

The effect of androgen on the retention of extinction memory after conditioned taste aversion in mice

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Abstract Conditioned taste aversion (CTA) induced by the application of a novel taste such as sodium saccharin (Sac) as the conditioned stimulus (CS) and a malaise-inducing agent as the unconditioned stimulus (US), results in acquisition of CTA memory to Sac. In contrast, CTA is extinguished by repeated presentations of the CS without the US, resulting in acquisition of the extinction memory. We examined the effects of androgenic hormones on acquisition and retention of extinction memory in mice. We gonadectomized sexually immature mice and continuously administered androgens to these animals. After sexual maturation, the mice underwent a conditioning period followed by an extinction period. Retrieval tests revealed that the androgen-treated group showed significantly greater retention of extinction memory than the non-treated group 5 weeks later, whereas such significant

difference was not observed in acquisition of extinction memory. These results demonstrate the enhancing effect of androgens on retention of extinction memory.

Keywords Acquisition · Retention · Androgen · Conditioned taste aversion memory · Extinction memory

Introduction

Conditioned taste aversion (CTA) is a robust and long-lasting memory that occurs after a single dose of a novel conditioned stimulus (CS) that is followed by a malaise-inducing agent as the unconditioned stimulus (US), resulting in avoidance of the taste employed as the CS [1–4]. The CTA paradigm is thought to serve as a model for the neural mechanisms underlying an animal's preference or aversion for a particular food item.

After CTA learning, the extinction of CTA is induced by repeated presentation of the CS without the US. This phenomenon is known as the extinction. Recent studies have demonstrated that extinction is a process of relearning [5], resulting in the acquisition and consolidation of a new memory, the so-called extinction memory [6, 7]. The retrieval of conditioned memory (the CS–US association) is blocked by the extinction memory, indicating that the CTA memory is not actually discarded from the brain by the extinction [5, 8–12].

The difference between the acquisition and retention processes of memory has been demonstrated by a number of reports using the local blockade of *N*-methyl-D-aspartate receptor [6, 7], protein synthesis of β -adrenergic receptor [13, 14] in brain regions related to CTA memory or the extinction memory, the intraperitoneal injection of anesthetics, such as propofol or the α 2-adrenergic blocker,

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yohimbine, [15, 16] or a point mutation strategy in mice [17].

When sexually mature or immature male mice (C57BL/6) underwent the conditioning period followed by the extinction period, mature mice showed higher retention of the extinction memory than immature mice, but not acquisition of the extinction memory in our laboratory [18]. In contrast, no differences were observed in acquisition and retention of CTA memory between sexually immature and mature males [18]. We hypothesized that the age-related difference of male mice in retention of the extinction memory was caused by the androgenic hormone, testosterone, during the sexual maturation (or pubertal) period. To test this hypothesis, we examined the effects of testosterone on the acquisition and retention of the extinction memory by continuous administration of testosterone to gonadectomized sexually immature male and female mice.

Experiment 1: confirmation of sexual maturity in mice

It is generally considered that the sexual maturation is initiated between 5 and 7 weeks in mice. We attempted to confirm whether 5-weeks-old mice are sexually immature and 7-weeks-old mice are sexually mature by weight measurement of sexual organs (gonad, spermatheca and uteri), because the development of spermatheca is dependent on the testicular androgen [19].

Materials and methods

Animals

We purchased male and female C57BL/6 mice from an animal supply company (Sankyo Lab Service, Tokyo, Japan). Mice were group-housed upon arrival and throughout the experiment in plastic wire and mesh cages (300 mm × 200 mm × 120 mm) in a room with controlled temperature (23 °C) and humidity (70 %), on a 12:12 h light/dark cycle (lights turned on at 07:00 and off at 19:00). Mice had ad libitum access to food (dry pellets, MF; Oriental Yeast, Osaka, Japan), and tap water was provided ad libitum except during water-restriction training, conditioning, and extinction periods as described below. All experiments in this study were performed in accordance with the Guidelines for the Care and Use of Laboratory Animals (National Institute of Health, 1985) and Guiding Principles for the Care and Use of Animals in the field of the Physiological Sciences (Physiological Society of Japan, 2003). All procedures were approved by the Animal Experiment Committee of Japan Women's University. All efforts were made to minimize subject discomfort and the number of animals used.

Surgical procedures: weights measurement of gonad and genitalia

After injection of a lethal dose of anesthesia, we removed the testes and spermatheca in males (18–30 g) or the ovary and uteri in females (13–24 g) in 5-, 7-, and 9-weeks-old mice (males: $n = 5$ each group; females: $n = 4$ each group) and measured their weight to examine the degree of sexual maturity.

Data analysis

The degree of sexual maturity was analyzed by a one-way analysis of variance (ANOVA). Post hoc analyses were performed using Tukey–Kramer multiple comparison tests.

In all experiments, results are expressed as the mean ± standard error of the mean (SEM) and the significance level of all statistical analyses was set at $p < 0.05$. All data analyses were conducted using the StatView software (SAS Instruments).

Results

Significant differences were observed in spermatheca weight (per 10 g body weight) (5-weeks-old: 0.042 ± 0.002 g; 7-weeks-old: 0.075 ± 0.003 g; 9-weeks-old: 0.075 ± 0.005 g, $F_{2,12} = 16.735$, $p < 0.0005$, one-way ANOVA; 5- vs. 7-weeks-old, 5- vs. 9-weeks-old, $p < 0.05$, Tukey–Kramer's test) but not testis weight (per 10 g body weight) (5-weeks-old: 0.070 ± 0.004 g; 7-weeks-old: 0.077 ± 0.005 g; 9-weeks-old: 0.077 ± 0.002 g, $F_{2,12} = 1.372$, $p = 0.291$, one-way ANOVA) among 5-, 7- and 9-weeks-old male mice. In female mice, uterine weight (per 10 g body weight) significantly differed (5-weeks-old: 0.013 ± 0.001 g; 7-weeks-old: 0.031 ± 0.003 g; 9-weeks-old: 0.029 ± 0.004 g, $F_{2,9} = 11.631$, $p < 0.005$, one-way ANOVA; 5- vs. 7-weeks-old, 5- vs. 9-weeks-old, $p < 0.05$, Tukey–Kramer's test), but the weight of ovaries (per 10 g body weight) did not (5-weeks-old: 0.003 ± 0.0003 g; 7-weeks-old: 0.003 ± 0.0002 g; 9-weeks-old: 0.002 ± 0.0001 , $F_{2,9} = 1.895$, $p = 0.206$, one-way ANOVA) among 5-, 7- and 9-weeks-old female mice. These results indicate that spermatheca and uterine weights of 5 weeks old significantly differed from those of 7 weeks old. Therefore, we judged from these results that male and female mice sexually matured between 5 and 7 weeks old.

