In vitro and in vivo physiology of low nanomolar concentrations of \( \text{Zn}^{2+} \) in artificial cerebrospinal fluid

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Artificial cerebrospinal fluid (ACSF), i.e., brain extracellular medium, which includes \( \text{Ca}^{2+} \) and \( \text{Mg}^{2+} \), but not other divalent cations such as \( \text{Zn}^{2+} \), has been used for in vitro and in vivo experiments. The present study deals with the physiological significance of extracellular \( \text{Zn}^{2+} \) in ACSF. Spontaneous presynaptic activity is suppressed in the stratum lucidum of brain slices from young rats bathed in ACSF containing 10 nM \( \text{ZnCl}_2 \), indicating that extracellular \( \text{Zn}^{2+} \) modifies hippocampal presynaptic activity. To examine the in vivo action of 10 nM \( \text{ZnCl}_2 \), on long-term potentiation (LTP), the recording region was perfused using a recording electrode attached to a microdialysis probe. The magnitude of LTP was not modified in young rats by perfusion with ACSF containing 10 nM \( \text{ZnCl}_2 \), compared to perfusion with ACSF without \( \text{Zn}^{2+} \), but attenuated by perfusion with ACSF containing 100 nM \( \text{ZnCl}_2 \). Interestingly, the magnitude of LTP was not modified in aged rats even by perfusion with ACSF containing 100 nM \( \text{ZnCl}_2 \), but enhanced by perfusion with ACSF containing 10 mM \( \text{CaEDTA} \), an extracellular \( \text{Zn}^{2+} \) chelator. The present study indicates that the basal levels of extracellular \( \text{Zn}^{2+} \), which are in the range of low nanomolar concentrations, are critical for synaptic activity and perhaps increased age-dependently.

Divalent cations such as \( \text{Ca}^{2+} \) and \( \text{Mg}^{2+} \) play key roles for synaptic neurotransmission. Among divalent cations, \( \text{Ca}^{2+} \) concentration is the highest in the brain and is approximately 1.2 mM in the cerebrospinal fluid (CSF) and brain extracellular fluid in the adult rats. The influx of extracellular \( \text{Ca}^{2+} \) into neurons is essential for strengthening of synaptic connections between neurons, i.e., synaptic plasticity such as long-term potentiation (LTP). Approximately 2 mM \( \text{Ca}^{2+} \) is added to conventional artificial cerebrospinal fluid (ACSF) based on essentiality of intracellular \( \text{Ca}^{2+} \) signaling in neurons and glial cells, which is induced by the influx of extracellular \( \text{Ca}^{2+} \). In contrast, excess influx of extracellular \( \text{Ca}^{2+} \) into neurons leads to the pathophysiological process of neurodegeneration associated with glutamate excitotoxicity.

Zinc serves as zinc metalloproteins and is also essential for synaptic neurotransmission. Furthermore, a subclass of glutamatergic neurons is zincergic and is stained by Timm’s sulfide-silver method, which stains histochemically reactive zinc in the presynaptic vesicles. \( \text{Zn}^{2+} \) is co-released with glutamate in activity-dependent manner and play a role as a signal factor as well as \( \text{Ca}^{2+} \). It has been reported that synaptic \( \text{Zn}^{2+} \) signaling is critical for LTP induction in the hippocampus. At mossy fiber-CA3 pyramidal cell synapses and Schaffer collateral-CA1 pyramidal cell synapses, which are zincergic, extracellular \( \text{Zn}^{2+} \), which is increased by LTP induction, serves for intracellular \( \text{Zn}^{2+} \) signaling and is involved in learning and memory. Even at medial perforant pathway-dentate granule cell synapses, which are non-zincergic, intracellular \( \text{Zn}^{2+} \) signaling, which originates in the internal stores containing \( \text{Zn}^{2+} \), is involved in memory acquisition and retention.

