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Letter

Effects of a repeated low dose of LiCl injection under conditioned taste/flavor aversion using xylene

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ABSTRACT — We examined whether repeated injections with low-doses lithium chloride (LiCl) as unconditioned stimulus (US) established conditioning as applied conditioned taste aversion (CTA) experiment, using xylene solution as a conditioned stimulus (CS). In the conditioning procedure, water-deprived male rats were exposed to xylene solution for 30 min, followed by LiCl or saline injection. As a two-bottle test, xylene solution and usual drink water were simultaneously provided to rats on the next day of the conditioning and measured each consumption volume. Conditioning and two-bottle test were repeated eight times respectively by turns. Groups of no treatment and sham injection after xylene ingestion were added to verify the effects of external contexts on establishment of CTA. Results indicate that the CTA was gradually established when the US was repeatedly presented even if the US was very low concentration, and the organic solvent functioned as CS even if it was not so desirable for animals. External contexts, such as handling and the 'pain' induced by injection, did not affect the establishment of the CTA in the present study. Although xylene was used as solution in the present study and defined as flavor stimulus, gas should be used to examine the effects of odor stimulus.

Key words: Conditioned taste aversion (CTA), Rat, Lithium chloride (LiCl), Xylene

INTRODUCTION

Conditioned taste aversion (CTA) is a prominent field of research in behavioral sciences (Braveman and Crane, 1977). The CTA occurs when an ingested tastant or flavor is followed by toxicosis induced by drugs (Garcia and Ervin, 1968). When the tastant or flavor is subsequently presented to rats, the consumption of the tastant or flavor stimulus becomes less. In this associative learning paradigm, the conditioned stimulus (CS) is gustatory stimulus provided by the tastant or flavor and is assumed to have become associated with aversive properties related to sickness. The unconditioned stimulus (US) is the malaise induced by toxic agents (Schou, 1958a, 1958b), and the conditioned response (CR) is the suppression of tastant or flavor intake.

In the general CTA experimental paradigm using animals, lithium chloride (LiCl) is currently the most widely used emetic drug as the US. It causes nausea without dangerous side effects (Provenza *et al*., 1994), and has a short (about 6 hr in rats) biological half-life (Hatfield *et al*., 2001). Even if methods of administrating LiCl differ (for example, mixed in food, orally, or bolus, subcutaneous or intraperitoneal injections), LiCl appears equally effective in creating an aversion in subjects (Cross-Mellor *et al*., 2004). Nachman and Ashe (1973) showed a dose response curve using various volumes and concentrations of LiCl (0.15-0.65 M) with a single injection against sucrose solution. The dose-response curve indicated that a very strong aversion occurs at a dose of 3.0 mEq/kg and that the threshold dose for producing an aversion was approximately 0.15 mEq/kg. They concluded that the aversion was dependent on the absolute quantity of LiCl, not on the concentration or volume of solution. Although the concentration of LiCl for establishing the CTA with a single intraperitoneal injection (i.p.) usually ranges from 0.3 M (Malone and Cox, 1971) to 0.15 M (Ossenkopp, *et al*., 2011; Parker and MacLeod, 1991), some researchers have examined effects of lower levels of LiCl. Parker *et al*. (1988b) reported that 50.2 mg/kg LiCl produces a CTA after a single saccharin-lithium conditioning trial. Apparently, the US strength is asymptotic at

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50.2 mg/kg. In another experiment three kinds of doses of LiCl (12.2, 25.4, and 50.2 mg/kg) were repeatedly exposed to examine the probability of establishment of CTA (Parker, 1982). As the result, rats intraperitoneally administrated 12.2 mg/kg LiCl showed avoidance against sucrose solution after the second conditioning among 6 injections and drank the solution again after 7 hr from introduction of the extinction. Results implied that 12.2 mg/kg LiCl induced weak avoidance against sucrose solution because of the CTA procedure, indicating that a very low dose (12.2 mg/kg) of LiCl was almost the lowest concentration inducing LiCl-sucrose associative learning (Parker, 1982). In the series of CTA experiments using low-dose LiCl, Parker (1988b) clarified that the lower the concentration of LiCl, the more difficult establishment of CTA becomes. However, the effect in repeated administrations of LiCl lower than the lowest concentration that established CTA in a single dose is still not known.

