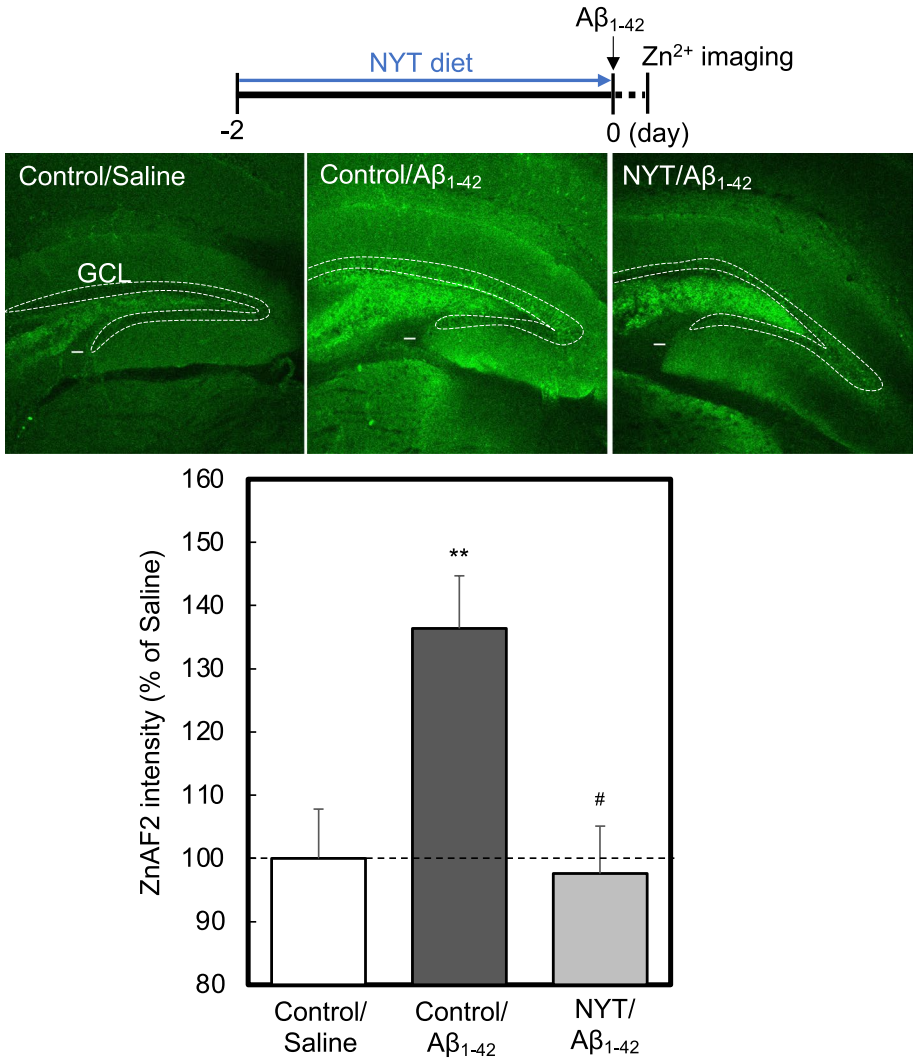

Fig. 2 Administration of NYT diet cancels intracellular Zn^{2+} level increased by $A\beta_{1\text{-}42}$. Intracellular ZnAF-2 fluorescence was determined in the granule cell layer (GCL) of mice 1 h after ICV injection of $A\beta_{1\text{-}42}$ (upper). Bar; 50 μm. The data (mean ± SEM) indicate the rate (%) of ZnAF-2 fluorescence after $A\beta_{1\text{-}42}$ injection to that after saline (vehicle) injection that was indicated as 100% (lower). **, $p < 0.01$, vs. saline: #, $p < 0.05$, vs. Aβ (Tukey's test). control/saline, 32 slices from 8 mice; control/Aβ, 29 slices from 8 mice; NYT/Aβ, 16 slices from 4 mice. Bar; 50 μm

water for 2 min. The staining solution was prepared from a 0.01% stock solution of FJB that was made by adding 10 mg of the dye powder to 100 ml of distilled water. The stock solution and 0.1% 4′,6-diamidino-2-phenylindole (DAPI) in distilled water were diluted with 0.1% acetic acid vehicle, resulting in a final dye concentration of 0.0004% FJB and 0.0001% DAPI in the staining solution. The staining solution was prepared within 10 min of use. The slides were bathed in the staining solution for 30 min and were rinsed for 2 min in each of three distilled water washes. Excess water was briefly removed by using a paper towel. The slides were placed at 50 °C for drying. The dry slides were twice immersed in xylene for 2 min before coverslipping with DPX, a non-aqueous, non-fluorescent plastic mounting media. FJB-positive cells in the unit area were measured in the dentate granule cell layer with a confocal laser-scanning microscopic system (Ex/Em: 480 nm/525 nm).