Experiment 2: acquisition of CTA memory or extinction memory in sexually mature intact mice

We examined the effect of sex on acquisition of CTA memory and extinction memory in sexually mature intact mice of 7 weeks old as confirmed in Experiment 1.

Materials and methods

Animals

Mice were housed individually upon arrival and throughout the experiment in individual plastic wire and mesh cages (235 mm × 165 mm × 125 mm). The experimental room was identical to that in Experiment 1. Behavioral procedures began at 10:00 in the light phase of the 12:12 h light/dark cycle (lights turned on at 07:00 and off at 19:00). The two sex groups were as follows: intact males (*n* = 7) and intact females (*n* = 6) in Table 1.

Behavioral procedures

Following a water-restriction training period at 6 weeks old, mice underwent the conditioned taste aversion (CTA) period and the extinction period at 7 weeks old (Fig. 1a, Non-operated group).

Water-restriction training period

At the beginning of the behavioral experiment, all mice were placed on a 20 h water-deprivation schedule, which allowed only 4 h of access to distilled water (DW) for 6 days. During this time, the amount of DW consumed during the first 10 min was measured (Fig. 1a, b). Mice were provided with just one bottle during all CTA procedures.

CTA period

Mice underwent the CTA period after the water-restriction training period (Fig. 1a). All mice were offered 0.5 % saccharin (Sac) for 10 min as the CS. Twenty minutes after the termination of drinking, all animals received the US by an intraperitoneal (i.p.) injection of the malaise-inducing agent lithium chloride (LiCl, 0.15 M, 2 % body weight) (Fig. 1b). Water was not supplied to mice between the CS and US. Mice were allowed access to water for 3.5 h after injection with LiCl but were then placed on a 20 h water-deprivation schedule. All mice underwent this conditioning for 3 days (C1–C3).

Extinction period

Mice underwent the extinction period after the CTA period (Fig. 1a). Mice were offered the CS (0.5 % Sac) for 10 min in the absence of follow-up with the US for 7 successive days (E1–E7) (Fig. 1a, b). Sac intake was measured for 10 min. Following access to water for 3 h and 50 min after exposure to Sac, mice were then placed on the 20 h water-deprivation schedule again.

Data analysis

Pre-conditioning control values of standard DW intake (SDW) are the mean of DW intake for the final 3 days of the water-restriction training period. Total Sac intake (%)

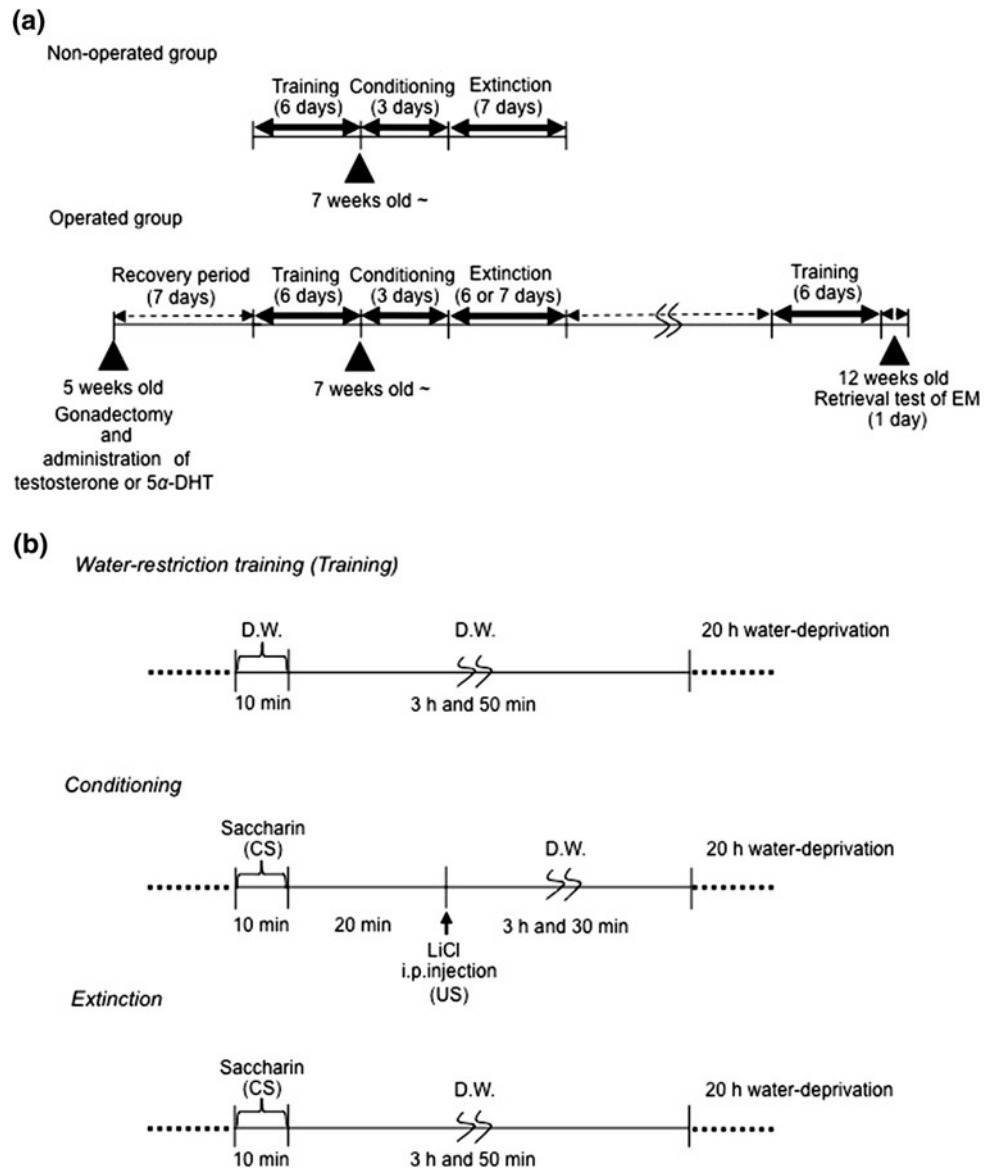
Table 1 Groups in Experiments 2, 3, and 4

