Fear conditioning and extinction distinctively alter bidirectional synaptic plasticity within the amygdala of an animal model of post-traumatic stress disorder

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A B S T R A C T

Synaptic plasticity in the amygdala plays an essential role in the formation and inhibition of fear memory; however, this plasticity has mainly been studied in the lateral amygdala, making it largely uninvestigated in other subnuclei. Here, we investigated long-term potentiation (LTP) and long-term depression (LTD) in the basolateral amygdala (BLA) to the medial division of the central amygdala (CEm) synapses of juvenile C57BL/6N (B6) and 129S1/SvImJ (S1) mice. We found that in naïve B6 and S1 mice, LTP was not induced at the BLA to CEm synapses, whereas fear conditioning lowered the threshold for LTP induction in these synapses of both B6 and S1 mice. Interestingly, fear extinction disrupted the induction of LTP at the BLA to CEm synapses of B6 mice, whereas LTP was left intact in S1 mice. Both low-frequency stimulation (LFS) and modest LFS (mLFS) induced LTD in naïve B6 and S1 mice, suggesting that the BLA to CEm synapses express bidirectional plasticity. Fear conditioning disrupted both types of LTD induction selectively in S1 mice and LFS-LTD, presumably NMDAR-dependent LTD was partially recovered by fear extinction. However, mLFS-LTD which has been known to be endocannabinoid receptor 1 (CB1R)-dependent was not induced after fear extinction in both mouse strains. Our observations suggest that fear conditioning enhances LTP while fear extinction diminishes LTP at the BLA to the CEm synapses of B6 mice with successful extinction. Considering that S1 mice showed strong fear conditioning and impaired extinction, strong fear conditioning in the S1 strain may be related to disrupted LTD, and impaired extinction may be due to constant LTP and weak LFS-LTD at the BLA to CEm synapses. Our study contributes to the further understanding of the dynamics of synaptic potentiation and depression between the subnuclei of the amygdala in juvenile mice after fear conditioning and extinction.

1. Introduction

Fear conditioning and extinction are forms of associative learning often used to assess fear memory formation and inhibition in the laboratory. Fear conditioning and extinction have been used to understand the etiology of post-traumatic stress disorder (PTSD) because exaggerated fear conditioning and impaired fear extinction have been reported as PTSD symptoms (Pitman, 1988; Wessa and Flor, 2007; Wicking et al., 2016). PTSD is included in the "Trauma- and Stressor-Related Disorders" category in DSM-5 and it develops after a terrifying event is experienced. Patients with PTSD repeatedly and persistently re-experience a traumatic event owing to the recurrent visits of the fearful memory (American Psychiatric Association, 2013). Although PTSD is a common psychiatric disorder, an effective treatment for PTSD has not yet been

discovered because the etiology of PTSD remains unclear.

To study the neural mechanisms underlying PTSD, researchers have been taking advantage of an inbred mouse strain, 129S1/SvImJ (S1), which displays impaired fear extinction after normal fear conditioning (Hefner et al., 2008; Whittle et al., 2010; Cazares et al., 2019; Gunduz-Cinar et al., 2019). Previously, we reported that compared to C57BL/6N (B6) mice that exhibit normal fear conditioning and extinction, S1 mice show an increased number of c-Fos positive cells in the medial division of the central amygdala (CEm) following contextual fear extinction (Park and Chung, 2019). Similarly, S1 mice exhibited increased c-Fos expression in the CEm after auditory fear extinction retrieval compared to B6 mice (Whittle et al., 2010). Considering that the final output area of the amygdala is the CEm, which projects to brain areas including the periaqueductal gray (PAG), bed nucleus of the stria terminalis (BNST), and hypothalamus that are known to mediate anxiety

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	Abbreviations		
		ASIC1a	acid-sensing ion channel-1a subunit
LTP	long-term potentiation	US	unconditioned stimulus
LTD	long-term depression	CS	conditioned stimulus
BLA	basolateral amygdala	ISI	inter-stimulus interval
CEm	medial division of the central amygdala	aCSF	artificial cerebrospinal fluid
LFS	low-frequency stimulation	eEPSC	evoked excitatory postsynaptic current
mLFS	modest low-frequency stimulation	HFS	high-frequency stimulation
PTSD	post-traumatic stress disorder	FC	fear conditioning
PAG	periaqueductal gray	Ext	extinction
BNST	bed nucleus of the stria terminalis	NMDAR	NMDA receptor
LA	lateral amygdala	CB1	cannabinoid receptor 1
BA	basal amygdala	PPR	paired pulse ratio
MMP-9	metalloproteinase-9	T-LA	thalamic lateral amygdala
		C-LA	cortical lateral amygdala

and/or fear responses (LeDoux et al., 1988; Tovote et al., 2015), we postulated that the abnormally excited CEm in S1 mice may mediate constant fear responses even after fear extinction.

Long-term potentiation (LTP) and long-term depression (LTD) have been studied as cellular mechanisms of amygdala-dependent learning primarily in the basolateral amygdala (BLA). In rodent brain slices, LTP and LTD can be induced in the BLA by stimulating inputs from the cortex, thalamus, or adjacent cells (Chapman et al., 1990; Li et al., 1998; Wang and Gean, 1999; Tully et al., 2007). In behaving animals, fear conditioning has been known to induce LTP in the BLA in *in vivo* or *ex vivo* conditions (McKernan and Shinnick-Gallagher, 1997; Rogan et al., 1997). Additionally, the lateral amygdala (LA), which receives conditioned stimuli-related information, has been shown to indirectly influence the CEm by sending information to it via the basal amygdala (BA) (Maren, 2001; Babaev et al., 2018).

A few studies have examined LTP and LTD at the BLA to CEm synapses which contain both the input and output areas of the amygdala. Matrix metalloproteinase-9 (MMP-9) cleaves components of the extracellular matrix and has been known to be involved in hippocampal LTP as well as hippocampus-dependent learning and memory (Nagy et al., 2006; Wojtowicz and Mozrzymas, 2010). MMP-9 is also involved in amygdala-dependent learning and memory. MMP-9 knockout mice show disrupted LTP at the BLA to CEm synapses (Knapska et al., 2007; Gorkiewicz et al., 2015). In addition, genetic variants in human ortholog of Acid-sensing ion channel-1a subunit (ASIC1a) gene were reported to be associated with amygdala dysfunction (Smoller et al., 2014). ASIC1a knockout mice exhibit disrupted fear conditioning and impaired LTP at the BLA to CEm synapses (Wemmie et al., 2003; Chiang et al., 2015). For LTD, stress was shown to disrupt LTD induction at the BLA to CEm synapses (Li et al., 2018). However, the effect of fear conditioning and extinction on LTP and/or LTD induction at the BLA to CEm synapses remains elusive. Considering that more c-Fos expression was found in the CEm of S1 mice than that of B6 mice after fear extinction (Park and Chung, 2019), the plasticity occurring at the BLA to CEm synapses in S1 mice may differ from that in B6 mice.