In vivo long-term potentiation (LTP) recording

Male rats anesthetized with chloral hydrate (400 mg/kg) were 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 inserted to stimulate the perforant pathway of

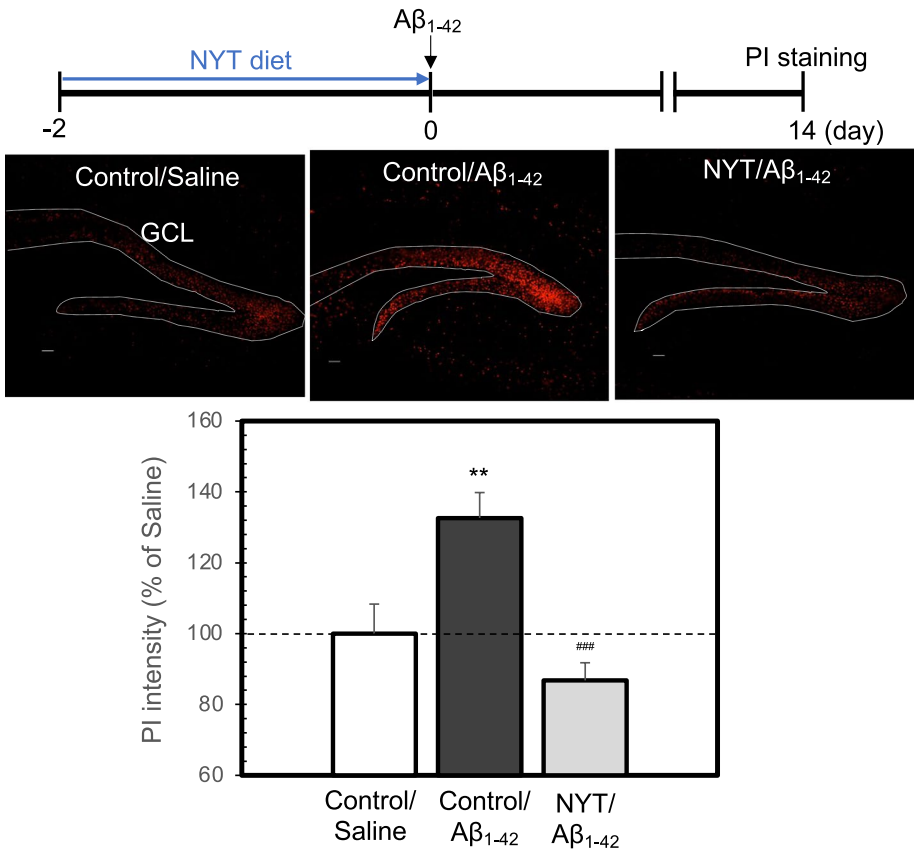

Fig. 3 Neuronal death assessed by PI staining is rescued by NYT diet. PI fluorescence was measured in the granule cell layer (GCL) of mice surrounded by the dotted line 14 days after ICV injection of $A\beta_{1\text{-}42}$ (upper). Bar; 50 μm. The data (mean ± SEM) indicate the rate (%) of PI fluorescence after $A\beta_{1\text{-}42}$ injection to that after saline (vehicle) injection that was indicated as 100% (lower). **, $p < 0.01$, vs. control/saline, ###, $p < 0.001$, vs. control/Aβ (Tukey's test). control/saline, 8 slices from 3 mice; control/Aβ, 8 slices from 3 mice; NYT/Aβ, 14 slices from 5 mice

anesthetized rats and to record in the dentate granule cell layer, respectively, as reported previously [5, 8]. After stable baseline recording for at least 30 min, $A\beta_{1\text{-}42}$ (25 μM) in saline was locally injected into the dentate granule cell layer of anesthetized rats at the rate of 0.25 μl/min for 4 min via an injection cannula attached to a recording electrode. LTP was induced by delivery of high-frequency stimulation (HFS; 10 trains of 20 pulses at 200 Hz separated by 1 s) 1 h after injection and recorded for 60 min.

