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Brief fear preexposure facilitates subsequent fear conditioning

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ABSTRACT

Post-traumatic stress disorder (PTSD) is an anxiety disorder that occurs following an unexpected exposure to a severe psychological event. A history of a brief trauma is reported to affect a risk for future PTSD development; however, little is known about the mechanisms by which a previous trauma exposure drives the sensitivity to a late-coming trauma. Using a mouse PTSD model, we found that a prior foot shock enhances contextual fear conditioning. This shock-induced facilitation of fear conditioning (*i.e.*, priming effect) persisted for 7 days and was prevented by MK801, an *N*-methyl-D-aspartate receptor antagonist. Other types of trauma, such as forced swimming or tail pinch, did not induce a priming effect on fear conditioning. Thus, a trauma is unlikely generalized to modify the sensitivity to other traumatic experiences. The behavioral procedure employed in this study may be a useful tool to elucidate the etiology of PTSD.

1. Introduction

Fear is a normal defensive psychological reaction in which animals and humans may protect themselves from uncertain dangers. However, inappropriate regulation of fear causes anxiety disorders. One of the anxiety disorders is post-traumatic stress disorder (PTSD), which is triggered by a sudden experience of a severe traumatic event. PTSD is characterized by mental re-experiences of the traumatic event, avoidance of stimuli that may be related to the trauma, and symptoms of increased arousal, such as heightened startle and palpitation (DSM-V, 2013). The sensitivity to PTSD varies among individuals; even the same stressor may induce PTSD in some people but may not induce it in others. A number of risk factors have been implicated to contribute to the vulnerability to PTSD development. A recent study has suggested that one of the risk factors is a past trauma experience (Ozer et al., 2003). However, the mechanisms by which a memory of a previous stressor is stored in the neuronal circuitry and thereafter facilitates the formation of PTSD are not fully understood. This is, in part, because of a lack of animal models for history-dependent modulations of the PTSD development.

Fear conditioning is a behavioral test that is widely used to measure the strength of aversive memory. In a typical test of contextual fear conditioning, mice or rats that received aversive electric foot shocks in a chamber show "freezing" behaviors when they are placed in the same chamber that does not deliver foot shocks any longer. Thus, the fear conditioning paradigm captures some aspect of PTSD. In this study, we used the fear conditioning test and sought to establish an experimental animal model for trauma-induced sensitization of PTSD. Specifically, we examined the effect of a brief fear preexposure on subsequent fear conditioning. We found that mice that had received a prior single foot shock showed higher freezing responses, compared to intact mice. Because this phenomenon resembles a prior trauma-induced increase in the sensitivity to PTSD in humans, we further scrutinized this experimental model.

2. Materials and methods

2.1. Animal ethics

Experiments were performed with the approval of the animal experiment ethics committee at the University of Tokyo (approval number: P24-10) and according to the University of Tokyo guidelines for the care and use of laboratory animals. All efforts were made to minimize the animals' suffering and the number of animals used.

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2.2. Animals and drugs

Male C57BL/6J mice (SLC, Shizuoka, Japan) of 4–6 weeks old were housed under conditions of controlled temperature and humidity (23 ± 1 °C, 55 ± 5%), maintained on a 12:12-h light/dark cycle, and had access to food and water *ad libitum*. All behavioral tests were performed between 8 AM and 2 PM. Mice were acclimated by daily handling for 5 days before the behavioral experiments. MK801 (TOCRIS Bioscience, Bristol, UK), an antagonist of NMDA receptor, was diluted in saline and intraperitoneally injected at a dose of 1.0 mg/kg 30 min prior to the priming shock. We chose a relatively high dose (1.0 mg/kg) of MK801, because MK801 at a dose of 0.1 mg/kg is reported to be insufficient to inhibit the acquisition of contextual fear memory, whereas 1.0 mg/kg MK801 inhibited it (Gould et al., 2002).

2.3. Behavioral procedures

Fear conditioning was performed in a shock chamber consisting of a plastic box (18 cm in width, 11 cm in depth, and 11 cm in height) with transparent walls and a metal grid floor connected to a shock scrambler (SGA-2010, O'HARA, Tokyo, Japan). On day 1, mice were placed in a shock chamber, and a single electrical shock (1 mA, 2 s; priming shock) was immediately delivered to floor metal grids. Then, the mice were quickly removed from the chamber. All these procedures were completed within 10 s. Mice in the Conditioning-only group were placed in the chamber, but they did not receive the priming shock. The animals were soon returned to their home cages. This immediate foot shock is known to induce no contextual fear conditioning, because the animals cannot relate the aversive experience to the environmental context (Landeira-Fernandez et al., 2006). On day 2 (conditioning session), the mice in the Conditioning-only group and the Priming-shock + Conditioning group were allowed to freely explore the chamber for 5 min. During the following 4 min, they received 4 foot shocks (1 mA, 1 s; conditioning shock) at an interval of 1 min. Mice in the Priming-shock-only group were placed in the shock chamber but did not receive the conditioning shock. On day 3 (test session), the animals were re-placed in the same chamber for 5 min without any foot shocks. During both the fear conditioning and the test session, the animals were monitored at 2 Hz using a top-view digital camera. Freezing was automatically identified, using custom-made MATLAB routine (Sakaguchi et al., 2012; Ishikawa et al., 2014). After denoise, the mouse body was binarized at each video frame, and the body motion was detected by calculating the number of pixels in which the binary values flipped from 0 to 1 or from 1 to 0 between two successive video frames. Freezing time was defined based on the total number of frames in which the number of the pixel changes was below the threshold. The threshold was determined so that the calculated freezing time was comparable to that obtained manually by three well-trained observers.

