Regular Article

Effects of Oral Calcium Dosage and Timing on Ethanol-Induced Sensitization of Locomotion in DBA/2 Mice

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Ethanol (EtOH) dosage, frequency, and paired associative learning affect the risk of alcoholism. Recently, Spanagel *et al.* reported that acamprosate calcium (Acam Ca) prescribed for alcoholism exerts an anti-relapse effect *via* Ca. Ca is contained in foods, sometimes consumed with alcohol. Therefore, we investigated the association among oral Ca ingestion, EtOH-induced locomotor sensitization, and plasma Ca levels on how to consume Ca for moderate drinking. We used DBA/2 CrSlc mice, and CaCl₂ as water-soluble Ca salts. For pre-administration, elemental Ca (50, 75, 100, or 150 mg/kg, *per os* (*p.o.*)) or water for control was administered 1 h before EtOH (2 g/kg, 20 v/v (%) EtOH in saline) administration intraperitoneal (*i.p.*) for locomotor sensitization or for plasma Ca level changes. For post-administration, elemental Ca (100 mg/kg) was administered 1 h after EtOH. Moreover, we employed bepridil and the dopamine D1 antagonist, SCH-23390 to further examine the mechanism of EtOH-induced sensitization. The locomotor sensitization segmentalized for 300 s had two peaks (0–90 s and 180–300 s). Pre-administration of Ca (50, 75, and 100 mg/kg) significantly reduced the 0–90-s peak, selectively blocked by SCH-23390, but “non-dose dependently” as Ca 150 mg/kg did not have this effect. Bepridil blocked the suppressive effect of pre-administration of Ca (100 mg/kg). The effective pre-doses of Ca (50–100 mg/kg) maintained plasma Ca basal levels against EtOH-induced decrease of Ca. On the contrary, post-administration of Ca inversely led to significant promotion of sensitization of both locomotor peaks. Oral Ca intake had diverse effects on EtOH-induced sensitization depending on Ca dosage and timing.

Key words alcoholism; acamprosate calcium; bepridil; ethanol; dopamine antagonist

Heavy and long-term alcohol consumption is associated with an increased risk of more than 200 diseases such as liver cirrhosis, cancer, pancreatitis, depression, and dementia.¹⁾ Disease burden is related to amount of alcohol consumption and drinking patterns (drinking frequency during the week and in relation to food consumption)^{1,2)} On the contrary, many epidemiological studies have also suggested that moderate alcohol consumption reduces the risk of mortality and of some diseases, such as cardiovascular diseases^{3,4)} and dementia.⁵⁾ Moderate drinking is implicated in health and QOL. For clarifying the influence of moderate alcohol consumption on health, we have reported the risk of reward by moderate-dose/long-term EtOH administration⁶⁾ and the relation between moderate/sustained alcohol intake and health during the lifespan in rodents.⁷⁾

Acamprosate calcium (Acam Ca) is widely used as an anti-relapse medication along with therapy in the treatment of alcohol dependence.⁸⁾ Acam Ca has an anti-relapse effect and suppresses alcohol reward preclusively following intraperitoneal (*i.p.*)⁹⁾ and *per os* (*p.o.*) administrations in rodents.⁶⁾ The mechanism of action of acamprosate is believed to block glutamatergic *N*-methyl-D-aspartate receptors and activate gamma-aminobutyric acid type A receptors.^{10–12)} Recently, Spanagel *et al.* reported that Acam Ca prescribed for alcoholism exerts an anti-relapse effect *via* Ca.¹³⁾ Ca signaling regulation plays an important role in neuronal cells, although Ca contribution on the anti-relapse effects of Acam Ca remains controversial.^{14,15)} Changes in intracellular Ca dynamics by alcohol stimulation have been observed during the development

of alcohol-induced psychological and physical dependence.^{16,17)} The resting concentration of the Ca ion in the cytoplasm is maintained around 100 nM, reported as 20000- to 100000-fold lower than the extracellular concentration.¹⁸⁾

On the contrary, plasma Ca levels are maintained within very narrow limits.¹⁹⁾ EtOH intake itself dose-dependently decreases blood Ca levels, (extracellular concentration for neurons) because of its effects on the parathyroid hormone (PTH), osteocalcin and the excretion of Ca into urine.^{20–23)} Ca contribution for EtOH-induced rewarding behaviors seems to be complicated in view of both extracellular and intracellular Ca signaling.

Ca is contained both in some medicines and in foods, sometimes eaten together with drinking. When we ingest Ca, the concentration of plasma Ca levels is temporarily elevated, while it is decreased by EtOH. We would like to know whether oral Ca intake would be beneficial for moderate drinking. Therefore, we investigated the relationship among oral Ca dosage and timing on EtOH-induced sensitization as a phenotypic behavior, and the changes in plasma Ca levels to obtain information on how to consume Ca for moderate drinking.

We used ethanol-induced sensitization of locomotor activity, a model of neurobehavioral plasticity implicated in addiction that occurs with repeated drug use.²⁴⁾ Many drugs of abuse stimulate locomotor activity and produce sensitization defined as an increase in the reinforcement strength of a drug following repeated exposure.^{6,25–27)} The results of EtOH-induced sensitization were associated with those of the conditioned place preference test in DBA/2.⁶⁾ A significant positive correlation

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was observed between sensitization of dopaminergic neuronal activity and behavioral sensitization induced by ethanol.²⁸⁾

EtOH-induced sensitization is regulated by dopamine D1 receptors.^{29–31)} Further, EtOH-induced behaviors are accompanied by an elevation of intracellular Ca.^{17,32–34)}

We previously observed two peaks in locomotor activity for 300 s induced by EtOH (2 g/kg) administration.⁶⁾ We speculated that both peaks in locomotion are developed in relation to the time-dependent changes in the function of the dopamine D1 receptor by repeated EtOH administration. Therefore, we investigated not only effects of oral Ca administration on the two peaks of locomotor activity, but also the blockage of the peaks with the dopamine D1 antagonist, SCH-23390.

This study is, to our knowledge, the first report of a relationship among Ca dosage and timing of administration and plasma Ca concentration for EtOH-induced sensitization in mice.

MATERIALS AND METHODS

Subjects We used DBA/2 mice in this study, as DBA/2 mice rapidly express a robust sensitization to EtOH.^{6,35,36)} Six-week-old DBA/2 CrSlc male mice (Japan SLC, Inc., Hamamatsu, Japan) were acclimated to the animal facility for 2 weeks before testing. The animals were group-housed (6 per cage) with *ad libitum* access to distilled water and standard chow (CRF-1, Charles River Laboratories, Yokohama, Japan). The animal facility was maintained at 23±1°C with 55% humidity and a 12-h/12-h light/dark cycle.

All experiments were approved by the Institutional Animal Care and Use Committee of SAPPORO BREWERIES LTD. (permit numbers 2015-002, 2016-001, and 2017-001).

Reagents EtOH and CaCl₂ 2 hydrate were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan) as a special grade chemical. Ca channel blocker, bepridil hydrochloride, and dopamine antagonist, R(+)-SCH-23390 hydrochloride, were obtained from Sigma-Aldrich (St. Louis, MO, U.S.A.). Acam Ca was prepared from Regtect® (Nippon Shinyaku Co., Ltd., Kyoto, Japan) by micronization with a force mill, followed by 3-time sifting and suspension in distilled water.

Locomotor Sensitization Tests Repeated exposure to alcohol and drugs of abuse enhances the motor-stimulant response termed behavioral sensitization, which has a significant positive correlation with dopaminergic neuronal activity.^{24–28)}

For EtOH-induced sensitization, we used a plastic black box (30×15×15 cm; Brain Science Idea Inc., Osaka, Japan) consisting of two chambers, a black chamber and a white chamber, divided by a sliding door.⁶⁾ Locomotor activity (locomotor distance) in the black area of the chamber was measured using the ANY-maze Video Tracking System (Stoelting Co., Wood Dale, IL, U.S.A.).⁶⁾

Before the sensitization tests, each mouse was placed in the black chamber in all experiments for 300 s once per day for 3 consecutive days as habituation.