Therefore, we investigated the induction of LTP and LTD at the BLA to CEm synapses of juvenile B6 and S1 mice to determine whether synaptic plasticity at those synapses would be altered by fear conditioning and extinction. Furthermore, we explored whether those alterations in the synaptic plasticity of the BLA to CEm synapses would be different in the fear extinction-impaired S1 mice.

2. Methods and materials

2.1. Ethics declarations

All experiments were carried out in compliance with the recommendations outlined in the National Institutes of Health's Guide for the Care and Use of Laboratory Animals. All experimental procedures were approved by the Institutional Animal Care and Use Committee of Konkuk University, Seoul, Korea.

2.2. Animals

The S1 mice were acquired from Jackson Laboratory (Bar Harbor, USA) and maintained by sibling-sibling mating. The B6 mice were obtained from Orient Bio (a branch of Charles River, Gapyeong, Korea). All mice were group-housed (4–5 per cage) in a controlled room (temperature, 23 ± 1 °C; humidity, 50 ± 10 %) with a 12/12-h light/dark cycle (lights on at 7 a.m.). Food and water were provided *ad libitum*. Male mice aged 3–4 weeks were used in the study and were handled for 5 days before the start of the experiments. When we conducted brain slice electrophysiology, mice were 4–6 weeks old after handling and behavioral experiments.

2.3. Auditory fear conditioning and extinction

After 5 days of handling (3 min/day), auditory fear conditioning and extinction procedures were conducted. In the conditioning protocol, after 5 min of acclimation, mice were presented with three pairings of a neutral tone (conditioned stimulus [CS], 75 dB, 10,000 Hz, 30 s) with a foot shock (unconditioned stimulus [US], 0.6 mA, 2 s, overlapped and terminated with the CS), along with 20–40 s of random inter-stimulus intervals (ISIs). During this protocol, the mice were placed in a hexahedral conditioning chamber having a width of 18 cm, depth of 18 cm, and height of 30 cm (H10-11M-TC, Coulbourn Instruments, PA, USA). After the last foot shock, the mice were kept in the conditioning chamber for another 30 s and then moved back to their home cages. After 24 h, fear extinction was performed. The mice were subjected to the novel extinction context (acrylic hexagonal prism with an apothem of 11 cm and height of 29 cm). After 2 min of acclimation, a US-free CS (a neutral tone, 75 dB, 10,000 Hz, 30 s) was delivered 30 times, along with 30 s of ISIs. After the last tone, the mice were retained in the extinction context for another 30 s and then returned to their home cages. The same extinction protocol was conducted for two consecutive days. Freezing behaviors were manually analyzed by observing whether mice moved or not (except for respiration) every 2 s.

2.4. Slice preparation and electrophysiology

Brain slices were obtained from naïve mice, mice at 24 h after fear conditioning, and mice at 24 h after fear extinction. To acquire the brain slices, mice were deeply anesthetized with isoflurane, and coronal brain slices (350 μm thickness) containing the amygdala were prepared using a vibratome (Leica VT 1000S) in an ice-cold sucrose dissection buffer (in mM: 212 sucrose, 3 KCl, 26 NaHCO$_3$, 1.25 NaH$_2$PO$_4$, 7 MgCl$_2$, and 10 glucose) bubbled with 95 % O$_2$/5 % CO$_2$ gas. The brain slices were then transferred to a chamber filled with artificial cerebrospinal fluid (aCSF; in mM: 1 NaH$_2$PO$_4$, 26.2 NaHCO$_3$, 118 NaCl, 2.5 KCl, 11 glucose, 2

CaCl$_2$, and 1 MgCl$_2$) bubbled with 95 % O$_2$/5 % CO$_2$ gas and warmed to 35 °C for recovery. After 45 min of recovery, the brain slices were stored at room temperature until recordings were performed.

Electrophysiological data were filtered at 2 kHz and sampled at 10 kHz using Axopatch 200B (Molecular Devices) and WinLTP 2.10 software (The University of Bristol). Whole-cell voltage clamp recordings were conducted with 3–5 MΩ glass electrodes filled with a K-gluconate-based internal solution (in mM: 120 K-gluconate, 5 NaCl, 1 MgCl$_2$, 0.2 EGTA, 10 HEPES, 2 MgATP, and 0.1 NaGTP; pH 7.2 with KOH) for LTP induction or a Cs methanesulfonate-based internal solution (in mM: 115 Cs methanesulfonate, 20 CsCl, 10 HEPES, 2.5 MgCl$_2$, 0.6 EGTA, 5 QX-314, 4 Na$_2$-ATP, 0.4 Na$_2$-GTP, and 10 Na-phosphocreatine; pH 7.2 with CsOH) for LTD induction. Evoked excitatory postsynaptic currents (eEPSCs) were isolated by adding picrotoxin (50 μM) to the aCSF and collected from the neurons in the Cem at a holding potential of −70 mV by stimulating the BLA with a bipolar stimulating electrode (CE2C55, FHC). After 5 min of baseline recording, LTP was induced by high-frequency stimulation (HFS, 100 pulses at 100 Hz), or LTD was induced by low-frequency stimulation (LFS, 600 pulses at 1 Hz or modest LFS, mLFS, 120 pulses at 12 Hz). For calculating the magnitude of LTP or LTD, the mean eEPSCs amplitudes collected during the 20–25

min period after LTP or LTD induction were divided by the mean eEPSCs amplitudes collected during the first 5 min of baseline recording. All recordings were acquired at 30–32 °C and series resistance and membrane resistance were monitored throughout the experiments. Recording data with a >20 % change in the series resistance and/or membrane resistance were discarded before analysis.