So far, the solution to which animal originally shows disgust has rarely been used as CS under the CTA experimental paradigm. Most CSs used in the CTA experiments have been saccharin (De Brugada *et al*., 2003a), sucrose (Zalaquett and Parker, 1989), or any other sweetened solution (Parker, 1984) to which rats innately show palatability, or neutral for rats, such as NaCl (Scalera *et al*., 1997) or vinegar (Best *et al*., 1985). Recently, it is reported that some disgust solutions, for example a volatile organic, such as xylene, toluene, or formaldehyde, is suspected to induce mobility, showing a highly sensitive physical condition (Sorg *et al*., 2004). One hypothesis in literature insists that the sensitivity is induced by repeated exposure to ultra-low doses of these chemicals with mechanism of the CTA learning. However, the CTA experiment with repeated exposure to ultralow doses of these organic compounds has been rarely conducted thus far. Sorg, Swindell, and Tschirgi (2004) investigated behavioral sensitization induced by a repeated inhalation of a low dose of formaldehyde (FA) (2 ppm, 1 hr/day x 5 days/week x 4 weeks). As a result, it was revealed that repeated low-level formaldehyde exposure produced enhanced fear conditioning to odor in rats. Although experiments revealed behavioral modification showing higher sensitivity against formaldehyde than before, the experiment was not within the context of CTA. Then, we examined whether repeated injections with low-doses (0.03 M or 0.003 M) lithium chloride (LiCl) as an unconditioned stimulus (US) established a conditioning as an applied conditioned taste aversion (CTA) experiment, using xylene solution as a conditioned stimulus (CS). Hypotheses 1. Very low doses of LiCl might function if they are repeatedly administered. Hypothesis 2. If some usage of organic solvents is availably carried out, they can be used as CS. In our experiment, volume of injection was fixed as 1 mL, and low concentration of LiCl were used for repeated exposure. Accumulative volume of LiCl was nearly equivalent to the minimum volume of LiCl that established the CTA in a single injection. In the present study, external contexts, such as 'handling', or 'pain' by the injection, which affected the result of the CTA, were also examined.

MATERIALS AND METHODS

Animals and experiment schedule

Male Sprague-Dawley rats, 4 weeks of age, were purchased from Charles River Japan (Yokohama, Japan). They were housed individually in suspended stainless steel cages in a vivarium maintained at a temperature of 20°C with a 12:12 light cycle (8:00 hr-20:00 hr was the light cycle) the Japan National Institute of Occupational Safety and Health (JNIOSH). Food was available ad libitum throughout the experiment. After 5-day-acclimation, a water deprivation was introduced. For the first 5 days of the water deprivation, a water tube connected to the home cage was opened for 30 min from 16:00 hr. During the next 3 days, a glass drink bottle filled with usual drink water was provided for 30 min per day for training animal to drink water from the glass bottle in the home cage. Then a 2-trial preference phase, in which two bottles containing usual drink water and xylene solution were simultaneously presented to animals for 30 min in their home cages, was conducted. This phase was induced to examine whether animals showed any preference to the drink water or xylene solution. After this phase, the first trial of the conditioning was started. About 24 hr after the conditioning, the first trial of the two-bottle test was induced. Combination of the conditioning and the two bottle test was repeated in turn 8 times in total. The experimental schedule is summarized in Table 1. At the start of conditioning, animals' weights ranged from 285 to 332 g. During the experiment, we followed guidelines for the care and use of laboratory animals set forth by the Institutional Animal Care and Use Committee of the JNIOSH. All efforts were made to minimize animal suffering and to use a minimal number of animals. Throughout the experiment, water consumption and body weight of each rat were measured every day.

Preparation of the US (injection) and the CS (xylene solution)

Concentrations of 0.03 and 0.003 M lithium chloride (LiCl, Wako Pure Chemical Industries Ltd, Osaka, Japan,

Table 1. Schedule of the experiment.

Weeks of age	4					5								6						…		
	1	2	3	4	5	1	2	3	4	5	1	2	3	1	2	1		1		8		8
	Acclimation					Water deprivation								Pre-test		Conditioning		Two-bottle test		Conditioning		Two-bottle test
Arrival						Drink from tube attached to home cage (30 min)			Bottle training-drink from a water drink bottle (30 min)					Xylene solution and usual drink water presentation		Presentation of xylene solution (30min) + injection		Xylene solution and usual drink water presentation		Presentation of xylene solution (30min) + injection		Xylene solution and usual drink water presentation

After arrival, animals were housed individually in a home cage for 5 days for acclimation. A water deprivation was introduced. For the first 5 days of the water deprivation, a water tube connected to the home cage was opened for 30 min. During the next 3 days, a glass drink bottle filled with usual drink water was provided for 30 min per day for training animal to drink water from the glass bottle in the home cage. Then a 2-trial preference phase, in which two bottles containing usual drink water and xylene solution were simultaneously presented to animals for 30 min in their home cages, was conducted. This phase was induced to examine whether animals showed any preference to the drink water or xylene solution. After this phase, the first trial of the conditioning was started. About 24 hours after the conditioning, the first trial of the two-bottle test was induced. The combination of the conditioning and the two bottle test was repeated in turn eight times in total.

