High K^+-induced Increase in Extracellular Glutamate in Zinc Deficiency and Endogenous Zinc Action

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To understand the physiological significance of endogenous zinc release under excess excitation in the hippocampus, in the present study, high K^+-induced increase in extracellular glutamate and endogenous zinc action against its increase were examined in young rats fed a zinc-deficient diet for 2 weeks. When the ventral hippocampus was perfused by 100 mM KCl, the extracellular concentration of glutamate was more increased in zinc-deficient rats than the control rats. Calcium orange signal in mossy fiber boutons was more increased in slices from zinc-deficient rats after delivery of tetanic stimuli (100 Hz, 5 sec) to the dentate granule cell layer. The decrease in FM4-64 signal, which is a measurement of exocytosis, in mossy fiber boutons was also enhanced in slices from zinc-deficient rats. These results suggest that the abnormal increase in extracellular glutamate in the hippocampus induced with high K^+ in zinc deficiency is due to the enhancement of exocytosis associated with affected Ca^{2+} mobilization. The expression of GLT-1, a glial glutamate transporter, in the hippocampus was higher in zinc-deficient rats, suggesting that GLT-1 protein increased serves to maintain the basal concentration of extracellular glutamate, which was not different between the control and zinc-deficient rats. On the other hand, the increase in extracellular glutamate concentration induced with high K^+ was enhanced in the presence of 1 mM CaEDTA, a membrane-impermeable zinc chelator, in both the control and zinc-deficient rats. It is likely that zinc released from glutamatergic neurons serves to suppress glutamate release under excess excitation in the hippocampus.

Key words —— zinc, neuromodulator, hippocampus, glutamate, exocytosis, *in vivo* microdialysis

INTRODUCTION

Zinc is en essential nutrient for brain development and brain function.[1,2] Approximately 50% of the world population is estimated to be at risk of zinc deficiency.[3] Approximately 90% of the total brain zinc is in zinc metalloproteins, in which zinc is catalytic, coactive (or cocatalytic) and structural.[4] The rest is histochemically reactive zinc, which predominantly exists in the presynaptic vesicles. The hippocampus is enriched with zinc-containing glutamatergic neurons that sequester the zinc in the presynaptic vesicles and release it in a calcium- and impulse-dependent manner.[5–9] All giant boutons of hippocampal mossy fibers contain the zinc in the presynaptic vesicles.[10] The zinc is released with glutamate and serves as an endogenous neuromodulator.[11,12] Zinc may negatively modulate glutamate release from neuron terminals.[13–15]

Dietary zinc deficiency causes brain dysfunctions such as impairment of learning and memory.[16–18] Zinc deficiency also causes anorexia[19] and activates the hypothalamic-pituitary-adrenal (HPA) axis.[20,21] Serum corticosterone concentration is significantly increased in young mice and rats after 2-week zinc deprivation. The increase in corticosterone secretion is observed prior to the decrease in extracellular zinc concentration in the brain in zinc deficiency.[21,22] In contrast, serum zinc concentration of zinc-deficient rats is less than 50% of that of the control.[21] It is possible that the decrease in serum zinc concentration is linked to the change in corticosterone secretion in zinc deficiency. Glucocorticoids increase cytosolic free calcium (Ca^{2+}) concentration in cultured hippocampal neurons.[23,24] The findings that basal Ca^{2+} levels in the hippocampal cells are higher in slices from young mice and rats after 2-week zinc deprivation, suggesting that intracellular Ca^{2+} dyshomeostasis in the hippocam-

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pus is associated with glucocorticoid secretion in zinc deficiency.[21,25] Moreover, Stein-Behrens *et al.*[26] reports that physiological elevation of glucocorticoids potentiates extracellular glutamate accumulation in the hippocampus. The homeostasis of extracellular glutamate is maintained through exocytosis and reuptake by glutamate transporters. It is possible that exocytosis is affected by intracellular Ca^{2+} dyshomeostasis in zinc deficiency, because the increase in intracellular Ca^{2+} is the trigger for exocytosis. On the other hand, the action of glucocorticoids in reuptake by glutamate transporters is unknown.