Object recognition memory

Rats were placed for 10 min into an open field, which was a 70 × 60 cm arena surrounded by 70 cm high walls, made of a black-colored plastic. Twenty-four hours after open field exploration, $A\beta_{1\text{-}42}$ in saline was bilaterally injected via injection cannulas into the dentate granule cell layer of unanesthetized rats in the same manner as in vivo LTP recording section [5, 8]. One hour later, training was performed by placing each rat into the field, in which two identical objects were placed in two adjacent corners, 15 cm from the walls. Rats explored the objects for 5 min. One hour later, the rats explored the open field for 3 min in the presence of one familiar (A) and one novel (B) object. 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

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 ± standard error. The results of statistical analysis are described in every figure legend.

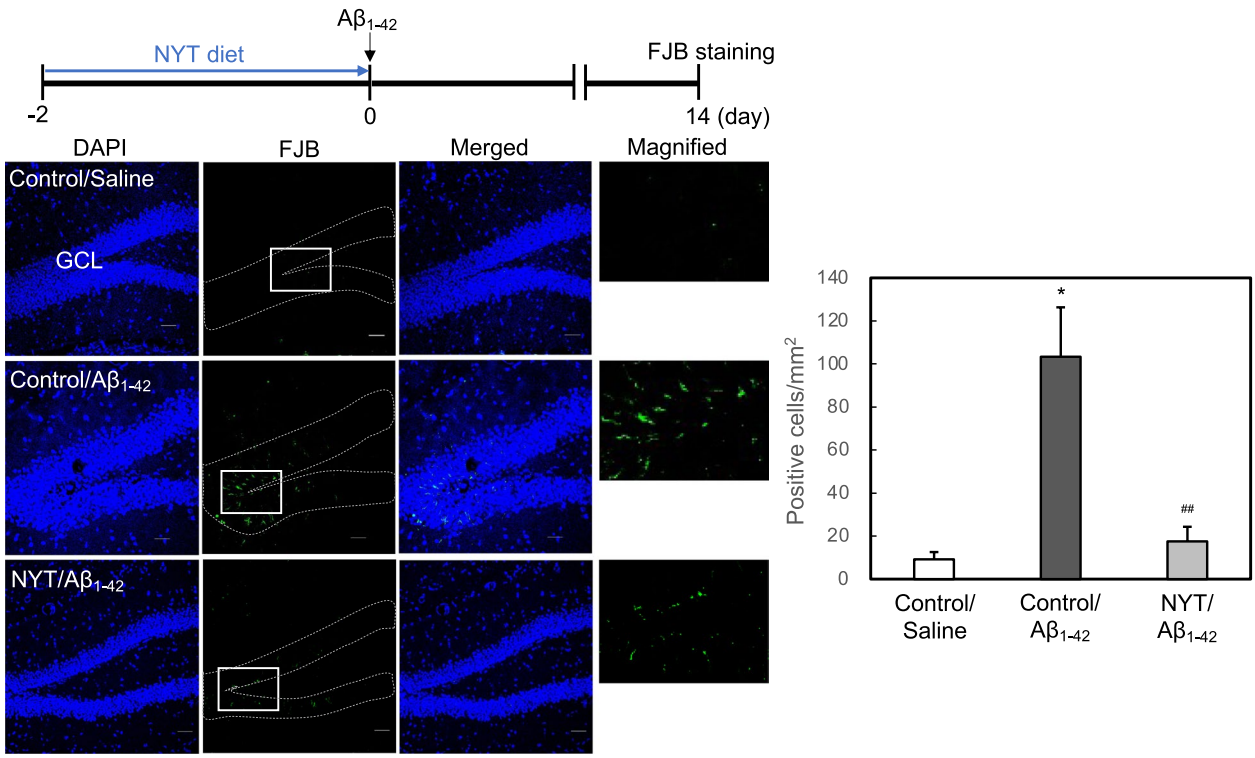

Fig. 4 Neuronal death assessed by FJB staining is rescued by NYT diet. Brain slices were stained with FJB and DAPI. FJB fluorescence was measured in the granule cell layer (GCL) of mice surrounded by the dotted line 14 days after ICV injection of $A\beta_{1\text{-}42}$ (left). Magnified images are the areas from the white square in FJB images. Bar; 50 μm. FJB positive cell number was counted in the GCL. The data (mean ± SEM) indicate FJB-positive cells in the unit area after injection of vehicle or $A\beta_{1\text{-}42}$ (right). *, $p < 0.05$, vs. control/saline, ##, $p < 0.01$, vs. control/Aβ (Tukey's test). control/saline, 12 slices from 4 mice; control/Aβ, 36 slices from 12 mice; NYT/Aβ, 10 slices from 4 mice

Results

NYT-induced MT synthesis reduces Zn^{2+} level increased by $A\beta_{1\text{-}42}$

