ORIGINAL ARTICLE

Genetic and environmental influences on one-trial conditioned context aversion in mice

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Abstract

Anticipatory nausea (AN) is caused by an association between contextual cues and the experience of nausea (the side effects of chemotherapy or radiation treatment) and it develops predominantly in female patients undergoing chemotherapy. Preclinical studies in rodents show that the administration of an illness-inducing agent in the presence of novel contextual cues can cause conditioned context aversion (CCA) and this has been proposed to model AN. The literature also suggests that brief pre-exposure to a novel context prior to shock delivery is critical in the development of contextual fear conditioning in rodents (a phenomenon known as Immediate Shock Deficit), but this has not been assessed in CCA. The aim of present study was to develop a CCA paradigm to assess this in outbred (CD1) and inbred (C57BL/6J) mice and evaluate potential sex differences. The results revealed that a single conditioning trial in which a distinctive context was paired with LiCl-induced illness was sufficient to elicit a conditioned response in both female and male CD1 outbred mice, but not in C57BL/6J inbred mice. In addition, CCA was facilitated when animals had prior experience with the context. Finally, outbred female mice showed longer and more robust retention of CCA than male mice, which parallels clinical findings. The results indicate the importance of using CD1 outbred mice as an animal model of AN as well as examining sex differences in the CCA paradigm. Similar findings in humans encourage the future use of this novel CCA preclinical mouse model.

KEYWORDS
anticipatory nausea, cancer, chemotherapy, conditioned context aversion, contextual learning, mice, one-trial conditioning, pre-exposure, sex differences, strain differences

1 | INTRODUCTION

Anticipatory nausea (hereafter, AN) is the most common and distressing side effect of chemotherapy treatment.[1-4] AN causes a significant reduction in patients' quality of life and it is the main reason for the discontinuation of the treatment.[5] The development of AN is thought to result from classical conditioning.[6] After cancer patients experience one or more nausea-inducing chemotherapy sessions (unconditioned stimulus [US]), the contextual cues (originally neutral stimuli), including the chemotherapy equipment, hanging ornaments on the walls, the clinic's smells and sounds etc., become conditioned stimulus (CS) that later trigger AN as a conditioned response.[7,8] In the laboratory, the conditioned context aversion (CCA) paradigm has become a valuable preclinical tool for modeling AN in chemotherapy patients.[9-12]

CCA is elicited by administering an agent that induces malaise such as lithium chloride (LiCl) in the presence of contextual cues which endow the context with conditioned properties.[13] In other words, after one or more (usually many) context-LiCl pairings, the context elicits nausea in the absence of LiCl, which is phenomenologically similar to what is observed in patients. Formation of CCA has been shown in rats as reduced explorative behavior, the lying-on-belly behavior, gaping,[14] and suppressed consumption of fluids,[13,15,16] and in mice as reduced water intake in the presence of contextual cues.[17,18]

Epidemiological studies show that AN occurs more frequently in female patients.[19–21] However, previous preclinical studies have exclusively used male animals preventing the exploration of sex differences in the CCA paradigm.[22–24] Due to the predominant use of male animal research subjects in CCA, clinical sex differences observed in cancer patients remain elusive. Also, the use of only male subjects in preclinical studies limits their translational value. Therefore, both sexes should be used especially in animal models where a sex difference is expected.

Furthermore, laboratory rats have been the main subjects of previous CCA studies.[8,13] Only two studies with the CCA paradigm have used genetically heterogeneous mice, a cross between C57BL/6J (B6) and DBA/2J (D2) strains as well as a cross between large and small strains.[17,18] In the current study, we compared two commonly used inbred and outbred mouse strains, C57BL/6J and CD1.The genetic differences underlying behaviors such as CCA can be investigated with inbred strains. However, outbred mice have more genetic variation and thus may be more useful for modeling the phenomenon in human populations. While many CCA studies have focused on rats, they overlook potential genetic differences underlying CCA. Although C57BL/6J and CD1 mice have been used in different learning tasks, to the best of our knowledge, there are no previous studies examining and comparing the development of CCA across these strains.

In rodents, the tendency to acquire aversions to contextual stimuli after the induction of illness has been documented in studies that conduct at least three or four conditioning trials in rats,[10,13,25,26] and mice.[17,18] Even though a single conditioning trial is important for investigating the pharmacological and neural basis involved in CCA, there are no previous studies investigating the development of CCA with a single conditioning trial. The use of multi-trial procedures to study CCA (and the dearth of observations of one-trial CCA) may be due to difficulties in contextual processing with limited exposure to the to-be-conditioned context. For example, Blanchard and colleagues[27] observed little learning in a single-trial contextual fear conditioning paradigm when they administered a shock immediately after placing the animals in the to-be-conditioned context (also see Reference 28). However, a short period of exposure to the context before shock administration (so-called pre-exposure) resulted in a freezing response during a subsequent test. In another study, Wiltgen et al. revealed that brief pre-exposure to a novel context prior to shock delivery is critical for enabling the development of contextual fear conditioning in rodents.[31] Thus, the literature suggests that the development of contextual fear conditioning in rodents may depend on some pre-exposure to a novel context prior to shock delivery. This phenomenon, which is commonly referred to as immediate shock deficit, has multiple causes. The consensus is that brief exposure to the context facilitates learning and conditioned response by increasing discriminability[29] and framing adequate context representations[30,31] in various behavioral paradigms, as previously mentioned. This is particularly the case in contextual fear conditioning studies (see References 32, 33). However, this effect has not been assessed and compared between inbred and outbred strains in the CCA paradigm.