Group designation	Sex	Number of mice	Surgery	Dose of androgen (mg/tube)	CTA (CS + US)
Experiment 2					
Sex groups	Male	7	Intact	–	Sac + LiCl
	Female	6	Intact	–	Sac + LiCl
Experiment 3					
T-dose groups	Male	9	Gonadectomy	0	Sac + LiCl
	Male	4	Gonadectomy	0.2	Sac + LiCl
	Male	5	Gonadectomy	0.5	Sac + LiCl
	Male	10	Gonadectomy	1.0	Sac + LiCl
	Male	11	Gonadectomy	2.0	Sac + LiCl
Non-CTA/T-dose groups	Male	5	Gonadectomy	0	Sac + NaCl
	Male	5	Gonadectomy	0.5	Sac + NaCl
DHT-dose groups	Male	5	Gonadectomy	0	Sac + LiCl
	Male	6	Gonadectomy	0.05	Sac + LiCl
	Male	9	Gonadectomy	0.1	Sac + LiCl
Blood T groups	Male	7, 8, 9, 10 weeks:3 each	Gonadectomy	0.5	–
Experiment 4					
Female T-dose groups	Female	8	Gonadectomy	0	Sac + LiCl
	Female	10	Gonadectomy	0.5	Sac + LiCl

T: testosterone, DHT: 5 α -dihydrotestosterone

Fig. 1 Experimental protocols for examining the effects of androgens on CTA memory and extinction memory.

a Experimental overview. We implanted gonadectomized mice with a hormone tube (one per individual) containing different doses of testosterone (0–2.0 mg/tube) or 5 α -DHT (0–0.1 mg/tube) at 5 weeks old. After acquisition of CTA memory at 7 weeks old, mice underwent an extinction period. At 12 weeks old, they underwent a retrieval test of the extinction memory (EM). **b** The water-restriction training (Training) period consisted of a 20 h water-deprivation schedule and 4 h of access to distilled water (DW). On conditioning days all mice were offered a conditioned stimulus (CS) for 10 min, and then experienced malaise induced by an unconditioned stimulus (US). In contrast, mice were offered the CS for 10 min without the US during extinction days. On conditioning and extinction days, all mice were placed on a 20 h water-deprivation schedule, and Sac intake was measured for 10 min



on conditioning (C1–C3) and extinction (E1–E7) days were calculated as percentages against SDW, because saccharin intake on C1 is highly influenced by degree of neophobia to CS as a novel taste [16, 20].

Because E1 was the day after the final conditioning day (C3), we used Sac intake on E1 as the parameter for acquisition of CTA memory. Moreover, we used Sac intake on E7 as the parameter for acquisition of extinction memory. Comparisons of two independent groups were analyzed using two-tailed unpaired *t* tests. We also used data of the Sac intake from C1 to E1 or from E1 to E7 to analyze whether the CTA memory or the extinction memory is acquired or not by repeated-measures two-way ANOVAs (group \times day during the CTA process from C1 to E1, or group \times day during the extinction process from E1 to E7) for each experiment.

Results

Between intact males and females, the two-way (sex \times day) repeated-measures ANOVAs revealed main effects of day (during the CTA process: $F_{1,3} = 36.244$, $p < 0.0001$; during the extinction process: $F_{1,6} = 40.454$, $p < 0.0001$; Fig. 2), but no main effects of sex (the CTA process: $F_{1,11} = 0.615$, $p = 0.450$; the extinction process: $F_{1,11} = 0.144$, $p = 0.711$) or sex \times day interaction (the CTA process: $F_{1,3} = 1.814$, $p = 0.164$; the extinction process: $F_{1,6} = 0.643$, $p = 0.696$). In the main effect of day, a post hoc test revealed significant decrease during the CTA process (C1 vs. C2, C3 or E1, C2 vs. C3 or E1, $p < 0.05$, respectively) and significant increase during the extinction process (E1 vs. E2–E7, E2 vs. E4–E7, E3 vs. E5–E7, E4 vs. E6 or E7, E5 vs. E7, $p < 0.05$, respectively). These

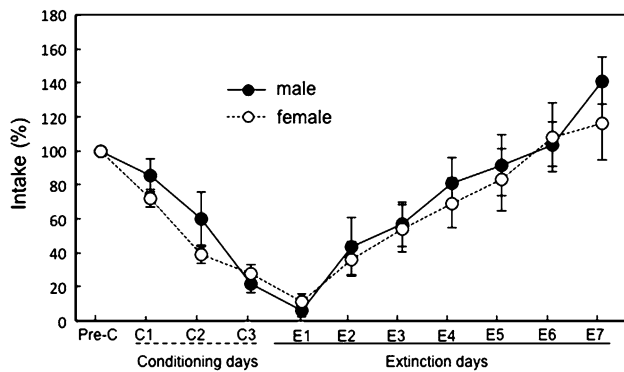


Fig. 2 Acquisition of CTA memory or extinction memory in intact mice. The sex difference was not observed in acquisition of CTA memory or extinction memory in two sex groups. *male, female* intact mice. Pre-C: pre-conditioning days calculated SDW. C1–C3: the three conditioning days. E1–E7: the seven extinction days. Data are presented as means \pm SEMs. (in this and other figures)

statistical results suggest that two sex groups acquire the CTA memory or the extinction memory and that acquisition processes are not influenced by the sex. Furthermore, no sex differences were observed in Sac intake on E1 ($p = 0.433$, t test) or E7 ($p = 0.346$, t test) in intact mice of both sexes (Fig. 2). In our present study, we confirmed that sex difference was not observed in acquisition of CTA memory or the extinction memory in sexually mature mice at 7 weeks old.

