*Exp. Anim. 65(4), 353–361, 2016*

**—Original—**

# Influences of yokukansankachimpihange on aggressive behavior of zinc-deficient mice and actions of the ingredients on excessive neural exocytosis in the hippocampus of zinc-deficient rats

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**Abstract:** We examined the effect of Yokukansankachimpihange (YKSCH), a form of Yokukansan containing parts of two herbaceous plants, Citrus Unshiu Peel (Chimpi) and Pinellia Tuber (Hange), on aggressive behavior of mice housed individually. Mice were fed a zinc-deficient diet for 2 weeks. In a resident-intruder test, the cumulative duration of aggressive behavior was decreased in zinc-deficient mice administrated drinking water containing YKSCH (approximately 300 mg/kg body weight/day) for 2 weeks. We tested mice for geissoschizine methyl ether (GM), which is contained in Uncaria Hook, and 18β-glycyrrhetinic acid (GA), a major metabolite of glycyrrhizin contained in Glycyrrhiza, which were contained in YKS and YKSCH. In hippocampal slices from zinc-deficient rats, excess exocytosis at mossy fiber boutons induced with 60 mM KCl was attenuated in the presence of GA (100–500 $\mu$M) or GM (100 $\mu$M). The intracellular $Ca^{2+}$ level, which showed an increase induced by 60 mM KCl, was also attenuated in the presence of GA (100–500 $\mu$M) or GM (100 $\mu$M). These results suggest that GA and GM ameliorate excess glutamate release from mossy fiber boutons by suppressing the increase in intracellular $Ca^{2+}$ signaling. These ameliorative actions may contribute to decreasing the aggressiveness of mice individually housed under zinc deficiency, potentially by suppressing excess glutamatergic neuron activity in the hippocampus.

**Key words:** aggressive behavior, social isolation, Yokukansankachimpihange, Yokukansan, zinc deficiency

## Introduction

Dementia is a syndrome of progressive deterioration of memory, other cognitive abilities, and functional impairment. Alzheimer's disease (AD) is the most common causes of dementia and is characterized by core symptoms such as cognitive deficits and behavioral and psychological symptoms of dementia (BPSD) such as aggression, hallucinations, disturbed behavior, and agitation [20]. BPSD are a serious problem for caregivers; there is a positive correlation between their severity and the care burden. Therapy for BPSD is considered to be as important as therapy for the core symptoms [18, 30]. Among the BPSD, agitation and aggression are observed in more than 60% of patients with dementia [14] and are frequently the primary cause of hospitalization

*(Received 14 March 2016 / Accepted 21 April 2016 / Published online in J-STAGE 30 May 2016)*

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*Abbreviations: Yokukansan (YKS), behavioral and psychological symptoms of dementia (BPSD), Yokukansankachimpihange (YKSCH), geissoschizine methyl ether (GM), 18β-glycyrrhetinic acid (GA), Alzheimer's disease (AD), hypothalamic-pituitary-adrenal (HPA)*

or institutionalization [22].

It has been reported that Yokukansan (YKS), a traditional Japanese herbal medicine, is effective for improving BPSD [8, 13, 17]. YKS is also effective for improving behavioral and psychological symptoms of Parkinsonian dementia and sleep disturbance in patients with dementia with Lewy bodies [10, 21]. On the other hand, it is estimated that disturbance of the glutamatergic neurotransmitter system underlies BPSD as well as core symptoms [5]. Excess secretion of glucocorticoids from the adrenal, which occurs via the enhanced activity of the hypothalamic-pituitary-adrenal (HPA) axis, is a well-known feature of AD [3, 4] and affects the glutamatergic neurotransmitter system [23]. When the brain is chronically exposed to a high concentration of glucocorticoids, cognitive function is affected and dendrite remodeling of neurons is induced in the hippocampus and prefrontal cortex [1]. There are correlations between enhanced HPA axis activity and dementia severity or hippocampal volume loss in individuals with probable AD [2].

HPA axis activity is readily enhanced by dietary zinc deficiency, which affects the glutamatergic neurotransmitter system [23, 24]. It has been reported that cognitive dysfunction and neuropsychological symptoms such as anxiety, depression, and aggression are observed in zinc-deficient mice and rats; aggressive behavior is observed in zinc-deficient mice, and depressive behavior is observed in both zinc-deficient mice and rats. It is estimated that elevation of the blood glucocorticoid level underlies the behavioral abnormality under zinc deficiency [24, 25]. Therefore, zinc-deficient mice and rats may be animal models that can be used to examine the efficacy of drugs on BPSD.