Susceptibility to kainate-induced seizures is altered after 2-week zinc deprivation, while extracellular zinc in the hippocampus seems to be sufficiently increased under excess excitation.[22] To understand the physiological significance of endogenous zinc release under excess excitation in the hippocampus, in the present study, high K^+-induced increase in extracellular glutamate and endogenous zinc action against its increase were examined in young rats fed a zinc-deficient diet for 2 weeks.

MATERIALS AND METHODS

Chemicals —— FM4-64, an indicator of presynaptic activity, was purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.). ZnAF-2 DA, a membrane-permeable zinc indicator, was kindly supplied from Daiichi Pure Chemicals Co., LTD (Tokyo, Japan). These fluorescent indicators were dissolved in dimethyl sulfoxide (DMSO) and then diluted with artificial cerebrospinal fluid (ACSF), which was composed of 124 mM NaCl, 2.5 mM KCl, 2.0 mM $CaCl_2$, 1.0 mM $MgCl_2$, 1.25 mM NaH_2PO_4, 26 mM $NaHCO_3$ and 10 mM D-glucose (pH 7.3). To facilitate cellular uptake of indicators, cremophore EL Sigma-Aldrich was added to DMSO solutions (the final concentration, 0.02%).

Experimental Animals and Diets —— Male Wistar rats (3 weeks old) were purchased from Japan SLC (Hamamatsu, Japan). They were housed under the standard laboratory conditions (23 ± 1°C, 55 ± 5% humidity) and had access to tap water and food *ad libitum*. Control (52.8 μg Zn/kg) and zinc-deficient (0.37 μg Zn/kg) diets were purchased from Oriental Yeast Co. Ltd. (Yokohama, Japan). Feeding the zinc-deficient diet was begun at 4 weeks of age. The lights were automatically turned on at 8:00 and off at 20:00. All experiments were performed in accordance with the guidelines for the care and use of laboratory animals of the University of Shizuoka, Japan.

***In Vivo* Microdialysis** —— A guide tube (CMA Microdialysis, Solna, Sweden) was implanted into the brain of the control and zinc-deficient rats. The rats were anesthetized with chloral hydrate (400 mg/kg) and individually placed in a stereotaxic apparatus. The skull was exposed, a burr hole was drilled, and a guide tube was implanted into the right hippocampus (AP –5.6 mm, ML +4.6 mm, VD +6.0 mm), base on the coordinate of the brain map.[27] The guide tube was secured with dental cement and screws. After the surgical operation, each rat was housed individually.

Forty-eight hours after implantation of the guide tube, a microdialysis probe (3-mm membrane CMA 12 probe, CMA Microdialysis) was inserted into the hippocampus of chloral hydrate-anesthetized rats through the guide tube. The hippocampus was preperfused with ACSF (127 mM NaCl, 2.5 mM KCl, 1.3 mM $CaCl_2$, 0.9 mM $MgCl_2$, 1.2 mM Na_2HPO_4, 21 mM $NaHCO_3$ and 3.4 mM D-glucose, pH 7.3) at 5.0 μl/min for 60 min to stabilize the region, perfused for 60 min in the same manner to determine the basal concentrations of extracellular glutamate, γ-amino butyric acid (GABA) and zinc, and perfused with 100 mM KCl in ACSF at 5.0 μl/min for 40 min to determine the change in their concentrations by neuronal depolarization. The perfusate was collected every 20 min. In another experiment, 60 min after the preperfusion with ACSF, perfusate was changed with 1 mM CaEDTA in ACSF. Sixty minutes later, the hippocampus was perfused with 100 mM KCl in ACSF containing 1 mM CaEDTA for 40 min in the same manner.

HPLC Analysis —— The perfusate samples were analyzed for glutamate and GABA contents by high-performance liquid chromatography (HPLC) [column, CAPCELL PAK C18 UG120A (1 mm × 150 mm) (Shiseido Co. Ltd., Tokyo, Japan); mobile phase, 0.1 M potassium dihydrogen phosphate, 0.1 M di-sodium hydrogen phosphate, 10% acetonitrile, 0.5 mM EDTA-2Na, 3% tetrahydrofuran, pH 6.0] using the pre-column derivatization technique with o-phthaldialdehyde and a fluorescence detector (NANOSPACE SI-2, Shiseido Co. Ltd.).