\( \text{Zn}^{2+} \) concentration in the brain extracellular fluid, which is estimated to be approximately 10 nM in the adults, is much lower than \( \text{Ca}^{2+} \) concentration. Thus, much less attention has been paid to essentiality of \( \text{Zn}^{2+} \) in brain extracellular fluid for synaptic function. ACSF, i.e., brain extracellular medium, without \( \text{Zn}^{2+} \) has been used for in vitro and in vivo experiments. It is possible that not only neuronal excitation but also synaptic plasticity such as LTP is modified in brain slices bathed in ACSF without \( \text{Zn}^{2+} \), in which original physiology might not appear. Clarifying the action of extracellular \( \text{Zn}^{2+} \) in the range of physiological concentrations is important to understand synaptic function precisely and also to understand the bidirectional action of \( \text{Zn}^{2+} \) under physiological and pathological conditions. Low nanomolar concentrations of \( \text{Zn}^{2+} \) are more physiologically relevant than...
micromolar concentrations of Zn$^{2+}$ widely used, which are often neurotoxic. To assess the physiological range of extracellular Zn$^{2+}$ concentration, in the present study, the action of low nanomolar concentrations of Zn$^{2+}$ added to ACSF was examined in both in vitro and in vivo experiments using young and old rats.

**Results**

**Spontaneous presynaptic activity.** Spontaneous presynaptic activity, which was determined by the attenuation of FM4-64 fluorescence, was assessed in the stratum lucidum containing CA3 mossy fiber boutons of slices bathed in agents in ACSF (Fig. 1). FM4-64 fluorescence intensity was almost the same among four groups at the start of observation (time 0 sec) (upper images in Fig. 1B). Presynaptic activity in brain slices bathed in ACSF without Zn$^{2+}$ was significantly higher than that in brain slices bathed in 10 nM ZnCl$_2$ in ACSF, which was comparable with presynaptic activity in brain slices bathed in ACSF without Zn$^{2+}$ under inhibition of neuronal depolarization in the presence of TTX (Fig. 1B). Presynaptic activity in brain slices bathed in 1 nM ZnCl$_2$ was almost the same as that bathed in ACSF without Zn$^{2+}$.

FM4-64 fluorescence intensity was almost the same between slices bathed in ACSF and ACSF containing 10 nM CuCl$_2$ or ACSF containing 10 nM FeCl$_3$ at the start of observation (time 0 sec) (upper images in Fig. 2). Presynaptic activity in brain slices was not modified by bathing in 10 nM CuCl$_2$ or 10 nM FeCl$_3$ in ACSF (Fig. 2).

**Intracellular levels of Ca$^{2+}$ in the hippocampus.** Variations in the intracellular levels of Ca$^{2+}$ were compared between brain slices prepared with conventional ACSF without Zn$^{2+}$ and bathed in ACSF containing 10 nM ZnCl$_2$. Intracellular Ca$^{2+}$ levels determined with calcium orange were also almost the same in the stratum radiatum, stratum lucidum and dentate molecular layer between the two groups (Fig. 3).

**In vivo dentate gyrus LTP.** To pursue physiological range of extracellular Zn$^{2+}$ concentration, in vivo LTP induction was compared between local perfusion of the recording area with ACSF without Zn$^{2+}$ and ACSF containing Zn$^{2+}$. LTP was not attenuated under perfusion with 10 nM ZnCl$_2$, but significantly attenuated under perfusion with 100 nM ZnCl$_2$ (Fig. 4A).

Adlard *et al.* report that metal chaperones for zinc and copper, i.e., clioquinol and PBT2, prevent normal age-related cognitive decline and demonstrate that the metal chaperones are effective for preventing the...
zinc-mediated cognitive decline that is observed in aging and diseases, suggesting that extracellular Zn$^{2+}$ concentration in the hippocampus is potentially changed along with aging. When LTP was induced under perfusion with 100 nM ZnCl$_2$ in aged rats, it was not attenuated unlike the case of young rats (Fig. 4A and B). Interestingly, LTP was significantly enhanced under perfusion with 10 mM CaEDTA, an extracellular Zn$^{2+}$ chelator, in aged rats (Fig. 4B), although no effect of 10 mM CaEDTA on LTP is reported in young rats$^{15}$.