Mice received Ca salts or distilled water by the *p.o.* route 1 h before EtOH administration in consideration of Ca absorption.³⁷⁾ We used CaCl₂ as water-soluble Ca salts. Ca doses were calculated as elemental Ca from CaCl₂. EtOH (2.0 g/kg) was used based on the dose-dependence to induce locomotor sensitization.⁶⁾ EtOH is rapidly absorbed into the blood in

a dose-dependent manner, after which it readily crosses the blood-brain barrier and reaches the brain. We, therefore, chose the *i.p.* route to elucidate the relationship between alcohol dosage and EtOH-induced sensitization.

Locomotor activity was recorded for 300 s (segmentalized 30 s) after EtOH or saline administration.

Effects of Ca Dosages before EtOH Administration on EtOH-Induced Sensitization Ca Dosages 50, 100, and 150 mg/kg (Locomotor Experiment 1)

We investigated whether the Ca dosage, before EtOH administration, affected the development of sensitization. Mice (*n*=34) were assigned to 4 groups (pre-water group: *n*=8, pre-Ca50 group: *n*=9, pre-Ca100 group: *n*=8, and pre-Ca150 group: *n*=9). Each mouse received a different dose of Ca (pre-water group: distilled water, pre-Ca50 group: Ca 50 mg/kg, pre-Ca100 group: Ca 100 mg/kg, pre-Ca150 group: Ca 150 mg/kg, administered by *p.o.*). One hour later, EtOH (2 g/kg, 20 v/v (%) EtOH in saline) was administered (*i.p.*). The sensitization tests were consecutively performed for 5 d (Admin 1–5, Admin 6–10 and Admin 11–15) with withdrawal-intervals of 2 d (weekends).

Additionally, we examined whether pretreatment with Ca itself has an effect on locomotor activity (pre-water_saline group and pre-Ca 150_saline group). Mice (*n*=8) in each group received distilled water or Ca 150 mg/kg by *p.o.*, respectively. One hour later, saline was administered (*i.p.*) and we measured locomotor activities (Admin 1–15).

Ca Dosage 75 mg/kg (Locomotor Experiment 2: Confirmation of Locomotor Experiment 1)

We investigated the effects of Ca (75 mg/kg) pre-administration which is the mean between 50 and 100 mg/kg to confirm the results of experiment 1. Mice (*n*=18) were assigned to 2 groups (pre-water group and pre-Ca75 group, each group; *n*=9). Each mouse received distilled water (pre-water group, *p.o.*) or Ca (75 mg/kg) (pre-Ca75 group, *p.o.*). One hour later, EtOH (2 g/kg, 20 v/v (%) EtOH in saline) was administered (*i.p.*). The sensitization tests were performed consecutively for 5 d (Admin 1–5 and Admin 6–10) with a withdrawal-interval of 2 d (weekend), and consecutively for 4 d (Admin 11–14 and Admin 15–18) with a withdrawal-interval of 3 d (weekend plus national holiday).

Antagonism by Ca Channel Blocker (Bepridil) against the Effects of Pre-administration of Ca (Locomotor Experiment 3)

L-Type and N-type Ca channels are engaged in ethanol-induced behaviors and neurochemical responses.^{38,39)} We investigated whether the Ca channel blocker prevented the effects of Ca on EtOH-induced sensitization. We chose a non-selective Ca channel blocker, bepridil. Mice (*n*=25) were assigned to three groups (pre-water group: *n*=8, pre-Ca100 group: *n*=9, and pre-Ca100+Bep group, *n*=8). Each mouse received distilled water (pre-water group, *p.o.*) or Ca 100 mg/kg (pre-Ca100 group, *p.o.*), or “Ca (100 mg/kg) and bepridil (50 mg/kg) mixture solution” (pre-Ca100+Bep group, *p.o.*). One hour later, EtOH (2 g/kg, 20 v/v (%) EtOH in saline) was administered (*i.p.*). The sensitization tests were consecutively performed for 5 d (Admin 1–5 and Admin 6–10) with a withdrawal-interval of 2 d (weekend).

Effects of Ca (100 mg/kg) after EtOH Administration on EtOH-Induced Sensitization (Locomotor Experiment 4)

We investigated whether post-administration of Ca showed

similar effects with pre-administration of Ca on the development of sensitization. Mice ($n=18$) were assigned to 2 groups (post-water group and post-Ca100 group, each group; $n=9$). Each mouse received EtOH (2 g/kg, 20 v/v (%) EtOH in saline, i.p.) and we recorded locomotor activity for 300 s. One hour after EtOH administration, each mouse received distilled water (post-water group) or Ca 100 mg/kg (post-Ca100 group). The sensitization tests were consecutively performed for 5 d (Admin 1–5, Admin 6–10, and Admin 11–15) with withdrawal-intervals of 2 d (weekends).

Inhibition by Dopamine D1 Antagonist of the Development of EtOH-Induced Sensitization (Locomotor Experiment 5)

There were two peaks (0–90 s and 180–300 s) of locomotor activity during the 300 s measurement of locomotion induced by EtOH. We investigated whether Ca-effective peak locomotion was concerned with the dopamine D1 receptor.

Mice ($n=36$) were assigned to 4 groups (saline–saline group, saline–EtOH group, D1–saline group, and D1–EtOH group, each group; $n=9$). Mice received saline (saline–saline group and saline–EtOH group, i.p.) or SCH-23390 (0.01 mg/kg) (D1–saline group and D1–EtOH group, i.p.). Thirty minutes later, each group was administered saline (saline–saline group and D1–saline group, i.p.) or EtOH (2 g/kg, 20 v/v (%) EtOH in saline, saline–EtOH group and D1–EtOH group, i.p.). The sensitization tests were consecutively performed for 5 d (Admin 1–5, Admin 6–10, Admin 11–15, and Admin 16–20) with withdrawal-intervals of 2 d (weekends).

Measurement of Total Ca Levels in Plasma after the Sensitization Tests Blood was sampled from the tail veins of the mice. After centrifugation at 3000 rpm for 5 min, the Ca concentration was determined by the Ca Assay Kit LS (CA31M, Metallogenics Co., Ltd., Chiba, Japan). We evaluated the relative changes of plasma Ca levels by Ca and EtOH administrations among the same ages after the sensitization tests.

Effects of Acam Ca Administration on Ca Levels in Plasma

We used twelve mice (39 weeks old). The Ca dose in Acam Ca (600 mg/kg) corresponded to elemental Ca (60 mg/kg). Before Acam Ca (600 mg/kg) administration (*p.o.*), the first blood was sampled. One hour later, the second blood was sampled for Ca measurement.

Effects of Ca before EtOH Administration on Ca Levels in Plasma

Mice ($n=36$, 14 weeks old) were assigned to 4 groups (water–saline group, water–EtOH group, Ca–saline group and Ca–EtOH group, each group; $n=9$). Blood was sampled from all mice and just after sampling, CaCl_2 solution (Ca 150 mg/kg, Ca–saline group and Ca–EtOH group) or distilled water (water–saline group and water–EtOH group) was administered (*p.o.*). One hour later, saline (water–saline group and Ca–saline group) or EtOH (2 g/kg, 20 v/v (%) EtOH in saline, water–EtOH group and Ca–EtOH group) was administered (i.p.) and mice were placed into the sensitization apparatus as was performed for the locomotor activity measurement. Five minutes later, the second blood was sampled.