On the basis of the evidence that YKS ameliorates aggressive behavior and excess glutamatergic neuron activity of zinc-deficient animals [26, 29], in the present study, we examined the effect of Yokukansankachimpihange (YKSCH), which is a form YKS combination containing parts of two herbaceous plants, Citrus Unshiu Peel (Chimpi) and Pinellia Tuber (Hange), on aggressive behavior of zinc-deficient mice housed individually. The effect of two compounds, which are contained in both YKS and YKSCH, on excess glutamate exocytosis was also examined in hippocampal slices prepared from zinc-deficient rats.

## Materials and Methods

*Chemicals and drugs*

Control and zinc-deficient diets were obtained from Oriental Yeast Co., Ltd. (Tokyo, Japan), and the zinc contents of the diets were 52.8 mg Zn/kg and 0.37 mg Zn/kg, respectively. YKSCH was kindly provided in the form of dried powder extract by Tsumura & Co. (Tokyo, Japan). The quality of YKSCH was assured based on the prescribed range of index components. The drug was manufactured from a dried extract of the following mixture crude drugs: JP Pinellia Tuber (5.0 g), JP Atractylodes Lancea Rhizome (4.0 g), JP Poria Sclerotium (4.0 g), JP Cnidium Rhizome (3.0 g), JP Uncaria Hook (3.0 g), JP Citrus Unshiu Peel (3.0 g), JP Angelica Root (3.0 g), JP Bupleurum Root (2.0 g), and JP Glycyrrhiza (1.5 g) (JP: The Japanese Pharmacopoeia). To administer YKSCH to mice at a dose of approximately 300 mg/kg body weight/day, YKSCH was dissolved in distilled water (1.5 mg/ml) and administered as drinking water. The concentration of YKSCH was calculated from the averaged intake volume (6.4 ml/day/mouse) of the drinking water, which was not changed in the presence of YKSCH. 18β-glycyrrhetinic acid (GA), a major metabolite of glycyrrhizin contained in Glycyrrhiza, and geissoschizine methyl ether (GM), contained in Uncaria Hook, were obtained from Tsumura & Co. (Tokyo, Japan). Calcium orange AM (a membrane-permeable calcium indicator) and FM4-64 (an indicator of presynaptic activity) were purchased from Molecular Probes, Inc. (Eugene, OR, USA) and Sigma-Aldrich Corporation (St. Louis, MO, USA), respectively. These indicators were dissolved in dimethyl sulfoxide (DMSO) and then diluted with artificial cerebrospinal fluid (ACSF), which consisted of 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).

*Experimental animals*

Male ddY mice (3 weeks old) and male Wistar rats (3 weeks old) were purchased from Japan SLC (Hamamatsu, Japan). Mice were individually housed (one mouse per cage), and rats were housed in groups (five rats per cage) (23 ± 1°C, 55 ± 5% humidity). The mice and rats had free access to water and food. Administration of the zinc-deficient diet and water containing YKSCH was begun at 4 weeks of age and finished at 6 weeks of age. The lights were automatically turned on

at 8:00 h and off at 20:00 h. All experiments were performed in accordance with the Guidelines for the Care and Use of Laboratory Animals of the University of Shizuoka, which are in accordance with the American Association for Laboratory Animal Science and the guidelines laid down by the NIH (*NIH Guide for the Care and Use of Laboratory Animals*) in the USA The Animal Experiment Committee of the University of Shizuoka approved all protocols for animal experiments (approval numbers: 136043 for rats; 136044 for mice).

*Resident-intruder test*

Three-week-old mice were housed individually for 1 week and then fed a zinc-deficient diet and YKSCH as a drinking water for 2 weeks. For use as intruders, three-week-old mice, which were housed in a group of five, were fed the control diet and water for 3 weeks. The resident–intruder test was carried out after intake of the zinc-deficient diet and YKSCH for 2 weeks. An intruder mouse was individually placed in the cages of the resident mice in the resident–intruder test. Behaviors of both resident and intruder mice were measured for 5 min. Biting attacks and wrestling of resident mice were assessed as aggressive behavior, while tail rattle, lateral threat, and pursuit were not assessed as aggressive behavior. The tests were performed four times, and the same mice were not used in each test.

*Hippocampal slices preparation*

Rats were anesthetized with ether and then decapitation. The brain was immediately excised and immersed in ice-cold choline-ACSF, which consisted of 124 mM choline chloride, 2.5 mM KCl, 2.5 mM $MgCl_2$, 1.25 mM $NaH_2PO_4$, 0.5 mM $CaCl_2$, 26 mM $NaHCO_3$, and 10 mM glucose (pH 7.3), to inhibit excessive neuronal excitation. Transverse hippocampal slices (400 $\mu$m) were prepared by using a vibratome ZERO-1 (Dosaka, Kyoto, Japan) in ice-cold choline-ACSF. The slices were then placed in ACSF at 25°C for at least 1 h. All solutions used in the experiments were serially bubbled with 95% $O_2$ and 5% $CO_2$.