> 97 % pure) dissolved with saline were prepared for animals in two conditioning groups. Saline only was used for injection control animals. LiCl and saline were intraperitoneally injected into animals as the US. As the CS, p-xylene (Wako Pure Chemical Industries Ltd., > 97 % pure) water solution was provided to animals. Xylene solution was made as follows: 1 L of xylene solution, 100 µL *p*-xylene was dissolved with tap water in a glass bottle, was shaken overnight. Then 200 mL of the solution was taken out from bottom of the bottle with the extension tube of an intravenous drip, and shaken overnight again after being diluted to 2 L. The xylene solution, expected concentration of it was 8.6 ppm, was subdivided into glass drink bottles and presented to animals as the CS.

Conditioning

When the conditioning started, all animals were 6 weeks of age. The conditioning and the two-bottle test were conducted in their home cages. Animals were randomly assigned to one of the following groups: (1) the xylene solution was presented without injection (INTACT, n = 6), (2) the xylene solution was presented and the saline was injected as a control for injection (SALINE, n = 6), (3) the xylene solution was presented and conducted a sham injection (SHAM, n = 6), (4) the xylene solution was presented and 0.003 M LiCl was injected (0.003 M LiCl, n = 6), and (5) the xylene solution was presented and 0.03 M LiCl was injected (0.03 M LiCl, n = 5). In the conditioning, a weight of xylene solution with each glass drink bottle was pre-measured and provided to each animal for 30 min. After the 30-min presentation of xylene solution, 0.03 and 0.003 M LiCl were immediately intraperitoneally injected into animals in the 0.03 M LiCl and the 0.003 M LiCl groups, respectively. Saline was intraperitoneally administered to animals in the SALINE group. All injection volumes were 1 mL/head. In the INTACT group, animals stayed in their home cage. Animals in the SHAM were taken out from the home cages immediately after the presentation of the xylene solution. They were handled, and were touched upon their abdomen with an empty and needleless syringe to examine effects of 'handling' and 'touching abdomen' on establishment of the CTA. After these treatments, SHAM animals were returned to home cages. Injected rats were returned to their home cage immediately after the injection. About 2 hr after the conditioning, all animals were allowed access to a pre-weighted drink glass bottles filled with usual drink water for 30 min for preventing dehydration. The xylene solution and the usual drink water intakes were measured by weighing the bottle before and at the end of 30 min consumption. The experimental conditions are summarized in Table 2.

Two-bottle test

Twenty-four hours after the conditioning, the two-bottle test was conducted to assess whether animals showed aversion to the xylene solution. In the two-bottle test, bottles with the xylene solution and with the usual drink water were simultaneously provided for 30 min in their home cages. Relative consumption of the xylene solution