Zinc Concentration —— The perfusate samples (50 μl) were diluted with 2% nitric acid (100 μl). Analysis of the samples in triplicate was conducted using a flameless atomic absorption spectrophotometer (Shimadzu AA6800F, Kyoto, Japan). Zinc

concentration in the samples was determined by a calibration curve prepared from zinc standard solution (Woko Pure Chemical Industries, Ltd., Osaka, Japan).

Brain Slice Preparation —— Control and zinc-deficient rats 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_2PO_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 at 25°C for at least 30 minutes. All solutions used in the experiments were continuously bubbled with 95% O_2 and 5% CO_2.

Calcium and Zinc Imaging —— To image intracellular zinc and calcium, the brain slices were loaded with 10 μM ZnAF-2DA and 10 μM calcium orange AM for 30 min and then transferred a chamber filled with ACSF to wash out unincorporated extracellular ZnAF-2DA and calcium orange AM for at least 30 min. The brain slices were transferred to a recording chamber filled with 10 μM 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), an antagonist of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA)/kainate receptors, in ACSF (3 ml) to block postsynaptic excitation. The fluorescence of ZnAF-2 (excitation, 488 nm; monitoring, 505–530 nm), which was used to image mossy fiber synapses, and calcium orange (excitation, 543 nm; monitoring, above 560 nm) was measured in the hippocampal mossy fiber boutons with a confocal laser-scanning microscopic system LSM 510 META (Carl Zeiss, Göttingen, Germany), equipped with the inverted microscope (Axiovert 200 M, Carl Zeiss).

Electrical stimuli (100 Hz, 5 sec, 100 μA, 200 μs/pulse) were delivered to the dentate granule cell layer through a tungsten electrode. The fluorescence of calcium orange was measured in mossy fiber boutons by using the confocal laser-scanning microscopic system LSM 510 at the rate of 1 Hz through a 20 × objective.

Exocytosis with FM4-64 —— The brain slices were transferred to an incubation chamber filled with ACSF containing 10 μM ZnAF-2DA, allowed to stand at 25°C for 30 min, transferred a chamber filled with ACSF to wash out extracellular ZnAF-2DA for at least 30 min, transferred to an incubation chamber filled with ACSF containing 5 μM FM4-64 and 45 mM KCl, allowed to stand at 25°C for 90 sec, transferred a chamber filled with ACSF to wash out extracellular FM4-64 and transferred to a recording chamber filled with ACSF containing 10 μM CNQX. The fluorescence of FM 4-64 (excitation, 488 nm; monitoring, above 650 nm) and ZnAF-2 was measured with the confocal laser-scanning microscopic system LSM 510 META at the rate of 1 Hz through a 20 × objective. Fifty seconds later, electrical stimuli (10 Hz, 180 sec, 100 μA, 200 μs/pulse) were delivered to the dentate granular cell layer through a tungsten electrode. In the case of no electrical stimulation, the slices were allowed to stand for 180 sec. Attenuation of FM 4-64 fluorescence (destaining) by presynaptic activity was measured with the confocal laser-scanning microscopic system LSM 510 META in the same manner. At the end of the experiments, complete depolarization-induced destaining was evoked by single strong stimuli (100 Hz, 18 sec, 100 μA, 200 μs/pulse). Region of interest was set in mossy fiber boutons double-labeled with FM4-64 and ZnAF-2. The activity-dependent component of FM4-64 fluorescence in the mossy fiber boutons was measured for each punctum by subtracting its residual fluorescence intensity (< 10% of initial intensity) measured after the strong electrical stimulation that produced maximal destaining. FM4-64 signal was then normalized by the maximal fluorescence intensity before the electrical stimulation.