*Presynaptic activity (exocytosis)*

Presynaptic activity was assessed by using FM4-64 as reported previously [11, 32]. The slices were placed in an incubation chamber filled with ACSF containing 5 $\mu$M FM4-64 and 45 mM KCl at 25°C for 90 s, placed in a chamber filled with ACSF to wash out extracellular FM4-64, and placed in a recording chamber filled with ACSF containing 10 $\mu$M 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), an antagonist of AMPA/kainate receptors, to prevent recurrent activity. The fluorescence of FM4-64 was measured with an LSM 510 META confocal laser-scanning microscopic system (excitation, 488 nm; monitoring, above 650 nm) (Carl Zeiss), which was equipped with an inverted microscope, at the rate of 1 Hz through a 10× objective to observe attenuation (destaining) of FM4-64 fluorescence elicited by presynaptic activity. The slices were stimulated with 60 mM KCl after measuring the basal levels of FM4-64 florescence for 30 s. The activity-dependent component of FM4-64 fluorescence at mossy fiber boutons was measured for each punctum by subtracting the residual fluorescence intensity (<10% of initial intensity) determined 240 s after stimulation with KCl and then normalized by the maximal fluorescence intensity before stimulation with KCl.

*Intracellular calcium imaging*

The slices were immersed in 10 $\mu$M calcium orange AM for 30 min and then placed in a chamber filled with ACSF to wash out the indicator for at least 30 min. The slices were then placed in a recording chamber filled with ACSF. The fluorescence of calcium orange was measured in the stratum lucidum of the CA3 in the hippocampus with an LSM 510 META confocal laser-scanning microscopic system (excitation, 543 nm; monitoring, above 560 nm).

*Statistical analysis*

All data were expressed as means ± standard error and statistically analyzed by using the GraphPad Prism 5 software. One-way ANOVA with Dunnett's test was used to make multiple comparisons with the control group, and Student's *t*-test was used for comparison of the means of paired data as indicated in the figure legends.

## Results

*Effect of YKSCH on aggressive behavior*

Social isolation-induced aggressive behavior is facilitated in zinc-deficient mice [25]. In the present study, we examined the effect of YKSCH on social isolation-induced aggressive behavior of mice, which were fed a zinc-deficient diet and YKSCH-containing water. There were no significant differences in the body weight and

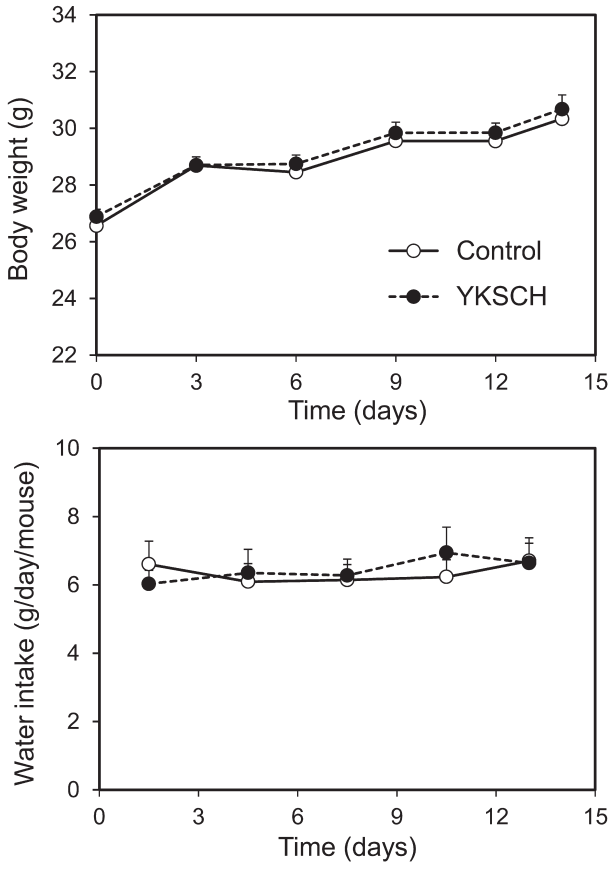


**Fig. 1.** Effect of YKSCH administration on body weight and water intake of zinc-deficient mice. Isolated mice were fed a zinc-deficient diet + water (control) or a zinc-deficient diet + YKSCH-containing water for 2 weeks. Each point and line (mean ± SEM) represents body weight (control, n=27–32; YKSCH, n=27–32) and water intake (control, n=12–16; YKH, n=11–16).

water intake between the control and YKSCH-treated mice (Fig. 1).