2.5. Statistical analysis

All study data are presented as mean ± standard error of the mean. Statistical analysis was conducted using GraphPad Prism 8 (San Diego, CA, USA). The magnitude of LTP or LTD in each mouse strain was assessed using a one-way analysis of variance (ANOVA) followed by a post-hoc test, Tukey's multiple comparisons test. Two-way ANOVA was used to analyze the strain-by-behavioral condition interactions and Sidak's post hoc multiple comparisons test was performed to confirm the differences within and between each mouse strain. Two-tailed Student's *t*-test was conducted to determine LTP or LTD induction within each strain and to compare the magnitudes of LTP or LTD between the strains. Before conducting the statistical tests, normality tests were carried out in advance and normally distributed data were statistically analyzed. Statistical significance was set at p-value <0.05.
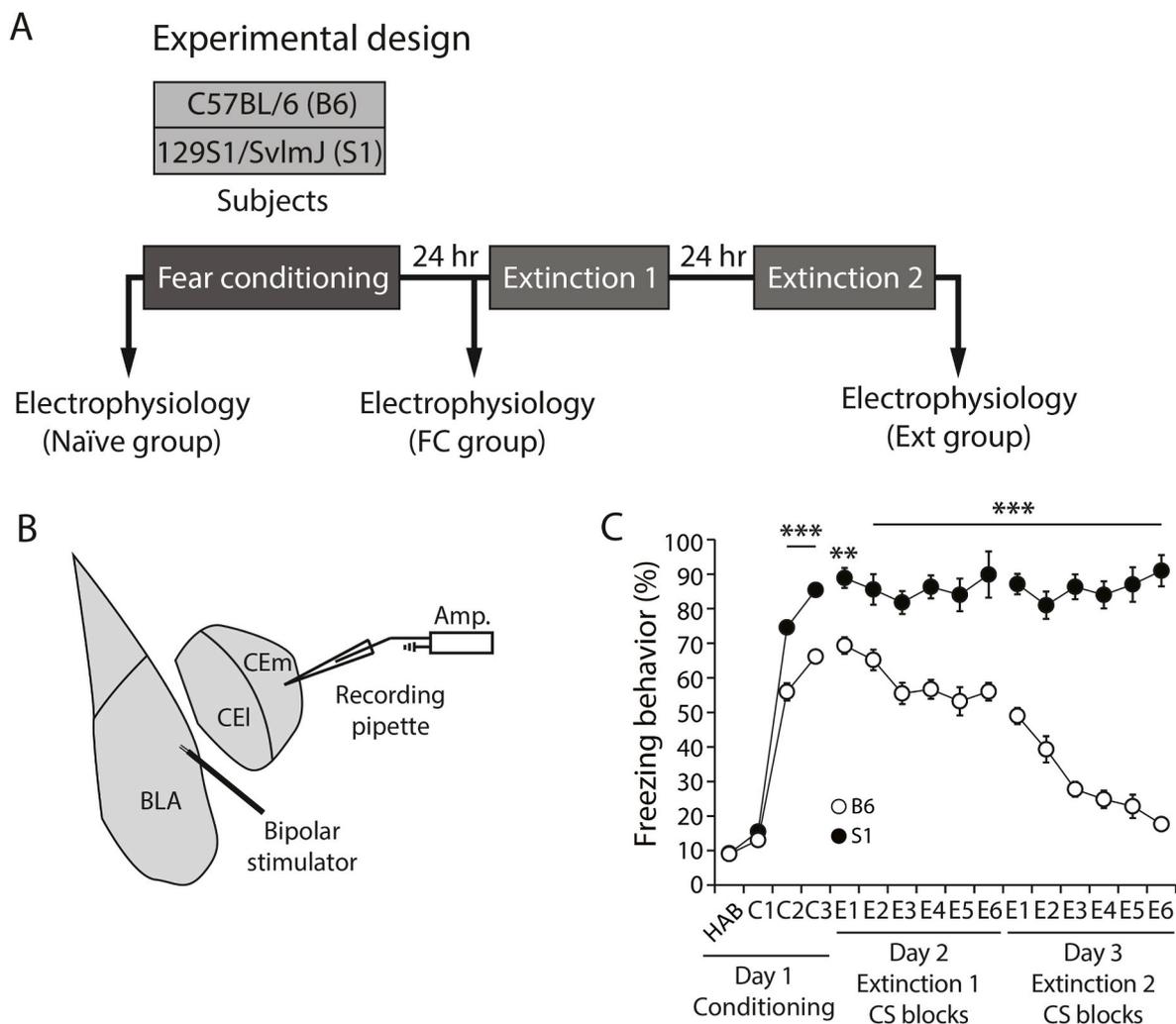
Fig. 1. **Experimental scheme for exploring synaptic plasticity at the BLA to CEm synapses in B6 mice and fear extinction-impaired S1 mice.** (A) Experimental design. (B) Recording configuration. EPSCs were evoked and recorded at the BLA to CEm synapses by placing a bipolar stimulating electrode in the BLA and a recording pipette in the CEm. (C) Both B6 and S1 mice showed intact fear conditioning, with S1 mice exhibiting more freezing during the second and third tones. S1 mice exhibited impaired fear extinction when compared with B6 mice (N = 13 in FC group [conditioning only] and N = 10 in Ext group [conditioning + extinction] in B6 mice; N = 13 in FC group and N = 12 in Ext group in S1 mice). Each extinction block consists of five extinction trials. BLA, basolateral amygdala; CEm, medial division of the central amygdala; EPSC, excitatory postsynaptic current; FC, fear conditioning; Ext, fear extinction. **p < 0.01, ***p < 0.001. N indicates the number of animals.

3. Results

To investigate whether fear conditioning and extinction would alter synaptic plasticity at the BLA to CEm circuit and whether this synaptic plasticity would be altered in a fear extinction-impaired animal model, we recorded the activity of CEm neurons while stimulating the BLA inputs before experimentation (naïve group), after fear conditioning, and after fear extinction in B6 and S1 mice. The glutamatergic neurons in the BLA project to the CEm both directly, and indirectly through GABAergic neurons in the lateral division of the central amygdala (CEl) (Janak and Tye, 2015; Fenster et al., 2018). As we applied PTX in

advance to block GABAergic receptors and isolate eEPCSs, we are selectively recording the activity in the direct BLA to CEm circuit as the activity in the indirect BLA - CEl - CEm circuit is blocked by PTX (Fig. 1A and B).