The present two experiments examined whether pre-exposure to a novel context (training context) facilitates CCA learning in inbred and outbred strains of mice. Based on the reported effects of pre-exposure in the context fear conditioning literature, it was hypothesized that pre-exposure to a novel context prior to a conditioning trial will facilitate the development of CCA in both inbred and outbred mice. The experiments also investigated the acquisition and the retention of LiCl-induced CCA in outbred CD1 male and female mice. Based on the reported effects of sex differences in AN incidence in humans, it was hypothesized that the development of CCA and its retention would be stronger in females than in male mice.

2 | EXPERIMENT 1a

In Experiment 1a, C57BL/6 inbred male mice were tested to see whether pre-exposure to the context (CS) had any impact on the development of CCA.

2.1 | Subjects

The subjects were 48 twelve-week-old male C57BL/6 inbred mice that were divided into two groups: pre-exposed ($n = 24$; LiCl = 12 and NaCl = 12) and non-pre-exposed ($n = 24$; LiCl = 12 and NaCl = 12). Each of these two groups was subdivided into two further groups based on the conditioning history (LiCl vs. NaCl). A 12/12 h light/dark cycle (lights on from 07.00 to 19.00 h) was maintained in the colony room, and the temperature was kept at 24°C ±1. All experiments were conducted during the light phase of the cycle. There was no natural light in the room. Mice were housed in the colony room as referred to Context A (please see detailed explanation in Apparatus section). Mice had ad libitum access to tap water and pellets except when they were water-restricted, as described below. The protocol used in these experiments was approved by the Animal Ethics Committee of Middle East Technical University (protocol number: 21/1).

2.2 | Materials and methods

2.2.1 | Apparatus

Two different contexts were used in the experiments: a control context (home cages) and a conditioning context (conditioning cages). The

home cages were located in the colony room and are referred to as Context A; the conditioning cages were located in the conditioning room and were referred to as Context B. The colony room (Context A) was under normal housing conditions. This room had a 12/12 h light/dark cycle with no natural lighting. Each mouse housed individually in a Eurostandard Type II long standard cage with transparent walls, wood shavings, and a standard plastic bottle. The conditioning room (Context B) was a small separate room. In this room, a 60 W dim red lamp, 75 dB white noise, and lemon oil were used to generate a distinctive/novel environment, and present throughout pre-exposure and conditioning sessions. Although the cages used in the novel context were the same size as the home cages, the floor was covered with cat litter instead of wood shavings, and the cage walls were striped with black and white bands. We have previously shown that decanting water from plastic bottles into glass bottles immediately before conditioning trials has no significant impact on the rate of water consumption from US-paired containers. Thus, we confidently claimed that the conditioned context aversion is independent of any difference in the taste of water in plastic versus glass bottles.[17] In our current study, we used the same manipulation as in our previous studies and green-colored glass bottles with red tape were used in the novel context instead of the regular plastic bottles used in the home context.

Table 1 depicts the experimental timeline. Animals underwent six phases: habituation, water acclimation, pre-exposure, conditioning, recovery, and retention, in that order.

Habituation

At the beginning of the experiment, all mice experienced the habituation phase in Context A. During the habituation stage, each mouse was handled for 3 min per day on three consecutive days. A saline injection was used during the habituation phase to accustom the mice to the pain caused by the injection needle.[34] The saline injections were given intraperitoneally on the last day of habituation. After the mice were injected with saline, the water restriction started at 17.30 on the same day.

Water acclimation

During the water acclimation phase, mice were trained to drink water promptly. After undergoing 16.5 h of water deprivation

(beginning at 17.30 on the last day of habituation), mice had access to water in their home cages via their regular plastic tubes for 30 min on two separate occasions: 10.00–10.30 and 17.00–17.30. This procedure was repeated on three consecutive days during the acclimation phase.

Pre-exposure

On the last day of water acclimation, mice in the pre-exposure group were exposed for 5 min to the Context B. Pre-exposure took place 24 h before the conditioning trial. The animals in the non-pre-exposed group stayed in their home cages (Context A) during this phase.

Conditioning

Following the water acclimation and pre-exposure phases, a single conditioning trial was carried out (Table 1). The CCA conditioning trial was conducted in Context B. The mice were transferred to the room individually during the conditioning trial. In this room, all mice were exposed to Context B for 5 min so that they would be familiar with this environment before injection. Five minutes after entering Context B, the animals in the experimental groups received an intraperitoneal injection of LiCl (6 mEq/kg), and the control groups received the same volume 0.9% NaCl. After the injection, the mice stayed in Context B for an extra 15 min to complete a 20-min CCA trial. After the conditioning trial ended, subjects were returned to their home cages in the colony room. Water consumption of each mouse was measured by weighing the water bottles before and after the 20-min conditioning trial.

Recovery period

Prior to the retention test, a 2-day recovery period was given during which mice had access to water between 10.00–10.30 and 17.00–17.30, just as they did during the water acclimation sessions (Table 1).

Retention

Following the completion of the recovery period (72 h after conditioning), the animals were placed in Context B for 15 min for a retention test (Table 1). The strength of the CCA was assessed by measuring the subjects' water intake in Context B. The water consumption of each mouse was recorded.

TABLE 1   Experimental procedure.