Western Blot —— The brains were immediately removed from diethyl ether-anesthetized rats and then placed in ice-cold ACSF for 1 min. The left hippocampus was rapidly removed by blunt dissection and put on a glass slide in contact with ice. The hippocampus was homogenized in a buffered, isotonic protease-inhibitor solution (150 mM NaCl, 50 μg/ml aprotonin, 2 mM phenylmethylsulfonyl fluoride, 200 μM leupeptin, 1 mM EDTA and 20 mM Tris, pH 7.4), followed by centrifugation at 1000 *g* for 10 min at 4°C. The supernatant was centrifuged at 20000 *g* for 30 min at 4°C. The resulting pellet, a crude membrane fraction, was lysed in a buffer (1% sodium dodecyl sulfate (SDS), 1 mM EDTA and 10 mM Tris, pH 7.4) and then centrifuged at 16000 *g* for 5 min at 4°C. The resulting supernatant was used as the source of membrane proteins. Protein concentrations in the samples were determined by using a BCA Protein Assay Kit (Pierce Biotechnology, Rockford, IL, U.S.A.). The samples were

solubilized by adding 0.25 volume of a loading buffer (8% SDS, 40% glycerol, 0.004% bromophenol blue, 24% beta-mercaptoethanol and 200 mM Tris, pH 6.8) and denatured by heating at 95°C for 5 min. Equal amounts of proteins were resolved by SDS-PAGE (10% gel), transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, U.S.A.), then blocked for overnight at 4°C with 5% skim-milk in a Tris-buffered saline containing Tween-20 (TTBS) (0.1% Tween-20, 150 mM NaCl, and 20 mM Tris, pH 7.5). Commercially available affinity-purified antibody to GLT-1 (0.4 μg/ml 5% skim-milk in TTBS, Alpha Diagnostic international, San Antonio, TX), a glial glutamate transporter, was applied to the membranes for 2 hr at room temperature. Polyclonal anti-actin antibody (1 : 10000 in 5% skim-milk in TTBS, Sigma-Aldrich) was applied simultaneously with anti-GLT-1 antiserum. The antibodies were detected by ECL-chemiluminescence after incubation with horseradish peroxidase-conjugated secondary antibody (1 : 5000 in 5% skim-milk in TTBS, GE healthcare UK limited, Buckinghamshire, U.K.). Optical densities of the protein bands were analyzed by a LAS-3000 imaging system (Fuji Photo Film Co., Ltd., Tokyo). For quantitative analysis of GLT-1 protein, the ratio of the density of GLT-1 to that of actin was calculated, and the ratio of the control group, in which rats were fed a control diet, was expressed as 100%.

Statistical Analysis —— Student's *t*-test was used for comparison of the means of unpaired data. For multiple comparison, analysis of variance (ANOVA) followed by Fisher's Protected Least Significant Difference (PLSD) was performed.

RESULTS

High K^+-induced Increase in Extracellular Glutamate

The basal concentrations of extracellular glutamate, GABA and zinc in the hippocampus before perfusion with 100 mM KCl were not appreciably different between the control (body weight; 132 ± 2 g) and zinc-deficient (body weight; 83 ± 2 g) rats (Fig. 1). Extracellular glutamate concentration during perfusion with 100 mM KCl was significantly higher in zinc-deficient rats. Extracellular GABA and zinc concentrations during perfusion with 100 mM KCl were also higher in zinc-deficient rats, although there was no significant difference between the control and zinc-deficient rats.

Calcium Signaling in Mossy Fiber Boutons and Exocytosis

The basal Ca^{2+} levels monitored with fura-2 are significantly higher in the hippocampal CA3 of brain slices prepared from zinc-deficient rats,[21] suggesting that intracellular Ca^{2+} homeostasis in the hippocampus is affected by 2-week zinc deprivation. It is possible that the affected Ca^{2+} homeostasis influences neuronal excitation in the hippocampus. To check the change in Ca^{2+} levels in mossy fiber boutons after depolarization, tetanic stimuli at 100 Hz for 5 sec were delivered to the dentate granule cell layer in the presence of CNQX (Fig. 2). In both the control and zinc-deficient rats, calcium or-