Experiment 3: effects of testosterone on acquisition and retention of extinction memory in males

The difference in retention of the extinction memory was observed between sexually immature and mature males [18]. The results of Experiment 1 indicate that 5-weeks-old mice are sexually immature and 7-weeks-old mice are sexually mature. Having certain levels of testosterone during sexual maturation period might facilitate retention of the extinction memory compared to the gonadectomized control group. Thus, we manipulated testosterone levels by gonadectomy and implantation of several different doses of testosterone at 5 weeks old to investigate the effect of testosterone on retention of the extinction memory during sexual maturation period.

Testosterone is aromatized to a form of estrogen, 17β -estradiol [21–24], and the estrogen is known to modulate the plasticity of the memory process [25–30]. Therefore, to exclude the hypothesis that estrogen affects acquisition or retention of the extinction memory, we also examined the effects of 5α -dihydrotestosterone (5α -DHT), which is a more potent androgen that cannot be aromatized to 17β -estradiol.

Materials and methods

Animals

Male mice for the behavioral experiment were housed individually upon arrival and throughout the experiment in individual plastic wire and mesh cages, as described in Experiment 2. Mice for assay of blood testosterone level were group-housed in cages, as described in Experiment 1. The experimental condition was identical to those of Experiments 1 and 2.

Procedures

Surgical procedures: administration of androgens At 5 weeks old, male mice (18–23 g) were gonadectomized and received implantation of testosterone under anesthesia with pentobarbital sodium (50 mg/kg; Dainippon Sumitomo Pharma, Osaka, Japan). We incised skin and muscle to remove testes, and implanted a silicon tube (outer diameter 2.0 mm; inner diameter 1.5 mm; Taiyo Kogyo, Tokyo, Japan) using silicone adhesive (TSE3941, Momentive Performance Materials Japan, Tokyo, Japan) and containing several different doses of testosterone (0, 0.2, 0.5, 1.0; or 2.0 mg; Wako Pure Chemical Industries, Osaka, Japan) or 5α -DHT (0, 0.05 or 0.1 mg; Wako Pure Chemical Industries). We filled only crystalline testosterone or 5α -DHT in the 3 to 5 mm silastic tube by the small funnel made from the tip of Pasteur pipette.

Behavioral procedures The mice underwent a water-restriction training period (6 days), the CTA period (3 days) and the extinction period (7 days) as described in Experiment 2 (Fig. 1a, Operated group), following at least a week of postoperative recovery period in which all mice were offered water and food ad libitum. In addition, to confirm that the CTA memory is acquired by association of CS with US and the Sac intake is not influenced by administration of testosterone, some animals received the normal saline solution (0.15 M NaCl, 2 % body weight, Non-CTA groups) instead of the US by an i.p. injection of the malaise-inducing agent LiCl (0.15 M, 2 % body weight, CTA groups) (Fig. 1b).

Five weeks after acquisition of CTA memory or the extinction memory (at 12 weeks old), mice underwent the retrieval test of extinction memory.

Retrieval test of extinction memory

Experimental groups (T-dose groups, Non-CTA T-dose group, and DHT-dose groups in Table 1) were subjected to a retrieval test of extinction memory at 12 weeks old after the water-restriction training period (6 days) under the 20 h

water-deprivation schedule (Fig. 1a, b). Mice were offered the CS for 10 min and Sac intake was measured.

Assay of blood testosterone level

At 7–10 weeks old, we collected blood from hearts under anesthesia by syringe with a 23G injection needle (TERUMO, Tokyo, Japan) in the tube-implanted male mice with 0.5 mg testosterone/tube at 5 weeks old. Blood samples were collected in Eppendorf tubes, centrifuged, and the serum was stored at -80°C until radioimmunoassay by contract clinical trial organization (SRL, Tokyo, Japan).

Data analysis

The most of analysis methods were identical to those of Experiment 2. We also assessed the retention rate of the extinction memory as follows: Retention rate of extinction memory = (Sac intake on the retrieval test/Sac intake on E7) \times 100. The effect of androgen on the retention rate of the extinction memory and the blood testosterone levels of mice with 0.5 mg/tube were analyzed by a one-way ANOVA.

Results

Acquisition of CTA memory or extinction memory in male mice

Sac intake decreased with each successive day during the CTA process (C1–E1) (Fig. 3a). The two-way [Testosterone (T)-dose group \times day] repeated-measures ANOVAs revealed main effect of day (during the CTA process: $F_{4,3} = 75.579$, $p < 0.0001$), but no main effect of T-dose group (0, 0.2, 0.5, 1.0 and 2.0 T-dose groups: $F_{4,34} = 2.558$, $p = 0.056$) or T-dose group \times day interaction ($F_{4,12} = 0.870$, $p = 0.5789$). In the main effect of day during the CTA process, a post hoc test revealed a significant decrease between all days (from C1 to E1: $p < 0.05$, respectively). Moreover, we observed no difference in Sac intake on E1 among the five T-dose groups ($F_{4,34} = 1.777$, $p = 0.156$, one-way ANOVA). These statistical results suggest that five T-dose groups acquired the CTA memory by consumption of the CS (Sac) after three times of pairing with the US at all doses of testosterone and the acquisition process was not affected by testosterone.