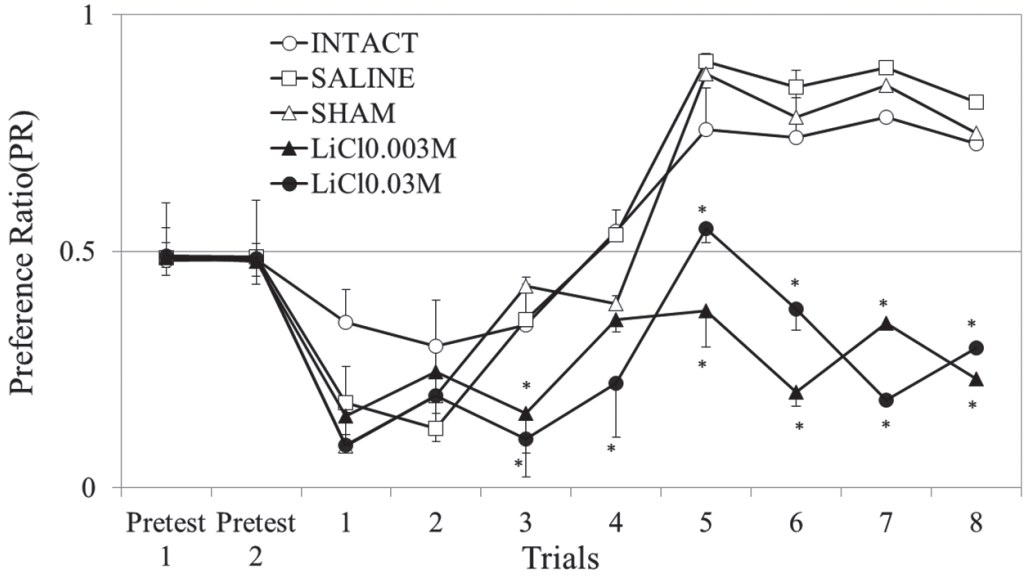

Fig. 1. Group means ± S.E. of preference ratios of xylene solution during 30 min on pretests 1 and 2, and trials 1-8 of the two-bottle test. Horizontal axis means trials of 30 min-two bottle test, vertical axis means preference ratio of xylene solution calculated as follows; the volume consumed from the bottles containing the xylene solution was divided by all drink consumption (the drink volume of solution of xylene plus usual drink water). White circle indicates preference ratio of animals in INTACT group, presentation of the flavor (xylene) solution only (n = 6). White triangle means that of animals in SALINE group, the flavor presentation paired with saline injection (n = 6). White square means SHAM, presentation of the flavor with sham injection (n = 6). Black triangle and circle indicate 0.003 M LiCl, the flavor presentation paired with 0.003 M LiCl injection (n = 6), and 0.03 M LiCl, flavor presentation paired with 0.03 M LiCl injection (n = 5), respectively. * means $p < 0.05$ (vs. SALINE).

Table 2. Summary of experimental groups and CTA procedure.

Groups	N	CS	US	
(1) INTACT	6	Xylene solution	-	Presentation of xylene solution only
(2) SALINE	6	Xylene solution	Saline	Presentation of xylene solution and saline injection
(3) SHAM	6	Xylene solution	-	Holding body, and touch to abdomen with syringe without needle
(4) LiCl 0.03 M	5	Xylene solution	LiCl 0.003 M	Presentation of xylene solution and 0.03M LiCl injection
(5) LiCl 0.003 M	6	Xylene solution	LiCl 0.03 M	Presentation of xylene solution and 0.03M LiCl injection

(1) presentation of the flavor (xylene) solution only (INTACT), (2) the flavor presentation paired with saline injection (SALINE), (3) presentation of the flavor with sham injection (SHAM), (4) the flavor presentation paired with 0.003 M LiCl injection (0.003 M LiCl), and (5) flavor presentation paired with 0.03 M LiCl injection (0.03 M LiCl).

was defined as preference ratio (PR). The PR was calculated as follows; bottles' weights after the test were measured with a burette with a graduation of 0.01 g, and consumptions of xylene and usual drink water were calculated each. Then the consumption volume of the xylene solution was divided by total drink consumption (the drink volume of the xylene solution plus that of the usual drink water). When PR of LiCl injection group was statistically smaller than that of the animals in the SALINE group, we judged that the conditioning was established. In order to examine the effects of external contexts, which were the 'handling' and the 'sham injection', the PR of the SHAM was compared with that of the SALINE group. In addition, to examine the effect of the 'pain' of the injection the PR of the SALINE and that of the INTACT were compared. The combination of the conditioning and the two-bottle test was repeated eight times.

Statistical analysis

Body weights on the third days of acclimation and on 5-day bottle training, on the first, the fourth, and the eighth days of two-bottle test days, and three days after the end of experiment, were separately analyzed by one-way analysis of variance (ANOVA). Also 30-min water consumptions on same days of body weight measurements were analyzed by repeated two-way ANOVA test. Main effects were experimental treatments (handling for SHAM, injections for saline and LiCl) and the repeated factor was days. PRs on days of pre-test were separately analyzed with one-way ANOVA. Post hoc comparisons were made with Dunnett's test following significant main or interaction effect by ANOVA to determine which treatment group(s) was/were different from the control (SALINE) group. A 5 x 8 mixed-factor ANOVA test for factors of experimental treatments (injection for SALINE and two LiCl groups, handling for SHAM group and no handling for INTACT group) and trials were used to evaluate effects. Separate one-way between-groups ANOVAs were conducted for the factor of experimental treatments on each trial. Post hoc comparisons were made with Newman-Keuls analyses following significant main or interactive ANOVA effects to determine which treatment groups were different from the control (SALINE) group. The level of statistical significance was set at $p < 0.05$ (two-tailed). Data was expressed as mean values ± S.E. All statistical analyses were performed with SPSS statistical package 19.0 software (IBM Inc., Chicago, USA).