**Discussion**
Zn$^{2+}$-deficient ACSF has a negative impact on in vitro brain slice preparation and experiments. Hippocampal excitability is attenuated in brain slices pretreated with ACSF containing 20 nM ZnCl$_2$ for 1 h, compared with those pretreated with conventional ACSF without Zn$^{2+}$ $^{20}$. An opposite effect on extracellular Zn$^{2+}$ is observed between in vitro and in vivo LTP induction; in vitro CA1 LTP is enhanced in hippocampal slices bathed in 5 μM

**Figure 2.** No effect of 10 nM CuCl$_2$ and 10 nM FeCl$_3$ on spontaneous mossy fiber exocytosis of young rats. FM4-64 fluorescence images in the CA3 after loading FM4-64 into slices bathed in ACSF and ACSF containing 10 nM Cu$^{2+}$ or ACSF containing 10 nM Fe$^{3+}$ (time 0 sec) (upper). Each point and line (mean ± SEM) represents the changes in FM4-64 fluorescence in the stratum lucidum (SL) of brain slices bathed in ACSF (n = 10), 10 nM CuCl$_2$ (n = 6), and 10 nM FeCl$_3$ (n = 6) after loading FM4-64 (time 0), which is expressed as 100%. (n = 6) (lower).

**Figure 3.** Intracellular Ca$^{2+}$ imaging in the hippocampus of young rats with calcium orange AM. Intracellular calcium orange fluorescence was imaged to estimate the basal levels of cytosolic Ca$^{2+}$ in the hippocampus of brain slices bathed in ACSF (n = 7) and 10 nM ZnCl$_2$ in ACSF (n = 7). SR, stratum radiatum; SL, stratum lucidum; DML, dentate molecular layer (left). Each bar and line (the mean ± SEM) represents fluorescent intensity in the SR, SL, and DML.
ZnCl₂ by using a recording electrode attached to a microdialysis probe 14. The evidence suggests that original 10 nM Zn²⁺ is significantly suppressed in the stratum lucidum of brain slices from young rats bathed in ACSF containing study performed focused on the concentration of 10 nM. Spontaneous presynaptic activity assessed with FM4-64 aged rats by perfusion with ACSF containing 10 mM CaEDTA, while it is not modified in young rats under the perfusate during the perfusion is higher in aged rats than in young rats. The magnitude of LTP was enhanced in slice preparations are poorly suitable for research on roles of endogenous Zn²⁺.

Figure 4. Action of extracellular Zn²⁺ and extracellular Zn²⁺ chelator in dentate gyrus LTP of young and aged rats. (A) Recording region was perfused with ACSF in young rats and then 10 nM (n = 5) and 100 nM (n = 4) ZnCl₂ in ACSF as shown by the shaded bar and HFS (10 trains of 20 pulses at 200 Hz separated by 1 s) was delivered at time 0 min (left). Each bar and line (mean ± SEM) represents the averaged PS amplitude of the last 10 min (time 50–60 min) (middle). **p < 0.001, vs. control (ACSF) (n = 10) (Tukey’s test). Representative fEPSP recordings at time −70 (black line), −30 (grey line) and 50–60 min (red line) are shown (right). (B) Recording region was perfused with ACSF in aged rats and then 100 nM (n = 18) and 10 mM CaEDTA (n = 7) ZnCl₂ in ACSF as shown by the shaded bar and HFS (10 trains of 20 pulses at 200 Hz separated by 1 s) was delivered at time 0 min (left). Each bar and line (mean ± SEM) represents the averaged PS amplitude of the last 10 min (time 50–60 min) (middle). *p < 0.05, vs. control (ACSF) (n = 26) (Tukey’s test). Representative fEPSP recordings at time −70 (black line), −30 (grey line) and 50–60 min (red line) are shown (right).

**ZnCl₂**, while *in vivo* CA1 LTP is attenuated under local perfusion of the recording region with 0.1–1 μM ZnCl₂ by using a recording electrode attached to a microdialysis probe 14. The evidence suggests that original synaptic activity is modified in slice experiments using ACSF without Zn²⁺ and that addition of Zn²⁺ to ACSF is necessary to prevent modification of synaptic function.

