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Enhancement of hippocampal mossy fiber activity in zinc deficiency and its influence on behavior

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Abstract The extracellular concentration of glutamate in the hippocampus is increased by hippocampal perfusion with CaEDTA, a membrane-impermeable zinc chelator, suggesting that the activity of glutamatergic neurons in the hippocampus are influenced by the extracellular concentrations of zinc. In the present study, the relationship between the extracellular concentrations of zinc and mossy fiber activity in the hippocampus was examined in mice and rats fed a zinc-deficient diet for 4 weeks. Timm's stain, by which histochemically reactive zinc in the presynaptic vesicles is detected, was attenuated in the hippocampus in zinc deficiency. The extracellular signal of ZnAF-2, a membrane-impermeable zinc indicator, was also lower in the hippocampal CA3, suggesting that the basal extracellular concentrations of zinc are lower maintained in zinc deficiency. To check mossy fiber activity after 4-week zinc deprivation, the decrease in the signal of FM4-64, an indicator of presynaptic activity (exocytosis), at mossy fiber synapses was measured under the condition of spontaneous depolarization. The decrease was significantly facilitated by zinc deficiency, suggesting that the basal exocytosis at mossy fiber synapses is enhanced by zinc deficiency. On the other hand, the increase in anxiety-like behavior was observed in the open-field test after 4-week zinc deprivation. The present study demonstrates that the decrease in the basal extracellular concentrations of zinc may be linked to the enhancement of the basal mossy fiber activity in zinc deficiency. This decrease seems to be also involved in neuropsychological behavior in zinc deficiency.

Keywords Synaptic zinc · Mossy fiber · Exocytosis · Hippocampus · Zinc deficiency · Neuropsychological behavior

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Introduction

Zinc concentration in the hippocampus (approximately 300 μM) is relatively high in the brain (Takeda et al. 2001). The hippocampus possesses zinc-containing glutamatergic neurons that sequester zinc in the presynaptic vesicles and release it in a calcium- and impulse-dependent manner (Frederickson 1989). All giant boutons of mossy fibers contain zinc in the presynaptic vesicles, while approximately 45% of Schaffer collaterals is zinc-positive (Sindreu et al. 2003). The zinc, which is stained by Timm's sulfide-silver method, is histochemically reactive and estimated to be loosely bound to endogenous ligands and ionic form. Zinc serves as an endogenous

neuromodulator (Smart and Xie 1994). The extracellular concentration of glutamate in the hippocampus is increased by hippocampal perfusion with CaEDTA, a membrane-impermeable zinc chelator, while that of γ-amino butyric acid (GABA) is decreased (Takeda et al. 2004). Zinc may serve as a negative feedback factor against glutamate release in the hippocampus (Minami et al. 2006; Takeda et al. 2007a). Moreover, zinc may suppress excess excitation of mossy fiber synapses (Takeda et al. 2007b). The crosstalk to calcium seems to be involved in the negative modulations of zinc against glutamatergic neuron activity. However, excess release of zinc from mossy fibers is neurotoxic to postsynaptic neurons (Koh et al. 1996; Choi and Koh 1998; Lee et al. 1999: Weiss et al. 2000; Suh et al. 2004).

Zinc homeostasis in the brain is strictly maintained by the brain barrier system, i.e., the blood-brain and blood-cerebrospinal fluid barriers (Takeda 2000, 2001). Serum zinc concentration is significantly decreased to approximately 30% of the control rats after 1-week zinc deprivation (Takeda et al. 2007c), whereas the total brain zinc concentration is not decreased even after 12-week zinc deprivation. However, zinc concentration in the hippocampus is significantly decreased after 12-week zinc deprivation (Takeda et al. 2001). Furthermore, zinc concentrations in the extracellular fluid and the synaptic vesicle, which are responsive to zinc deficiency, are decreased in young mice and rats even after 4-week zinc deprivation (Takeda et al. 2003a, b).