2.4. Forced swimming and tail pinch

Mice were subjected to two aversive stressors, *i.e.*, the forced swimming and the tail pinch. The forced swimming is widely used as a behavioral despair model that produces acute stress (Porsolt et al., 1977). Individual mice were forced to swim inside a vertical Plexiglas cylinder (innerϕ = 12 cm). The water temperature was 22 ± 1 °C, the depth was 15 cm, and the above-water wall height was 8 cm. The mice were kept in the water for 15 min until they spent 60% of their time in immobility. The immobility time was evaluated using a video-based automatic detection system (Ishikawa et al., 2014). The mice were then returned to their home cage. The water was changed for each mouse. For the tail pinch, an artery clip (4.5 cm in length) was placed on the base of the tail for 5 s. Mice that did not try to remove the tail pinch within 1 s were excluded from the following experiments.

2.5. Elevated-plus maze

The mice were placed in the center of a maze with four arms arranged in the shape of a plus sign. The maze consisted of a central quadrangle (8 cm in width and 8 cm in length), two opposing open arms (25 cm in length and 8 cm in width), and two opposing closed arms. These four arms were identical in size, but the closed arms were equipped with 25-cm-high walls at both the sides and the far end. The floorboard was made of white plastic, the walls were made of opaque gray plastic, and the floorboard was elevated 25 cm above the ground. At the beginning of each trial, the mice were placed on the central quadrangle facing an open arm. The movements of the mice during a period of 5 min were recorded by a camera positioned above the center of the maze. The number of entries into the open arms and the closed arms and the time spent in each arm were manually determined. An entry into an arm was defined as placement of the four paws on that arm (Kumakura et al., 2010). The time spent in the open arms was expressed as a percentage of the total time spent in the open and closed arms, *i.e.*, open-arm time/(open-arm time + closed-arm time) × 100. The number of open arm entries were expressed as a percentage of the total arm entries, *i.e.*, open-arm entries/(open-arm entries + closed-arm entries) × 100. Mice whose total number of entries into arms was less than 10 were excluded.

2.6. Acetic acid-induced writhing test

Pain sensitivity was evaluated by referring to acetic acid-induced writhing responses. Acetic acid (0.9%) was intraperitoneally injected at a volume of 10 ml/kg into mice, and they were placed in a vertical Plexiglas cylinder (inner ϕ = 12 cm) 5 min before the test. Mice were habituated to these cylinders for 30 min before injection. Their movements were video-recorded for 10 min. A stretching behavior of the hind limbs accompanied by a contraction of the abdominal muscles was defined as a writhing response. From each video, a total of 30 time periods (5 s in length) were extracted at an interval of 20 s. A blinded observer judged whether the writhing behaviors were present or absent in these 5-s video clips. The writhing frequency was expressed as the percentage of the video clips with writhings to the total 30 videos.

2.7. Plasma corticosterone concentration

The blood was collected from the abdominal inferior vena cava of anesthetized mice with diethyl ether, incubated at 37 °C for 30 min, and centrifuged at 3000 × *g* for 10 min. The supernatant was collected as blood serum. The blood was obtained within 15 min after acute stresses, during which corticosterone is reported to exhibit the peak response (Anisman et al., 1998; Shanks et al., 1990). The concentration of corticosterone was measured with Corticosterone ELISA kit (Assaypro).

2.8. Statistical analyses

Statistical analyses were performed using R software. All data are demonstrated as means ± SEMs. Student's *t*-test was used for comparisons between two independent groups. One-way analysis of variance (ANOVA) and Tukey's *post hoc* test were used for comparisons among more than two independent groups. Statistical significance was set at $P < 0.05$.