Dose-Dependent Effects of Ca before EtOH Administration on Ca Levels in Plasma

Mice ($n=36$, 24 weeks old) were assigned to 4 groups (water group, Ca 50 mg group, Ca 100 mg group and Ca

150 mg group, each group; $n=9$). Blood was sampled from all mice and just after sampling, each mouse received a different dose of Ca (water group: distilled water, Ca50 group: Ca 50 mg/kg, Ca100 group: Ca 100 mg/kg, Ca150 group: Ca 150 mg/kg) (*p.o.*). One hour later, EtOH (2 g/kg, 20 v/v (%) EtOH in saline) was administered (i.p.) and mice were placed into the sensitization apparatus as was performed for the locomotor activity measurement. Five minutes later, the second blood was sampled.

Effects of Ca after EtOH Administration on Ca Levels in Plasma

Mice ($n=18$, 20 weeks old) were assigned to 2 groups (post-water group and post-Ca100 group, each group; $n=9$). Blood was sampled from all mice and just after sampling, each mouse received EtOH (2 g/kg, 20 v/v (%) EtOH in saline) administration (i.p.) and 1 h later, received distilled water (post-water group) or Ca 100 mg/kg (post-Ca 100 group) (*p.o.*). One hour after water or Ca administration, (approximately 2 h after the first blood sampling), the second blood was sampled for plasma Ca measurement.

Data Analysis SPSS software 10.0.7J for Windows (IBM Co., Armonk, NY, U.S.A.) and Microsoft Excel 2013 (Microsoft Corporation, Redmond, WA, U.S.A.) were used for all statistical analyses. Data in the text and figures are presented as mean $\pm$ standard error of the mean (S.E.M.).

Locomotor Sensitization

Between-group comparisons (Locomotor experiments 1, 2, 3, and 4) were performed by two-way ANOVA followed by Tukey's *post-hoc* test for multiple comparisons. A three-way ANOVA was applied for Locomotor experiment 5 [pretreatment (saline and dopamine D1 antagonist, SCH-23390), treatment (saline or, EtOH), and administration time on locomotor activity], followed by Bonferroni's *post-hoc* test. Within group comparisons before and after the withdrawal-intervals (between admin 5 and admin 6, and admin 10 and admin 11 in Locomotor experiment 5) were performed by unpaired *t*-test.

Plasma Ca Concentration

Within-group comparisons for plasma Ca concentration were performed by paired *t*-test. Between groups comparisons were performed by one-way ANOVA followed by Tukey's *post-hoc* test.

All data are expressed as means $\pm$ standard error (S.E.) A *p*-value <0.05 was considered to be statistically significant.

RESULTS

Plasma Ca Levels

Effects of Acam Ca on Plasma Ca Levels

The average plasma Ca levels were 2.2 ± 0.03 mM ($n=12$) and 2.4 ± 0.04 mM before and 1 h after Acam Ca (600 mg/kg) administration, respectively. There was significant change as per the paired *t*-test ($p=0.017$) before and after Acam Ca (600 mg/kg, about 60 mg/kg as elemental Ca) administration.

Effects of Ca before EtOH Administration on Plasma Ca Levels

The results are shown in Fig. 1. There were no significant changes in plasma Ca levels between the before and after administrations in the water–saline group ($p=0.372$), but significant changes between the before and after administrations were noted in the Ca–saline group ($p=0.017$). On the contrary, EtOH administration significantly reduced plasma Ca levels

in the water–EtOH group ($p=0.019$) but tended to increase plasma Ca levels by pre-administration of Ca (150 mg/kg) in the Ca–EtOH group ($p=0.100$).

Dose-Dependent Effects of Ca before EtOH Administration on Plasma Ca Levels

We next examined plasma Ca levels before Ca administration and after EtOH administration at three Ca doses. The dose-dependent changes of plasma Ca levels are shown in Figs. 2A and 2B. The plasma Ca concentration was significantly reduced by EtOH in the water group ($p<0.001$). On the contrary, by pre-administration of all Ca doses (50, 100, and

150 mg/kg), there were no significant differences between the before and after administrations ($p=0.649$, $p=0.474$, and $p=0.114$, respectively) as shown in Fig. 2A. The changes in plasma Ca concentration are shown in Fig. 2B. The increase of Ca in the plasma after EtOH administration varied dose-dependently with the Ca dosage of pre-administration. The Ca change in the water group was different from that in the Ca150 group ($p=0.061$). These results suggested that pre-administration of Ca maintains the basal levels of Ca against EtOH-induced decrease of Ca.

Effects of Ca after EtOH Administration on Plasma Ca Levels

The changes in plasma Ca concentration were -0.08 ± 0.04 and 0.04 ± 0.03 mM in the post-water and the post-Ca100 groups, respectively. There was no significant difference in the within-group comparison in both groups ($p>0.05$). The between-group comparison of changes in plasma Ca was significantly different ($p=0.023$).

Locomotor Sensitization

Effects of Ca Dosages before EtOH Administration on EtOH-Induced Sensitization. Ca Dosages 50, 100, and 150 mg/kg, Locomotor Experiment 1

We investigated whether the Ca dosage before EtOH administration affected the development of sensitization. Mice ($n=34$) were assigned to 4 groups (pre-water group; $n=8$, pre-Ca50 group; $n=9$, pre-Ca100 group; $n=8$, and pre-Ca150 group; $n=9$). The locomotor activity for 300 s (segmentalized 30 s) of Admins 1, 5, 10, 12, and 15 in each group is illustrated in Fig. 3A (water group), 3B (pre-Ca50 group), 3C (pre-Ca100 group), and 3D (pre-Ca150 group). With repeated EtOH administrations, the first locomotor peak appeared at 0–90 s and the second peak at 180–300 s, and both peaks increased with progression of sensitization in the 4 groups, as shown in Figs. 3A–D. Most of first locomotion peaks (0–90 s) were over 4.0 m in the water and pre-Ca150 groups, but not in the pre-Ca50 and pre-Ca100 groups. The 0–300-s locomotor sensitization is illustrated in Fig. 3E. We performed a two-way ANOVA to assess the effects of Ca on EtOH-induced locomotor activity. The administration [$F(14, 433)=44.3$, $p<0.001$] and Ca dosage [$F(3, 433)=8.59$, $p<0.001$] had significant effects on locomotor

Fig. 1. Effects of Ca (150 mg/kg) Administration before EtOH (2 g/kg) Administration on Plasma Ca Levels

Mice ($n=36$, 14 weeks old) were assigned to 4 groups (water–saline group, water–EtOH group, Ca–saline group, and Ca–EtOH group, each group; $n=9$). Blood was sampled from all mice and just after sampling, Ca 150 mg/kg (Ca–saline group and Ca–EtOH group) or distilled water (water–saline group and water–EtOH group) was administered (*p.o.*). One hour later, saline (water–saline group and Ca–saline group) or EtOH (2 g/kg, 20 v/v (%) EtOH in saline) (water–EtOH group and Ca–EtOH group) was administered (*i.p.*) and mice were placed into the sensitization apparatus as was performed for the locomotor activity measurement. Five minutes later, the second blood was sampled. *: $p<0.05$, paired *t*-test.

Fig. 2. Pre-administration of Ca Protects EtOH-Induced Decrease in Plasma Ca Levels

Mice ($n=36$, 24 weeks old) were assigned to 4 groups (water group, Ca 50 mg group, Ca 100 mg group, and Ca 150 mg group, each group; $n=9$). Blood was sampled from all mice and just after sampling, each mouse received a different dose of Ca (water group: distilled water, Ca50 group: Ca 50 mg/kg, Ca100 group: Ca 100 mg/kg, Ca150 group: Ca 150 mg/kg) (*p.o.*). One hour later, EtOH (2 g/kg, 20 v/v (%) EtOH in saline) was administered (*i.p.*) and mice were placed into the sensitization apparatus as was performed for the locomotor activity measurement. Five minutes later, the second blood was sampled. (A) Plasma Ca levels before and after EtOH administration. ***: $p<0.001$, paired *t*-test. (B) Changes in plasma Ca levels before and after EtOH administration. *p*-value, Tukey's *post-hoc* test.