Days 1–4	Days 5–7	Day 7	Day 8	Day 9–10	Day 11
Habituation context A	Water acclimation context A	Pre-exposure context B	Conditioning context B	Recovery context A	Retention context B
Handling and saline injection to accustom mice to the injection needle	Acclimation to drink water under a restricted schedule	Brief pre-exposure to the novel context to reduce neophobia, stress, and novelty	A single conditioning trial	Water access on two occasions per day	Water consumption test lasting 15 minutes

Note: *Experimental timeline*. The experiment consisted of six phases: habituation, water acclimation, pre-exposure, conditioning, recovery, and retention. The number of days during the retention phase varied by experiment.
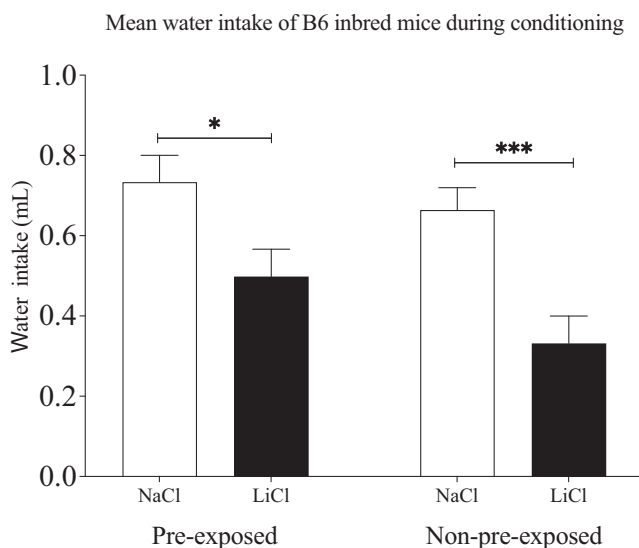
2.3 | Statistical analyses

Statistical analyses were performed using Prism GraphPad (Version 9). The water intake during conditioning and retention tests were assessed using an analysis of variance (ANOVA). Planned contrasts were performed independent of the statistical significance obtained from the F tests using Fisher's LSD test.[35] Confidence level was set to 95% for differences to be considered as significant ($p < 0.05$).

2.4 | Results

2.4.1 | Conditioning

Figure 1 shows the average water intake during conditioning of pre-exposed and non-pre-exposed groups of male C57BL/6J male mice given either LiCl or NaCl. Water intake during conditioning was lower for animals injected with LiCl relative to those which received NaCl. The water intake data was analyzed with a 2 (pre-exposure: pre-exposed vs. non-pre-exposed) × 2 (conditioning: NaCl vs. LiCl) facto-rial ANOVA. This analysis revealed a main effect of Conditioning, $F (1, 44) = 19.07$, $p < 0.001$, no main effect of Pre-exposure, $F (1, 44) = 3.294$, $p = 0.074$, and no interaction between these factors, $F (1, 44) = 0.563$, $p = 0.457$. Fisher's LSD analysis showed that ani-mals in the pre-exposed LiCl ($M = 0.498$, SD $= 0.235$) group drank significantly less water compared to animals in the pre-exposed NaCl ($M = 0.733$, SD $= 0.233$) group during conditioning ($p = 0.014$). Simi-larly, in the non-pre-exposed Groups, animals that received LiCl ($M = 0.332$, SD $= 0.237$) drank significantly less water compared to animals that received NaCl ($M = 0.664$, SD $= 0.192$; $p < 0.001$). These results indicate that the LiCl injections during conditioning induced ill-ness and reduced water consumption.

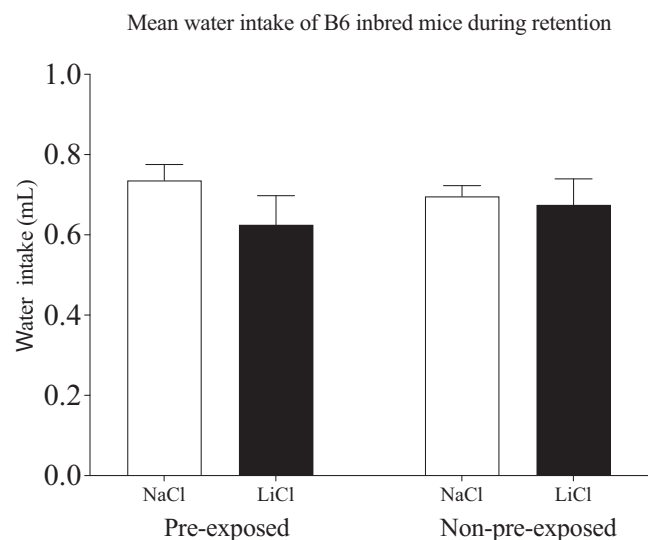### 2.4.2 | Retention test

Figure 2 shows the average water intake during retention test of pre-exposed and non-pre-exposed groups of male C57BL/6J mice that received either LiCl or NaCl during conditioning. Water intake during retention was similar for animals injected with LiCl or NaCl. The water intake data was analyzed with a 2 (pre-exposure: pre-exposed vs. non-pre-exposed) × 2 (conditioning: NaCl vs. LiCl) factorial ANOVA. This analysis revealed no main effect of Conditioning $F (1, 44) = 1.481$, $p = 0.23$, pre-exposure, $F (1, 44) = 0.008$, $p = 0.927$, and no interaction between these factors, $F (1, 44) = 0.692$, $p = 0.41$. Fisher's LSD analysis showed that animals in the Pre-exposed LiCl ($M = 0.625$, SD $= 0.251$) group displayed similar water intake to animals in the pre-exposed NaCl ($M = 0.736$, SD $= 0.137$) group during retention trial ($p = 0.154$). Similarly, in the Non-pre-exposed Groups, animals that received LiCl ($M = 0.675$, SD $= 0.224$) drank similar amounts of water compared to animals that received NaCl ($M = 0.696$, SD $= 0.093$; $p = 0.787$). These results indicate that none of the animals, regardless of whether they were pre-exposed to Context B before the conditioning trial, developed CCA.