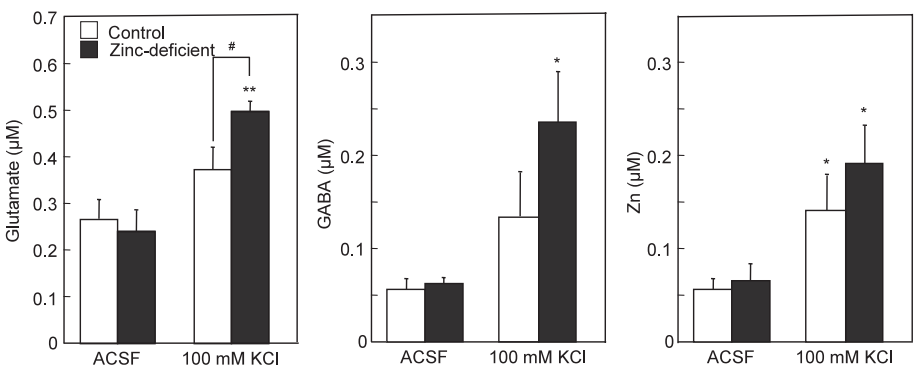

Fig. 1. Extracellular Concentrations of Glutamate, GABA and Zinc in the Hippocampus under Perfision with 100 mM KCl

Rats were fed a control or zinc-deficient diet. Twelve days later, a guide tube was surgically implanted into the brain. Forty-eight hours after implantation, the ventral hippocampus was perfused with ACSF for 60 min and then 100 mM KCl in ACSF for 40 min. The concentrations of glutamate, GABA and zinc in the perfusate were measured every 20 min. The ACSF represents the mean of 3 samples before perfusion with 100 mM KCl. The 100 mM KCl represents the mean of 2 samples during perfusion with 100 mM KCl. Each column with a vertical line represents the mean ± SEM ($n = 6$). *, $p < 0.05$; **, $p < 0.01$, *v.s.* ACSF (the basal level); #, $p < 0.05$, *v.s.* control.

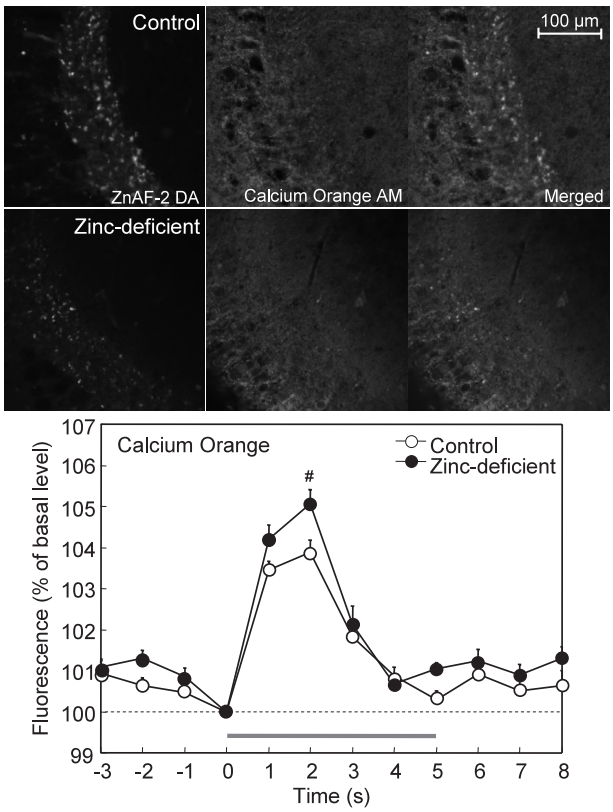

Fig. 2. Increase in Calcium Signals in Mossy Fiber Boutons after Tetanic Stimulation in the Presence of an AMPA/Kainate Receptor Antagonist

Brain slices were prepared from rats fed a control or zinc-deficient diet for 2 weeks and double-stained with ZnAF-2DA and calcium orange AM (upper panel). Tetanic stimuli at 100 Hz for 5 sec were delivered to the dentate granule cell layer immersed in 10 μM CNQX in ACSF. Calcium orange signals were monitored by a confocal laser-scanning microscopic system LSM 510. Region of interest (ROI) was set at mossy fiber synapses, which were imaged with ZnAF-2. Five ROI per slice were averaged. Note that the changes in signal intensities of ZnAF-2 and calcium orange by zinc deficiency could not be shown in the confocal images, because the imaging was performed under each optimal condition. The shaded bar represents the period of tetanic stimulation. The data represent the ratio (%) of fluorescence signals to a basal fluorescence signal just before the stimulation, which was expressed as 100%. Each point and line represents the mean ± SEM (12 slices). #, $p < 0.05$, *v.s.* control.