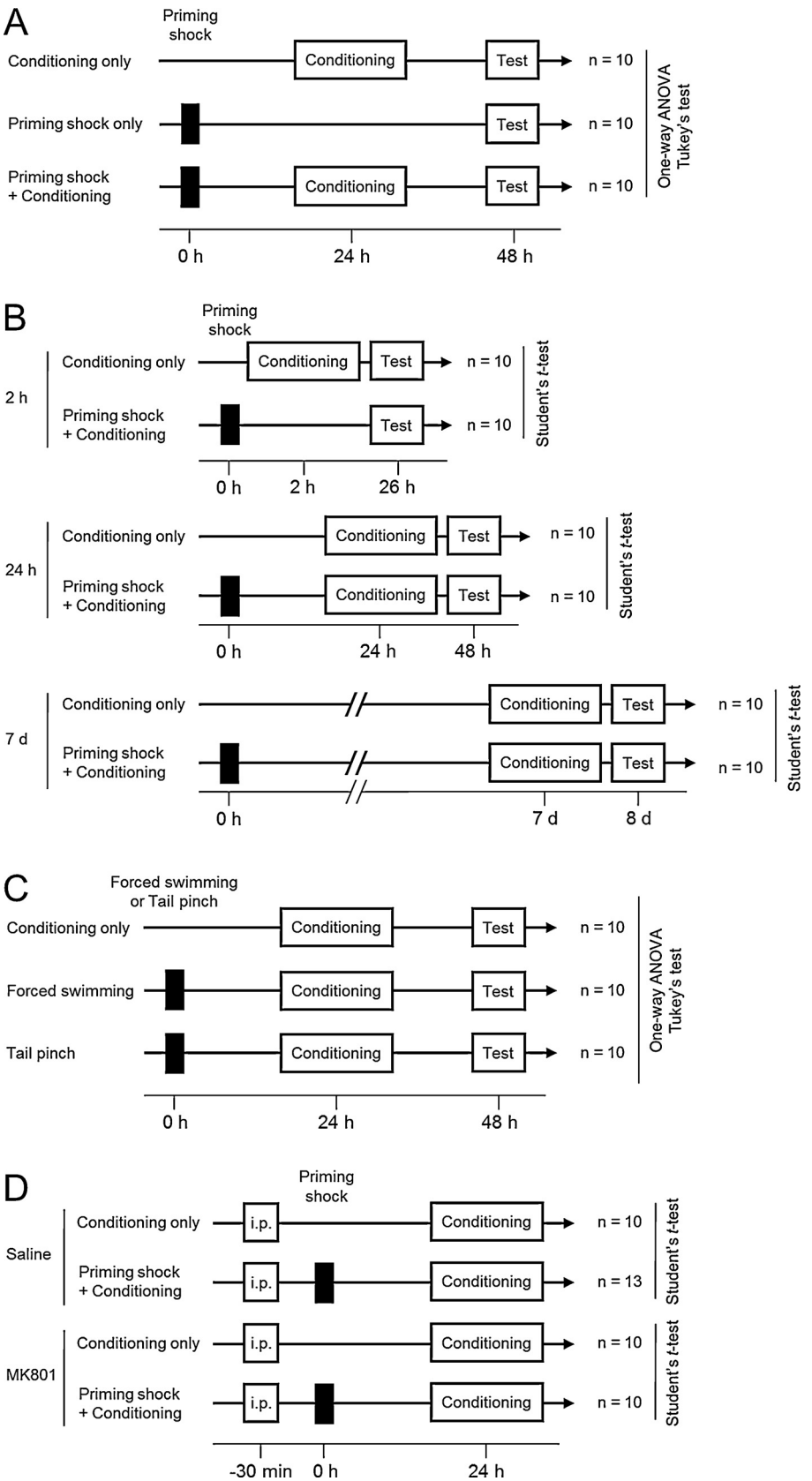

Fig. 1. Experimental paradigms. (A) We examined whether the priming shock enhances subsequent fear response and learning. See Fig. 2. (B) We examined how long the effect of the priming shock remains. See Fig. 3. (C) We examined other aversive stressors instead of the priming shock also enhance fear conditioning. See Fig. 5. (D) We examined whether the priming is dependent on NMDA receptor activity. See Fig. 6. *n* indicates the number of mice.