3 | EXPERIMENT 1b

Experiment 1a showed that pre-exposure to the CS did not poten-tiate CCA learning in C57BL/6J inbred mice, in fact there was no CCA development in these mice. Experiment 1b was conducted using the same procedure to investigate the effect of pre-exposure on CCA learning in CD1 outbred mice. The strain was deliberately chosen to see if outbred mice are sensitive to developing CCA.

FIGURE 1 20-min water intake during conditioning in C57Bl/6J inbred male mice ($n = 12$ for each group). All data depicted as mean ± SEM. *$p < 0.05$, ***$p < 0.001$.

FIGURE 2 15-min water intake during the retention test in C57Bl/6J inbred male mice ($n = 12$ for each group). All data depicted as mean ± SEM.

3.1 | Subjects

Forty eight 12-week-old CD1 outbred male mice were divided into four groups: pre-exposed LiCl, pre-exposed NaCl, non-pre-exposed LiCl and non-pre-exposed NaCl ($n = 12$ for each group). CD1 mice underwent the same procedural steps for conditioning and retention as those described in Experiment 1a.

3.2 | Method

The same method was used in Experiment 1b.

3.3 | Results

3.3.1 | Conditioning

Figure 3 shows the average water intake during conditioning of pre-exposed and non-pre-exposed groups of male CD1 mice given either LiCl or NaCl during conditioning. Water intake during conditioning was lower for the animals injected with LiCl relative to those which received NaCl; however, this difference was significant only for the non-pre-exposed animals. The water intake data was analyzed with a 2 (pre-exposure: pre-exposed vs. non-pre-exposed) × 2 (conditioning: NaCl vs. LiCl) factorial ANOVA. This analysis revealed a main effect of Conditioning, $F (1, 44) = 10.96$, $p = 0.002$, no main effect of pre-exposure, $F (1, 44) = 0.02$, $p = 0.889$, and no interaction between these factors, $F (1, 44) = 1.578$, $p = 0.216$. Fisher's LSD analysis revealed that animals in the Pre-exposed LiCl ($M = 0.46$, SD $= 0.221$) and pre-exposed NaCl ($M = 0.606$, SD $= 0.243$) groups had similar water intakes during conditioning ($p = 0.153$). In the Non-

pre-exposed Groups, animals that received LiCl ($M = 0.361$, SD $= 0.236$) drank significantly less water compared to animals that received NaCl ($M = 0.685$, SD $= 0.28$; $p = 0.002$). Overall, we only observed significant differences between the experimental and control non-pre-exposed groups, which likely results from the novelty of both the context and LiCl in these groups. Pre-exposed Groups were familiarized with the context and this may have attenuated the effect of LiCl on water consumption.

3.3.2 | Retention test

Figure 4 shows the average water intake during retention test of pre-exposed and non-pre-exposed groups of male CD1 mice that received either LiCl or NaCl during conditioning. Water intake during conditioning was lower for animals injected with LiCl relative to those which received NaCl, but only when animals were pre-exposed to the conditioning cages. The water intake data was analyzed with a 2 (pre-exposure: pre-exposed vs. non-pre-exposed) × 2 (conditioning: NaCl vs. LiCl) factorial ANOVA. This analysis revealed a main effect of Conditioning $F (1, 44) = 5.052$, $p = 0.03$, no main effect of Pre-exposure, $F (1, 44) = 1.093$, $p = 0.302$, and no interaction between these factors, $F (1, 44) = 0.474$, $p = 0.495$, $p = 0.41$. Fisher's LSD analysis revealed that animals in the Pre-exposed LiCl ($M = 0.327$, SD $= 0.331$) group drank significantly less water compared to animals in the Pre-exposed NaCl ($M = 0.608$, SD $= 0.044$) group during retention trial ($p = 0.044$). However, in the Non-pre-exposed Groups, animals that had received LiCl ($M = 0.493$, SD $= 0.367$) drank a similar amount of water compared to animals that had received NaCl ($M = 0.642$, SD $= 0.312$; $p = 0.276$). These results suggest that brief exposure to the conditioning context facilitates the development of CCA.
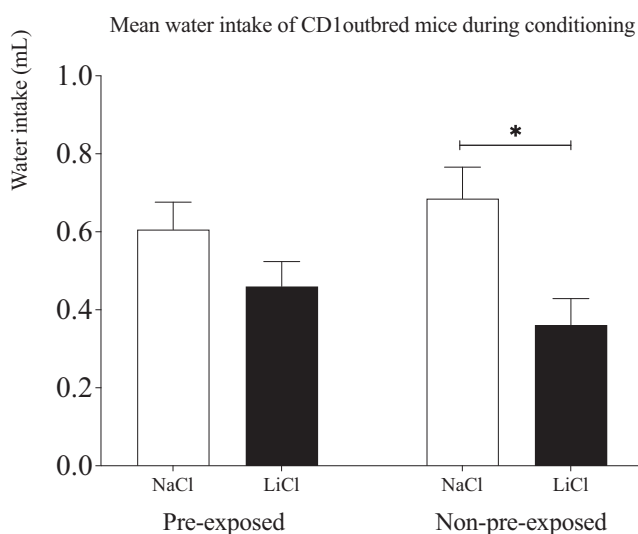
FIGURE 3   20-min water intake during the conditioning in CD1 outbred male mice ($n = 12$ for each group). All data depicted as mean ± SEM **$p < 0.01$.
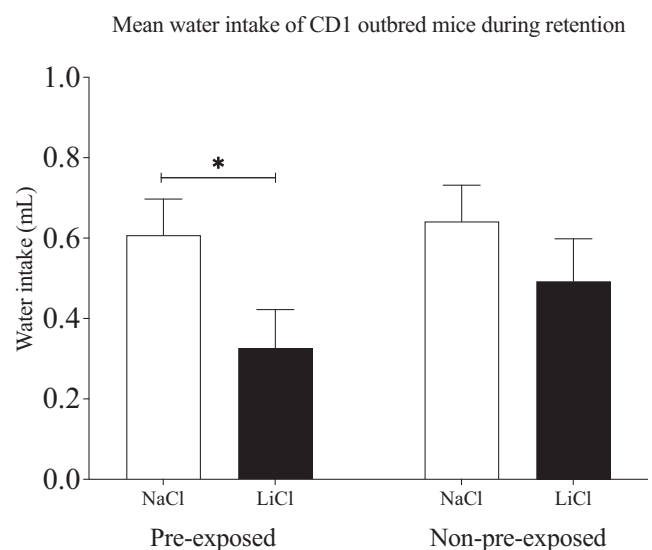
FIGURE 4   15-min water intake during the retention test in CD1 mice ($n = 12$ for each group). All data depicted are mean ± SEM. * $p < 0.05$.