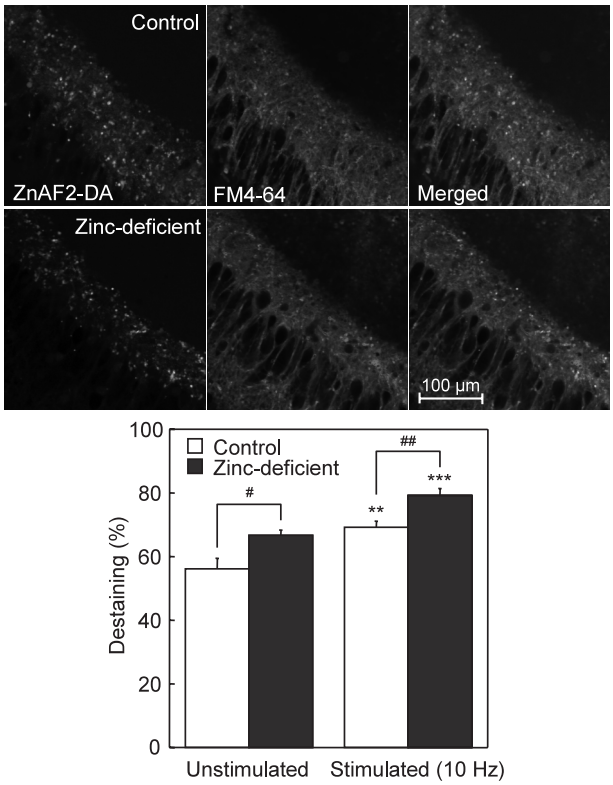

Fig. 3. Exocytosis at Mossy Fiber Boutons

Brain slices were prepared from rats fed a control or zinc-deficient diet for 2 weeks. Giant boutons of mossy fibers were double-labeled with FM4-64 and ZnAF-2DA (Upper panel). Tetanic stimuli at 10 Hz for 180 sec were delivered to the dentate granule cell layer and then single strong stimuli at 100 Hz for 18 sec were delivered to the same position. ROI was set at giant boutons of mossy fibers. Five ROI per slice were averaged. Note that the changes in signal intensities of FM4-64 and ZnAF-2 by zinc deficiency could not be shown in the confocal images, because the imaging was performed under each optimal condition.The activity-dependent component of FM4-64 signal was measured for each punctum (1 sec) by subtracting its residual fluorescence intensity (< 10% of initial intensity) measured after the strong electrical stimulation. FM4-64 signal was then normalized by the maximal fluorescence intensity before tetanic stimulation at 10 Hz. As a control experiment, tetanic stimuli at 10 Hz for 180 sec were not delivered to the dentate granule cell layer and then treated in the same manner. The data (the mean ± SEM) represent the percentage of the decreased FM4-64 signal (destaining) 180 sec after tetanic stimulation at 10 Hz (no stimulation in the case of the control experiment) (12 slices). **, $p < 0.01$, ***, $p < 0.001$, *v.s.* unstimulated; #, $p < 0.05$, ##, $p < 0.01$, *v.s.* control.

ange signal was increased in mossy fiber boutons after tetanic stimulation. The increase was more in zinc-deficient rats. Because CNQX completely blocks the increase in calcium orange signal in the CA3 pyramidal cell layer during tetanic stimulation (100 Hz, 5 sec),[28] the increase in calcium orange signal in the presence of CNQX may originate from mossy fiber boutons.