As mentioned previously, S1 mice display disrupted fear extinction following strong fear conditioning when compared to B6 mice (Fig. 1C) (Whittle et al., 2010; Park and Chung, 2019). Both B6 and S1 mice were well trained to the CS and a significant strain-by-trial interaction in freezing time ($F_{(2, 138)} = 17.55$, $p < 0.001$, two-way ANOVA) was demonstrated, with S1 mice showing longer freezing time during the second ($p < 0.001$) and third tones ($p < 0.001$). S1 mice failed to
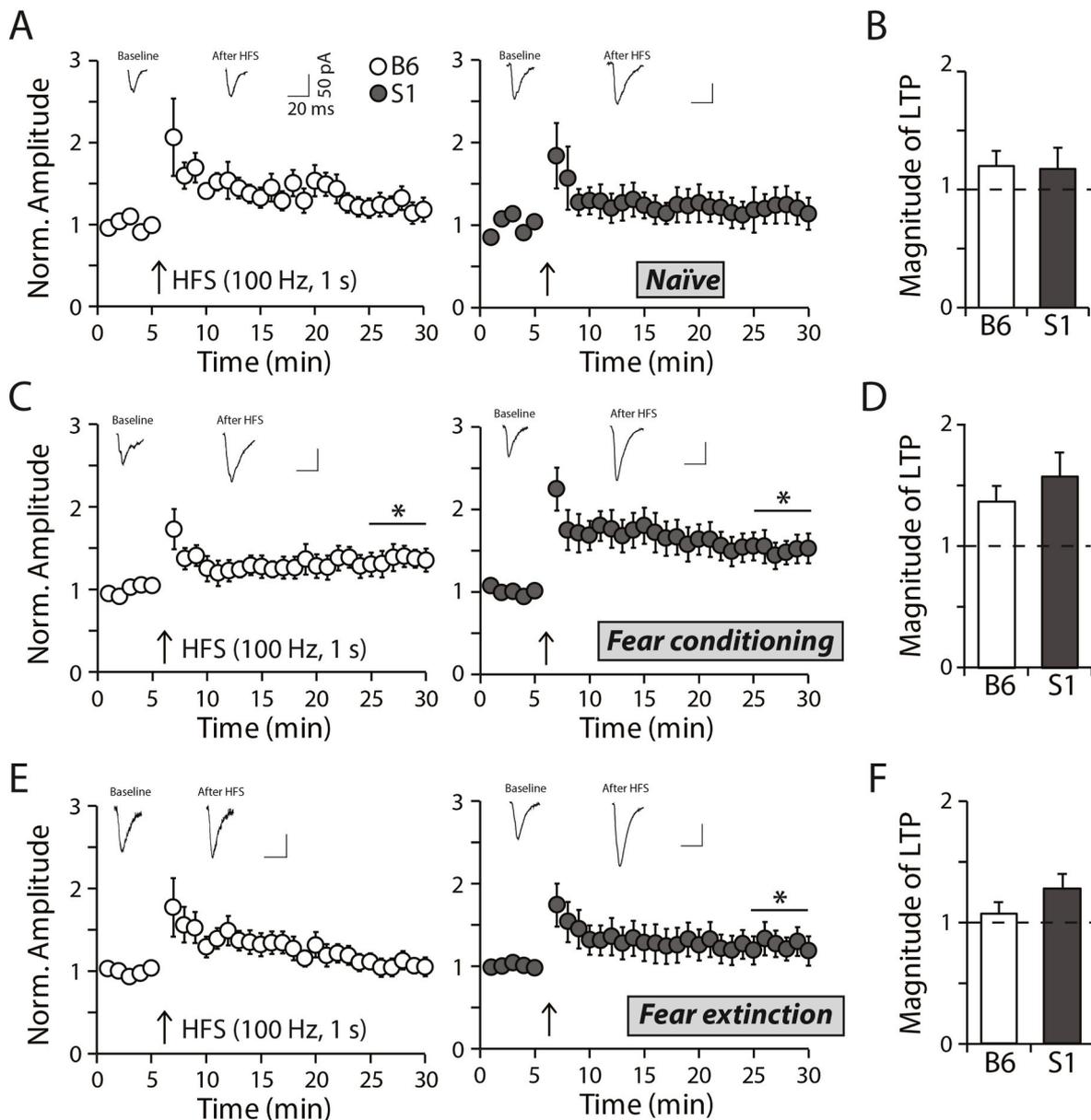
Fig. 2. Fear conditioning lowered the threshold for LTP induction at the BLA to CEm synapses in both B6 and S1 mice and fear extinction disrupts LTP in B6 but not in S1 mice. (A) A single HFS failed to induce LTP at the BLA to CEm synapses in both B6 (N = 8, n = 11; p = 0.138) and S1 (N = 6, n = 9; p = 0.392) mice (magnitude of LTP relative to baseline: 119.9 ± 12.8 % in B6 mice; 117.4 ± 18.1 % in S1 mice). (B) The magnitudes of LTP were similar between B6 and S1 mice (p = 0.912). (C) After fear conditioning, the same HFS showed significant induction of LTP at the BLA to CEm synapses in both B6 (N = 7, n = 12; p = 0.031) and S1 (N = 7, n = 15; p = 0.023) mice (magnitude of LTP relative to baseline: 136.3 ± 13.6 % in B6 mice; 157.2 ± 19.8 % in S1 mice). (D) The magnitudes of LTP were comparable between B6 and S1 mice (p = 0.528). After fear extinction, the induction of HFS-LTP was disrupted in B6 mice (N = 5, n = 9; p = 0.470), whereas S1 mice (N = 5, n = 12; p = 0.045) showed constant induction of LTP in the BLA to CEm circuit (magnitude of LTP relative to baseline: 106.9 ± 9.9 % in B6 mice; 123.0 ± 3.4 % in S1 mice). (F) The magnitude of LTP was not significantly different between B6 and S1 mice (p = 0.202). Calibration: 20 ms, 50 pA. LTP, long-term potentiation; BLA, basolateral amygdala; CEm; medial division of the central amygdala; HFS, high-frequency stimulation. *p < 0.05. N indicates the number of animals and n indicates the number of neurons.

extinguish the learned fear, whereas B6 mice successfully extinguished the CS-evoked fear during fear extinction as two-way ANOVA analysis revealed a significant strain-by-trial interaction in freezing time ($F_{(11, 240)}$ = 13.22, p < 0.001).

Brain slices containing the amygdala were acquired at three different time points (before conditioning, 24 h after conditioning, and 24 h after extinction). After fear conditioning or extinction mice stayed in their home cages for 24 h for memory consolidation to occur. Then we recorded eEPSCs in the CEm neurons by stimulating the BLA neurons with a bipolar stimulating electrode (Fig. 1B).