Susceptibility to kainate-induced seizures is significantly enhanced in young mice and rats after 4-week zinc deprivation (Takeda et al. 2003a). It is possible that the decrease in the extracellular concentrations of zinc is linked to susceptibility to epileptic seizures in zinc deficiency, because zinc is an endogenous blocker of *N*-methyl-D-aspartate (NMDA) receptors that are critical for glutamatergic neuron activity. Calcium influx via NMDA receptors plays a key role for glutamatergic neuron activation (Westbrook and Mayer 1987; Vogt et al. 2000; Molnár and Nadler 2001). NMDA receptor function is important for not only cognitive behavior but also psychological behavior (Morris et al. 1986; Guilarte and Chen 2007). Thus, it is also possible that unblocking of NMDA receptors with zinc alters the behavioral response in zinc deficiency.

Approximately 50% of the world population does not get adequate zinc (Brown et al. 2001). Zinc deficiency in children is a nutritional and health problem in both developing and developed countries (Prasad 1983; Sandstead 1995; Gibson 1998; Penland 2000). The evidence from experimental animals indicates that zinc deprivation during periods of early development critically affects brain function and behavior, in addition to brain development (Halas et al. 1983,1986). Lethargy (reduced activity and responsiveness) is a characteristic in zinc deficiency (Golub et al. 1995). However, the mechanism of neurochemical changes associated with behavioral abnormality in zinc deficiency is unknown. In the present study, to pursue the involvement of the decrease in the extracellular concentrations of zinc in the hippocampus in abnormal behavior in zinc deficiency, the basal mossy fiber activity, which might be responsive to extracellular concentrations of zinc, was checked after 4-week zinc deprivation. The neuropsychological behavior was also evaluated in the open-field test.

Materials and methods

Chemicals

Control (44 mg Zn/kg) and zinc-deficient (2.7 mg Zn/kg) diets were purchased from Oriental Yeast Co. Ltd. (Yokohama, Japan). Artificial cerebrospinal fluid (ACSF) used as a perfusate 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). FM4-64, an indicator of presynaptic activity, was purchased from SIGMA (St. Louis, MO). ZnAF-2 and ZnAF-2 DA, a membrane-impermeable and membrane-permeable zinc indicator, were kindly supplied from Daiichi Pure Chemicals Co., Ltd. (Tokyo, Japan). These fluorescent indicators were dissolved in dimethyl sulfoxide (DMSO) and then diluted with ACSF. To facilitate cellular uptake of membrane-permeable indicators, cremophore EL (Sigma) was added to DMSO solutions (the final concentration, 0.02% v/v).

Experimental animals

Male ddY mice and Wistar rats (both 3 weeks old) were purchased from Japan SLC (Hamamatsu,

Japan). Feeding the zinc-deficient diet was begun at 4 weeks of age. They were housed under the standard laboratory conditions (23 ± 1°C, 55 ± 5% humidity) and had access to tap water and diet ad libitum. The lights were automatically turned on at 8:00 and off at 20:00. All experiments were performed in accordance with the Japanese Pharmacological Society guide for the care and use of laboratory animals.

Timm's sulfide-silver staining

Mice fed the control or zinc-deficient diet for 4 weeks were deeply anesthetized with chloral hydrate, and then perfused transcardially with 0.1% (w/v) Na_2S in phosphate buffer (pH 7.4). The brains were excised and immersed in 4% (w/v) paraformaldehyde in 0.1 M sodium phosphate buffer (pH 7.4) for 24 h and then in 10–30% (w/v) sucrose for 72 h. Coronal 30 μm sections were prepared in a cryostat at −20°C. Timm's staining was performed according to the procedure described previously (Danscher 1981).

Brain slice preparation

Mice and rats were fed the control or zinc-deficient diet for 4 weeks. They were deeply 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 min. All solutions used in the experiments were continuously bubbled with 95% O_2 and 5% CO_2.

Extracellular zinc imaging and recording

Brain slices from the control and zinc-deficient mice were transferred to a chamber for observation filled with ACSF (2 ml) containing 10 μM ZnAF-2, continuously bubbled with 95% O_2 and 5% CO_2, and mounted on the stage of an inverted microscope (Diaphot TMD 300, Nikon, Tokyo, Japan). Fluorescence intensity was measured with an Argus-50/CA system (Hamamatsu Photonics, Hamamatsu, Japan) with a cooled CCD camera (excitation, 490; dichroic beam splitter, 505 nm; the rate, 1 Hz) at 25°C.