Sac intake increased during the extinction process (E1–E7) (Fig. 3a). The excessive dose of testosterone such as 2.0 mg/tube tended to inhibit the acquisition of extinction memory. The two-way repeated-measures ANOVAs revealed main effect of day (during the extinction process: $F_{4,6} = 54.463$, $p < 0.0001$), but no effect of T-dose group ($F_{4,34} = 2.391$, $p = 0.070$) or T-dose group \times day

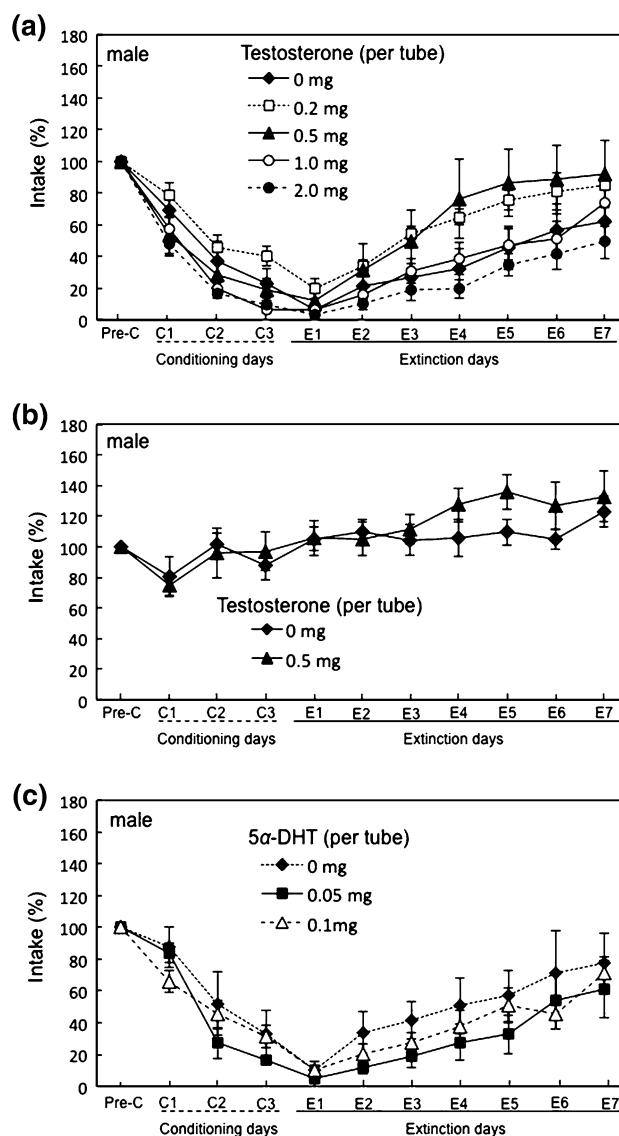


Fig. 3 Acquisition of CTA memory or extinction memory in male mice. **a** The effect of testosterone in five T-dose groups. **b** The effect of testosterone in two Non-CTA/T-dose groups. **c** The effect of 5 α -DHT in three DHT-dose groups. Neither testosterone nor 5 α -DHT affected the acquisition process of the CTA memory or the extinction memory in male mice

interaction ($F_{4,24} = 1.171$, $p = 0.272$). In the main effect of day, a post hoc test revealed significant increase during the extinction process (E1 vs. E2–E7, E2 vs. E4–E7, E3 vs. E5–E7, E4 vs. E6 or E7, E5 vs. E7, $p < 0.05$, respectively). Moreover, Sac intake on E7 did not differ among five T-dose groups ($F_{4,34} = 1.246$, $p = 0.310$, one-way ANOVA). These statistical results suggest that five T-dose groups acquired the extinction memory by repeated presentation of the CS without US at all testosterone doses and the acquisition process was not influenced by testosterone.

To confirm that the CTA memory is acquired by association of CS with US and Sac intake is not influenced by

administration of testosterone, we compared the Sac intakes of two Non-CTA/T-dose groups (Fig. 3b) with those of two CTA/T-dose groups (0 T-dose and 0.5 T-dose groups; Fig. 3a) and found that Sac intake on E1 significantly differed among the four groups ($F_{3,20} = 68.940$, $p < 0.0001$, one-way ANOVA). A post hoc test revealed that two Non-CTA/T-dose groups exhibited significantly higher Sac intake than did both CTA groups (Non-CTA/0 T-dose or/0.5 T-dose group vs. 0 T-dose or 0.5 T-dose group, $p < 0.0001$, respectively). In two Non-CTA/T-dose groups, CTA was never induced. These results suggest that the decrease of Sac intake was occurred by association of CS with US in absence or presence of testosterone.

Moreover, a two-way (Non-CTA/T-dose group \times day) repeated-measures ANOVAs revealed main effect of day (during the CTA process: $F_{1,3} = 5.591$, $p < 0.01$; during the extinction process: $F_{1,6} = 3.225$, $p < 0.05$; Fig. 3b), but no main effect of Non-CTA/T-dose group (the CTA process: $F_{1,8} = 0.001$, $p = 0.971$; the extinction process: $F_{1,8} = 0.842$, $p = 0.386$) or Non-CTA/T-dose group \times day interaction (the CTA process: $F_{1,3} = 0.514$, $p = 0.677$; the extinction process: $F_{1,6} = 1.536$, $p = 0.187$). In the main effect of day, a post hoc test revealed significant increase between days (during the CTA process: C1 vs. C2 or E1; during the extinction process: E1 vs. E7, E2 vs. E7; $p < 0.05$, respectively). The Sac intakes of two Non-CTA/T-dose groups clearly increased from C1 to E7 as contrasted those of two CTA/T-dose groups. These results may be due to acquisition of a safe taste memory to CS [31]. The Sac intake also did not significantly differ between two Non-CTA/T-dose groups, which was also confirmed by *t* tests (Sac intake on E1: $p = 0.979$; Sac intake on E7: $p = 0.623$). These results demonstrate that Sac intake was not affected by chronic administration of testosterone.