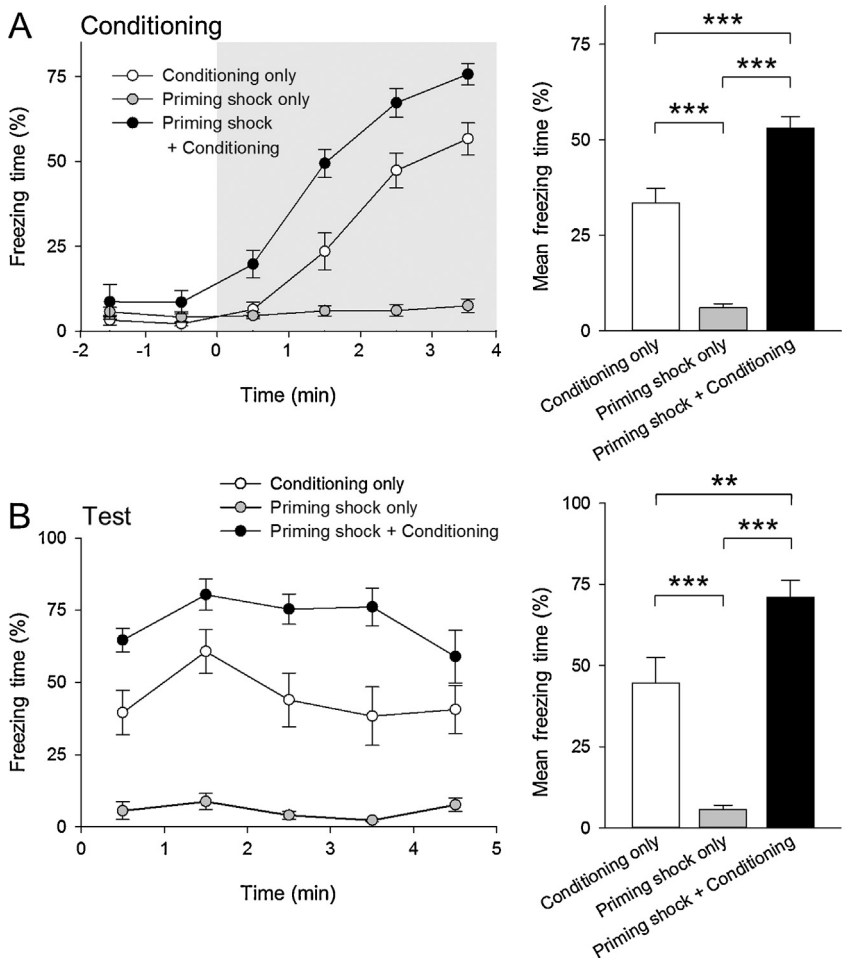

Fig. 2. Priming shock facilitates subsequent fear conditioning. (A) Left: freezing time during the conditioning session at 24 h are expressed as percentages of the time spend for freezing to the total time (1 min each). Mice were placed in a chamber and received repetitive foot shocks for the latter 4 min (gray area). Right: the freezing time percentages during the 4-min shock period are shown as the means ± SEMs. (B) The time course of freezing time (left) and the mean ± SEM freezing time (right) during the test session at 48 h. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, Tukey's test after one-way ANOVA; $n = 10$ mice each.

3. Results

3.1. Priming shock enhances subsequent fear conditioning

We examined the effect of a single foot shock on the following fear conditioning. In this first part of the experiments, a total of 30 mice were randomly divided into three groups, *i.e.*, 'Conditioning only', 'Priming shock only', and 'Priming shock + Conditioning' (Fig. 1A).

During the conditioning session, the Conditioning-only group exhibited a gradual increase in their freezing time in the course of the conditioning (Fig. 2A left). The Priming-shock + Conditioning group also exhibited a gradual development of freezing behaviors, but it spent a longer time for freezing, compared to the Conditioning-only group (Fig. 2A; $Q_{3,27} = 6.57$, $P = 2.3 \times 10^{-4}$, Tukey's test; $F_{2,27} = 60.6$, $P = 1.0 \times 10^{-10}$, one-way ANOVA, $n = 10$ mice each). This result indicates that the priming shock augmented the freezing responses. The mice in the Priming-shock-only group did not exhibit significantly stronger freezing responses than the Conditioning-only mice did, suggesting that the priming shock *per se* induced no contextual fear conditioning. During the test session, both the Conditioning-only group and the Priming-shock + Conditioning group exhibited stronger freezing responses than the Priming-shock-only group (Fig. 2B; Conditioning-only: $Q_{3,27} = 7.01$, $P = 9.8 \times 10^{-5}$, Tukey's test; $F_{2,27} = 35.4$, $P = 2.8 \times 10^{-8}$, one-way ANOVA; Priming-shock + Conditioning: $Q_{3,27} = 11.8$, $P = 1.6 \times 10^{-9}$, Tukey's test), and the Priming-shock + Conditioning group exhibited stronger freezing responses than the Conditioning-only group (Fig. 2B; $Q_{3,27} = 4.82$, $P = 5.6 \times 10^{-3}$, Tukey's test). Thus, priming shock-induced facilitation of freezing responses persisted for at least 24 h. In other words, the priming shock not only enhanced the instantaneous freezing responses during the conditioning but also produced a stronger long-term fear memory.

Next, to examine how long the effect of the priming shock remains, we changed the time interval between the priming shock and the conditioning shock. A priming shock was delivered 2 h, 24 h, or 7 d before the fear conditioning (Fig. 1B). For all intervals, the Priming-shock + Conditioning group showed a longer freezing time than the Conditioning-only group during the conditioning session (Fig. 3A; 2 h: $t_{18} = 2.79$, $P = 1.2 \times 10^{-2}$; 24 h: $t_{18} = 3.91$, $P = 1.0 \times 10^{-3}$; 7 d: $t_{18} = 4.96$, $P = 1.0 \times 10^{-4}$; Student's *t*-test, Priming shock + Conditioning *versus* Conditioning-only of the corresponding cohort, $n = 10$ mice each). The same results were obtained for the test sessions (Fig. 3B; 2 h: $t_{18} = 3.05$, $P = 6.9 \times 10^{-3}$; 24 h: $t_{18} = 2.80$, $P = 1.2 \times 10^{-2}$; 7 d: $t_{18} = 2.64$, $P = 1.7 \times 10^{-2}$, Student's *t*-test, Priming shock + Conditioning *versus* Conditioning-only mice of the corresponding cohort, $n = 10$ mice each). These results indicate that the facilitatory effect of the priming shock was formed within 2 h and that its potency was maintained for at least 7 d.