Mossy fiber activity

The brain slices from the control and zinc-deficient rats 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 s, transferred a chamber filled with ACSF to wash out extracellular FM4-64 and transferred to a recording chamber filled with ACSF containing 10 μM 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), an antagonist of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA)/kainate receptors, which blocks postsynaptic neuronal excitation. The fluorescence of FM 4-64 (excitation, 488 nm; monitoring, above 650 nm) and ZnAF-2 (excitation, 488 nm; monitoring at 505–530 nm), which was used to identify mossy fiber synapses, were measured for 230 s with a confocal laser-scanning microscopic system LSM 510 META at the rate of 1 Hz through a 20× objective to observe attenuation of FM 4-64 fluorescence (destaining) based on spontaneous presynaptic activity. At the end of the experiments (230 s later), complete depolarization-induced destaining was evoked by application of single strong stimuli (100 Hz, 18 s, 100 μA, 200 μs/pulse) to the dentate granular cell layer through a tungsten electrode. Region of interest (ROI, around 5 μm in diameter) was set in mossy fiber synapses double-labeled with FM4-64 and ZnAF-2. The activity (spontaneous depolarization)-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 just after the start of the measurement.

Open-field test

Behavior and locomotor activity of mice fed the control or zinc-deficient diet for 4 weeks were

examined in the open-field test. Each mouse was placed in an arena (90 × 90 × 90 cm) made of a black-colored wooden box, in which it has never been placed ($n = 11$). The arena was illuminated with three overhead lights (40 W each). Behavior of each mouse in the arena, which was recorded with a video camera, was observed for 30 min.

Statistical analysis

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

Results

Extracellular zinc levels in zinc deficiency

Zinc ion and zinc loosely bound to endogenous ligands are stained by Timm's sulfide-silver method. When zinc levels in the presynaptic vesicles were evaluated by Timm's stain, the stain in the hippocampus and other brain regions was attenuated in mice (the mean body weight, 22.3 g; the mean body weight of control mice, 41.5 g) fed a zinc-deficient diet for 4 weeks (Fig. 1), in agreement with the previous data (Takeda et al. 2003a). The decreased rate of the body weight after 4-week zinc deprivation was almost the same between mice and rats, when feeding the zinc-deficient diet was begun at 4 weeks of age. Timm's stain was also similarly attenuated in both mice and rats after 4-week zinc deprivation (Takeda et al. 2003a, b). It seems that the effect of dietary zinc deficiency is not significantly different between mice and rats.

Imaging extracellular zinc in the hippocampus with ZnAF-2 was performed after application of ZnAF-2 to brain slices. The dentate hilus and stratum lucidum, in which mossy fibers exist, was imaged immediately after the application, suggesting that zinc released from mossy fibers is imaged with ZnAF-2 (Fig. 2a). However, extracellular ZnAF-2 signals in both regions were lower in zinc deficiency. When the imaging was continued for 20 s, the signal intensity of ZnAF-2 were not significantly changed in both slices from the control and zinc-deficient mice (data not shown). ZnAF-2 signals in the stratum lucidum and stratum radiatum were also lower in zinc deficiency 20 s after the application (Fig. 2b).

Exocytosis at mossy fiber boutons

Exocytosis at mossy fiber boutons was evaluated by using FM4-64, a fluorescent styryl dye. FM4-64 is taken up into presynaptic vesicles in an activity-dependent manner and the signal is decreased by presynaptic activity, because FM-4-64 signal originates from vesicular membrane-bound form and the membrane-bound FM-4-64 is released from the membranes by presynaptic activity (exocytosis)