3.1. Fear conditioning facilitates LTP and successful fear extinction prevents LTP at the BLA to CEm synapses

For LTP induction at the BLA to CEm synapses, a well-established LTP protocol employing HFS (100 pulses at 100 Hz) was used. In the amygdala brain slices that were obtained before any behavioral manipulation (naïve group), a single HFS was not sufficient to induce LTP at the BLA to CEm synapses of both B6 and S1 mice (p = 0.138 in B6 mice; p = 0.391 in S1 mice; between B6 and S1 mice, p = 0.912; Fig. 2A and B). However, following fear conditioning, the same HFS was sufficient to induce LTP at the BLA to CEm synapses (p = 0.031 in B6 mice; p = 0.023 in S1 mice; Fig. 2C), with no difference between the strains (p = 0.528; Fig. 2D).
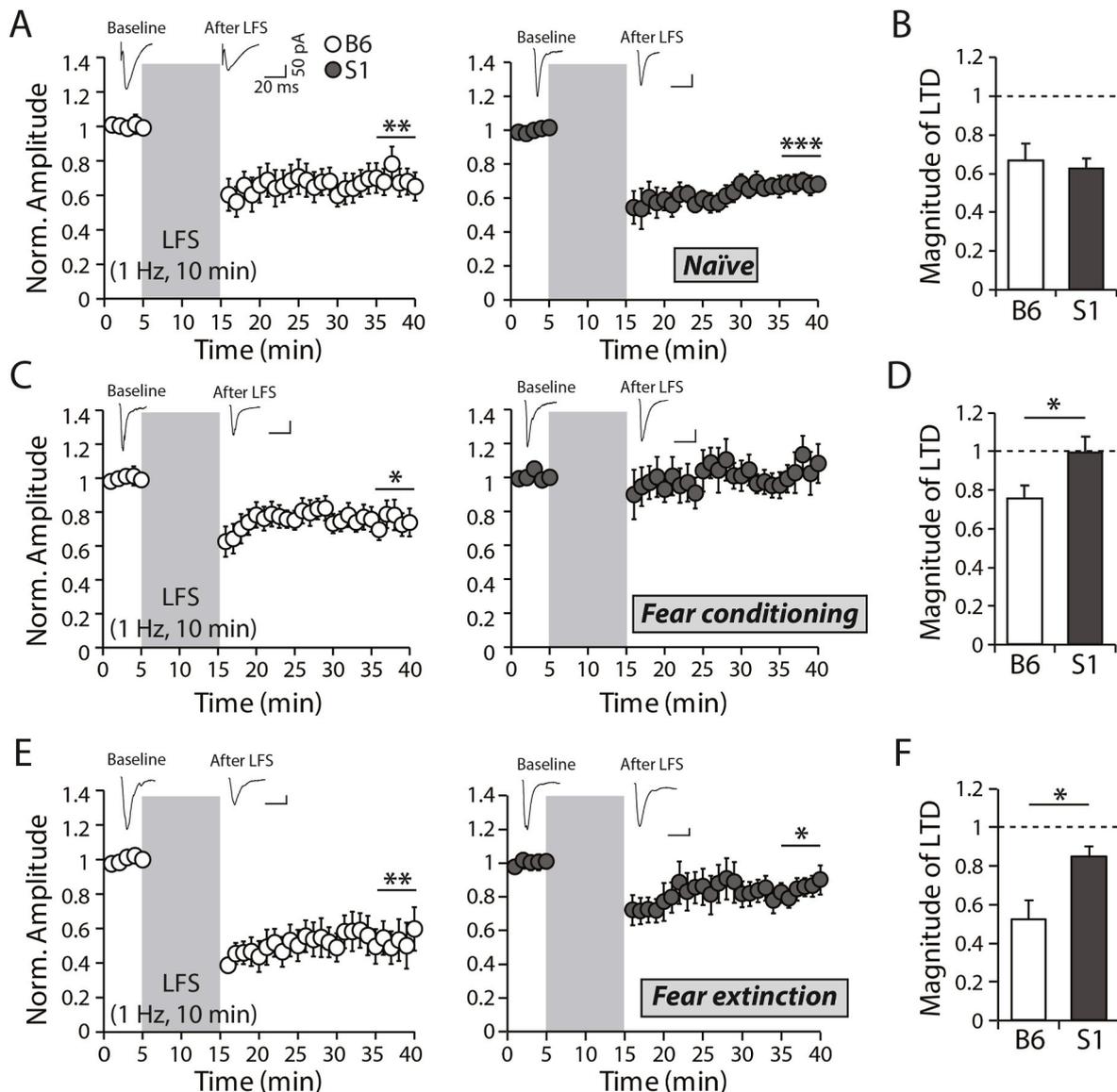
Fig. 3. Fear conditioning impairs LFS-LTD and fear extinction induces weak LFS-LTD in S1 mice at the BLA to CEm synapses. LTD was successfully induced by LFS in both B6 (N = 7, n = 10; p = 0.009) and S1 mice (N = 6, n = 11; p < 0.001) at the BLA to CEm synapses (magnitude of LTD relative to baseline: 66.8 ± 8.7 % in B6 mice; 62.6 ± 5.0 % in S1 mice). (B) The magnitudes of LTD were not different between B6 and S1 mice (p = 0.967). (C) Fear conditioning impaired the induction of LFS-LTD at the BLA to CEm synapses in S1 mice (N = 6, n = 11; p = 0.846), while B6 mice (N = 6, n = 11; p = 0.020) showed consistent LTD (magnitude of LTD relative to baseline: 75.5 ± 6.8 % in B6 mice; 99.6 ± 8.3 % in S1 mice). (D) The magnitude of LTD in S1 mice was significantly smaller than that in B6 mice (p = 0.036). (E) After fear extinction, LFS-LTD was restored in S1 mice (N = 7, n = 10; p = 0.016), while B6 mice (N = 5, n = 9; p = 0.008) consistently showed LTD induction at the BLA to CEm synapses (magnitude of LTD relative to baseline: 52.5 ± 9.7 % in B6 mice; 84.7 ± 5.6 % in S1 mice). (F) However, the magnitude of LTD in S1 mice was significantly smaller than that in B6 mice (p = 0.049). Calibration: 20 ms, 50 pA. LFS, low-frequency stimulation; LTD, long-term depression; BLA, basolateral amygdala; CEm, medial division of the central amygdala; *p < 0.05, **p < 0.01, ***p < 0.001. N indicates the number of animals and n indicates the number of neurons.