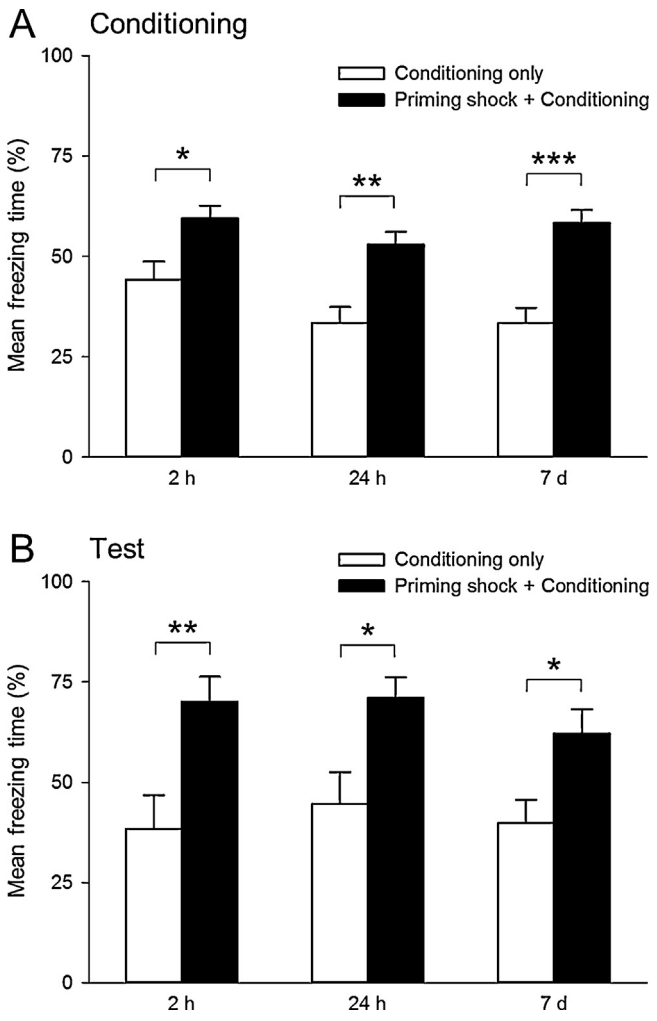

Fig. 3. The effect of a priming shock is long-lasting. (A,B) The freezing time percentages are shown as the means ± SEMs during the conditioning (A) and test (B) sessions. White bars and black bars represent Conditioning only mice and Priming shock + Conditioning mice, respectively. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, Student's *t*-test, $n = 10$ mice each.

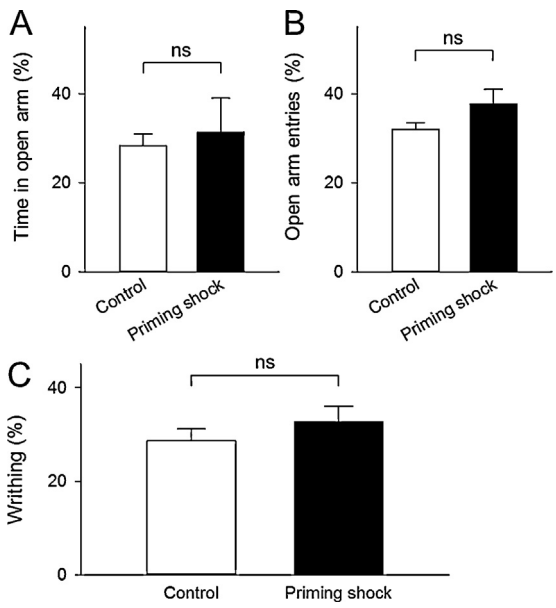

Fig. 4. Priming shock does not affect basal anxiety level or pain threshold. (A,B) The mean ± SEM percentages of the time spent in the open arms to the total 5-min observation period (A) and the percentage of the number of the open-arm entries to the total arm entry number (B) in the elevated-plus maze test in mice that received a single foot shock 24 h before the maze test (Priming shock, $n = 9$ mice) or did not received any foot shock (Control, $n = 12$ mice). $^{ns}P > 0.05$, Student's *t*-test. (C) Percentage of the numbers of the 5-s periods with acetic acid-induced writhing responses in mice that received a single foot shock 24 h before the writhing test (Priming shock, $n = 10$ mice) or did not receive any shock (Control, $n = 10$ mice). $^{ns}P > 0.05$, Student's *t*-test.