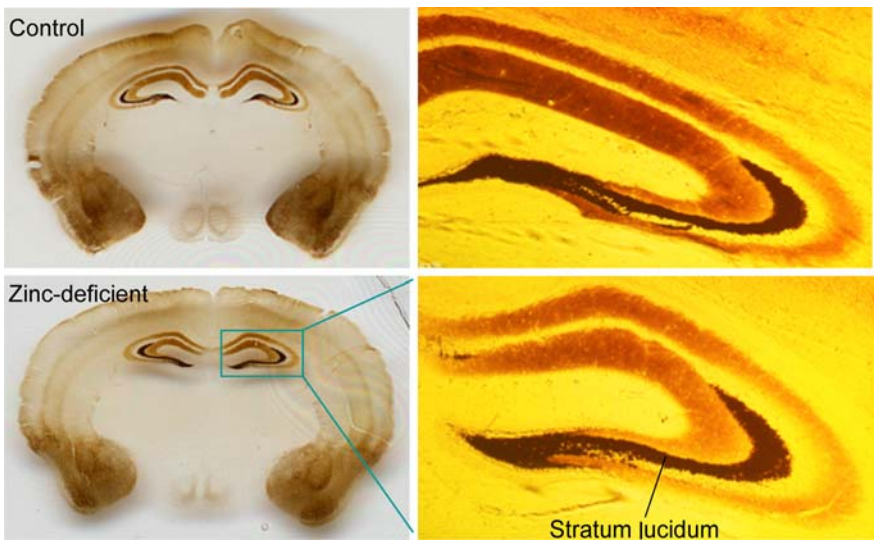

Fig. 1 Timm's staining. Coronal slices for Timm's staining were prepared from mice fed a control or zinc-deficient diet for 4 weeks ($n = 4$). The hippocampus is magnified in the right-hand panels

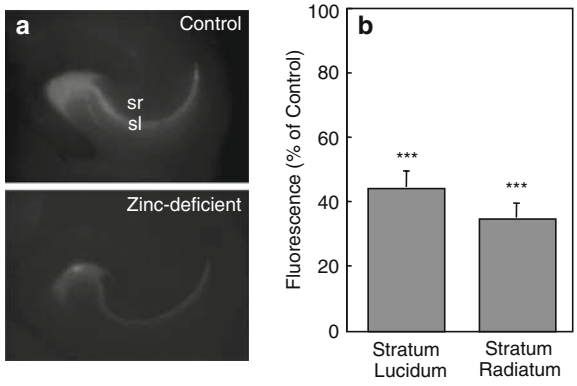

Fig. 2 Imaging of extracellular zinc in the hippocampus with ZnAF-2. (**a**) The fluorescence of ZnAF-2 was imaged immediately after application of ZnAF-2 to brain slices, which were prepared from the control and zinc-deficient mice. (**b**) The fluorescence intensity in the stratum lucidum (sl) and stratum radiatum (sr) was measured by using an Argus-50/CA system and fluorescence intensity per pixel was averaged. Each bar and line (the mean ± SEM) represent the ratio (%) of fluorescence intensity of zinc-deficient group to that of the control group 20 s after application of ZnAF-2 (10 slices). *** $P < 0.001$ versus the control

(Klingauf et al. 1998, Zakharenko et al. 2001). The decrease in FM4-64 signal, which represents the extent of exocytosis, was measured at mossy fiber synapses double-stained with ZnAF-2 DA and FM4-64 (Fig. 3). To evaluate exocytosis under the condition of spontaneous depolarization, an electrical stimulation to facilitate exocytosis was not applied to the dentate granule cells. The decrease in FM4-64 signal was significantly facilitated in slices from rats (the mean body weight, 103 ± 2.6 g; the mean body weight of control rats, 195 ± 7.3 g) fed the zinc-deficient diet for 4 weeks.

Open-field test

To check behavioral abnormality in zinc deficiency, the control and zinc-deficient mice were subjected to the open-field test (Fig. 4). The frequency of line crossing 0–10 min after the start of the test was significantly more in the control mice than in zinc-deficient mice. The frequency of line crossing was significantly decreased in the control mice during the test, whereas it was almost unchanged in zinc-deficient mice. On the other hand, the frequency of grooming was significantly increased in the control mice during the test, whereas it was also almost unchanged in zinc-deficient mice. It is likely that anxiety-like behavior is increased by zinc deficiency.