To clarify the physiological range of extracellular Zn²⁺ concentration, the action of low nanomolar concentrations of Zn²⁺ in ACSF was examined in both *in vitro* and *in vivo* experiments. On the basis of the estimated concentration of extracellular Zn²⁺ under the basal (static) condition, which is approximately 10 nM 17, the present study performed focused on the concentration of 10 nM. Spontaneous presynaptic activity assessed with FM4-64 is significantly suppressed in the stratum lucidum of brain slices from young rats bathed in ACSF containing 10 nM Zn²⁺, but not in ACSF containing 10 nM Cu²⁺ or 10 nM Fe³⁺, indicating that hippocampal presynaptic activity is enhanced in brain slices prepared with ACSF without Zn²⁺. Micromolar Zn²⁺ also suppresses hippocampal mossy fiber exocytosis 5. It is likely that Zn²⁺ dose-dependently suppresses presynaptic activity in the hippocampus. On the other hand, the basal levels of intracellular Ca²⁺ determined with calcium orange were not modified in the hippocampus of brain slices prepared with ACSF without Zn²⁺. Suh et al. 23 report that acute brain slice preparations are poorly suitable for research on roles of endogenous Zn²⁺ released from zincergic neurons. Vesicular zinc determined by Timm’s sulfide-silver method is lost during slice preparation and slice incubation; *in vitro* Zn²⁺ release is reduced to about 25% of *in vivo* Zn²⁺ release. It is likely that extracellular Zn²⁺ is physiologically critical in the range of low nanomolar concentrations for *in vitro* slice experiments from young animals.

To examine the action of 10 nM Zn²⁺ on *in vivo* LTP induction, the recording region was perfused using a recording electrode attached to a microdialysis probe. The magnitude of LTP was not significantly modified in young rats by perfusion with ACSF containing 10 nM Zn²⁺, compared to perfusion with ACSF without Zn²⁺, but attenuated by perfusion with ACSF containing 100 nM Zn²⁺. Because 100 nM Zn²⁺ also attenuates CA1 LTP 14, this concentration of Zn²⁺ is beyond the range of physiological concentration in young rats as the basal concentration of extracellular Zn²⁺. The present study indicates that extracellular Zn²⁺ modulates LTP at low nanomolar concentrations in young rats. Interestingly, the magnitude of LTP was not modified in aged rats by perfusion with ACSF containing 100 nM Zn²⁺. The perfusate reaches the equilibrium state with the brain extracellular fluid containing Zn²⁺, resulting in the perfusate (ACSF) with Zn²⁺. It is possible that Zn²⁺ concentration in the perfusate during the perfusion is higher in aged rats than in young rats. The magnitude of LTP was enhanced in aged rats by perfusion with ACSF containing 10 mM CaEDTA, while it is not modified in young rats under the
same condition. It is likely that extracellular Zn$^{2+}$ concentration is increased age-dependently and that 100 nM Zn$^{2+}$ is in the range of physiological concentration in aged rats. Extracellular Zn$^{2+}$ may suppressively modulate LTP induction, especially in aged rats.

Zinc concentration in the CSF is reported to be 150–380 nM$^{25–27}$. If it is the same as zinc concentration in the brain extracellular fluid, a large portion of zinc is not free ion in the brain extracellular fluid under the basal condition. At zincergic synapses, extracellular Zn$^{2+}$ levels are dynamically changed by the degree of Zn$^{2+}$ activity-dependently released from neuron terminals, which is required for learning and memory via synaptic plasticity$^{14}$. Even at non-zincergic synapses, extracellular Zn$^{2+}$ may serve as a pool for Zn$^{2+}$ release from the internal stores, which is also required for learning and memory$^{13}$. In conclusion, the present study indicates that original neurophysiology may not appear in ACSF without Zn$^{2+}$. The basal levels of extracellular Zn$^{2+}$, which are in the range of low nanomolar concentrations, are critical for synaptic activity and perhaps increased age-dependently. Homeostasis of Zn$^{2+}$ in both extracellular and intracellular compartments is still poorly understood and the understanding is important to search a strategy for preventing Zn$^{2+}$-mediated cognitive decline.