It is possible that the abnormal increase in Ca^{2+} levels in mossy fiber boutons after depolarization alters mossy fiber activity. Exocytosis at mossy fiber boutons was evaluated using a fluorescent styryl dye FM4-64. FM4-64 signal, which originates from vesicular membrane-bound FM4-64, is decreased by presynaptic activity.[29,30] The decrease in FM4-64 signal by no stimulus (spontaneous depolarization) and tetanic stimuli at 10 Hz for 180 sec was measured in mossy fiber boutons in the presence of CNQX (Fig. 3). The decrease in FM4-64 signal under both conditions was significantly enhanced by zinc deficiency.

GLT-1 Protein Expression

Extracellular glutamate concentration is depen-

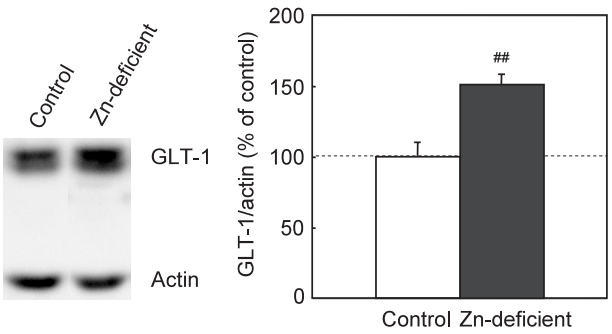

Fig. 4. GLT-1 Protein Expression

The brains were excised from rats fed a control and zinc-deficient diet for 2 weeks. The crude membrane fraction extracted from the hippocampus was subjected to western blotting (left-side). The ratio of the density of gel band corresponding to GLT-1 to that corresponding to actin was calculated, and the data was expressed as percentage of the control group, which was expressed as 100% (right-side) (n = 5). ##, $p < 0.005$, *v.s.* control.

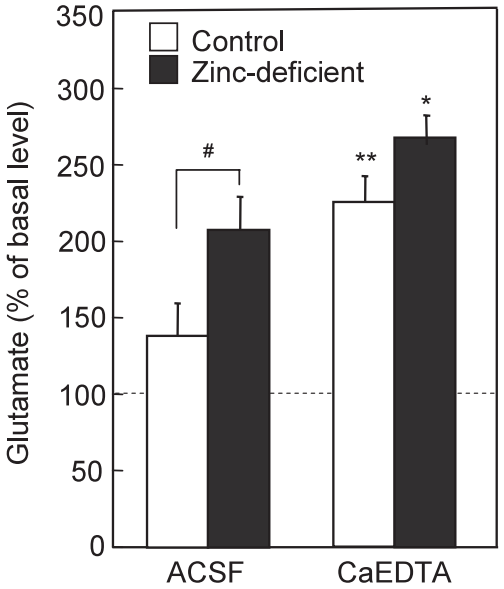

Fig. 5. Effect of CaEDTA on the Increase in Extracellular Glutamate in the Hippocampus under Perfusion with 100 mM KCl

Rats were fed a control or zinc-deficient diet. Twelve days later, a guide tube was surgically implanted into the brain. Forty-eight hours after implantation, the ventral hippocampus was perfused with ACSF for 60 min and then 100 mM KCl in ACSF for 40 min (ACSF group). To examine the effect of chelating extracellular zinc, the ventral hippocampus was also perfused with 1 mM CaEDTA in ACSF for 60 min and 100 mM KCl + 1 mM CaEDTA in ACSF for 40 min (CaEDTA group). The concentration of glutamate in the perfusate was measured every 20 min. Each column with a vertical line (the mean ± SEM) represents the ratio (%) of the concentration (the mean of 2 samples) during stimulation with 100 mM KCl to the basal concentration before the stimulation (the mean of 3 samples), which was expressed as 100% (n = 6). *, $p < 0.05$, **, $p < 0.01$, *v.s.* ACSF; #, $p < 0.05$, *v.s.* control.

dent on glutamate release (exocytosis) and glutamate uptake by its transporters. When GLT-1 protein level was compared between the control and zinc-deficient rats, GLT-1 protein level in the hippocampus was significantly higher in zinc-deficient rats (Fig. 4).