To evaluate aggressive behavior of isolated zinc-deficient mice after the intake of YKSCH for 2 weeks, the resident-intruder test was performed. Approximately 60% of all the mice were aggressive zinc-deficient mice, in agreement with previous data (the rate of aggressive mice fed a control diet,<10%) [29]. The rate of mice that exhibited aggression against intruder mice was not significantly different between the control and YKSCH-treated mice (Fig. 2A). The latency in performance of aggressive behavior was also not different between the control and YKSCH-treated mice (Fig. 2B). In contrast, the cumulative duration of aggressive behavior was significantly decreased in YKSCH-treated mice (Fig. 2C).

*Effects of GA and GM on presynaptic activity*

To examine the effects of GA and GM on excess presynaptic activity induced with high $K^+$, exocytosis at mossy fiber terminals was evaluated by using FM4-64. The fluorescence of FM4-64 taken up into presynaptic vesicles is attenuated in an activity–dependent manner. Because FM4-64 fluorescence originates from vesicular membrane-bound FM4-64, the fluorescence is attenuated by release from the vesicular membranes induced with presynaptic activity [11, 32]. Attenuation of FM4-64 fluorescence in the stratum lucidum, which contains mossy fiber terminals, was significantly suppressed in the hippocampal slices immersed in 100–500 $\mu$M GA (Fig. 3) or 100 $\mu$M GM (Fig. 4).

*Effects of GA and GM on increase in intracellular $Ca^{2+}$*

Excess presynaptic activity induced with high $K^+$ is mediated by the influx of extracellular $Ca^{2+}$ into the presynaptic terminals. The changes in intracellular $Ca^{2+}$ level were assessed with calcium orange AM. The increase in intracellular $Ca^{2+}$ level induced with high $K^+$ was suppressed in the presence of 100–500 $\mu$M GA (Fig. 5) or 100 $\mu$M GM (Fig. 6).

## Discussion

It has been reported that YKS is beneficial to therapy for BPSD [8, 17]. The action mechanisms of YKS on BPSD have been examined by using experimental animals such as zinc-deficient mice. YKS ameliorates aggressive behavior of zinc-deficient mice housed individually [25, 29] and excess glutamate exocytosis at mossy fiber terminals in hippocampal slices from zinc-deficient rats [26]. On the other hand, attention has recently been paid to therapy for BPSD with YKSCH, a form of YKS containing Chimpi and Hange [12, 13]. In the present paper, we examined the effect of YKSCH on aggressive behavior of isolated zinc-deficient mice, a model of BPSD.

In the resident-intruder test, the cumulative duration of aggressive behavior was significantly decreased in zinc-deficient mice administered drinking water containing YKSCH. YKS reduces the rate of mice exhibiting aggressive behavior, but not the cumulative duration of aggressive behavior [25]. The data suggest that YKSCH is potentially beneficial to therapy for BPSD. The difference in action against aggressive behavior may be due to the additional parts of herbaceous plants, Chimpi and

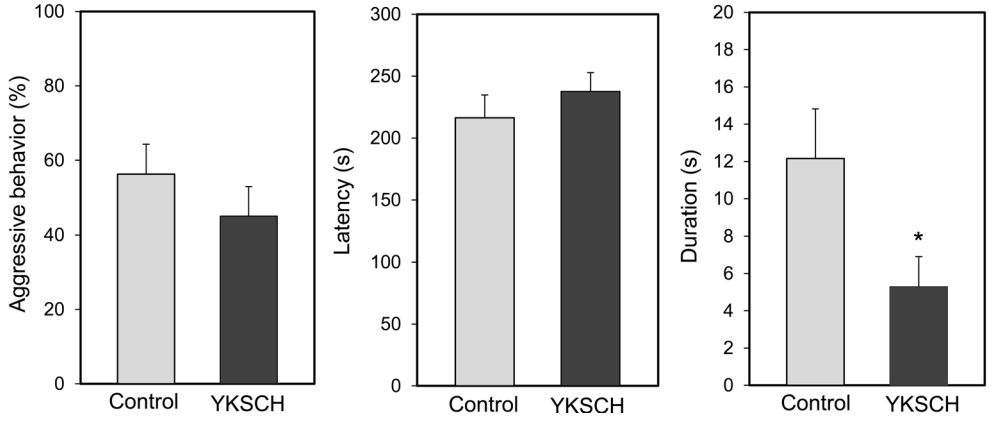


**Fig. 2.** Effect of YKSCH administration on aggressive behavior of zinc-deficient mice. Isolated mice were fed a zinc-deficient diet and YKSCH–containing water for 2 weeks. The resident–intruder test was performed as described in the materials and methods section. The tests were performed four times (n=8; total control and YKSCH (32 mice)). Each bar and line (mean ± SEM) represents the ratio (%) of mice that exhibited aggressive behavior to total mice, the time until start (latency) of aggressive behavior, and cumulative duration of aggressive behavior. **P*<0.01 (Student's *t*-test), vs. control.