**Experimental Procedures**

**Chemicals and animals.** Male Wistar rats were purchased from Japan SLC (Hamamatsu, Japan) and used as young (6–9 weeks of age) and aged (>60 weeks of age) rats. They were housed under the standard laboratory conditions (23 ± 1°C, 55 ± 5% humidity) and had access to tap water and food ad libitum. FM4–64, an indicator of presynaptic activity, and calcium orange AM, a membrane-permeable calcium indicator, were purchased from Sigma-Aldrich (St. Louis, MO) and Molecular Probes, Inc. (Eugene, OR), respectively. These indicators were dissolved in dimethyl sulfoxide (DMSO) and then diluted to artificial cerebrospinal fluid (ACSF) containing 119 mM NaCl, 2.5 mM KCl, 1.3 mM MgSO$_4$, 1.0 mM NaH$_2$PO$_4$, 2.5 mM CaCl$_2$, 26.2 mM NaHCO$_3$, and 11 mM D-glucose (pH 7.3). ZnCl$_2$, FeCl$_3$, and CuCl$_2$ were dissolved in purified water and prepared as 10 mM stock solutions. The extracellular Zn$^{2+}$ concentration is increased age-dependently and that 100 nM Zn$^{2+}$ is in the range of physiological concentration in aged rats. Extracellular Zn$^{2+}$ activity-dependently released from neuron terminals, which is required for learning and memory via synaptic plasticity$^{14}$. Even at non-zincergic synapses, extracellular Zn$^{2+}$ may serve as a pool for Zn$^{2+}$ release from the internal stores, which is also required for learning and memory$^{13}$. In conclusion, the present study indicates that original neurophysiology may not appear in ACSF without Zn$^{2+}$. The basal levels of extracellular Zn$^{2+}$, which are in the range of low nanomolar concentrations, are critical for synaptic activity and perhaps increased age-dependently. Homeostasis of Zn$^{2+}$ in both extracellular and intracellular compartments is still poorly understood and the understanding is important to search a strategy for preventing Zn$^{2+}$-mediated cognitive decline.

**Brain slice preparation.** Mice were anesthetized with ether and decapitated. The brain was quickly removed and immersed in ice-cold choline-ACSF containing 124 mM choline chloride, 2.5 mM KCl, 2.5 mM MgCl$_2$, 1.25 mM NaH$_2$PO$_4$, 0.5 mM CaCl$_2$, 26 mM NaHCO$_3$, and 10 mM glucose (pH 7.3) to suppress excessive neuronal excitation. Horizontal brain slices (400 μm) were prepared by using a vibratome ZERO-1 (Dosaka Kyoto, Japan) in an ice-cold choline-ACSF. Slices were then maintained in ACSF and ACSF containing 1–10 nM ZnCl$_2$, 10 nM CuCl$_2$, or 10 nM FeCl$_3$ at 25°C for 1 h. All solutions used in the experiments were continuously bubbled with 95% O$_2$ and 5% CO$_2$.

**Spontaneous exocytosis.** The brain slices bathed in ACSF and ACSF containing Zn$^{2+}$, Cu$^{2+}$, or Fe$^{3+}$ were transferred to an incubation chamber filled with each ACSF containing 5 μM FM4-64, 45 mM KCl, and 10 μM 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), an antagonist of AMPA/kainate receptors, allowed to stand at 25°C for 90 s and transferred a chamber filled with each ACSF to wash out extracellular FM4-64 and KCl for 15 min. Brain slices were transferred to a recording chamber filled with each ACSF containing 10 μM CNQX to prevent recurrent activity. FM4-64 fluorescence (excitation, 543 nm; emission, 640 nm) was measured with a confocal laser-scanning microscopic system LSM 510 META at the rate of 1 Hz for 300 s through a 10× objective. In another experiment, the brain slices bathed in ACSF was treated in the same manner for staining with FM4-64 and transferred to a recording chamber filled with 1 μM tetrodotoxin (TTX), a voltage-gated sodium channel blocker, in ACSF containing 10 μM CNQX to measure the change in FM4-64 fluorescence under inhibition of neuronal depolarization. Because FM4-64 fluorescence originates in vesicular membrane-bound FM4-64, FM4-64 fluorescence is attenuated by spontaneous presynaptic activity$^{28,29}$ (Fig. 1A). FM4-64 fluorescence was then normalized by the initial fluorescence intensity at the time 0 sec, which is expressed as 100%. The rate (%) of attenuated FM4-64 fluorescence at the time 300 sec was compared among groups bathed in ACSF and reagents in ACSF.