Discussion

Glutamate concentration in the brain extracellular fluid is estimated to be around 2 μM, while that in the synaptic vesicle of glutamatergic neurons is markedly high (~100 mM) (Meldrum 2000). Glutamate is involved in most aspects of brain functions such as cognition, memory, and learning (Fonnum 1984; Collingridge and Lester 1989; Headley et al. 1990). Thus, glutamate homeostasis in the brain extracellular fluid is critical for brain functions and linked to neuropsychological behavior. In zinc-containing glutamatergic neurons, its activity may be modulated by the extracellular concentrations of zinc (Minami et al. 2006; Takeda et al. 2007a). On the other hand, the extracellular concentration of zinc in the hippocampus is decreased in young mice and rats after 4-week zinc deprivation (Takeda et al. 2003a, b). It is possible that the decrease in the extracellular concentration of zinc alters zinc-containing glutamatergic neuronal activity in zinc deficiency, followed by neuropsychological behavior characterized by zinc deficiency. In the present study, the relationship between the extracellular concentrations of zinc and mossy fiber activity in the hippocampus was examined in mice and rats fed a zinc-deficient diet for 4 weeks.

Histochemically reactive zinc levels detected by Timm's staining were attenuated in the hippocampus in zinc deficiency. The extracellular signals of ZnAF-2, a zinc indicator, were also lower in the hippocampal CA3, suggesting that the basal extracellular concentrations of zinc are lower maintained in zinc deficiency. In an in vivo microdialysis experiment in kainate-treated young rats after 4-week zinc deprivation, the extracellular concentrations of zinc are less increased in the hippocampus after treatment with kainate, whereas the extracellular concentrations of glutamate are more increased (Takeda et al. 2003a). It is likely that the enhanced activity of glutamatergic neurotransmitter system in the hippocampus is linked to the decrease in the extracellular concentrations of zinc, because zinc is an endogenous blocker of NMDA receptors that are critical for glutamatergic neuron activity (Westbrook and Mayer 1987; Vogt et al. 2000; Molnár and Nadler 2001). To

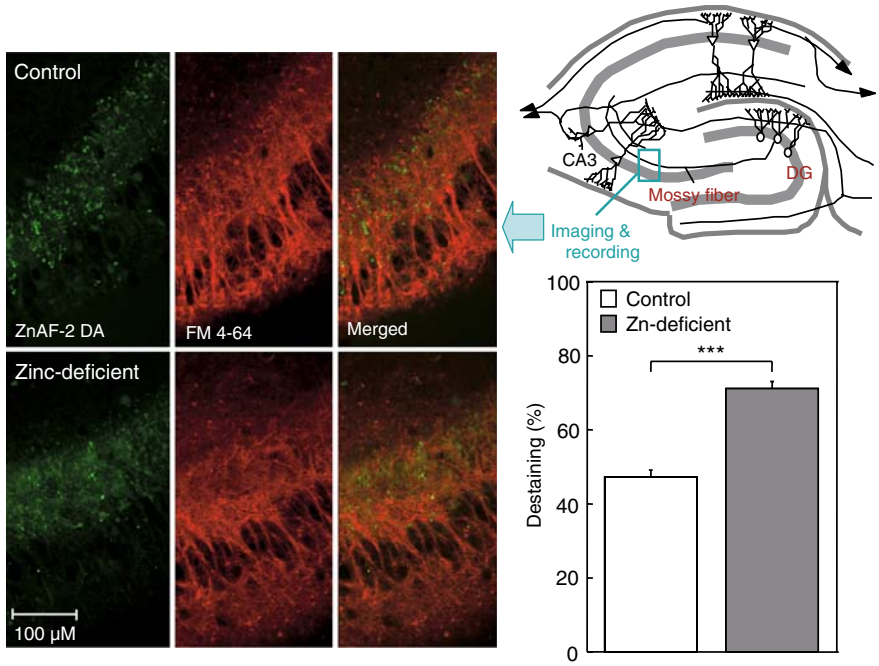

Fig. 3 Exocytosis at mossy fiber boutons. Brain slices were prepared from rats fed a control or zinc-deficient diet for 4 weeks. Giant boutons of mossy fibers were double-labeled with FM4-64 and ZnAF-2DA (left panels). ROI was set at double-labeled mossy fiber synapses. The activity (spontaneous depolarization)-dependent component of FM4-64 signal was measured for each punctum (1 s). The residual fluorescence intensity (<10% of initial intensity) measured after the strong electrical stimulation to the dentate granule cell layer (DG) was subtracted from fluorescence intensity based on spontaneous depolarization that was measured for 230 s. FM-64 signal was then normalized by the maximal fluorescence intensity just after the start of the measurement. The data (the mean ± SEM) represent the decreased signal (destaining) (%) 230 s after the start of the measurement (9 slices). *** $P < 0.001$ versus the control