4 | EXPERIMENTS 1a AND 1b DISCUSSION

The results of Experiment 1 show that one pairing of a distinctive context with LiCl-induced malaise did not result in CCA in C57BL/6J inbred mice—this was the case regardless of whether mice were pre-exposed or not pre-exposed to the conditioning Context B. This suggests that pre-exposure to the training context does not facilitate CCA learning in C57BL/6J inbred mice. However, one pairing of a distinctive context with LiCl-induced malaise did result in CCA development in CD1 outbred mice, but only if the animals had prior experience of the context; no such effect was observed in the non-pre-exposed group. The results in CD1 outbred mice replicate in CCA findings in context fear conditioning,[28,30] a finding that to our knowledge has not been previously documented. This suggests that both genetic background (i.e., strain) and environmental factors (i.e., pre-exposure to the conditioning context) have an impact on the acquisition of CCA in laboratory mice.

5 | EXPERIMENT 2a

Experiment 2a was carried out in male CD1 mice to assess the duration of retention, which consisted of multiple tests at 72-h intervals. Retention tests were conducted until there was no significant difference between the LiCl and NaCl groups.

5.1 | Subjects

Twenty-eight male CD1 mice were divided into an experimental (LiCl, $n = 14$) and a control group (NaCl, $n = 14$).

5.2 | Method

The conditioning procedures and the retention tests were the same as those used in the previous experiments. The retention tests were carried out at three-day intervals to investigate the duration of retention of CCA in male outbred mice.

5.3 | Statistical analyses

Statistical analyses were performed using GraphPad Prism (Version 9.0). The primary tool for statistical analyses were t-test and repeated-measures ANOVA. Fisher's LSD test was used for multiple comparisons. Statistical significance was defined as $p < 0.05$.
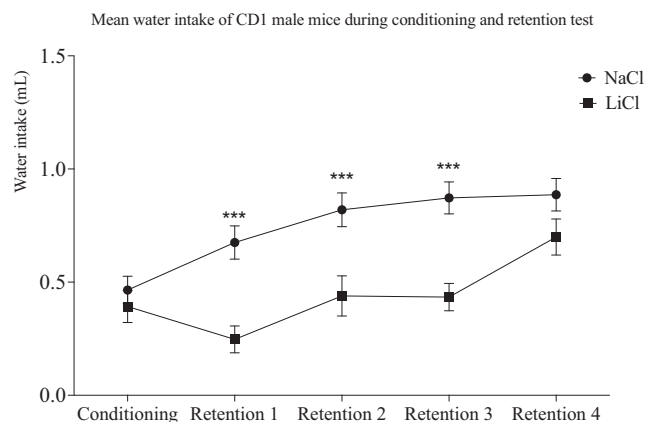
5.4 | Results

5.4.1 | Conditioning

Figure 5 shows the average water intake during conditioning of male CD1 mice given either LiCl or NaCl during conditioning. Water intake during conditioning was similar for animals injected with LiCl or NaCl, in line with the findings in pre-exposed mice of Experiment 1b. The water intake data was analyzed with a t-test (Conditioning: NaCl vs. LiCl). This analysis revealed no significant difference in water intake between LiCl ($M = 0.392$, SD $= 0.263$) and NaCl ($M = 0.465$, SD $= 0.229$) groups during conditioning in CD1 male outbred mice, $t (26) = 0.781$, $p = 0.442$. Although LiCl-treated animals consumed a similar amount of water as NaCl-treated animals during conditioning, this result is likely due to the reduced novelty of the pre-exposed context.

5.4.2 | Retention tests

Figure 5 shows the average water intake during retention tests of male CD1 mice that received either LiCl or NaCl during conditioning. Water intake during the first three retention trials was lower for the animals injected with LiCl relative to those which received NaCl. The water intake data was analyzed with a 2 (Conditioning: NaCl vs. LiCl) $\times$ 4 (Retention Tests: Retention Test 1 vs. Retention Test 2 vs. Retention Test 3 vs. Retention Test 4) factorial ANOVA. This analysis revealed a main effect of Conditioning $F (1, 104) = 48.21$, $p < 0.001$, Retention Test, $F (3, 104) = 6.947$, $p < 0.001$, $p = 0.302$, but no interaction between these factors, $F (3, 104) = 1.281$, $p = 0.285$. The Fisher's LSD tests showed that the water intake of LiCl group was significantly lower than NaCl group in Retention Test 1 (LiCl: $M = 0.247$, SD $= 0.223$ vs. NaCl: $M = 0.675$, SD $= 0.275$, $p < 0.001$), Test 2 (LiCl: $M = 0.439$, SD $= 0.333$ vs. NaCl: $M = 0.82$, SD $= 0.279$, $p < 0.001$), and Test 3 (LiCl: $M = 0.434$, SD $= 0.227$ vs. NaCl: $M = 0.872$, SD $= 0.264$, $p < 0.001$). However, there was no significant difference between the LiCl ($M = 0.699$, SD $= 0.298$) and NaCl ($M = 0.886$, SD $= 0.269$) groups in Retention Test 4 ($p = 0.073$). The results of the first three retention tests indicate that experimental male mice developed CCA, and this waned with repeated tests.