**Intracellular levels of Ca$^{2+}$.** To assess the basal levels of intracellular Ca$^{2+}$, the brain slices in ACSF and ACSF containing 10 nM ZnCl$_2$ were placed for 30 min in each ACSF containing 5 μM calcium orange AM, transferred to a chamber filled with each ACSF to wash out extracellular calcium orange AM for 15 min, and transferred to a recording chamber filled with each ACSF. The fluorescence of calcium orange (excitation, 543 nm; monitoring, above 560 nm) was measured with a confocal laser-scanning microscopic system LSM 510 META for 30 sec at the rate of 1 Hz through a 10× objective.

**In vivo LTP.** Male rats were anesthetized with chloral hydrate (400 mg/kg) and placed in a stereotaxic apparatus. A bipolar stimulating electrode and a monopolar recording electrode made of tungsten wire attached to an microdialysis probe (E-A-I-12-01, Eicom Co., Kyoto) were positioned stereotaxically so as to selectively stimulate the perforant pathway while recording in the dentate gyrus under local perfusion with agents in ACSF (127 mM NaCl, 2.5 mM KCl, 0.9 mM MgCl$_2$, 1.2 mM NaH$_2$PO$_4$, 1.3 mM CaCl$_2$, 21 mM NaHCO$_3$, and 3.4 mM D-glucose (pH 7.3)). The electrode stimulating the perforant pathway was positioned 8.0 mm posterior to the bregma, 4.5 mm lateral, 3.0–3.5 mm inferior to the dura. A recording electrode was implanted ipsilaterally 4.0 mm posterior to the bregma, 2.3–2.5 mm lateral and 3.0–3.5 mm inferior to the dura. All the stimuli were biphasic square wave pulses (200 μs width) and their intensities were set at the current that evoked 40% of the maximum population spike (PS) amplitude. Test stimuli (0.05 Hz) were delivered at 20 s intervals to monitor PS amplitude. At the beginning of the experiments, input/output curves were generated by systematic variation of the stimulus current (0.1–5.0 mA) to evaluate synaptic potency. After stable baseline recording under perfusion with ACSF for at least 30 min, PS amplitudes were measured under perfusion with agents in ACSF for 60 min and LTP was induced by...
delivery of high-frequency stimulation (HFS; 10 trains of 20 pulses at 200 Hz separated by 1 s) and recorded for 60 min. PS amplitudes (test frequency: 0.05 Hz) were averaged over 120-second intervals and expressed as percentages of the mean PS amplitude measured during the 15-min baseline period (from −75 min to −60 min) perfused with ACSF prior to LTP induction, which was expressed as 100%. PS amplitudes for the last 10 min were also averaged and represented as the magnitude of LTP.

**Statistical analysis.** For statistical analysis, Student’s t-test was used for comparison of the means of unpaired or paired two-data. For multiple comparisons, differences between the control and 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 assessed by one-way ANOVA followed by post hoc testing using the Tukey’s test (the statistical software, unpaired or paired two-data. For multiple comparisons, differences between the control and 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 each figure legend. In Figs 1, 2 and 3, "n“ means the number of slices. The experiments have separately done in triplicate to confirm the reproducibility and all slices used in the present study were analyzed statistically. In Fig. 4, “n“ means the number of rats.

**Ethics Statement.** All experiments were performed in accordance with the Guidelines for the Care and Use of Laboratory Animals of the University of Shizuoka that 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. The ethics committee of the University of Shizuoka has approved all experimental protocols (The approval number, 136043).

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Author Contributions
Planned experiments: H.T., and A.T. Performed the Experiments: R.N., Y.S., M.S., Y.K. and M.O. Analyzed data: Y.S., H.T. and A.T. Wrote the paper: H.T., and A.T. All authors reviewed the manuscript.

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