In addition, a two-way [5α -DHT (DHT)-dose group \times day] repeated-measures ANOVAs revealed main effect of day (during the CTA process: $F_{2,3} = 56.576$, $p < 0.0001$; during the extinction process: $F_{2,6} = 20.339$, $p < 0.0001$; Fig. 3c), but no main effect of DHT-dose group (0, 0.05 and 0.1 DHT-dose groups, the CTA process: $F_{2,17} = 0.767$, $p = 0.480$; the extinction process: $F_{2,17} = 0.847$, $p = 0.446$) or DHT-dose group \times day interaction (the CTA process: $F_{2,6} = 1.791$, $p = 0.120$; the extinction process: $F_{2,12} = 0.471$, $p = 0.928$). In the main effect of day, a post hoc test revealed significant decrease during the CTA process (C1 vs. C2, C3 or E1, C2 vs. E1, C3 vs. E1, $p < 0.05$, respectively) and significant increase during the extinction process (E1 vs. E3–E7, E2 vs. E5–E7, E3 vs. E6 or E7, E4 vs. E7, E5 vs. E7, $p < 0.05$, respectively). These results indicate that 5α -DHT had no effect on the acquisition process of either CTA memory or extinction memory at any dose. Results for Sac intake on E1 ($F_{2,17} = 0.760$, $p = 0.483$, one-way ANOVA) and E7 ($F_{2,17} = 0.259$,

$p = 0.775$, one-way ANOVA) among the three DHT-dose groups were similar to those obtained for five T-dose groups. These results indicate that the acquisition of both types of memories were unaffected by androgens under our experimental conditions.

Retention of extinction memory in male mice

We compared the results obtained by five T-dose groups on the retrieval test of the extinction memory at 12 weeks old. A one-way ANOVA detected a significant difference in the retention rate of the extinction memory among five T-dose groups ($F_{4,34} = 3.328$, $p < 0.05$). A post hoc test revealed that the 0.5 T-dose group exhibited a significantly higher retention rate of the extinction memory than mice implanted with other doses (0.5 vs. 0 or 2.0 T-dose groups: $p < 0.05$, respectively; Fig. 4a). In contrast, the effect of testosterone 2.0 mg/tube was the same as that of testosterone 0 mg/tube. In the Non-CTA group, the high retention rates of the extinction memory were observed in both Non-CTA/T-dose groups and no significant difference was observed between two Non-CTA/T-dose groups (84.79 ± 5.97 and 92.46 ± 10.91 %, respectively, $p = 0.555$, *t* test, data not shown as graph).

5α -DHT caused similar enhancing action. The retention rate of the extinction memory significantly differed among three DHT-dose groups ($F_{2,17} = 3.973$, $p < 0.05$, one-way ANOVA; Fig. 4b) and a post hoc test revealed that the retention rate of the extinction memory in the 0.05 DHT-dose group significantly differed from that of the 0 DHT-dose group ($p < 0.05$).

Assay of blood testosterone level in implanted males at 0.5 mg/tube

The behavioral experiment indicated that testosterone at 0.5 mg/tube had a reinforcing effect of retention of the extinction memory. Therefore, we examined whether or not the dose of testosterone at 0.5 mg/tube as an optimal dose was equivalent to the physiologically appropriate value of the normal testosterone level in male mice.

No significant difference was observed among the 7-, 8-, 9-, and 10-weeks-old mice gonadectomized at 5 weeks old and chronically administered 0.5 mg testosterone/tube (7-weeks-old: 2.50 ± 0.40 ng/mL; 8-weeks-old: 2.07 ± 0.29 ng/mL; 9-weeks-old: 1.83 ± 0.15 ng/mL; 10-weeks-old: 1.67 ± 0.59 ng/mL, $F_{3,8} = 0.843$, $p = 0.508$, one-way ANOVA).

Experiment 4: effects of testosterone on acquisition and retention of extinction memory in females

The results of Experiment 3 indicated that testosterone enhanced retention of the extinction memory in male mice

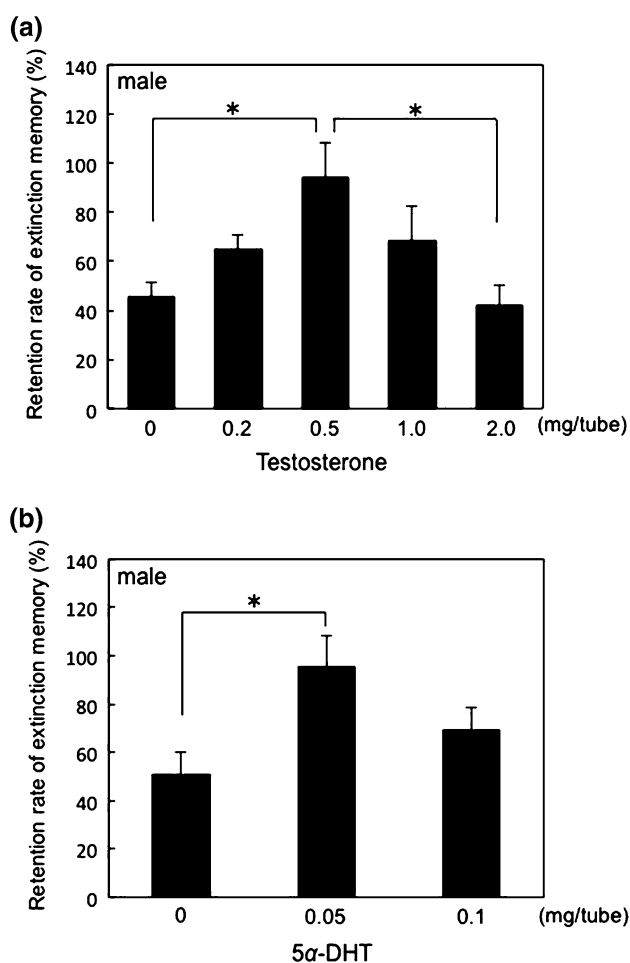


Fig. 4 Retention of extinction memory in male mice. **a** The effect of various doses of testosterone. **b** The effect of various doses of 5 α -DHT. Androgens (testosterone: 0.5 mg/tube; 5 α -DHT: 0.05 mg/tube) significantly enhanced the retention of extinction memory in male mice. In contrast, the retention rate of extinction memory in the 2.0 T-dose group was the same degree as that of the 0 T-dose group. * $p < 0.05$

under the optimal dose of testosterone at 0.5 mg/tube. We then investigated the effects of testosterone on acquisition and retention of the extinction memory in females.