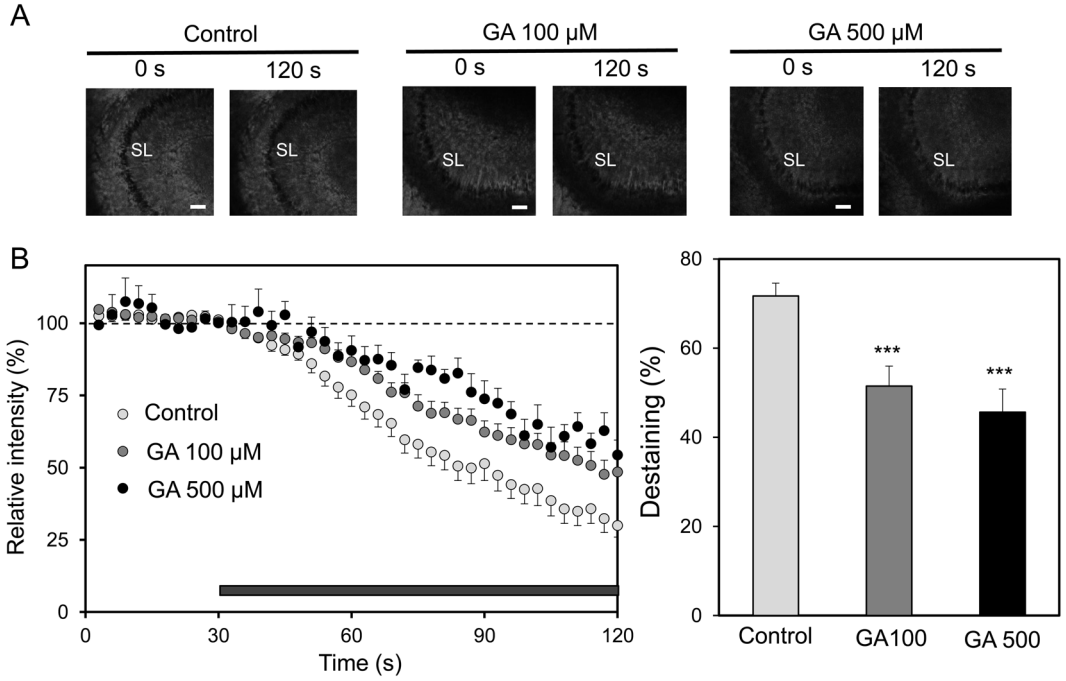


**Fig. 3.** Effect of GA on high $K^+$-induced exocytosis at mossy fiber boutons. Hippocampal slices (400 $\mu$m thickness) were prepared from rats fed a zinc-deficient diet and YKSCH–containing water for 2 weeks, labeled with FM4-64, immersed in CNQX + ACSF containing 100 $\mu$M or 500 $\mu$M GA for 15 min, and stimulated with 60 mM KCl (shaded bar) after measurement of the basal FM4-64 fluorescence for 30 s. (A) 0 s, basal images; 120 s, images 90 s after stimulation with KCl. Bar, 100 $\mu$m. To measure the decrease in FM4-64 fluorescence intensity at mossy fiber terminals, the region of interest (ROI, 5 regions) was set in the stratum lucidum (SL) of the CA3 region. (B) The data (mean ± SEM) represent the ratios (%) for each FM4-64 fluorescence intensity to the basal FM4-64 fluorescence intensity before stimulation with KCl, which was averaged and expressed as 100% (control, n=19; 100 $\mu$M GA, n=14; 500 $\mu$M GA, n=7). FM4-64 fluorescence was normalized by the maximal fluorescence intensity (the basal level) and the minimal fluorescence intensity 240 s after stimulation with KCl (left side). The data (mean ± SEM) represents the decreased FM4-64 fluorescence (destaining) (%) 90 s after KCl stimulation (right side). ****P*<0.001, vs. control (one-way ANOVA with Dunnett's test).