MTs is a candidate, which reduces intracellular Zn^{2+} level increased by $A\beta_{1\text{-}42}$, and newly synthesized MTs increase the capacity of capturing free Zn^{2+} [7, 8]. The present study was performed focused on the dentate granule cell layer because dentate gyrus neurons are the most vulnerable to $A\beta_{1\text{-}42}$ toxicity in the hippocampus described below [7]. MT level was elevated in the dentate granule cell layer 2 days after administration of NYT diet (Fig. 1). Intracellular Zn^{2+} level, which was assessed by ZnAF-2 fluorescence, was elevated 1 h after ICV injection of $A\beta_{1\text{-}42}$, while the increase was rescued by the pre-administration of NYT diet (Fig. 2). Because $A\beta_{1\text{-}42}$ is taken up into hippocampal cells including dentate gyrus neurons [18], the increase in intracellular Zn^{2+} induced by $A\beta_{1\text{-}42}$ is observed in the hippocampus, resulting in increase in ZnAF-2 intensity in the dentate gyrus by $A\beta_{1\text{-}42}$ in the present study.

Pre-intake of NYT diet rescues neuronal death

After ICV injection of $A\beta_{1\text{-}42}$, neuronal death is preferentially observed in dentate gyrus neurons in the hippocampus [7] because of the high uptake of $A\beta_{1\text{-}42}$ into dentate gyrus neurons [18]. We observed neuronal death in the dentate granule cell layer by using PI and FJB staining. PI fluorescence and FJB-positive cells were increased 14 days after ICV injection of $A\beta_{1\text{-}42}$, while both increases were rescued by the pre-administration of NYT diet (Figs.3 and 4).

Pre-intake of NYY diet rescues affected LTP and memory

In vivo LTP at the perforant pathway-dentate granule cell synapses was induced 1 h after local injection of $A\beta_{1\text{-}42}$ into the dentate granule cell layer via an injection cannula attached to a recording electrode. LTP attenuated by $A\beta_{1\text{-}42}$ was significantly ameliorated after oral administration of NYT diet for 2 days, while the amelioration was not complete (Fig. 5).