Fig. 3. Effects of Ca Dosage before EtOH Administration on EtOH-Induced Sensitization

Locomotor experiment 1 (Ca dosage (50, 100, 150 mg/kg) and Locomotor experiment 2 (Ca dosage 75 mg/kg). Mice ($n=34$) were assigned to 4 groups (pre-water group: $n=8$, pre-Ca50 group: $n=9$, pre-Ca100 group: $n=8$, and pre-Ca150 group: $n=9$). Each mouse received a different dose of Ca (pre-water group: water, pre-Ca50 group: Ca 50 mg/kg, pre-Ca100 group: Ca 100 mg/kg, pre-Ca150 group: Ca 150 mg/kg, administered by *p.o.*). One hour later, EtOH (2 g/kg, 20 v/v (%)) EtOH in saline) was administered (*i.p.*). The sensitization tests were consecutively performed for 5 d (Admin 1–5, Admin 6–10, and Admin 11–15) with withdrawal-intervals of 2 d (weekends). In the pre-water_saline group ($n=8$) and pre-Ca150_saline group ($n=8$), mice received distilled water or Ca 150 mg/kg by *p.o.*. One hour later, saline was administered (*i.p.*) and measured locomotor activities (Admin 1–15). Mice ($n=16$) were assigned to 2 groups (pre-water_saline group: $n=8$ and pre-Ca150_saline group: $n=8$). Mice in each group received distilled water or Ca 150 mg/kg by *p.o.*, respectively. One hour later, saline was administered (*i.p.*) and measured locomotor activities (Admin 1–15). A. Locomotor activity for 300s (segmentalized 30s) on Admins 1, 5, 10, 12, and 15 (Locomotor experiment 1, pre-water group). B. Locomotor activity for 300s (segmentalized 30s) on Admins 1, 5, 10, 12, and 15 (Locomotor experiment 1, pre-Ca50 group). C. Locomotor activity for 300s (segmentalized 30s) on Admins 1, 5, 10, 12, and 15 (Locomotor experiment 1, pre-Ca100 group). D. Locomotor activity for 300s (segmentalized 30s) on Admins 1, 5, 10, 12, and 15 (Locomotor experiment 1, pre-Ca150 group). E. Locomotor activity for 0–300s (segmentalized 30s) on Admins 1–15 (Locomotor experiment 1). Solid line; ●: pre-water group, △: pre-Ca50 group, ■: pre-Ca100 group, *: pre-Ca150 group. Broken line; +: pre-water_saline group, ◆: pre-Ca150_saline group. Pre-water group vs. pre-Ca100 group ($p=0.034$), pre-Ca150 group vs. pre-Ca50 group ($p=0.011$), and pre-Ca100 group vs. pre-Ca150 group ($p<0.001$), two-way ANOVA followed by Tukey's *post-hoc* test. F. Locomotor activity for 0–90s on Admin 1–15 (Locomotor experiment 1). Solid line; ●: pre-water group, △: pre-Ca50 group, ■: pre-Ca100 group, *: pre-Ca150 group. Broken line; +: pre-water_saline group, ◆: pre-Ca150_saline group. Pre-water group vs. pre-Ca50 group ($p=0.001$), pre-Ca100 group vs. pre-Ca50 group ($p=0.003$) and pre-Ca150 group vs. pre-Ca50 group ($p=0.014$) and pre-Ca100 group vs. pre-Ca150 group ($p=0.033$), two-way ANOVA followed by Tukey's *post-hoc* test. G. Locomotor activity for 0–300s on Admin 1–18 (Locomotor experiment 2). Mice ($n=18$) were assigned to 2 groups (pre-water group and pre-Ca75 group, each group; $n=9$). Each mouse received distilled water (pre-water group, *p.o.*) or Ca (75 mg/kg) (pre-Ca75 group, *p.o.*). One hour later, EtOH (2 g/kg, 20 v/v (%)) EtOH in saline) was administered (*i.p.*). The sensitization tests were performed consecutively for 5 d (Admin 1–5 and Admin 6–10) with a withdrawal-interval of 2 d (weekend), and consecutively for 4 d (Admin 11–14 and Admin 15–18), with a withdrawal-interval of 3 d. ●: pre-water group, ○: pre-Ca75 group. Group ($p<0.001$), administration ($p=0.972$), two-way ANOVA. H. Locomotor activity for 0–90s on Admin 1–18 (Locomotor experiment 2). ●: pre-water group, ○: pre-Ca75 group. Group ($p<0.001$), administration ($p<0.001$) and group vs. administration ($p=0.961$), two-way ANOVA. Pre: pre-administration.

activity, but there was no interaction between the administration and Ca dosage [$F(42, 433)=0.42, p=1.000$]. Using Tukey's *post-hoc* test to perform between-group comparisons, there was a significant difference between the water group and the pre-Ca100 group ($p=0.034$). Moreover, the pre-Ca150 group differed significantly from the pre-Ca50 group ($p=0.011$) and the pre-Ca100 group ($p<0.001$), but not the pre-water group ($p=0.253$). The results of the 0–90-s locomotor sensitization are shown in Fig. 3F. The administration [$F(14, 433)=28.1, p<0.001$] and Ca dosage [$F(3, 433)=7.82, p<0.001$] had significant effects on locomotor activity, but there was no interaction between the administration and Ca dosage [$F(42, 433)=0.65, p=0.957$]. Using Tukey's *post-hoc* test, the pre-water group differed significantly from both the pre-Ca50 group ($p=0.001$) and the pre-Ca100 group ($p=0.003$), but not from the pre-Ca150 group ($p=0.833$). The pre-Ca150 group was also significantly different from the pre-Ca50 group ($p=0.014$) and the pre-Ca100 group ($p=0.033$).

Thus, the Ca pre-dosage effect was not dose-dependent.

Oral water or Ca 150 mg/kg administration itself did not have an effect on locomotor activity (pre-water_saline group and pre-Ca150_saline group, Figs. 3E and 3F).

Ca Dosage 75 mg/kg (Locomotor Experiment 2)

As shown in Fig. 2B, the plasma Ca levels after EtOH administration did not change by pre-administration of Ca (50–100 mg/kg). We confirmed the effect of Ca on sensitization using Ca (75 mg/kg) as the mean dose between 50 and 100 mg/kg.

The 0–300-s locomotor sensitization and 0–90-s locomotor sensitization induced by EtOH are illustrated in Figs. 3G and 3H. Using a two-way ANOVA to assess the effects of groups and administrations (Admin 1–18) on EtOH-induced locomotor activity, the administration [$F(17, 281)=13.3, p<0.001$] and group [$F(1, 281)=13.0, p<0.001$] had significant effects on locomotor activity. However, there was no interaction between administration and group [$F(17, 281)=0.45, p=0.972$] in the 0–300-s locomotor sensitization (Fig. 3G). Moreover, in the 0–90-s locomotor sensitization, the administration [$F(17, 281)=6.45, p<0.001$] and group [$F(1, 281)=28.3, p<0.001$] had

significant effects on locomotor activity, but there was no interaction between administration and group [$F(17, 281)=0.48, p=0.961$] (Fig. 3H).