Intriguingly, after fear extinction, HFS did not induce any significant LTP in B6 mice as observed in the naïve group, whereas S1 mice consistently showed LTP at the BLA to CEm synapses after HFS (p = 0.470 in B6 mice; p = 0.045 in S1 mice; Fig. 2E). However, the magnitudes of LTP after fear extinction were not significantly different between the two strains (p = 0.202; Fig. 2F). One-way ANOVA confirmed that the magnitude of LTP was not significantly different among the 3 groups (naïve, fear conditioning, and fear extinction) in each strain (F $_{(2, 29)}$ = 1.332, p = 0.280 in B6 mice; F $_{(2, 33)}$ = 1.393, p = 0.263 in S1 mice). Considering these results, fear conditioning lowered the threshold for LTP induction at the BLA to CEm synapses, wherein LTP was induced by a single HFS in both B6 and S1 mice. Although the LTP of the BLA to CEm synapses disappeared after successful fear extinction in B6 mice, the LTP persisted in S1 mice owing to impaired extinction. These results suggest that fear conditioning enhances the synaptic efficacy of the BLA to CEm synapses, and successful extinction in B6 mice diminishes the synaptic strength between the BLA and CEm. In contrast, in S1 mice displaying disrupted extinction, HFS-induced LTP remained intact at the BLA to CEm synapses after extinction.

3.2. Fear conditioning impairs LFS-induced LTD selectively in S1 mice at the BLA to CEm synapses and these impairments persist even after extinction

It has been widely established that the capability for bidirectional plasticity, both LTP and LTD matters to completely understand the synaptic plasticity in a given circuit (Bear, 2003), thus we investigated the LTD induction before and after fear conditioning and extinction. The CEm activity during and after LFS (600 pulses at 1 Hz) showed successful LFS-induced LTD at the BLA to CEm synapses of naïve B6 and S1 mice (p = 0.009 in B6 mice; p < 0.001 in S1 mice; between B6 and S1 mice, p = 0.967; Fig. 3A and B).

However, after fear conditioning, LFS failed to induce LTD selectively in S1 mice, whereas B6 mice displayed constant LTD at the BLA to CEm synapses (p = 0.020 in B6 mice; p = 0.846 in S1 mice; Fig. 3C). S1 mice showed a significantly smaller magnitude of LFS-induced LTD than B6 mice (p = 0.036; Fig. 3D).

After fear extinction, LTD was restored in S1 mice, whereas B6 mice exhibited constant LTD at the BLA to CEm synapses (p = 0.008 in B6 mice; p = 0.016 in S1 mice; Fig. 3E). However, the magnitude of LTD in S1 mice remained significantly smaller than that in B6 mice (p = 0.049; Fig. 3F). One-way ANOVA showed that the magnitude of LTD does not significantly differ among the 3 groups (naïve, fear conditioning, and fear extinction) in B6 mice (F $_{(2, 27)}$ = 1.904, p = 0.168). The magnitude of LTD was significantly different among the 3 groups in S1 mice (F $_{(2, 29)}$ = 8.300, p = 0.001) and ensuing post-hoc test revealed that the magnitude of LTD in the fear conditioning group was significantly smaller than that of the naïve group in S1 mice (p = 0.001, Tukey's multiple comparisons test). These results demonstrate that LFS induced LTD at the BLA to CEm synapses in both naïve B6 and S1 mice. Fear conditioning impaired the induction of LFS-induced LTD selectively in S1 mice and extinction restored LTD at the BLA to CEm synapses in S1 mice regardless of its success; however, the magnitude of LTD in S1 mice was significantly smaller than that in B6 mice.

3.3. Fear conditioning and extinction modulate mLFS-induced LTD at the BLA to CEm synapses in B6 and S1 mice, distinctively

A previous study showed that two distinct types of LTD occur at the BLA to central amygdala (CeA) synapses. LFS (1 Hz, 10 min) induces NMDA receptor (NMDAR)-dependent LTD, while modest LFS (mLFS; 12 Hz, 10 s) induces cannabinoid receptor 1 (CB1)-dependent LTD (Li et al., 2018). Since we had observed impairment in LFS-induced LTD (Fig. 3), we examined whether fear conditioning and extinction alter mLFS-induced LTD as well and whether the alterations differ in S1 mice. The mLFS successfully induced LTD at the BLA to CEm synapses in both

strains of naïve mice (p = 0.004 in B6 mice; p = 0.015 in S1 mice; between B6 and S1 mice, p = 0.398; Fig. 4A and B). Additionally, we found that the paired-pulse ratio (PPR) was increased after mLFS, indicating that mLFS-induced LTD occurs via presynaptic alterations (p = 0.026 in B6 mice; p = 0.033 in S1 mice; between B6 and S1 mice, p = 0.372; Fig. 4A). Our results were consistent with a previous study which reported that mLFS induces LTD at the BLA to CEm synapses in a presynaptic manner via CB1 receptors (Li et al., 2018). In the case of mLFS-induced LTD after fear conditioning, mLFS-induced LTD was impaired in S1 mice, whereas it was intact in B6 mice (p = 0.010 in B6 mice; p = 0.237 in S1 mice; between B6 and S1 mice, p = 0.092; Fig. 4C and D). After fear extinction, both mouse strains showed no synaptic depression in response to mLFS (p = 0.615 in B6 mice; p = 0.170 in S1 mice; between B6 and S1 mice, p = 0.448; Fig. 4E and F), suggesting that mLFS-induced LTD may mediate fear extinction in B6 mice. These results demonstrate that fear conditioning leads to impairment of LFS- and mLFS-induced LTD selectively in S1 mice.

4. Discussion

In this study, we investigated the effect of fear conditioning and extinction on the synaptic plasticity of the BLA to CEm synapses in juvenile mice. The BLA and CEm are the major input and output of the amygdala, respectively (LeDoux et al., 1988; Ehrlich et al., 2009; Moscarello and Penzo, 2022). We found that B6 mice displayed typical fear extinction whereas S1 mice exhibited impaired extinction at those synapses after strong fear conditioning. In naïve B6 and S1 mice, HFS did not induce any LTP, whereas LFS and mLFS induced LTD at the BLA to CEm synapses. Interestingly, fear conditioning decreased the threshold for HFS-induced LTP in both B6 and S1 mice; however, LFS- and mLFS-induced LTD were selectively disrupted at the BLA to CEm synapses of S1 mice. After fear extinction, HFS induced LTP in S1 mice but not in B6 mice. Furthermore, in S1 mice, the LFS-induced LTD that was disrupted by fear conditioning was restored after fear extinction; however, the magnitude of LTD in S1 mice was significantly smaller than that in B6 mice (Fig. 5). Additionally, impaired mLFS-induced LTD was observed after fear extinction in both mouse strains. These observations suggest that 1) both LTP and LTD can be induced at the BLA to CEm synapses in the amygdala, and 2) stronger fear conditioning and impaired fear extinction in S1 mice are accompanied by altered synaptic plasticity in these synapses, with a tendency towards increased potentiation.