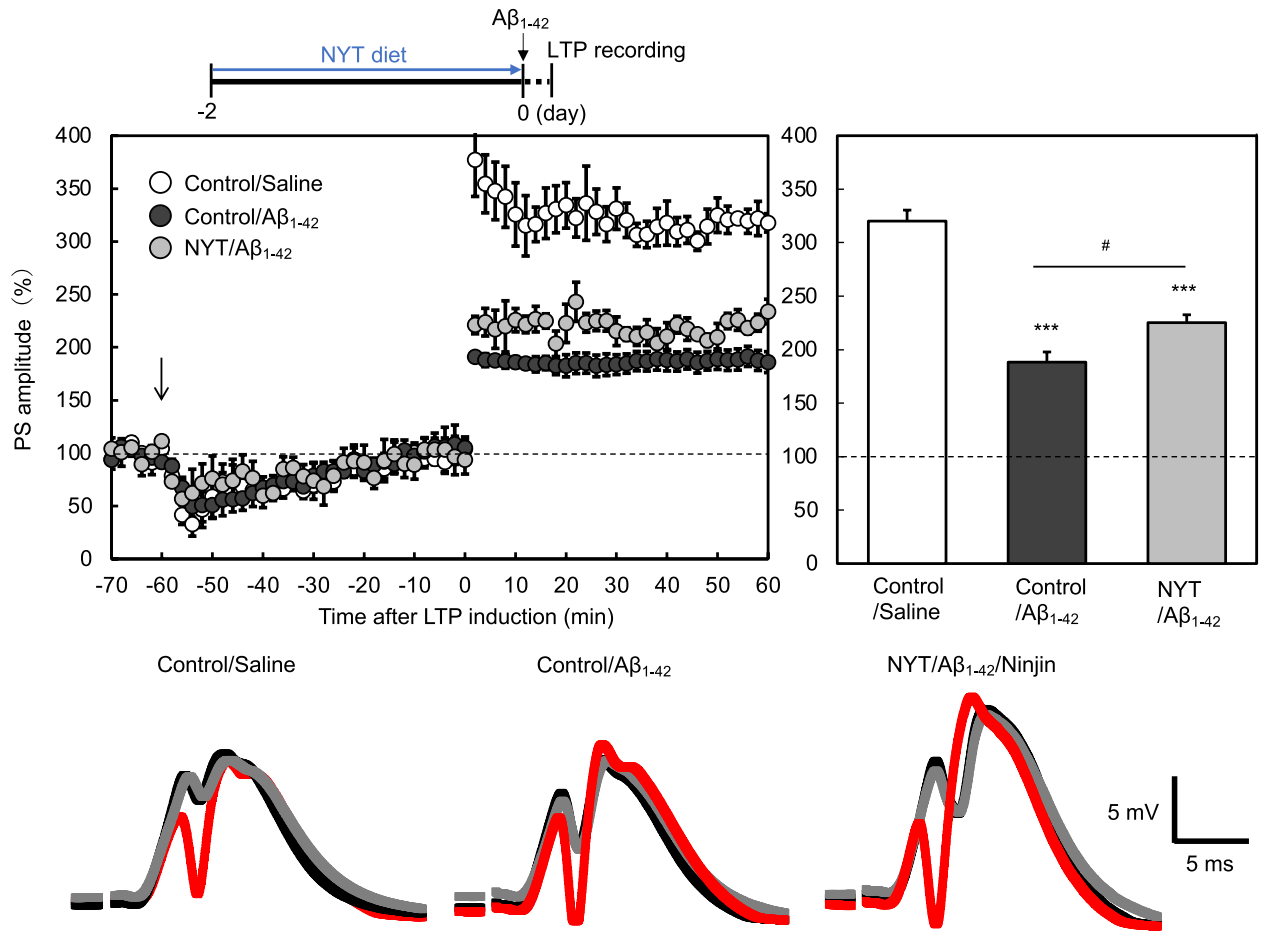

Fig. 5 Attenuated LTP by $A\beta_{1\text{-}42}$ is ameliorated by NYT diet. LTP recording was performed 2 days after administration of NYT diet to rats. LTP was induced 1 h after local injection of saline (vehicle) and $A\beta_{1\text{-}42}$ in saline into the dentate granule cell layer via an injection cannula as shown by the arrow (upper-left). Averaged PS amplitudes for the last 10 min were represented as the magnitude of LTP (upper-right). ***, $p < 0.001$, vs. control/saline; #, $p < 0.05$, vs. control/Aβ (Tukey's test). Representative fEPSP recordings are shown at the time -70 min (before injection; black line), -20 min (after injection; grey line) and 60 min (after tetanic stimulation; red line) (lower). control/saline, $n = 7$ rats; control/Aβ, $n = 7$ rats; NYT/Aβ, $n = 7$ rats

In vivo performant pathway LTP is linked with object recognition memory [5]. When training of the object recognition test was done 1 h after local injection of $A\beta_{1\text{-}42}$ into the dentate granule cell layer, the exploring time was not significantly affected by $A\beta_{1\text{-}42}$ injection and NYT diet administration (Fig. 6). One hour later, the exploring time during the test was not also affected by $A\beta_{1\text{-}42}$ injection and NYT diet administration (Fig. 6). In contrast, object recognition memory was impaired by $A\beta_{1\text{-}42}$ injection, while the impairment was rescued by the intake of NYT diet (Fig. 6).