3.2. Priming shock does not alter anxiety or pain

A previous study has demonstrated that patients with anxiety disorders showed stronger fear responses in fear conditioning than healthy controls (Lissek et al., 2005). Therefore, it is possible that the priming shock-facilitated freezing resulted from an increased basal anxiety level. To address this possibility, we examined the effect of a priming shock on basal anxiety using the elevated-plus maze. Mice were placed in a chamber and immediately received an electrical shock ('Priming shock' group), whereas control mice were placed in the same chamber but did not receive the shock. They are soon returned to their home cages. After 24 h, the mice were subjected to the elevated-plus maze. Control and Priming-shock mice did not significantly differ in the percentage of time spent in the open arms (Fig. 4A, $t_{19} = 0.43$, $P = 0.68$, Student's *t*-test, $n = 9 - 12$ mice) or the number of entries to the open arms (Fig. 4B, $t_{19} = 1.79$, $P = 8.9 \times 10^{-2}$, Student's *t*-test, $n = 9 - 12$ mice). These results suggest that a priming shock has no effect on general anxiety under free-moving conditions. Therefore, the priming shock-facilitated freezing is unlikely to be mediated by an increased basal anxiety level.

We also examined the possibility that the priming shock lowered the pain threshold and thereby enhanced fear conditioning. Mice of the Control and Priming-shock groups were intraperitoneally treated with 0.9% acetic acid. The severity of writhing responses, a putative pain-reflecting behavior, did not differ between two groups (Fig. 4C, $t_{18} = 0.96$, $P = 0.35$, Student's *t*-test, $n = 10$ mice each).

3.3. Other aversive stressors do not enhance subsequent fear conditioning

We next examined whether the priming shock can be replaced with other aversive experiences such as forced swimming or tail pinch (Fig. 1C). On day 1, mice were subjected to the forced swimming or the tail pinch, instead of a foot shock. On days 2 and 3, mice were subjected to fear conditioning and test sessions, respectively. Mice with either forced swimming or tail pinch did not exhibit an increase in freezing time compared to Conditioning-only mice during the conditioning (Fig. 5B; $F_{2,27} = 2.61$, $P = 9.2 \times 10^{-2}$, one-way ANOVA, $n = 10$ mice each) and the test (Fig. 5C; $F_{2,27} = 1.44$, $P = 0.25$, one-way ANOVA, $n = 10$ mice each). It is possible that the priming shock, forced swimming, and tail pinch differed in their intensities of aversion. We thus measured the plasma level of corticosterone after these stressors. A priming shock induced a smaller increase in plasma corticosterone compared to forced swimming (Fig. 5C; $Q_{4,\,12} = 5.67$, $P = 8.1 \times 10^{-3}$, Tukey's test; $F_{3,\,12} = 14.0$, $P = 3.2 \times 10^{-4}$, one-way ANOVA, $n = 4$ mice each), and the mice experienced forced swimming showed a higher corticosterone level than control mice or tail pinch-experienced mice (Fig. 5C; Control *versus* Forced swimming: $Q_{4,\,12} = 8.43$, $P = 3.3 \times 10^{-4}$; Forced swimming *versus* Tail pinch: $Q_{4,\,12} = 7.31$, $P = 1.2 \times 10^{-3}$, Tukey's test). Thus, different intensities of stressors cannot explain the fact that neither forced swimming nor tail pinch facilitated conditioned fear responses.