Antagonism by Ca Channel Blocker (bepridil) against the Effects of Pre-administration of Ca (Locomotor Experiment 3)

We investigated whether the Ca channel blocker prevented the effects of Ca on sensitization of locomotion induced by EtOH. Mice ($n=25$) were assigned to three groups (pre-water group: $n=8$, pre-Ca100 group: $n=9$, and pre-Ca100+Bep group: $n=8$). The 0–300-s locomotor sensitization and 0–90-s locomotor sensitization are illustrated in Figs. 4A and 4B, respectively. We conducted a two-way ANOVA to assess the effects of the 3 groups and administrations (Admin 1–10) on EtOH-induced locomotor activity. In the 0–300-s locomotor sensitization (Fig. 4A), the administration [$F(9, 216)=20.2, p<0.001$] had significant effects on locomotor activity, but there was no significant difference in group [$F(2, 216)=0.322, p=0.725$] and no interaction between the administration and group [$F(18, 216)=0.321, p=0.997$]. In the 0–90-s locomotor sensitization (Fig. 4B), the administration [$F(9, 216)=32.2, p<0.001$] and group [$F(2, 216)=8.11, p<0.001$] had significant effects on locomotor activity, but there was no interaction between the administration and group [$F(18, 216)=0.279, p=0.999$]. Using Tukey's *post-hoc* test, the pre-water group differed significantly from the pre-Ca100 group ($p=0.004$), but not the pre-Ca100+Bep group ($p=0.842$). Moreover, the pre-Ca100 group differed significantly from the pre-Ca100+Bep group ($p<0.001$). Pre-administration of bepridil significantly blocked the suppression effects of Ca (100 mg/kg) on 0–90-s locomotor sensitization, but not that of 0–300s.

Effects of Ca (100 mg/kg) after EtOH Administration on EtOH-Induced Sensitization (Locomotor Experiment 4)

We investigated whether Ca after EtOH administration showed similar effects as the pre-administration of Ca on EtOH-induced sensitization of locomotion. Mice ($n=18$) were assigned to 2 groups (post-water group and post-Ca100 group, each group; $n=9$). The locomotor activity for 300s (segmentalized 30s) of Admins 1, 5, 10, 12, and 15 in each group is

Fig. 4. Antagonism by Ca Channel Blocker (Bepridil) against the Effects of Pre-administration of Ca (Locomotor Experiment 3)

Mice ($n=25$) were assigned to 3 groups (pre-water group: $n=8$, pre-Ca100 group: $n=9$, and pre-Ca100+Bep group: $n=8$). Each mouse received distilled water (pre-water group, *p.o.*) or Ca 100 mg/kg (pre-Ca100 group, *p.o.*) or "Ca (100 mg/kg) and bepridil (50 mg/kg) mixture solution" (pre-Ca100+Bep group, *p.o.*). One hour later, EtOH (2 g/kg, 20 v/v (%) EtOH in saline) was administered (i.p.). The sensitization tests were consecutively performed for days (Admin 1–5, and Admin 6–10) with a withdrawal interval of 2 d (weekend). (A) Locomotor activity for 0–300s on Admin 1–10. ●: pre-water group, □: pre-Ca100 group, *: pre-Ca100+Bep group. Group ($p=0.725$), administration ($p<0.001$) and group vs. administration ($p=0.997$), two-way ANOVA. (B) Locomotor activity for 0–90s on Admin 1–10. ●: pre-water group, □: pre-Ca100 group, *: pre-Ca100+Bep group. Pre-water group vs. pre-Ca100 group ($p=0.004$) and pre-Ca100+Bep group ($p=0.842$), pre-Ca100 group vs. pre-Ca100+Bep group ($p<0.001$), two-way ANOVA followed by Tukey's *post-hoc* test. Pre: pre-administration. Bep: bepridil.

illustrated in Fig. 5A (post-water group) and 5B (post-Ca100 group). Most of the first locomotion peaks (0–90 s) were about 4.0 m in the water group in this experiment (Fig. 5A), but about 5.0 m in the post-Ca100 group (Fig. 5B).

Using a two-way ANOVA to assess the effects of groups and administrations (Admin 1–15) on EtOH-induced 300-s locomotor activity, the administration [$F(14, 235)=24.4$, $p<0.001$] and group [$F(1, 235)=30.4$, $p<0.001$] had significant effects on locomotor activity. However, there was no interaction between administration and group [$F(14, 235)=1.64$, $p=0.069$] in the 0–300-s locomotor sensitization. The 0–90-s locomotor sensitization and 180–300-s locomotor sensitization are illustrated in Figs. 5C and 5D. In the 0–90-s locomotor sensitization (Fig. 5C), the administration [$F(14, 235)=10.6$, $p<0.001$] and group [$F(1, 235)=14.7$, $p<0.001$] had significant effects on locomotor activity, but there was no interaction between administration and group [$F(14, 235)=1.37$, $p=0.170$]. In the 180–300-s locomotor sensitization (Fig. 5D), the administration [$F(14, 235)=26.6$, $p<0.001$] and group [$F(1, 235)=23.5$, $p<0.001$] had significant effects on locomotor activity, but there was no interaction between administration and group

[$F(14, 235)=0.801$, $p=0.667$]. Moreover, the 180–300-s locomotor activity in the post-Ca100 group was significantly reduced at the withdrawal-interval (admin 5 vs. admin 6, $p=0.035$, and admin 10 vs. admin 11, $p=0.036$) by unpaired *t*-test within the post-Ca100 group, but not in the post-water group ($p>0.05$).

The Ca (100 mg/kg) after EtOH administration significantly promoted both the 0–90 s and 180–300 s peaks of the EtOH-induced sensitization.

Inhibition by Dopamine D1 Antagonist of the Development of EtOH-Induced Sensitization (Locomotor Experiment 5)

The dopamine D1 receptor signaling system regulates ryanodine receptors, a group of Ca channels, which play a role in expression in EtOH dependence.³³ Activation of inositol 1,4,5-trisphosphate (IP3)-induced Ca^{2+} signaling by dopamine is mediated through D1 dopamine receptors.³⁴ We investigated whether the Ca-effective 0–90-s and 180–300-s locomotor activities were concerned with the dopamine D1 receptor.

Mice ($n=36$) were assigned to 4 groups (saline–saline group, saline–EtOH group, D1–saline group, and D1–EtOH

Fig. 5. Effects of Ca after EtOH Administration on the EtOH-Induced Sensitization (Locomotor Experiment 4)

Mice ($n=18$) were assigned to 2 groups (post-water group and post-Ca100 group, each group; $n=9$). Each mouse received EtOH (2 g/kg, 20 v/v (%) EtOH in saline) (i.p.) and we recorded locomotor activity for 300 s. One hour after EtOH administration, each mouse received distilled water (post-water group) or Ca 100 mg/kg (post-Ca100 group). The sensitization tests were consecutively performed for 5 d (Admin 1–5, Admin 6–10, and Admin 11–15) with withdrawal-intervals of 2 d (weekends). (A) Locomotor activity for 300 s (segmentalized 30 s) on Admins 1, 5, 10, 12 and 15 (post-water group). (B) Locomotor activity for 300 s (segmentalized 30 s) on Admins 1, 5, 10, 12 and 15 (post-Ca100 group). (C) Locomotor activity for 0–90 s on Admin 1–15. ●: post-water group, □: post-Ca100 group. Group ($p<0.001$), administration ($p<0.001$) and group vs. administration ($p=0.069$), two-way ANOVA. (D) Locomotor activity for 180–300 s on Admin 1–15. ●: post-water group, □: post-Ca100 group. Group ($p<0.001$), administration ($p<0.001$) and group vs. administration ($p=0.170$), two-way ANOVA. Within group comparison before and after withdrawal-interval (admin 5 vs. admin 6, and admin 10 vs. admin 11), *: $p<0.05$ by unpaired *t*-test. Post: post-administration.