Mean water intake of CD1 male mice during conditioning and retention test

FIGURE 5 20-min water intake during the conditioning and 15-min water intake during the retention tests in CD1 outbred male mice (LiCl, $n = 14$; NaCl, $n = 14$). All data depicted as mean ± SEM. *** $p < 0.001$.

6 | EXPERIMENT 2b

The results of Experiment 2a replicated those of Experiment 1b in revealing that CD1 outbred male mice developed a strong CCA to the context with only a single conditioning trial. This CCA was evident for three retention tests. Experiment 2b was designed to examine the acquisition and duration of retention of CCA but in female CD1 outbred mice.

6.1 | Subject

Twenty-seven female CD1 mice were divided into two groups: an experimental group (LiCl, $n = 14$) and a control group (NaCl, $n = 13$).

6.2 | Method

The conditioning procedures and retention tests were similar to those in the previous experiments. The retention tests were carried out at 3-day intervals to examine the duration of extinction, until there were no differences between groups.

6.3 | Results

6.3.1 | Conditioning

Figure 6 shows the average water intake during conditioning of female CD1 mice given either LiCl or NaCl. Water intake during conditioning was similar for animals injected with LiCl or NaCl. The water intake data was analyzed with a t-test (Conditioning: NaCl vs. LiCl). This analysis revealed no significant difference in water intake between the LiCl ($M = 0.593$, SD $= 0.352$) and NaCl ($M = 0.67$,

SD $= 0.249$) groups during conditioning in CD1 male outbred mice, $t (25) = 0.652$, $p = 0.52$.

6.3.2 | Retention tests

Figure 6 shows the average water intake during retention tests of female CD1 mice that received either LiCl or NaCl during conditioning. Water intake during the first eight retention trials was lower for the animals injected with LiCl relative to those which received NaCl. The water intake data was analyzed with a 2 (Conditioning: NaCl vs. LiCl) $\times$ 9 (Retention Tests: Retention Test 1 vs. Retention Test 2 vs. Retention Test 3 vs. Retention Test 4 vs. Retention Test 5 vs. Retention Test 6 vs. Retention Test 7 vs. Retention Test 8 vs. Retention Test 9) factorial ANOVA. This analysis revealed a main effect of Conditioning $F (1, 225) = 64.52$, $p < 0.001$, Retention Test, $F (8, 225) = 14.16$, $p < 0.001$, $p = 0.302$, but no interaction between these factors, $F (8, 225) = 0.297$, $p = 0.966$. Fisher's LSD tests revealed that water intake of the LiCl group was significantly lower than that of the NaCl group in Retention Test 1 (LiCl: $M = 0.308$, SD $= 0.19$ vs. NaCl: $M = 0.639$, SD $= 0.230$, $p = 0.006$), Test 2 (LiCl: $M = 0.372$, SD $= 0.231$ vs. NaCl: $M = 0.752$, SD $= 0.289$, $p = 0.002$), Test 3 (LiCl: $M = 0.55$, SD $= 0.276$ vs. NaCl: $M = 0.846$, SD $= 0.378$, $p = 0.015$), Test 4 (LiCl: $M = 0.58$, SD $= 0.256$ vs. NaCl: $M = 0.995$, SD $= 0.4$, $p < 0.001$), Test 5 (LiCl: $M = 0.663$, SD $= 0.337$ vs. NaCl: $M = 0.947$, SD $= 0.253$, $p = 0.019$), Test 6 (LiCl: $M = 0.819$, SD $= 0.241$ vs. NaCl: $M = 1.21$, SD $= 0.35$, $p = 0.001$), Test 7 (LiCl: $M = 0.816$, SD $= 0.242$ vs. NaCl: $M = 1.11$, SD $= 0.361$, $p = 0.015$), Test 8 (LiCl: $M = 0.92$, SD $= 0.239$ vs. NaCl: $M = 1.225$, SD $= 0.466$, $p = 0.012$). However, in Test 9, no significant difference was found between the LiCl ($M = 1.014$, SD $= 0.331$) and NaCl groups ($M = 1.216$, SD $= 0.430$; $p = 0.094$). These results indicate that experimental female mice developed robust CCA, which was evident for eight retention tests.

7 | EXPERIMENTS 2a AND 2b DISCUSSION

Experiment 2 revealed that outbred CD1 female and male mice showed a strong conditioned aversion to context after a single conditioning trial. Even though there is no previous research showing sex differences in water consumption among CD1 mice, there is a study showing that female rats consume more daily water than males.[36] Although we did not directly compare male and female mice in our experiments, we found that control females tended to consume more water than control males, a result which is consistent with McGivern's study.[36] In our study, when male and female mice were compared independently with treatment as a factor in retention tests, relative to NaCl-treated female mice, LiCl-treated female mice displayed suppressed water intake for eight retention tests. However, the significant difference in water intake between LiCl- and NaCl-treated male mice lasted for only four retention tests. These results suggest that the retention was longer in female outbred mice than male mice, in
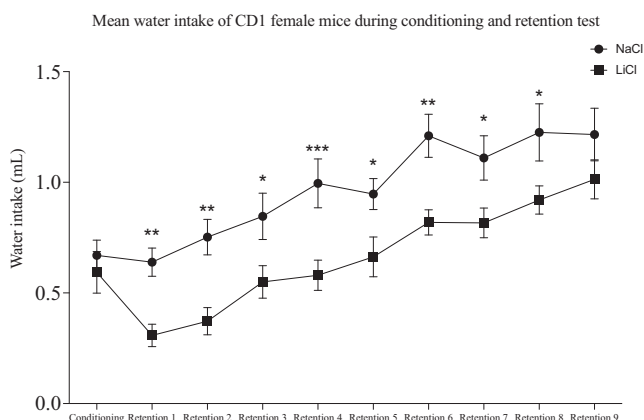
FIGURE 6 20-min water intake during the conditioning and 15-min water intake during the retention tests in CD1 outbred female mice (LiCl, $n = 14$; NaCl, $n = 13$). All data depicted as mean ± SEM. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