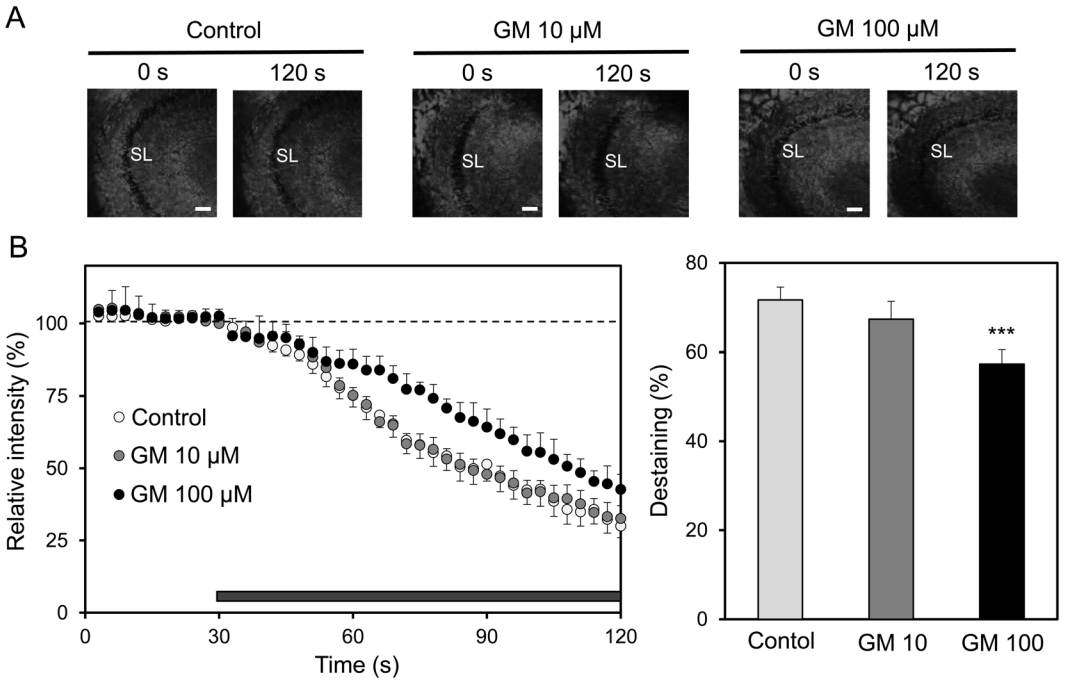


**Fig. 4.** Effect of GM on high $K^+$-induced exocytosis at mossy fiber boutons. Hippocampal slices (400 $\mu$m thickness) were prepared from rats fed a zinc-deficient diet and YKSCH–containing water for 2 weeks, labeled with FM4-64, immersed in CNQX + ACSF containing 10 $\mu$M or 100 $\mu$M GM for 15 min, and stimulated with 60 mM KCl (shaded bar) after the measurement of the basal FM4-64 fluorescence for 30 s. (A) 0 s, basal images; 120 s, images 90 s after stimulation with KCl. Bar, 100 $\mu$m. To measure the decrease in FM4-64 fluorescence intensity at mossy fiber terminals, five ROIs were set in the stratum lucidum (SL) of the CA3 region. (B) The data (mean ± SEM) represent the ratios (%) for each FM4-64 fluorescence intensity to the basal FM4-64 fluorescence intensity before stimulation with KCl, which was averaged and expressed as 100% (control, n=19; 10 $\mu$M GM, n=11; 100 $\mu$M GM, n=10). FM4-64 fluorescence was normalized by the maximal fluorescence intensity (the basal level) and the minimal fluorescence intensity 240 s after stimulation with KCl (left side). The data (mean ± SEM) represent the decreased FM4-64 fluorescence (destaining) (%) 90 s after KCl stimulation (right side). ***$P$<0.001, vs. control (one-way ANOVA with Dunnett's test).

Hange, in YKSCH. It is reported that Chimpi is a promising functional food for prevention of dementia such as AD [31] and that it has antianxiety-like effects [7]. On the other hand, zinc deficiency markedly suppresses the increase in body weight in young mice and rats [25, 26, 29]. Because body weight was found to be almost the same in the control and YKSCH-treated mice, it is likely that nutritional compensation is not involved in the actions of YKSCH on aggressive behavior.

On the other hand, it is possible that the common components in YKS and YKSCH are effective for reducing aggressive behavior of zinc-deficient mice housed individually. We tested the effects of GM and GA, which were contained in the YKS and YKSCH, on the increase in intracellular $Ca^{2+}$. GM is an indole alkaloid and a component of Uncaria Hook. It has been identified as the active component associated with the anti-aggressive actions of YKS [15]. GM reaches the brain parenchyma by passing through the blood–brain barrier after oral administration of YKS to the rats [6]. It has a partial agonistic action for serotonin (5-HT) 1A receptors and ameliorates aggressive behavior induced by isolation stress, and it reduces sociality in mice by stimulating 5-HT1A receptors [19]. Furthermore, GM ameliorates glutamate-induced neurotoxicity in the pheochromocytoma (PC12) cell line [9]. GA is the aglycone of glycyrrhizin and a component of Glycyrrhiza. It is called licorice root and is one of the common drugs in herbal medicines used clinically. Glycyrrhizin is metabolized to GA via β-glucuronidase activity in intestinal flora after oral administration [27] and is absorbed from the small intestine into the systemic circulation. Although the action of GA in the brain is poorly understood, its derivatives have ameliorative effects in rodent models