RESULTS

In group means of body weights on the third days of acclimation and bottle training, on the first, fourth and eighth days of the two-bottle test, and three days after the end of experiment, reliable differences were not found between groups throughout the experiment (data not shown). There were not different among groups in PRs on days of pre-test (Fig. 1). The repeated two-way ANOVA revealed that there were no experimental treatment effects on 30 min water intakes on the third days of acclimation and bottle training, on the first, fourth, and eighth days of tests, and three days after the end of experiment (data not shown).

Figure 1 shows the group mean of PR on trials 1-8 of the two-bottle test. A 5 x 8 mixed ANOVA revealed a significant experimental treatment effect $F(4, 24) = 14.699$, $p < 0.01$, trial effect, $F(7, 18) = 30.553$, $p < 0.01$ and experimental treatment x trial effect $F(7,48) = 56.027$, $p < 0.01$. A subsequent analysis of the experimental treatment effect by means of a Newman-Keuls test revealed that animals in two LiCl-injected groups drank significantly less of the xylene solution ($p < 0.05$) than the SALINE, SHAM, and INTACT groups. Since the experimental treatment x trial effect was statistically significant, subsequent single-factor ANOVAs indicated that the group differed on trials 4-8, Fs(4, 24) = 9.364, 39.426, 26.857, and 18.598 for trials 5, 6, 7 and 8, respectively (all *ps* < 0.01). On trials 1-4 there were no significant differences among groups; groups consumed a similar mean amount of xylene during trials 1-4. Newman-Keuls comparisons, for each of trials 4-8, revealed that animals in 0.03 M LiCl and 0.003 M LiCl groups drank significantly less xylene solution than animals in SALINE, INTACT, and SHAM groups ($p < 0.05$), and no other effects were significant; therefore, as measured by the 30 min consumption test on each trial, all CS groups showed equivalent strength CTAs.

DISCUSSION

Animals that were repeatedly injected with 1 mL of low dose LiCl (0.003 M or 0.03 M) acquired flavor aversion using a low concentration of xylene solution as the flavor CS in the CTA experimental procedure. Animals in all groups drank a similar amount of xylene solution and usual drink water on the first and the second trials in pre-test, and on the first and the second trials in the two-bottle test. However, compared with intake volumes of xylene solutions of animals in INTACT, SHAM, and SALINE groups, intake volumes of animals injected with 1 mL of 0.003 M and 0.03 M LiCl were significantly decreased from trial 5 (except trial 4) and 3, respectively. Intake volumes of xylene solution for animals in 0.03 M and 0.003 M groups were similar. These results indicate that LiCl injections may act as a chemical stressor, even if LiCl concentrations are very low. In Parker's study (1982, 1984), 2 mL of 0.15 M LiCl was used as a low dose to animals weighing 277-366 g; the absolute quantity of LiCl to them was about 10.57-13.96 mg in total of three trials. On the other hand, absolute quantities of LiCl injections used in a total of 8 trials in our experiment were about 9.15 and 0.915 mg for animals in 0.03 and 0.003 M LiCl, respectively. The quantity in higher dose (0.03 M) of LiCl in our study was close to that of the lowest quantity in Parker (1982). This result is suggested to support the report by Nachman and Ashe (1973) that the aversion was dependent on the absolute quantity of LiCl and not on the concentration or volume of solution even if the CTA procedure administrated low doses of LiCl repeatedly. CTA may be established at around 10 mg of LiCl, regardless of the frequency of adminis-