Fig. 6. Inhibition by Dopamine D1 Antagonist of the Development of EtOH-Induced Sensitization (Locomotor Experiment 5)

Mice ($n=36$) were assigned to 4 groups (saline-saline group, saline-EtOH group, D1-saline group, and D1-EtOH group, each group; $n=9$). Mice received saline (saline-saline group and saline-EtOH group, i.p.) or SCH-23390 (0.01 mg/kg) (D1-saline group and D1-EtOH group, i.p.). Thirty minutes later, each group was administered saline (saline-saline group and D1-saline group, i.p.) or EtOH (2 g/kg, 20 v/v (%)) EtOH in saline (saline-EtOH group and D1-EtOH group, i.p.). The sensitization tests were consecutively performed for 5 d (Admin 1-5, Admin 6-10, Admin 11-15, and Admin 16-20) with withdrawal-intervals of 2 d (weekends). (A) Locomotor activity for 300 s (segmentalized 30 s) on Admins 1, 3, and 5 (saline-EtOH group and D1-EtOH group). (B) Locomotor activity for 0-90 s on Admin 1-Admin 20. □: saline-saline group, ■: saline-EtOH group, ○: D1-saline group, ●: D1-EtOH group. Saline-EtOH group vs. D1-EtOH group, ***: $p<0.001$, three-way ANOVA followed by Bonferroni's *post-hoc* test. (C) Locomotor activity for 180-300 s on Admin 1-20. □: saline-saline group, ■: saline-EtOH group, ○: D1-saline group, ●: D1-EtOH group. Saline-EtOH group vs. D1-EtOH group, **: $p<0.01$, ***: $p<0.001$, three-way ANOVA followed by Bonferroni's *post-hoc* test. Admin: administration. D1: D1 antagonist (SCH-23390). EtOH: ethanol.

group, each group; pretreatment-treatment, $n=9$). The locomotor activity for 300 s (segmentalized 30 s) of Admins 1, 3, and 5 in the saline-EtOH group and D1-EtOH group is illustrated in Fig. 6A. From Admin 1-5, the first locomotor peak (0-90 s) was blocked by SCH-23390 (0.01 mg/kg), but not the second locomotor peak (180-300 s). The 0-90-s and 180-300-s locomotor sensitizations are illustrated in Figs. 6B and 6C.

In the locomotor activities (0-90 s) (Fig. 6B), a three-way ANOVA to assess the influence of pretreatment (saline and SCH-23390), treatment (saline and EtOH), and administration time on locomotor activity demonstrated significant main effects of pretreatment [$F(1, 622)=4601.3$; $p<0.001$], treatment [$F(1, 622)=1872.8$; $p<0.001$], and administration time [$F(19, 622)=5.17$; $p<0.001$], as well as significant interactions between pretreatment and treatment [$F(1, 622)=1406.6$; $p<0.001$], and pretreatment and administration time [$F(19, 622)=5.37$; $p<0.001$], treatment and administration time [$F(19, 622)=5.17$; $p<0.001$], and pretreatment, treatment and administration time [$F(19, 622)=4.69$; $p<0.001$]. Using Bonferroni's *post-hoc* test, the locomotor activity in the D1-saline group was significantly lower than that in the saline-saline group

($p<0.001$; Admin 1-20) at SCH-23390 (0.01 mg/kg). The locomotor activity in the D1-EtOH group was significantly lower than that in the saline-EtOH group ($p<0.001$; Admin 1-20), although a great deal of thought should be given to the dose of SCH-23390 used (Fig. 5B). The SCH-23390 (0.01 mg/kg) pre-dose blocked EtOH-induced locomotion (0-90 s).

In the locomotor activities (180-300 s) (Fig. 6C), a three-way ANOVA to assess the influence of pretreatment (saline and SCH-23390), treatment (saline and EtOH), and administration time on locomotor activity demonstrated significant main effects of pretreatment [$F(1, 622)=691.8$; $p<0.001$], treatment [$F(1, 622)=1550.3$; $p<0.001$], and administration time [$F(19, 622)=3.16$; $p<0.001$], as well as significant interactions between pretreatment and treatment [$F(1, 622)=63.4$; $p<0.001$], and pretreatment and administration time [$F(19, 622)=5.98$; $p<0.001$], treatment and administration time [$F(19, 622)=4.33$; $p<0.001$], and pretreatment, treatment and administration time [$F(19, 622)=8.10$; $p<0.001$].

Using Bonferroni's *post-hoc* test, the locomotor activity in the D1-saline group was significantly lower than that in the saline-saline group ($p<0.05$; Admin 1-20). On the contrary, the locomotor activity from Admin 1 to Admin 5 in the D1-EtOH

group did not significantly differ from that in the saline–EtOH group ($p > 0.05$, Fig. 6C) unlike the 0–90-s locomotion ($p < 0.001$, Fig. 6B). From Admin 6–20, there was a significant difference between the saline–EtOH group and the D1–EtOH group ($p = 0.008$; Admin 6, $p < 0.001$; Admin 7–20). The SCH-23390 (0.01 mg/kg) pre-dose did not block EtOH-induced locomotion in the early stage of sensitization (like Admin 1–5), but blocked it after the progression of sensitization.

DISCUSSION

Changes in Plasma Ca Levels by Ca (*p.o.*) and/or EtOH (*i.p.*)

In our previous study, Acam Ca (600 mg/kg; *p.o.*) 1 h before EtOH-conditioning significantly reduced the development of EtOH-induced CPP.⁶⁾ The molecular weight (MW) of elemental Ca per Acam Ca is about 1/10 (Ca: MW 40, Acam Ca: MW 400.48). At this dose, plasma Ca levels were significantly increased 1 h post administration. In other studies, using rodents, the doses of Acam Ca that significantly suppressed CPP or sensitization induced by EtOH 2 g/kg (*i.p.*) were 300 mg/kg (*p.o.*),⁴⁰⁾ 400 mg/kg (*i.p.*),⁴¹⁾ and 300 mg/kg (*i.p.*).⁹⁾ Since plasma Ca levels after Ca salt administration were rapidly increased by *i.p.* compared to *p.o.* administration,³⁷⁾ the Acam Ca dose in those reports may be sufficient for temporal changes in plasma Ca levels.

Next, we used CaCl_2 as water-soluble Ca salts. Although Ca is absorbed in the mammalian small intestine, there might be a difference in Ca absorption rate between Acam Ca and Ca salts with food additives.^{42–44)} Therefore, we first examined plasma Ca changes with a high dose of Ca (150 mg/kg). As shown in Fig. 1, plasma Ca levels significantly decreased by EtOH (2 g/kg, *i.p.*) administration as previously reported.^{22,23,45)} Plasma Ca levels were significantly increased 1 h after pre-administration of Ca (150 mg/kg), a period nearly equivalent to that required for maximum plasma Ca levels after oral Ca administration. Moreover, the pre-administration of Ca (150 mg/kg) prevented the decrease of plasma Ca by EtOH and kept the baseline plasma Ca levels.

As shown in Fig. 2A, pre-administration of Ca (50–150 mg/kg) prevented the decrease of plasma Ca levels induced by EtOH. The Ca levels in the plasma of EtOH groups were dose-dependently increased by pre-administration of Ca (Fig. 2B), even though plasma Ca levels are strictly controlled within very narrow limits.^{18,19)} The pre-administration doses of Ca to maintain the baseline of plasma Ca levels after EtOH 2 g/kg (*i.p.*) were between 50 and 100 mg/kg. From these results, the decrease of plasma Ca by EtOH could be prevented by pre-administration of Ca salts.

Next, we investigated whether Ca dosages (50–150 mg/kg) before EtOH (2 g/kg) administration blocked the development of sensitization.