LTP and LTD in the amygdala have been studied to understand the cellular mechanisms underlying fear conditioning and extinction. Considering that the LA of the BLA receives sensory inputs from the thalamus and cortex during fear conditioning, studies of synaptic plasticity in the amygdala have mainly been focused on the LTP between sensory inputs and the LA (McDonald, 1998; LeDoux, 2000).

The BLA receives information about the CSs and USs and relays the information to the CeA, which can be further divided into the CEm and the CEl. The CEm is the major output of the amygdala and sends inputs to the hypothalamus, PAG, and BNST that modulate defensive responses, such as freezing (LeDoux et al., 1988; Pape and Pare, 2010; Tovote et al., 2015). Lesions in either the BLA or CeA have been known to disrupt fear conditioning, suggesting both the BLA and CeA are required for fear conditioning (Hitchcock and Davis, 1986; Campeau and Davis, 1995; Cousens and Otto, 1998; Maren, 1999). However, only a handful of studies examined the synaptic plasticity in the BLA-CEm circuit where we investigated in the present study.

Genetic deletion of MMP-9 or administration of an MMP-9 inhibitor destabilizes LTP at the BLA to CEm synapses, which were investigated in the present study (Gorkiewicz et al., 2015). Additionally, the deletion of ASIC1a in all inhibitory neurons in mice has been reported to impair fear conditioning and disrupt LTP at the BLA to CEm synapses, suggesting that fear conditioning requires LTP induction in the BLA to CEm circuitry. Unlike our present study which used a single HFS, they induced
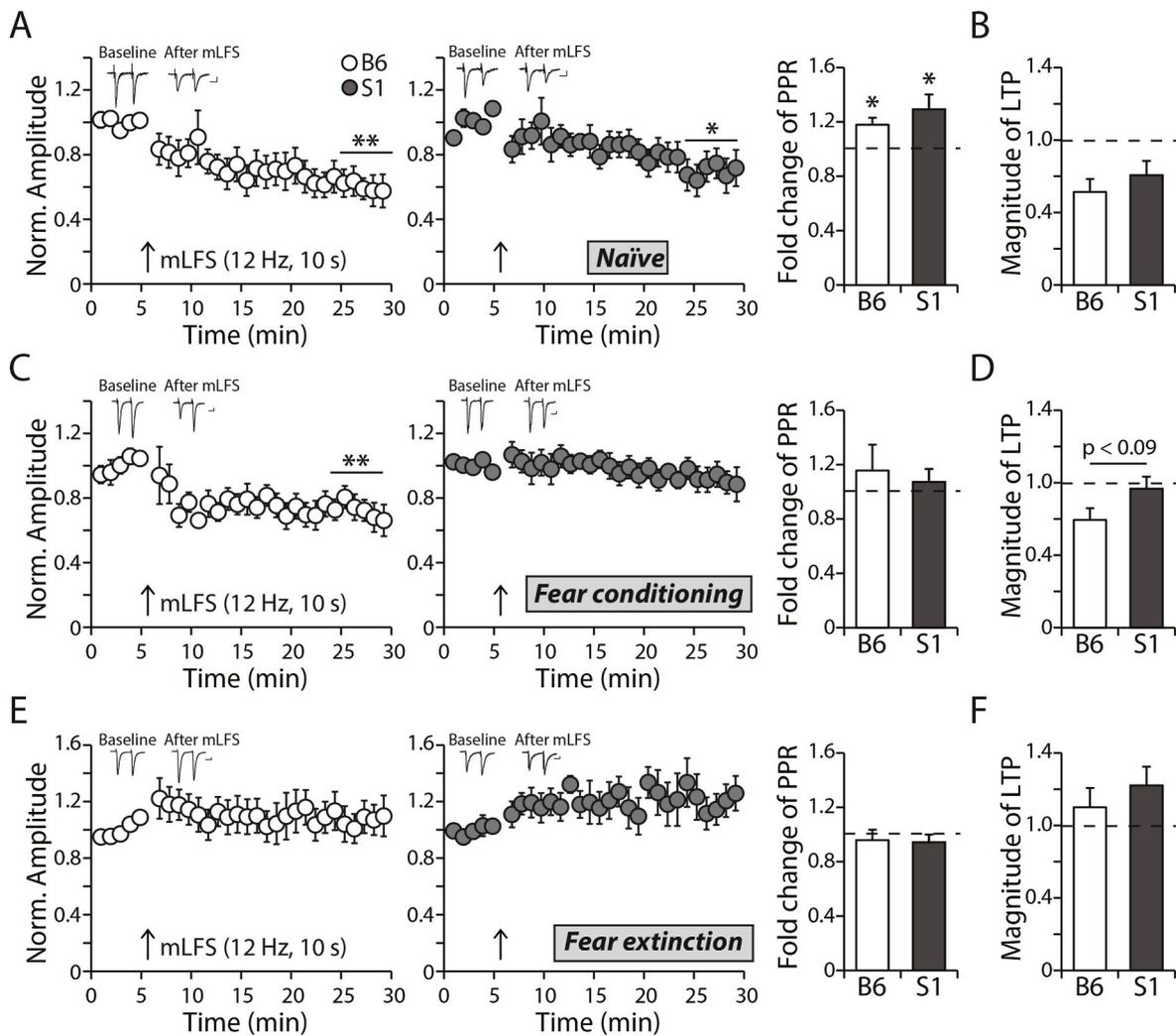
Fig. 4. Fear conditioning impairs mLFS-LTD in S1 mice and fear extinction impairs mLFS-LTD in both S1 and B6 mice at the BLA to CEm synapses. (A) mLFS successfully induced LTD in both B6 (N = 3, n = 6; p = 0.004) and S1 mice (N = 3, n = 7; p = 0.019) at the BLA to CEm synapses (magnitude of LTD relative to baseline: 60.0 ± 8.0 % in B6 mice; 70.8 ± 9.2 % in S1 mice). The paired-pulse ratio was also increased by mLFS (fold change of PPR relative to baseline: 118.1 ± 5.8 %, p = 0.026 in B6 mice; 130.1 ± 10.9 %, p = 0.033 in S1 mice). (B) The magnitudes of LTD were not different between B6 and S1 mice (p = 0.398). (C) Fear conditioning impaired the induction of mLFS-LTD at the BLA to CEm synapses in S1 mice (N = 3, n = 6; p = 0.237), whereas B6 mice (N = 4, n = 6; p = 0.010) showed consistent LTD (magnitude of LTD relative to baseline: 69.5 ± 7.5 % in B6 mice; 89.6 ± 7.8 % in S1 mice). (D) The magnitude of LTD showed a trend of significant difference between S1 and B6 mice (p = 0.092). (E) After fear extinction, mLFS-LTD was impaired in both mouse strains (N = 3, n = 7, p = 0.615 in B6 mice; N = 2, n = 6, p = 0.170 in S1 mice) at the BLA to CEm synapses (magnitude of LTD relative to baseline: 106.1 ± 11.6 % in B6 mice; 119.3 ± 12.1 % in S1 mice). (F) The magnitude of LTD was comparable between B6 and S1 mice (p = 0.448). Calibration: 20 ms, 50 pA. mLFS, modest low-frequency stimulation; LTD, long-term depression; BLA, basolateral amygdala; CEm, medial division of the central amygdala. *p < 0.05, **p < 0.01, ***p < 0.001.