Effect of CaEDTA on High K^+-induced Increase in Extracellular Glutamate

To examine the action of extracellular zinc increased by perfusion with 100 mM KCl (Fig. 1), the hippocampus was perfused with 100 mM KCl in the presence of 1 mM CaEDTA. The increase in extracellular glutamate concentration by perfusion with 100 mM KCl was significantly enhanced in the presence of CaEDTA, a membrane-impermeable zinc chelator, in both the control and zinc-deficient rats (Fig. 5).

DISCUSSION

Intracellular Ca^{2+} homeostasis in the hippocampus seems to be affected prior to alteration of zinc homeostasis in the brain, because extracellular zinc concentration in the brain is not decreased in young rats fed a zinc-deficient diet for 2 weeks, in addition to histochemically reactive zinc, which is detected by Timm's staining.[21,25] Susceptibility to kainate-induced seizures is altered in young rats after 2-week zinc deprivation.[22] It is possible that the affected Ca^{2+} homeostasis influences neuronal activity and seizure susceptibility. On the other hand, physiological significance of extracellular zinc increased under excess excitation is poorly understood. In the present study, high K^+-induced increase in extracellular glutamate and endogenous zinc action against its increase were examined in young rats fed a zinc-deficient diet for 2 weeks.

In global ischemia, the concentration of extracellular potassium transiently reaches 75 mM, followed by the increase in extracellular glutamate.[31] When the hippocampus was perfused by 100 mM KCl, the extracellular concentration of glutamate was more increased in zinc-deficient rats than the control rats. Calcium orange signal in mossy fiber boutons was more increased in slices from zinc-deficient rats after delivery of tetanic stimuli (100 Hz, 5 sec) to the dentate granule cell layer. The decrease in FM4-64 signal, which is a measurement of exocytosis, in mossy fiber boutons was also enhanced in slices from zinc-deficient rats. These results suggest that the abnormal increase in

extracellular glutamate in the hippocampus induced with high K^+ in zinc deficiency is due to the enhancement of exocytosis associated with affected Ca^{2+} mobilization. Ca^{2+} dyshomeostasis in the hippocampus seems to be associated with the abnormal secretion of glucocorticoid in zinc deficiency.[21] On the other hand, the expression of GLT-1, a glial glutamate transporter, in the hippocampus was higher in zinc-deficient rats, suggesting that GLT-1 protein increased serves to maintain the basal concentration of extracellular glutamate, which was not different between the control and zinc-deficient rats. It is possible that the increase in GLT-1 protein is a compensatory change against the enhancement of the basal exocytosis in zinc deficiency.

Synaptic Zn^{2+} serves as a negative feedback factor against glutamate release,[14, 15] while translocation of Zn^{2+} to postneurons leads to neurotoxic effects under excess excitation in neurological diseases such as ischemia and epilepsy.[32–35] Quinta-Ferreira and Matias[36, 37] report that Ca^{2+} influx into mossy fibers by tetanic stimulation is inhibited by endogenous zinc. A sustained increase in Ca^{2+} levels in CA3 pyramidal neurons is enhanced in the presence of 1–10 mM CaEDTA after regional delivery of 1 mM glutamate to dentate granule cells.[38] Côté *et al*.[39] report that the neurotoxic and neuroprotective actions of Zn^{2+} depend on its concentration and that this dual action is cell type specific. Lavoie *et al*.[40] report that intracellular zinc chelator influences hippocampal neuronal excitability in rats. To examine the physiological significance of synaptic Zn^{2+} in glutamate dynamics in the extracellular compartment under excess excitation, the hippocampus was perfused with 100 mM KCl in the presence of 1 mM CaEDTA. The increase in extracellular glutamate concentration induced with high K^+ was significantly enhanced in the presence of CaEDTA in both the control and zinc-deficient rats. Chelation of endogenous zinc by CaEDTA causes a significant increase in ischemic cell death in hippocampal slice cultures.[14] Therefore, it is likely that Zn^{2+} released from glutamatergic neurons serves to suppress glutamate release under excess excitation in the hippocampus.

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