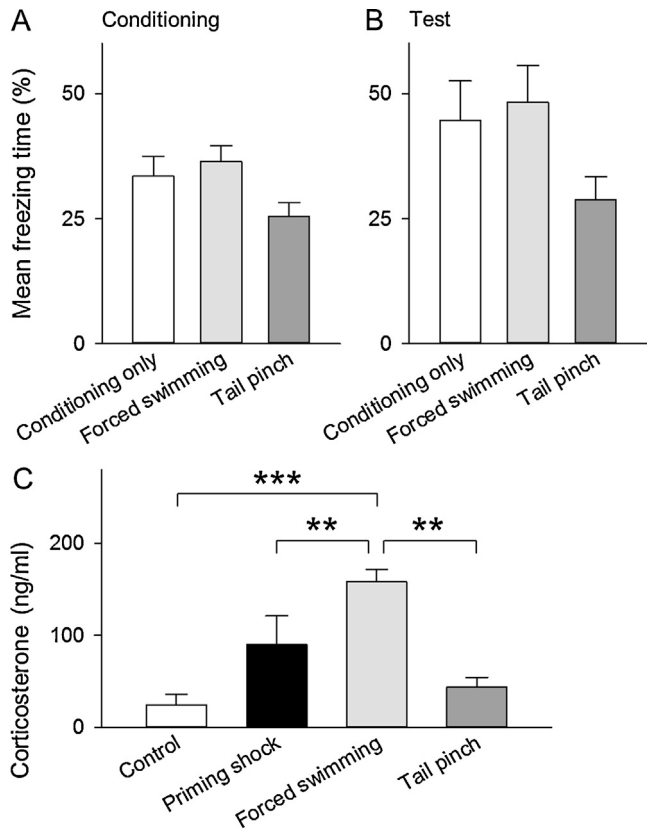

Fig. 5. Neither forced swimming nor tail pinch affects subsequent fear conditioning. (A and B) The mean ± SEM percentages of the freezing times of mice that received forced swimming or tail pinch 24 h before the conditioning, or 'Conditioning-only' mice are shown during the conditioning (A) and test (B) experiments. One-way ANOVA, $n = 10$ mice each. (C) Plasma corticosterone concentration after various acute stresses. $^{**}P < 0.01$, $^{***}P < 0.001$, Tukey's test after one-way ANOVA; $n = 4$ mice each.

3.4. MK801 inhibits the priming effect

NMDA receptors play an important role in the acquisition of fear (Campeau et al., 1992; Goosens and Maren, 2004). To examine the involvement of NMDA receptors in the priming effect, we intraperitoneally injected MK801, an NMDA receptor antagonist, 30 min before the priming shock (Fig. 1D). During the conditioning session, Saline-treated Priming-shock + Conditioning group exhibited a gradual increase in their freezing time (Fig. 6A left), and spent a longer time for freezing compared to the Conditioning-only group (Fig. 6A right, $t_{21} = 2.08$, $P = 2.1 \times 10^{-4}$, Student's t-test, $n = 10$–13 mice). However, MK801-treated Priming-shock + Conditioning group exhibited a similar development of freezing to the Conditioning-only group (Fig. 6B left), and did not exhibit stronger freezing responses (Fig. 6B right, $t_{18} = 2.10$, $P = 0.43$, Student's t-test, $n = 10$ mice each). These results suggest that the priming effect emerged through NMDA receptor-dependent neuronal plasticity.

4. Discussion

In the present study, we showed that a prior foot shock enhanced subsequent fear conditioning and that the priming shock effect lasted for 7 days. We furthermore demonstrated that other aversive shocks could not enhance fear conditioning and that this effect was inhibited by MK801, an NMDA receptor antagonist.

In contrast to our findings, Rau et al. (2005) has demonstrated that a prior single foot shock 24 h before contextual fear conditioning does not alter the freezing level during the context re-exposure. We believe that this apparent discrepancy is attributable to different strengths of aversive stimuli delivered to animals. To induce fear conditioning, we delivered a fewer foot shocks to subjects (4 shocks at 1 mA for 1 s), compared to Rau's conditions (15 shocks at 1 mA for 1 s in Experiment 4). More importantly, Rau et al. delivered a prior shock 192 s after placement to the shock chamber, whereas we used an immediate shock so that the shock *per se* does not induce contextual fear conditioning (Landeira-Fernandez et al., 2006). As a result, animals in Rau et al. (2005) exhibited an almost saturated level (approximately 80%) of freezing time even in the absence of prior foot shocks, and therefore, the priming shocks might be unable to induce a further increment in the freezing level. Another study has reported that a single prior shock has no effect on subsequent fear conditioning by one other single footshock applied in a different context (Rau and Fanselow, 2009). This discrepancy is probably due to the floor effect; they used only one shock as a weak unconditioned stimulus, which alone could not induce a conditioned fear response even in that study. Therefore, the enhancement of freezing responses by a prior foot shock did not appear; note that we delivered 4 shocks to induce fear conditioning, which alone could induce fear responses.

Other aversive stressors rather than the priming shock, such as forced swimming or tail pinch, did not facilitate fear conditioning 24 h after stressors. On the other hand, previous works have demonstrated that stress generally enhances aversive learning (Cordero et al., 2003; Fanselow and Bolles, 1979; Shors et al., 1992). We failed to find stress-induced enhancement of fear conditioning. Perhaps because we placed a long interval (24 h) from stress until the fear conditioning, the direct effect of stress might have disappeared before the start of fear conditioning. In general, stressors activate the hypothalamus-pituitary-adrenal axis and lead to secretion of glucocorticoids. Glucocorticoids, such as corticosterone in rodents, enhance the excitability of pyramidal neuron in the basolateral amygdala (Duvarci and Pare, 2007). The basolateral amygdala is a region critical for fear memory formation (LeDoux, 2000). It is reported that previous stress exposure attenuates GABAergic inhibition in basolateral amygdala, which leads to facilitation of fear conditioning (Rodriguez Manzanares et al., 2005). In the present study, however, increases in corticosterone induced by other stressors were not significantly lower than those by the priming shock; nonetheless, the other stressors did not mimic the effect of the priming shock. Thus, the enhanced fear conditioning cannot be explained by a glucocorticoid-induced, non-specific increase in the excitability of amygdala neurons, but our result implies that a stressor induces specific long-lasting plasticity in its relevant synaptic route and thereby exerts a specific effect on subsequent fear memory formation. In human, a prior trauma that enhances the sensitivity of PTSD development is not necessarily identical to the trauma that subsequently induce PTSD. Mice may have a less ability to integrate different traumatic events into a generalized fear experience.