accordance with clinical outcomes showing ANV occurs more severely in women than men. Experiment 2 also replicated the results of Experiment 1 suggesting that pre-exposure to context facilitates development of CCA in outbred mice.

8 | GENERAL DISCUSSION

The present series of experiments was conducted to evaluate strain and sex differences in CCA, a preclinical animal model of AN. We found that, following a single conditioning trial in which a distinctive context is paired with LiCl-induced illness is sufficient to elicit a conditioned response (i.e., CCA) in both female and male CD1 outbred mice, but not in C57BL/6J inbred mice, however this was only observed when mice had prior experience with the context. In addition, outbred female mice show more retention of CCA than male mice. In what follows, we will discuss each of these findings in turn.

8.1 | Pre-exposure

Pre-exposure to a stimulus has varying effects on learning depending on the duration and frequency of the exposure.[29,31–33] Brief pre-exposure to a stimulus can aid learning, a phenomenon referred to as facilitation, whereas long exposure to the same stimulus can impair learning, a phenomenon known as latent inhibition.[29] This relationship between pre-exposure and learning can be conceptualized as an inverted U-shaped curve with the optimal level of pre-exposure located at the top of the curve.[29,31] However, whether pre-exposing mice to a novel context facilitates CCA learning in both inbred and outbred strains of mice has not been assessed. In our experiments, although we did not find a significant interaction between pre-exposure and treatment, when CD1 mice were pre-exposed to the training context for 5 min 24 h before conditioning, the LiCl-treated CD1 mice displayed significantly lower water intake than the NaCl-treated CD1 mice at test. However, no significant difference was observed between LiCl- and NaCl-treated CD1 mice without pre-exposure, indicating that pre-exposure facilitated learning based on a single context–illness pairing. We did not observe a similar effect among C57BL/6J inbred mice; rather, LiCl- and NaCl-treated animals displayed similar water intakes at test independent of the pre-exposure state.

The pre-exposure effect may have occurred because the mice acquired a conjunctive representation of the context while exploring the environment.[30,37] When animals were subsequently conditioned, their previous experience with the context may have allowed them to better associate the context with the illness induced by LiCl injection. In the absence of pre-exposure, the experimental animals only had 5 min to explore the context during CCA conditioning, which may have been insufficient for the acquisition of a conjunctive representation of the context, or this may have been overshadowed by the administration of the LiCl injection. Previous experience with the context 24 h earlier may have allowed them to develop a representation of the context and later associate it with the illness induced by LiCl injection on the day of conditioning. Studies have also shown that rats display suppressed fluid consumption due to exploration behavior when they are introduced to a novel place,[38] hence another possible explanation for the pre-exposure effect in our studies, then, is that the prior experience with the context reduced novelty and exploratory behavior of the mice. In fact, we consistently observed that non-pre-exposed CD1 mice which received LiCl consumed less water than those receiving NaCl during the conditioning trial, and this difference was attenuated in pre-exposed CD1 mice.

8.2 | Strain differences

Research on the genetics of feeding and drinking behavior indicates that several genes play a role in metabolism and nutrition-related behaviors. In addition, mice from different genetic backgrounds show significant variation in body weight as well as food and water intake.[39–41] Some studies have found that C57BL/6J mice are not sensitive to water deprivation and their daily water consumption is, in general, relatively low. Therefore, in our study, the lack of significant differences between control and experimental groups in inbred C57BL/6J mice may be due to the C57BL/6J mice's insensitivity to water deprivation.[42]

The underlying basis of the strain differences in Experiment 1 may also result from variations in the response to LiCl—that is the C57BL/6J strain may not be sensitive to the aversiveness of the drug.[43,44] Research on the role of genetic determinants of susceptibility to LiCl-induced toxicity indicates that there are strain differences.[14,34,43–47] Evaluation of the time to death after a lethal dose of LiCl shows that C57BL/6J mice are 15x more resistant than the most susceptible strain, the 129/ReJ. Compared to six other strains of mice (129/ReJ, S.W., C3H/S, DBA/2, Balb/c), C57BL/6J also have the highest ability to eliminate Li^+ from the body, particularly through urination.[43] Consistent with this, it has been reported that DBA/2J mice develop stronger LiCl-induced taste aversion than C57BL/6J mice.[34] In another study, although there were no significant strain differences in the acquisition of an LiCl-induced taste aversion, during extinction C57BL/6J mice manifested an earlier (and hence faster) extinction of the aversion relative to the DBA/2J strain.[45] However, it has also been found that C57BL/6J and DBA/2J mice respond equally to illness induced by LiCl, as evidenced by their similar c-fos expression levels in the parabrachial nucleus, a brain region associated with learning unpleasant gustatory and visceral information. This data suggests that the strain differences in conditioned taste aversion are not simply due to DBA/2J mice experiencing more illness.[48] Note, however, that the aforementioned study evaluated c-fos expression following LiCl consumption rather than intraperitoneal injection, so this may explain the lack of sensitivity in the c-fos measure. Whatever the source of these differences, our results are by and large in agreement with the preexisting literature.