Discussion

MTs capture 7 equivalents of Zn^{2+} and become a chemical form of Zn_7MTs. The occupation of Zn^{2+}-binding sites in MTs is correlated with Zn^{2+} concentration [19, 20]. Intracellular MTs are mainly a chemical form of Zn_5MTs when intracellular Zn^{2+} is ~ 100 pM, an estimated basal concentration. In vivo K_d value of Zn^{2+} to $A\beta_{1\text{-}42}$ is in the range of ~ 3–30 nM, while that to MTs is ~ 1 pM [6]. Thus, it is estimated that MTs can capture free Zn^{2+} derived from $A\beta_{1\text{-}42}$ in the intracellular compartment. However, it was unclear whether the beneficial effect of NYT is directly linked with increased synthesis of MTs in the previous study [16]. When dexamethasone, an inducer of MT-I and MT-II, is intraperitoneally injected into rats once a day for 2 days, hippocampal MT level is significantly elevated 1 day after injection and reduces the increase in intracellular Zn^{2+} derived from $A\beta_{1\text{-}42}$, followed by rescuing the affected LTP [8]. $A\beta_{1\text{-}42}$-induced neurodegeneration is also rescued after the same treatment with dexamethasone [7]. On the basis of the data that the biological

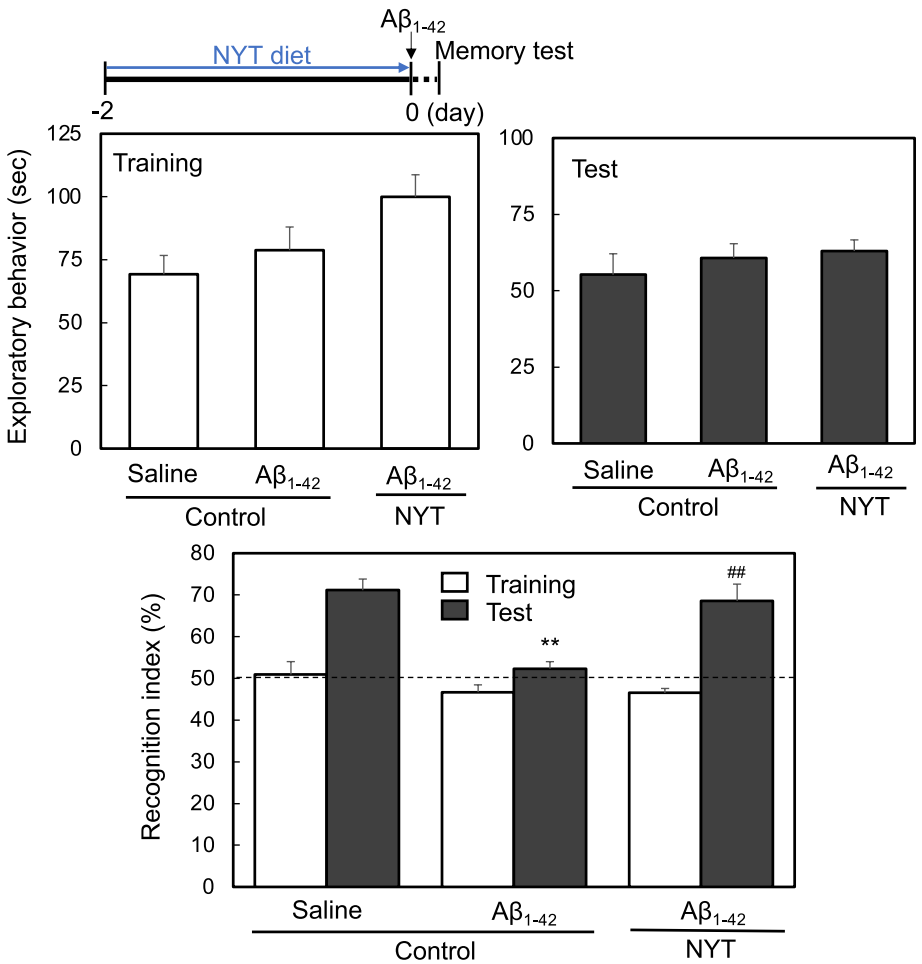

Fig. 6 Exploratory behavior in the training and test of object recognition. The memory test was performed 1 h after local injection of saline (vehicle) and $A\beta_{1\text{-}42}$ in saline into the dentate granule cell layer of rats via an injection cannula in the same manner as Fig. 5. The time of exploratory behavior in the field was measured in the training and test (middle). control/saline, $n = 6$ rats; control/Aβ, $n = 7$ rats; NYT/Aβ, $n = 7$ rats. One hour after training, the memory was evaluated as the recognition index (lower). **, $p < 0.01$, vs. control/saline in test; ##, $p < 0.01$, vs. control/Aβ in test (Tukey's test). control/saline, $n = 6$ rats; control/Aβ, $n = 7$ rats; NYT/Aβ, $n = 7$ rats

half-life of MTs is 18–20 h [17], it is estimated that the effective period of newly synthesized MTs on capturing toxic Zn^{2+} ferried by extracellular $A\beta_{1\text{-}42}$ is a few days when MT synthesis is induced by the intake of NYT diet.