tration or volume of a given dose. In Parker's experiments (1984), it was thought that 0.15 M LiCl induced a weak aversion, because the volume of consumption of saccharin solution in animals that were injected with 0.15 M LiCl was recovered quicker during the extinction procedure than that of animals that were administrated higher concentrations of LiCl. However, during conditioning/test, consumption of saccharin solution was avoided by animals injected with 0.15 M LiCl (0.15 mEq/kg) from the first trial of the CTA. In addition, it has been reported that concentration of 0.15 M LiCl was effective with a single dose to establish the CTA in rats (Nachman and Ashe, 1973). The CTA was also established in animals injected with 0.003 M LiCl in the present study. In this group, absolute volume was 0.915 mg of LiCl in total, and the consumption of xylene solution decreased from trial 5, indicating that the effective volume of LiCl injection might be much lower than 10 mg of LiCl. From point of view of Nachman and Ashe (1973), the minimum effective dose of LiCl could be 0.575 mg (0.115 mg x 5 trials). It seems that the illness induced by LiCl at the conditioning phase is slight since very-low-dose LiCl was administered to animal. Moreover, it seems that the illness could not be maintained between intervals (48 hr) of conditioning because the half-life of LiCl is about 30 min to 1 hr. Although this experiment's paradigm is the CTA, a process seems to be similar to usual classic conditioning. Mechanisms of associative learning for establishment of CTA may differ in the case where a single administration of LiCl can establish the CTA and in the case where repeated administration of LiCl is required. That is, the process of associative learning may vary according to the intensity of US. Further studies are needed to clarify this hypothesis.

While intake volumes of xylene solution for animals in INTACT, SHAM and SALINE groups were similar throughout the experiment, external contexts, which were 'handling' and 'pain' induced by injection in the present study, did not affect the CTA establishment. The result indicated that associative learning between LiCl injection and xylene solution was the only factor inducing the CTA. Results in the present study are consistent with the study that examined effects of some events to stimulate animals' external systems as disruptions for establishing the CTA (Holder *et al*., 1989). In the study by Holder *et al*. (1989), rats, given saccharin water followed by delayed illness, were exposed to events such as access to females, mild foot shocks, pain induced by intraperitoneal injection of hypertonic saline, and heat that changed both skin and core temperatures. Almost all of stimuli did not interfere with the acquisition or extinction of a taste aversion. Overall, both results emphasize the independence of internal system (i.e., the system that deals with internal events such as taste and illness) and the external system. Furthermore, associating of events related to internal system is not readily interfered with by events related to external system.

Previous experimental animal studies have reported that odor aversions were weaker than taste aversion (Bermudez-Rattoni, *et al*., 1988; Capaldi *et al*., 2004). However, results in the present study indicated that flavor stimulus (xylene) functions the same as CS under taste conditioned adverse learning. It is not surprising because previous studies reported that animals rapidly learn to avoid smells associated with malaise (Batsell and Best, 1992, 1993). Although xylene in the present study was used as water solution, if xylene is used as odor stimulus, it should be presented as gas. Because a chemical characteristic of xylene is insoluble in water and the concentration of xylene was below the detection limit value in the manufacture method in this experiment, xylene solution was defined as the 'odor' stimulus instead of as a taste or a flavor. Therefore, future studies should show the stimulus with gas. We would like to examine the method of conditioning that used the sense-of-smell stimulus with gas from now on. Heale *et al*. (1994) has reported that olfactory stimulation with toluene, xylene, and other organic solvents elicit a burst of 15-30 Hz fast waves, which were elicited by smells of predators, in the dentate gyrus of male rats, but not with other odorous substances, including food, rat vaginal secretions and rat excrement. Although the odor of xylene was novel for rats used in the present study, rats may innately have a negative preference for xylene.

In an experiment that uses a sense-of-smell (smell) stimulus of VOC, the stimulus should be presented as gas. A method of conditioning that uses VOCs as a gas stimulus could be established and examined in future experiments.

In conclusion, animals that were repeatedly injected with 1 mL of low dose LiCl (0.003 M or 0.03 M) acquired flavor aversion using a low concentration of xylene solution as the flavor CS in the CTA experimental procedure.

Repeated LiCl injections may act as a chemical stressor, even if LiCl concentrations are very low. The result is suggested that the aversion was dependent on the absolute quantity of LiCl and not on the concentration or volume of solution even if the CTA procedure administrated low doses of LiCl repeatedly.

While intake volumes of xylene solution for animals in INTACT, SHAM and SALINE groups were similar throughout the experiment, external contexts, which were

'handling' and 'pain' induced by injection in the present study, did not affect the CTA establishment. The result indicated that the associative learning between LiCl injection and xylene solution was the only factor inducing the CTA.

In an experiment that uses a sense-of-smell (smell) stimulus of VOC, the stimulus should be presented as gas. A method of conditioning that uses VOCs as a gas stimulus could be established and examined in future experiments.

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Conflict of interest---- The authors declare that there is no conflict of interest.

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