Locomotor Sensitization

Inhibitory Effects of Ca Dosages before EtOH Administration on EtOH-Induced Sensitization (Locomotor Experiments 1 and 2)

Locomotor sensitization had a significant positive correlation with dopaminergic neuronal activity.²⁸⁾ The measurement of locomotor activity after drug use such as cocaine and methamphetamine is normally examined for a certain period of time (*e.g.*, 5 min–2 h varied with tests); however, segmentalized changes in locomotor activity like in 30 s intervals have

not been reported. In our previous study,⁶⁾ we found two elevated peaks of locomotor activity during a 300-s measurement after repeated EtOH administrations. In this experiment, we found reproducible results that locomotor activity during the 300-s measurement had two peaks at 0–90 s and 180–300 s. With the progression of EtOH-induced sensitization, both locomotor peaks became larger, as shown in Figs. 3A–3D. The 0–90-s locomotor activity in the pre-Ca50 and pre-Ca100 groups were under 4.0 m of locomotor activity and lower than in the pre-water and pre-Ca150 groups.

There were no significant differences of both 0–300-s and 0–90-s locomotor activities between the pre-water and pre-Ca150 groups, as shown in Figs. 3E and 3F. On the contrary, the pre-administration of Ca (100 mg/kg) significantly reduced both 0–300-s and 0–90-s locomotor activity (Figs. 3E, 3F). Further, the sensitization of the first peak of 0–90 s, not the second peak of 180–300 s, was suppressed by pre-administration of Ca (50 mg/kg and Ca 100 mg/kg, Figs. 3A–3D and 3F). Moreover, the sensitization of the pre-Ca150 group was significantly different from that of the pre-Ca50 and pre-Ca100 groups (Fig. 3F). The suppressed effects of Ca on locomotor sensitization was not dose-dependent. The effective Ca doses corresponded to pre-administration doses of Ca 50–100 mg/kg, which prevented the decrease of plasma Ca levels by EtOH (2 g/kg) (Fig. 2B).

Additionally, we conducted pre-administration of Ca at the dose of Ca (75 mg/kg), as the mean between 50–100 mg/kg (Locomotor experiment 2) to confirm the hypothesis on the role of Ca on EtOH-induced sensitization. As shown in Figs. 3G and 3H, with pre-administration of Ca (75 mg/kg), the EtOH-induced sensitization was significantly reduced compared to that in the pre-water group in both the 0–300-s and 0–90-s locomotor sensitizations. These results confirmed those of Locomotor experiment 1. Moreover, the pre-administration of Ca was effective after the progression of sensitization (*e.g.*, since Admin 11) compared to the process of sensitization formation (*e.g.*, Admin 1–5). We believe that the optimal Ca dose against constant EtOH administration differs depending on the stage of sensitization progression and may be higher after the progression of sensitization.

Antagonism by Ca Channel Blocker (Bepridil) against Effects of Pre-administration of Ca (Locomotor Experiment 3)

To examine the effects of pre-Ca administration, we used the non-selective Ca channel blocker, bepridil (50 mg/kg). Bepridil hydrochloride is insoluble in water. Dimethyl sulfoxide is often used to make it soluble, but we were concerned about its toxicity in repeated administration (*i.p.*). To avoid the toxicity of dimethyl sulfoxide, bepridil was suspended in CaCl_2 solution (Ca 100 mg/kg). In this experiment, the 0–300-s sensitizations among the pre-water, pre-Ca100, and pre-Ca100+Bep groups were not significantly different (Fig. 4A). On the contrary, in the 0–90-s locomotor sensitization, Ca significantly blocked the sensitization induced by EtOH (Fig. 4B) similarly to Fig. 3F. Moreover, bepridil blocked the suppression effect of Ca (100 mg/kg) on EtOH-induced sensitization. From the results, we confirmed the effects of pre-administration of Ca on EtOH-induced sensitization of locomotion. From these results, we speculate that the first (0–90 s) locomotor peak may be especially influenced by change in plasma Ca levels induced by EtOH. The pre-administrations of Ca between 50

and 100 mg/kg maintained the baseline Ca levels even after EtOH administration, which may play a role in the significant reduction of sensitization.

In this experiment, the above results were obtained by usage of Ca100 mg/kg and bepridil (50 mg/kg), although Ca channel blockers attenuate ethanol-induced behaviors and neurochemical responses.^{38,39)} We conducted another experiment (water, Ca 75 mg/kg, Ca 75 mg/kg+bepridil 50 mg/kg; data not shown). In the Ca 75 mg/kg+bepridil group, the sensitization was more suppressed than in the Ca 75 mg/kg group. Therefore, the Ca pre-dosage for suppression of EtOH sensitization must be within a very strict range.

Effects of Ca after EtOH Administration on EtOH-Induced Sensitization (Locomotor Experiment 4)

We investigated whether Ca after EtOH administration showed similar effects on sensitization induced by EtOH as pre-administration of Ca. As shown in Figs. 5A and 5B, Ca 1 h after EtOH (2 g/kg) administration significantly promoted both the 0–90 s and 180–300 s peaks of the EtOH-induced sensitizations. The results of post-administration of Ca (Ca 100 mg/kg, Figs. 5C and 5D) were opposite to those of pre-Ca-administration (Ca 100 mg/kg, Figs. 3E and 3F), *i.e.*, the timing of Ca administration is very important for the sensitization.

We compared the effects of Ca on changes in plasma Ca levels between before and after Ca administration. Increase of plasma Ca levels in the post-Ca100 group (0.04 ± 0.03 mM) was not higher than that in the pre-Ca100 group (0.06 ± 0.07 mM, Fig. 2B). While it was difficult to explain the differences in plasma Ca levels between both timings in this experiment, we considered that there were dynamic changes in Ca signaling,^{18,19,32,33)} which may have optimal Ca levels after EtOH administration. It is well-known that the higher the EtOH doses, the more the sensitization is promoted.⁶⁾ EtOH administration dose-dependently arrests the decrease of plasma Ca levels.^{21,22,45)} Based on these reports and the results of Locomotor experiments 1–3, both EtOH doses and the following changes in plasma Ca levels by EtOH affected the progression of sensitization. We measured plasma Ca levels as total Ca. The active form of Ca is the free Ca ion and approximately half of total Ca is equivalent to the free Ca ion⁴⁶⁾; the rest of Ca is mainly albumin bound-Ca. The free Ca ion passes through the blood brain barrier⁴⁷⁾ and the extracellular Ca concentration in the brain depends on plasma Ca levels.⁴⁸⁾ By sustained stimulation of EtOH, the upregulation of L-type high voltage-gated Ca channels is induced in the cerebral cortex.⁴⁹⁾ Moreover, the intracellular Ca ion is elevated by increased type 1 IP3 receptor *via* facilitated mobilization of Ca ions from the intracellular Ca ion stores to the cytosol by EtOH stimulation.¹⁷⁾ Changes in intracellular Ca dynamics by alcohol stimulation may be observed *via* two routes during the development of EtOH-induced dependence, *i.e.*, route 1: Ca influx of exogenous Ca into the cytosol through Ca channels in the plasma membrane,⁴⁹⁾ and route 2: Ca release from the stores into the cytosol through Ca release channels mediated by IP3 receptors and ryanodine receptors.^{16,17)} In our results, only pre-administration of Ca (Ca dosage 50–100 mg/kg) significantly reduced the 0–90-s peak, but not the 180–300-s peak. The plasma Ca basal levels directly affected the Ca channels in plasma membrane (route 1) and the Ca dosage of pre-administration was important for maintenance of Ca basal levels (extracel-

lular Ca). In post-administration of Ca, both “Ca influx via Ca channels after stimulation by EtOH (route 1)” and “Ca release from Ca ion stores stimulated by both EtOH and increased Ca from route 1 (route 2)” might be promoted as a result of increase in intracellular Ca. We speculated that the 0–90-s peak and the 180–300-s peak were affected by route 1 and route 2, respectively. We should investigate whether intracellular Ca levels are changed in relation to plasma Ca levels after EtOH and/or oral Ca administrations.