8.3 | Sex differences

Studies in human participants have reported susceptibility to dizziness, nausea, and vomiting in women that can be caused by a variety of reasons.[49–57] Genetic, hormonal, and social factors may all contribute to the observed sex differences in behavioral responses to nausea-inducing treatments.[58] Post-chemotherapy AN has also been reported to occur more frequently in female patients than male patients.[7,21,59,60] Despite these reported sex differences, studies identifying the behavioral and neural mechanisms of CCA have focused mostly on male rodents.[13,15,25,61] In Experiment 2, we investigated the acquisition and retention of CCA in a sex-specific fashion. We found that both outbred female and male mice showed a robust conditioned aversion to the reinforced context after a single conditioning trial. However, female mice exhibited stronger resistance to extinction than male mice, a finding that supports the observed sex differences in the human population. The observed sex difference in the development of CCA may be due to estrogen levels.[62,63] Circulating levels of estrogen have been found to influence conditioned disgust behaviors in rats, as evidenced by the rats' increased aversive responses to the reinforced context during the period of proestrus compared to diestrus.[58] It also has been reported that nausea, malaise, and conditioned aversive responses in animals are enhanced by estrogen.[64] Estradiol has been found not only to cause a shift in the palatability of a sucrose solution but also induce taste avoidance when paired with a novel sucrose taste.[65] These results indicate that susceptibility to malaise-inducing agents in females is caused by endogenous levels of gonadal hormones. Examination of the effects of gonadal hormones on CCA may help explain sex differences in nausea-related behaviors. The assessment of hormonal effects on nausea could prompt further investigation on the sexual differentiation of neural systems and behavioral mechanisms related to aversion learning and provide additional information about clinical sex differences seen in humans. Establishing a comprehensive understanding of the risk factors that contribute to the development of AN in female patients would also provide a stronger foundation for addressing its symptoms.

8.4 | Single conditioning trial

Previous studies on context aversion have employed multiple conditioning trials to induce aversion.[9,13,25,61] In such experiments, the use of repeated trials is undesirable because animals receive repeated injections of illness-inducing agents such as LiCl or bacterial lipopolysaccharides, are water-restricted for long time periods, and extended recovery periods are needed.[13,25,61] The procedural advantages of conditioning animals with a single trial are numerous. First, post-training stabilization processes (so-called "consolidation") can be better targeted and selectively disrupted by pharmacological or behavioral interventions when only one training trial is sufficient for animals to develop a CR. Procedures that include multiple cycles of conditioning trials preclude the evaluation of various interventions targeting

different stages of memory formation because in each trial after the first one, animals learn new information which adds to the pre-existing memory strength. This makes the stages of memory formation indistinguishable from each other. This information is quite important for the development of interventions that target the strength of the CCA memory to reduce conditioned responses. Second, in vitro techniques such as the evaluation of immediate early gene expression to detect neural activation in different stages of memory formation are much more applicable to the single-trial conditioning method because repeated exposures to CS and US are avoided.

8.5 | Rat versus mice

The majority of CCA studies have been conducted with rats.[12,13] Using rat subjects, researchers have established that an environmental context can serve as a CS for illness. Rats display conditioned aversion to a context as evidenced by the suppressed consumption of flavored solution in a retention test as a consequence of pairing LiCl-induced illness with a novel context during conditioning.[13] Only two studies on the CCA paradigm have used genetically heterogeneous mice, a cross between the C57BL/6J (B6) and DBA/2J (D2) strains as well as a cross between large and small strains.[17,18] In these studies, the development of the context aversion conditioning paradigm in outbred mice without the use of a flavored solution was shown by using plain water, rather than the flavored solution that is offered in many CCA studies.[17,18] The results of our current experiments with outbred mice are consistent with those of our previous studies showing that mice can develop aversion to environmental cues without the use of a taste during test. Aversion was acquired after a single conditioning trial and we observed sex differences in retention.

9 | CONCLUSION

Our initial goal sought to investigate whether CCA was sensitive to exposure to the contextual environment before conditioning—a process known as pre-exposure training. Earlier studies in fear conditioning have revealed an elevation in contextual fear learning when subjects had prior exposure to the context. We observed a similar effect in the case of CCA, where we only observed successful CCA following a brief pre-exposure period of 5 min. Our finding that a single instance of pre-exposure results in CCA learning is particularly significant given its potential practical applications in clinical settings. While it is feasible to implement brief pre-exposure to enhance learning, employing a protracted pre-exposure may conversely lead to inhibition, thus potentially reducing, rather than increasing, anticipatory nausea—although at present this is speculative and requires empirical assessment. In subsequent experiments, we aimed to investigate sex differences in retention of conditioned contextual aversion learning. Interestingly, we found that female mice exhibited a longer duration

of conditioned responses compared to male mice. This finding has significant implications for translational research. Indeed, these results align with findings from human studies on anticipatory nausea, bolstering the potential for the use of this innovative mouse model in future research. This may, in turn, provide valuable insights into the pharmacological and neural mechanisms underlying these sex differences, thereby advancing our understanding and offering potential avenues for targeted therapeutic interventions.

One limitation of our study lies in the absence of a direct comparison between pre-exposed and non-pre-exposed CD1 females with respect to CCA. This gap in our research prevents us from drawing definitive conclusions about the potential differences or similarities in CCA responses between females and males. To obtain a more comprehensive understanding of CCA in CD1 females, future studies should aim to incorporate this comparison.

DATA AVAILABILITY STATEMENT

The raw data presented in this paper are available at: https://osf.io/zc8pr/?view_only=c3ea40acd3694417b21674e65aad8205.

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