NMDA receptors are critical for synaptic plasticity. Correlated presynaptic and postsynaptic activity triggers calcium influx *via* NMDA receptor channels and induces synaptic plasticity and memory formation (Malenka and Nicoll, 1999). In our study, priming shock-induced increases in freezing time were observed as long as 7 days after the priming shock, and this effect was inhibited by MK801. Therefore, the priming effect is likely shaped by NMDA receptor-dependent synaptic plasticity. Another possible explanation for MK801-induced cancelation of priming shock-facilitated freezing is that MK801 exerts an anti-nociceptive effect, as shown in an animal model of chronic pain (Fisher et al., 2000). However, the acute anti-nociceptive actions of NMDA receptor antagonists are conflicting. Some studies have demonstrated that a high dose

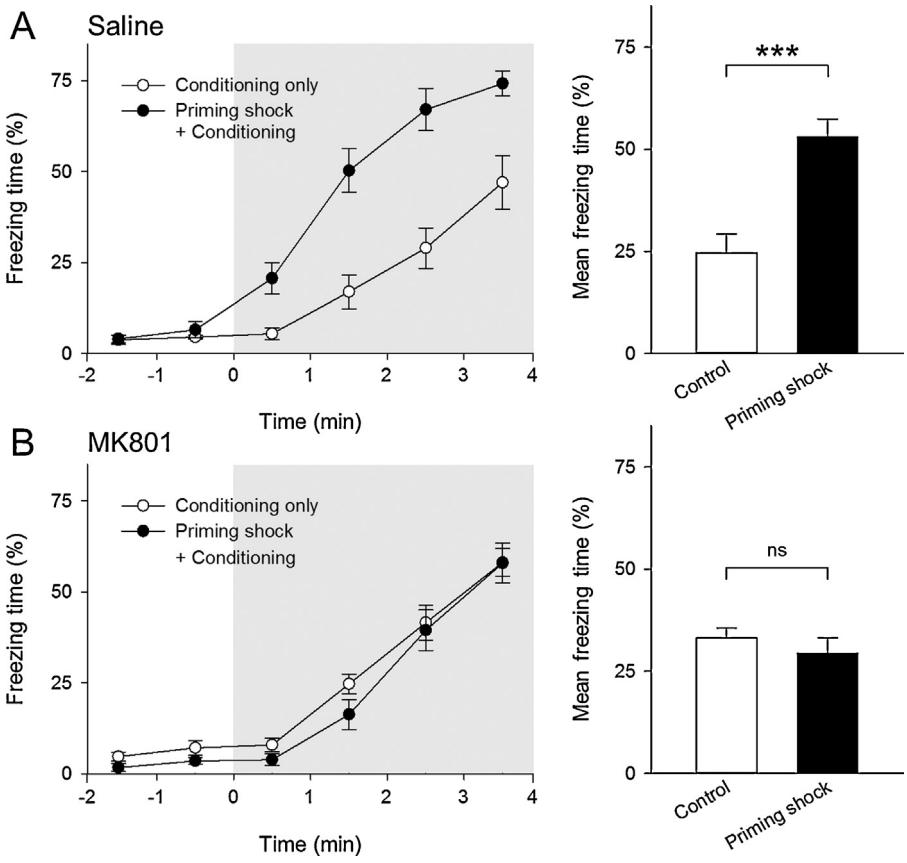

Fig. 6. The priming shock effect forms through NMDA receptor activity. (A and B) Left: freezing time during the conditioning session are expressed as percentages of the time spend for freezing to the total time (1 min each) in Saline-pretreated mice (A) and MK801-pretreated mice (B). Right: the freezing time percentagees during the 4-min shock period are shown as the means ± SEMs. $^{***}P < 0.001$, $^{ns}P > 0.05$, Student's *t*-test; Saline-conditioning only, $n = 10$; Saline-Priming shock + Conditioning, $n = 13$; MK801-Conditioning only, $n = 10$; MK801- Priming shock + Conditioning, $n = 10$.

of MK801 induces hyperalgesia in animal models of acute pain (Al-Amin et al., 2003; Schmidt et al., 2009). We used a relatively high dose (1.0 mg/kg) of MK801. Therefore, MK801-induced decrease in freezing time is more likely mediated by NMDA receptor-induced synaptic plasticity, rather than NMDA receptor-induced analgesia.

In conclusion, we demonstrated that a priming shock proved to enhance fear conditioning, consistent with human studies showing that a history of prior traumas is a risk factor for developing PTSD. In mice, this effect lasted for at least 7 days, was stressor-specific, and dependent on NMDA receptors. The behavioral test used in this study will provide a platform for our understanding of the neural mechanisms underlying the development of PTSD.

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