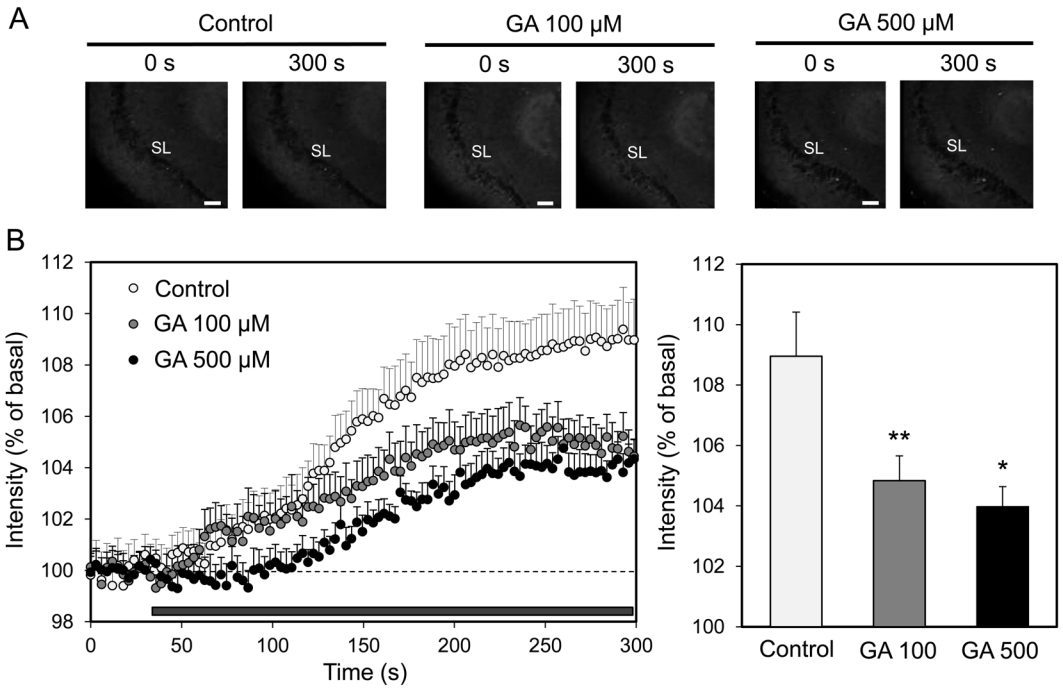


**Fig. 5.** Effect of GA on high $K^+$-induced increase in intracellular $Ca^{2+}$. Hippocampal slices were prepared from rats fed a zinc-deficient diet and YKSCH–containing water for 2 weeks, stained with calcium orange AM for 30 min, immersed in ACSF for at least 30 min, transferred in 100 $\mu$M or 500 $\mu$M GA in ACSF, and stimulated with 60 mM KCl for 270 s (shaded bar) after measurement of the basal calcium orange fluorescence for 30 s. (A) 0 s, basal images; 300 s, images 270 s after stimulation with KCl. Bar, 100 $\mu$m. Five ROIs were set in the stratum lucidum. Each point and line (the mean ± SEM) represents the rate (%) of fluorescence intensity after stimulation with KCl to the basal fluorescence intensity before stimulation, which was represented as 100% (control, n=7; 100 $\mu$M GA, n=9; 500 $\mu$M GA, n=7) (left side). (B) The data (mean ± SEM) represent averaged rates (%) of fluorescence intensity for the last 30 s after KCl stimulation (right side). *$P$<0.05, vs. control (one-way ANOVA with Dunnett's test). **$P$<0.01, vs. control (one-way ANOVA with Dunnett's test).

of AD and amyotrophic lateral sclerosis [28]. Mizoguchi *et al.* [16] reported that the immune response of 11β-hydroxysteroid dehydrogenase type-1, which is a molecule recognized by GA, is observed markedly in neurons, moderately in astrocytes, and scarcely in microglial cells in the hippocampus, indicating that GA acts via specific binding sites in the rat brain parenchyma. Based on these findings, it is possible that the actions of GM and GA contribute to decreasing the aggressiveness of zinc-deficient mice.