Animals

Female mice were housed individually upon arrival and throughout the experiment in individual plastic wire and mesh cages, as described in Experiment 2. The experimental condition was identical to those of Experiment 1 and 2.

Surgical procedures: administration of testosterone

At 5 weeks old, females (13–18 g) were gonadectomized, and then received implantation of a silicon tube containing

testosterone (0 or 0.5 mg) under anesthesia. The series of procedures was identical to those of Experiment 3. Experimental females (Female T-dose groups in Table 1) were offered water and food ad libitum during a week of the postoperative recovery period.

Behavioral procedures

The mice underwent a water-restriction training period (6 days), the CTA period (3 days), the extinction period (6 days) and the water-restriction training period prior to the retrieval test (6 days) followed by the retrieval test of the extinction memory as described in Experiment 3 (Fig. 1a). Data analysis was identical to those of Experiment 3.

Results

Acquisition of CTA memory and extinction memory in female mice

In the acquisition process of CTA memory (C1–E1), the two-way (Female T-dose group \times day) repeated-measures ANOVAs revealed main effect of day (during the CTA process: $F_{1,3} = 36.921$, $p < 0.0001$; Fig. 5) or Female T-dose group (Female 0 T-dose and Female 0.5 T-dose groups: $F_{1,16} = 6.685$, $p < 0.05$), but no Female T-dose group \times day interaction (the CTA process: $F_{1,3} = 1.898$, $p = 0.142$). In the main effect of day or Female T-dose group, a post hoc test revealed significant difference between all days (decrease from C1 to E1, $p < 0.05$, respectively) or between two Female T-dose groups ($p < 0.05$). The t test indicated no significant difference in Sac intake on E1 ($p = 0.103$). These statistical results suggest that testosterone may affect the acquisition process of CTA memory, but the Female 0 T-dose group finally acquired the CTA memory in same degree as that of the Female 0.5 T-dose group.

In the acquisition process of the extinction memory (E1–E6), the two-way (Female T-dose group \times day) repeated-measures ANOVAs revealed main effect of day (the extinction process: $F_{1,5} = 44.718$, $p < 0.0001$; Fig. 5), but no main effect of Female T-dose group ($F_{1,16} = 3.822$, $p = 0.068$) or Female T-dose group \times day interaction (the extinction process: $F_{1,3} = 1.651$, $p = 0.156$). In the main effect of day, a post hoc test revealed significant increase during the extinction process (E1 vs. E3–E6, E2 vs. E3–E6, E3 vs. E5 or E6, E4 vs. E6, E5 vs. E6, $p < 0.05$, respectively). The Sac intake on E6 also did not differ between the two Female T-dose groups ($p = 0.578$, t test). These statistical results suggest that testosterone did not affect the acquisition of the extinction memory.

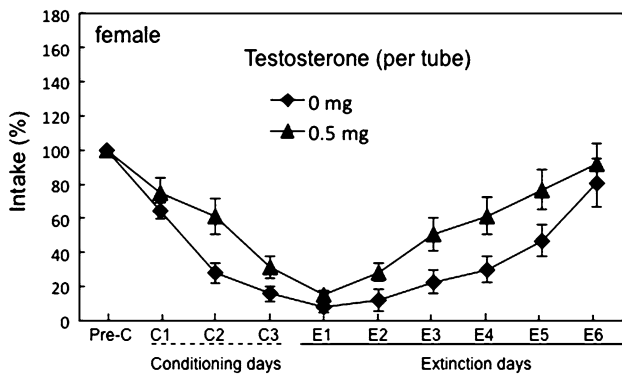


Fig. 5 Acquisition of CTA memory or extinction memory in female mice. The effect of testosterone in two Female T-dose groups. Testosterone did not affect the acquisition process of the CTA memory or the extinction memory in female mice

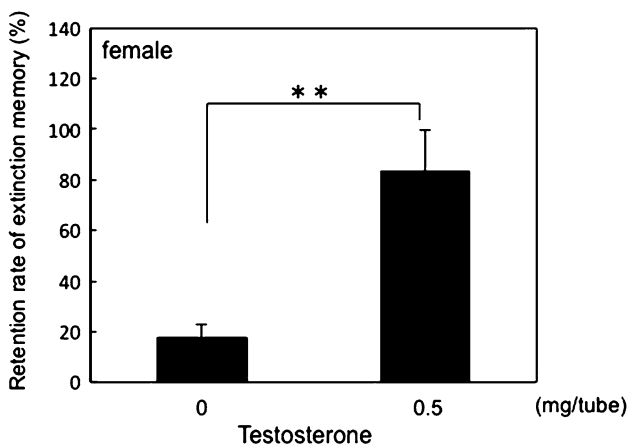


Fig. 6 Retention of extinction memory in female mice. Testosterone (0.5 mg/tube) enhanced the retention of extinction memory in female mice. $**p < 0.01$

Retention of extinction memory in female mice

The retrieval test of the extinction memory at 12 weeks old demonstrated that the Female 0.5 T-dose group exhibited significantly higher retention of the extinction memory than did the Female 0 T-dose group ($p < 0.05$, t test; Fig. 6).