The present study indicates that pre-administration of NYT diet for 2 days increases synthesis of MTs and may reduce intracellular Zn^{2+} toxicity derived from $A\beta_{1\text{-}42}$, resulting in protecting neuronal death by $A\beta_{1\text{-}42}$. It is likely that increased synthesis of MTs plays a key role for the protecting effect of NYT. $A\beta_{1\text{-}42}$-mediated neuronal death is rescued after co-injection of extracellular (CaEDTA) and intracellular (ZnAF-2DA) Zn^{2+} chelators [7], supporting that Zn^{2+} release from intracellular Zn-$A\beta_{1\text{-}42}$ complexes plays a key role for neuronal death.

Hippocampus-related memory of object recognition is affected when in vivo perforant pathway LTP is attenuated after local injection of $A\beta_{1\text{-}42}$ into the dentate granule cell layer [5, 7, 8]. In the present study, in vivo perforant pathway LTP, which was successfully recorded after local injection of $A\beta_{1\text{-}42}$ into the dentate granule cell layer as reported previously [5, 7, 8], was impaired by $A\beta_{1\text{-}42}$, while the impairment was ameliorated by pre-administration of NYT diet for 2 days. Furthermore, the pre-administration of NYT diet for 2 days rescued object recognition memory loss by $A\beta_{1\text{-}42}$, suggesting that increased synthesis of MTs plays a key role for the rescuing effect of NYT on memory loss by $A\beta_{1\text{-}42}$. $A\beta_{1\text{-}42}$-mediated impairments of LTP and memory are also rescued after co-injection of

extracellular (CaEDTA) and intracellular (ZnAF-2DA) Zn^{2+} chelators [5], supporting that Zn^{2+} release from intracellular Zn-$A\beta_{1\text{-}42}$ complexes plays a key role for hippocampal dysfunction.

There is no evidence on MT synthesis in the brain by Kampo medicines. NYT is traditionally used for the patients with insomnia, neurosis, and anorexia [21], suggesting that NYT components may pass through the blood–brain barrier and increase synthesis of MTs in the brain. Unfortunately, there is no evidence on inducers to facilitate MT synthesis in the brain, which are secure for the brain function. The reason is that most MT inducers are not taken up into the brain parenchyma cells because of impermeability against the blood–brain barrier [22]. Exogenous catecholamines including isoproterenol, which cannot pass through the blood–brain barrier, induces MTs in peripheral tissues, e.g., the liver and kidney [23–25]. Isoproterenol, an adrenergic β receptor agonist, enhances MT synthesis in the dentate gyrus and cancels neurodegeneration via intracellular Zn^{2+} toxicity after ICV co-injection of $A\beta_{1\text{-}42}$ and isoproterenol [26]. It is estimated that MT synthesis is enhanced by adrenergic β receptor-mediated signaling after the intake of NYT diet and contributes to ameliorating $A\beta_{1\text{-}42}$ toxicity in the brain. It is necessary to clarify NYT components to lead to adrenergic β receptor-mediated signaling.

Conclusion

The present study suggests that MT synthesis by NYT contributes to protecting neuronal death in the dentate gyrus and memory loss after exposure to $A\beta_{1\text{-}42}$. It is likely that MT synthesis by NYT components protectively act on hippocampal function.

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Not applicable

Authors' contributions
AT originally designed the concept and wrote the manuscript. HT, HT, DM, RT, YN, ET, SW, and MS performed the research and analytical experiments. AT and HT provided interpretation and discussion of the data. All authors read and approved the final manuscript.

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Availability of data and materials
All data supporting the conclusions are included in the manuscript.

Declarations

Ethics approval and consent to participate
The Ethics Committee for Experimental Animals has permitted the present study in the University of Shizuoka.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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