The actions of GA and GM were examined by focusing on excess excitation in the hippocampus, which was induced by zinc deficiency and potentially linked to aggressive behavior [24, 25]. Zinc deficiency elevates the blood glucocorticoid level in mice and rats and induces excess glutamatergic neuron activity. Because an *in vivo* hippocampal microdialysis experiment indicated that an abnormal increase in extracellular glutamate induced with 100 mM KCl was suppressed by administration of YKS to zinc-deficient rats [26], the actions of GA and GM on excess glutamate exocytosis induced by zinc deficiency were assessed in hippocampal slices from zinc-deficient rats. Excess exocytosis at mossy fiber boutons, which was induced with 60 mM KCl, was significantly attenuated in the presence of GA (100–500 $\mu$M) or GM (100 $\mu$M). The increase in intracellular $Ca^{2+}$ level induced with 60 mM KCl was also attenuated in the presence of GA (100–500 $\mu$M) or GM (100 $\mu$M). These results suggest that GA and GM ameliorate excess glutamate release from mossy fiber boutons by suppressing the increase in intracellular $Ca^{2+}$ signaling.

In conclusion, YKSCH reduced the cumulative duration of aggressive behavior in zinc-deficient mice housed individually. GA and GM may contribute to the reducing action, potentially by suppressing excess glutamatergic neuron activity.

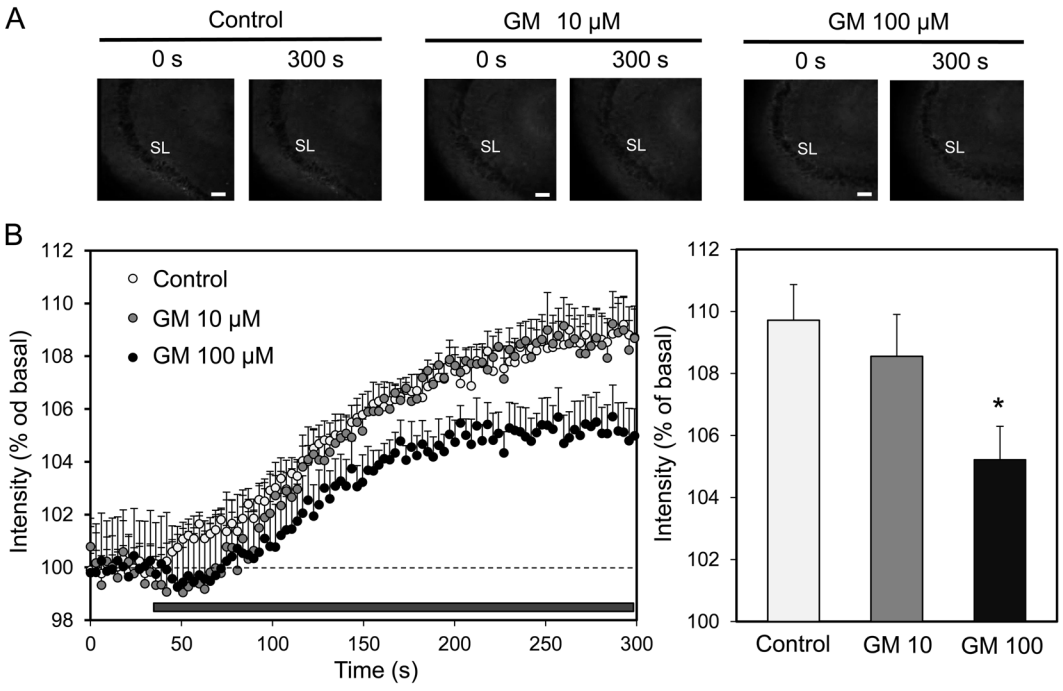


**Fig. 6.** Effect of GM on the high $K^+$-induced increase in intracellular $Ca^{2+}$. Hippocampal slices were prepared from rats fed a zinc-deficient diet and YKSCH–containing water for 2 weeks, stained with calcium orange AM for 30 min, immersed in ACSF for at least 30 min, transferred in 10 $\mu$M or 100 $\mu$M GM in ACSF, and stimulated with 60 mM KCl for 270 s (shaded bar) after measurement of the basal calcium orange fluorescence for 30 s. (A) 0 s, basal images; 300 s, images 270 s after stimulation with KCl. Bar, 100 $\mu$m. Five ROIs were set in the stratum lucidum. Each point and line (the mean ± SEM) represents the rate (%) of fluorescence intensity after stimulation with KCl to the basal fluorescence intensity before stimulation, which was represented as 100% (control, n=6; 10 $\mu$M GM, n=4; 100 $\mu$M GM, n=5) (left side). (B) The data (mean ± SEM) represent averaged rates (%) of fluorescence intensity for the last 30 s after KCl stimulation (right side). *$P$<0.05, vs. control (one-way ANOVA with Dunnett's test).

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