Discussion

We administered testosterone to the gonadectomized mice at 5 weeks old to test the hypothesis that the sexual maturity-related difference of retention of the extinction memory in males [18] was caused by testosterone. Our present results suggest that the certain levels of testosterone, such as the optimal dose observed in T-dose groups (Experiment 3), during the sexual maturation period may facilitate retention of the extinction memory.

Testosterone can be converted to 17β -estradiol by aromatase or 5α -DHT by 5α -reductase in its target cell [21–24]. Estrogen is known to modulate the plasticity of the memory process [25–30]. We showed that 5α -DHT, which is not aromatized to 17β -estradiol, also enhanced the retention of the extinction memory. These results suggest that the enhancing effect of testosterone on the retention of the extinction memory is not caused by estrogen derived from aromatized testosterone. However, the effects of 17β -estradiol on the extinction memory may not be completely excluded because we did not directly show that 17β -estradiol treatment was not effective.

Our results suggest that an optimal dose of testosterone can maximize its enhancing effect on the retention of extinction memory. A similar optimal dose was observed in the effect of 5α -DHT. We measured testosterone levels in the blood of mice implanted with 0.5 mg of testosterone and found that the most effective dose was equivalent to the physiologically appropriate value in male mice, compared with the normal testosterone level (approximately 1.5–2.0 ng/mL) in male mice [32]. A previous study has also demonstrated the enhancing effect of a physiologically appropriate dose of testosterone on memory mechanisms in the hippocampus [33]. Moreover, measurement of the concentrations of testosterone showed no difference at different time points of the experimental schedule. Thus, it is unlikely that the accumulative concentration of androgens cause enhancement of the retention rate of extinction memory.

The present results demonstrate that testosterone treatment in mice enhances the retention of the extinction memory but does not affect either the acquisition of CTA memory or the acquisition of extinction memory in mice. However, the excessive dose such as 2.0 mg testosterone may suppress the retention of the extinction memory. The reciprocal action between moderate and excessive dose in hormone has already been reported [34]. Our present result indicates that chronic and systemic testosterone treatment does not influence the acquisition of CTA memory and extinction memory but does the retention of extinction memory. Therefore, for extinction memory, the acquisition pathway may differ from the retention pathway, with a different pathway between acquisition and retention of the extinction memory being generally accepted [5, 13–17].

We also found that testosterone treatment facilitated retention of the extinction memory in females. The blood testosterone levels of females are generally close to 0 mg/mL. Therefore, the results with the Female T-dose group (Experiment 4) indirectly suggest that the sex difference exists in retention of the extinction memory. Although the biological meaning of testosterone-increased changes in the taste memory is unknown, our results (Experiment 4) that the behavior of females was masculinized by testosterone raise the possibility that brain regions related to the retention of

extinction memory of both sexes have an equal sensitivity to testosterone treatment during the sexual maturation period.

In contrast, we did not observe any sexual dimorphism in the acquisition of CTA memory or extinction memory in intact mice (Experiment 2). Chambers and Sengstake [35] found similar results using more than 20 h of water-deprivation, which was consistent with our experimental protocol. Ingram and Corfman [36] also reported that no sexual dimorphism in the acquisition of extinction memory in C57BL/6 mice was observed under strong aversion, which is similar to our CTA procedure.

On the other hand, previous studies using rats have reported that androgen treatments result in a slow extinction process [37–39], whereas we did not find any effects of androgen on acquisition of the extinction memory, the so-called extinction process, in the present study. This discrepancy may be attributable to the differences among protocols. Chambers et al. [37] reported dose-dependent prolongation of the number of extinction days under the water-deprivation protocol in adult rats implanted with several doses of testosterone. However, we did not observe significant differences in the extinction rates of mice implanted with various doses of testosterone during the sexual pre-maturation period.

Additionally, Foy and Foy [39] employed a 4 h CS–US interval, whereas we only used a 20 min interval. These authors [39] also mentioned that the effect of androgen on the extinction process was attenuated when using a short CS–US interval in rats. Moreover, Ingram and Corfman [36] reported a sex difference in the extinction process when acquisition of CTA memory was weak (e.g., one conditioning trial), but no sexual dimorphism was observed when acquisition was strong.

However, we cannot exclude the possibility that differences between the effect of androgens on the extinction process in the present and previous studies may be due to interspecific differences between rats and mice. A recent report demonstrated species differences between rats and mice in the signaling pathways within the central nervous system for the visceral illness response to LiCl [40]. Ingram and Corfman [36] observed sexual dimorphism in the extinction process after CTA acquisition under water-deprivation conditions in intact DBA/2 mice but not in intact C57BL/6 mice.

Androgens such as testosterone and 5 α -DHT are bound to and activate nuclear androgen receptors (ARs), and they affect neuronal function via gene transcription [41]. Additionally, ARs are expressed in the hippocampus, amygdala, and cerebral cortex in rodents [42], and levels of ARs in the brain are modulated by androgens [43]. Immunohistochemical analyses have also revealed the appearance of ARs in the amygdala and the ventral medial prefrontal cortex (vmPFC) [44, 45]. These two brain regions are related to CTA and extinction [3, 13, 14, 46–51], and

androgens are thought to act on synaptic plasticity in these regions. In the present study, the degree of extinction memory retention was enhanced by administration of androgens to gonadectomized male and female mice. Our preliminary experiments using a real-time PCR procedure revealed a dominant expression of ARs in both the amygdala and vmPFC in male mice during the sexual pre-maturation period [52]. These findings suggest that androgens, which act on both the amygdala and vmPFC of males during the sexual pre-maturation period, cause the sex difference in extinction memory retention. More detailed investigations pertaining to the effects of androgens on the amygdala and vmPFC may elucidate the molecular and cellular mechanisms underlying extinction memory retention. Experiments are underway in our laboratory to investigate whether testosterone affects brain regions which are involved in retention of extinction memory.

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Conflict of interest The authors declare no conflicts of interest that could be perceived as prejudicing the impartiality of the research reported.

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