Inhibition by Dopamine D1 Antagonist of the Development of EtOH-Induced Sensitization (Locomotor Experiment 5)

Intracellular Ca signaling¹⁷⁾ and the dopamine D1 receptor contribute to alcohol dependence.^{29–31)} Activation of inositol IP3-induced Ca^{2+} signaling by dopamine is mediated through D1 dopamine receptors.³⁴⁾ As mentioned above, pre-administration of Ca which blocked the reduction of Ca levels by EtOH, especially suppressed the 0–90-s peak (the first locomotor peak) of EtOH-induced sensitization (Figs. 3A–3D, 3F, 3H, and 4B), but not the second locomotor peak (180–300 s). On the contrary, post-administration of Ca promoted both peaks of EtOH-induced sensitization (Figs. 5A, 5B).

We investigated the role of dopamine D1 receptor in Ca-related effective 0–90 s and/or 180–300-s locomotion. As shown in Fig. 6A, the dopamine D1 antagonist (SCH-23390, 0.01 mg/kg) blocked only the first peak (0–90 s), not the second peak (180–300 s). The locomotor sensitization (0–90 s) induced by EtOH was blocked in Admin 1–20, although the locomotor activity in the D1-saline group was also blocked by the dose of SCH-23390 (0.01 mg/kg) (Fig. 6B). On the contrary, SCH-23390 did not block the second peak (180–300 s) from Admin 1 to Admin 5 but blocked it from Admin 6 to Admin 20 (Fig. 6C). From these results, both peaks were associated with the dopamine D1 receptor, but its contribution to the progression was changed by repeated administrations of EtOH. Moreover, in the D1–EtOH group the locomotor activities of the second peak were decreased at every weekend interval such as between Admin 5 and Admin 6, Admin 10 and Admin 11, and Admin 15 and Admin 16 (Fig. 6C). The results were contrary to those in the saline–EtOH group. The sensitivities of the dopamine D1 receptor were changed with the sensitization progression by repeated EtOH administrations and the washout period. They may be related to the results that the effects of the pre-administration of Ca could be observed only after the development of sensitization, but not in the early stage of sensitization from Admin 1 to Admin 5.

Both suppression (Figs. 3F, 3H) and promotion (Figs. 5C, 5D) of locomotor sensitization by Ca administration were remarkably observed after the progression of sensitization, together with co-occurring changes of sensitivity to dopamine D1 receptor in the 180–300 s peak (Fig. 6C). Moreover, locomotor activity was reduced during the withdrawal interval only in the post-Ca100 group (Fig. 5D). Nestler and Aghajanian reported that quantitative and functional changes in proteins affecting dopaminergic signals by repeated cocaine administrations are observed during withdrawal for a few days.^{50,51)} The effects of ethanol-induced sensitization by pre- and post-Ca administration may be more or less associated with the dopamine D1 receptor related changes in sensitivity, although further studies are needed to clarify the relation between extracellular and intracellular Ca levels.

In humans, drinking volume and drinking duration vary depending on the situation, personal habits, and other factors. Moreover, drinking is sometimes associated with eating, which affects the absorption rate of EtOH.⁵²⁾ Alcohol absorption of spirits was more rapid than that of beer or wine in a study with human participants.⁵³⁾ Therefore, the optimal Ca dose against plasma Ca drop by EtOH is inferred for the drinking. In the relationship between EtOH intake and plasma Ca, Petroianu *et al.* in a study with human participants reported that there was an inversely related diminution of serum Ca concentrations with increasing serum alcohol by acute EtOH ingestion.⁴⁵⁾ Moreover, Laitinen *et al.* reported Ca level changes after EtOH intake in human studies.^{22,23)} For example, they measured serum Ca concentrations in addition to blood alcohol concentration and blood PTH in humans before and at intervals up to 16 h after the ingestion of 1.2 to 1.5 g of EtOH/kg over a 3-h period (cross-over study: control was fruit juice). The blood alcohol peak was 4-h from drinking initiation. After a significant drop of PTH during the 3-h drinking period, the free Ca ion concentration significantly decreased from 1.18 ± 0.01 to 1.15 ± 0.01 mM in men, and from 1.20 ± 0.01 to 1.15 ± 0.01 mM in women after 6-h and the total Ca (about double concentration to that of the free Ca ion) presented a similar decrease.²³⁾ The decreased amount of total Ca after 8-h was 0.14 mM in that report²³⁾ and was similar to the water-EtOH group, in our study as shown in Figs. 1, 2A, and 2B (after EtOH 2 g/kg, i.p. administration). On the contrary, in the study of serum Ca concentration (total Ca) compared among four Ca salt supplements (Ca 900 mg/human participants), the serum peaks were obtained 4–6 h after Ca ingestion and the increased amount at the peak (peak-baseline) was about 0.07–0.15 mM.⁵⁴⁾ Therefore, we consider that plasma Ca changes in humans by EtOH or Ca supplement ingestion are realistically possible, although the Ca doses (Ca 50–150 mg/kg) of pre-administration were high in mice compared to human doses.

Moreover, phosphoric acid, a kind of acidic ingredient in soft drinks as a food additive affects Ca metabolism, such as inducing hypercalciuria.⁵⁵⁾ We may have to pay attention to both Ca and food components that cause Ca excretion.

Based on our results, we should pay attention to high Ca intake after heavy drinking, because blood alcohol level 1 h after EtOH (2 g/kg) administration remains high.⁵⁶⁾ Laitinen *et al.* reported that alcohol intake dose-dependently induces hypoparathyroidism, hypercalciuria, and hypermagnesuria.²²⁾ Repeated alcohol ingestion led to hypocalcemia. Nutritionally, Ca supplements should be prescribed for replenishment of Ca levels after EtOH consumption. Because Ca in foods is slowly released through the absorption process after food digestion, plasma Ca changes may be smaller than those observed with high dose Ca supplements. Ca should be supplied with food, or Ca supplements should be taken before drinking or the day after drinking.

Plasma Ca levels in patients with alcohol dependence are low compared to those of healthy people, because liver disease is often associated with abnormally low levels of Ca-binding albumin, and these patients may also have impaired vitamin D metabolism.⁵⁷⁾ Recently, Schuster *et al.* reported that lowered plasma Ca concentrations are a risk factor for relapse in patients with high alcohol intake, and especially in patients with increased craving.⁵⁸⁾

There may be differences in the optimal Ca doses and timing between healthy people and patients with alcohol dependence.

CONCLUSION

We investigated the association among oral Ca ingestion, EtOH-induced locomotor sensitization, and plasma Ca levels. The aim was to elucidate the association between Ca and EtOH in moderate drinking. Pre-administration of Ca (50, 75, and 100 mg/kg) significantly reduced the 0–90-s peak of sensitization of locomotor activity induced by EtOH, but not dose-dependently, as Ca (150 mg/kg) did not show the same effect. The effective Ca pre-doses (50–100 mg/kg) corresponded to those for maintaining basal plasma Ca levels against EtOH-induced decrease of Ca levels. On the contrary, post-administration of Ca inversely led to significant promotion of sensitization of both locomotor peaks. The effects of Ca on EtOH-induced sensitization and plasma Ca levels depended on the dose and the timing of administrations. Ca ingestion before and after EtOH administration induced diverse effects on EtOH rewarding effects.

Although further research is needed to determine the optimal dosage of Ca intake to balance losses after drinking in humans, we should take into consideration both the EtOH dosage and the timing of Ca intake.

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Conflict of Interest Chikako Shimizu and Youichi Tsuchiya are employees of SAPPORO HOLDINGS LTD. Yutaka Mitani is a retiree of SAPPORO HOLDINGS LTD. Toshitaka Nabeshima serves as a consultant to SAPPORO HOLDINGS LTD.

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