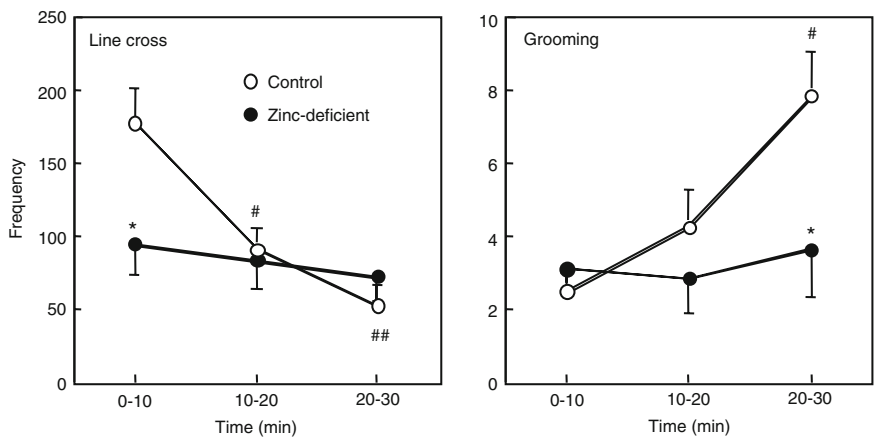

Fig. 4 Open-field test. Mice were fed a control or zinc-deficient diet for 4 weeks. The open-field test was performed in an arena for 30 min and the frequency of line crossing and grooming was added up every 10 min. Each point and line represents the mean ± SEM ($n = 11$). * $P < 0.05$ versus the control; # $P < 0.05$, ## $P < 0.01$ versus the point 0–10 min after the start of the test

check mossy fiber activity in zinc deficiency, the decrease in FM4-64 signal (exocytosis) at mossy fiber synapses was measured under the condition of spontaneous depolarization. The decrease was significantly facilitated by zinc deficiency, suggesting that the basal exocytosis at mossy fiber synapses is enhanced by zinc deficiency. The present study demonstrates that the decrease in the basal extracellular concentrations of zinc may be linked to the enhancement of the basal mossy fiber activity in zinc deficiency.

On the other hand, zinc deficiency causes anorexia, reduced gain in the body weight and growth retardation. It activates the hypothalamic-pituitary-adrenal (HPA) axis that is involved in stress response; serum corticosterone concentration is significantly increased by zinc deficiency (Chu et al. 2003). There are reports that the activation in the endocrine system influences glutamatergic neurotransmitter system. Glucocorticoids increase cytosolic free calcium (Ca^{2+}) concentration in cultured hippocampal neurons (Elliott et al. 1992, 1993). The basal Ca^{2+} levels detected with fluo-4FF are also significantly higher in hippocampal slices from zinc-deficient mice (Takeda et al. 2008). Stein-Behrens et al. (1994) reports that physiological elevation of glucocorticoids potentiates glutamate accumulation in the hippocampus. Thus, the increase in glucocorticoid secretion from the adrenal seems to be also linked to the enhancement of the basal mossy fiber activity in zinc deficiency. Furthermore, the activation of HPA axis, which is linked to the hippocamal function, influences neuropsychological behavior (Venero and Borrell 1999; Lee et al. 2002; Bianchi et al. 2003). In the open-field test, in the present study, the increase in anxiety-like behavior was observed in mice fed the zinc-deficient diet for 4 weeks. Anxiety-like behavior is also increased in zinc-deficient rats (Takeda et al. 2007c).

In conclusion, neuropsychological behavior characterized by zinc deficiency seems to be linked to the enhancement of the basal mossy fiber activity, in which the decrease in the extracellular concentrations of zinc in the hippocampus and the activation of the HPA axis may be involved.

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