LTP by pairing four trains of HFS (HFS x 4) and LTP was induced well even at the BLA-CEm circuit of wild-type mice (Chiang et al., 2015). Here, we discovered that fear conditioning enhances LTP induction induced by a single HFS at the BLA to CEm synapses, and this potentiation remains intact when extinction fails but not when it succeeds. These observations suggest that the CS-US association after fear conditioning facilitates the potentiation of the BLA to CEm synapses by lowering the threshold for potentiation, whereas extinction reverses this threshold back to the baseline to allow masking of the HFS-induced LTP in those synapses.

In the case of LTD, we investigated the two distinct types of LTD at the BLA to CeA synapses. We found that LFS (1 Hz, 10 min) and mLFS (12 Hz, 10 s) induce NMDAR-dependent and CB1-dependent LTD, respectively. Forced swim stress has been previously shown to selectively impair CB1-dependent LTD (Li et al., 2018). This suggests that NMDAR-dependent LTD at the BLA to CeA synapses might be involved in emotional learning, whereas CB1-dependent LTD could be related to

stress adaptation. In the present study, we found that fear conditioning and extinction did not affect LFS-LTD in B6 mice, suggesting that NMDAR-dependent LTD at the BLA to CEm synapses remains intact. However, strong fear conditioning disrupts LFS-LTD and impaired fear extinction induces weak LTD at the BLA to CEm synapses in S1 mice, suggesting that emotional learning and fear extinction require NMDAR-dependent LTD at the BLA to CEm synapses. CB1-dependent mLFS-LTD was also selectively impaired after fear conditioning in S1 mice. Interestingly, we observed that mLFS failed to induce LTD after fear extinction in both mouse strains. Fear conditioning and extinction have been known to increase plasma concentration of the stress hormone, corticosterone, wherein plasma corticosterone concentration has shown a positive correlation with fear responses (Cordero et al., 1998; Kelley et al., 2009; Ter Horst et al., 2012; Zeitlin et al., 2012; Tomizawa et al., 2013). The selective impairment of mLFS-induced LTD after fear conditioning in S1 mice may be caused by the different degrees of stress during fear conditioning. The relatively long freezing time of S1 mice

Bidirectional synaptic plasticity
at the BLA to CEm synapses
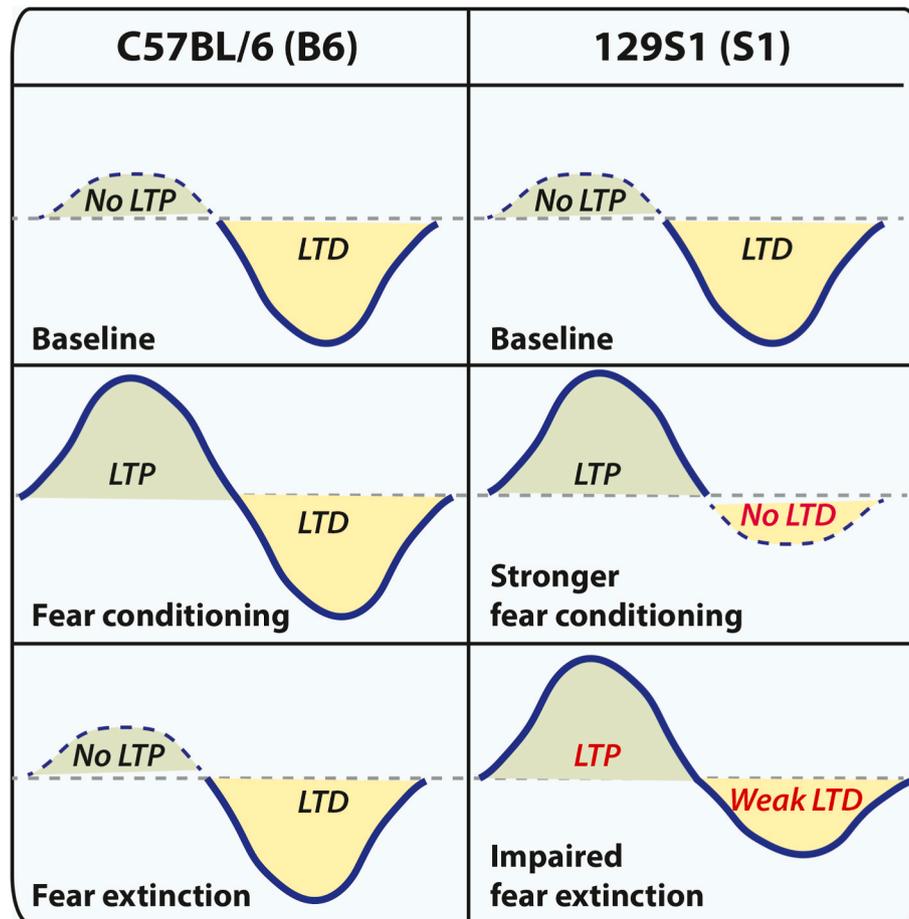
Fig. 5. Synaptic plasticity at the BLA to CEm synapses following fear conditioning and extinction in B6 and S1 mice. In naïve B6 and S1 mice, HFS failed to induce LTP while LFS induced LTD in the BLA to CEm circuit. Fear conditioning lowered the threshold for HFS-LTP in both mouse strains, and LTD was impaired selectively in S1 mice showing strong fear conditioning. After fear extinction, LTP was again disrupted selectively in B6 mice having normal fear extinction, whereas significantly weak LTD was restored in S1 mice with impaired fear extinction. BLA, basolateral amygdala; CEm, medial division of the central amygdala; HFS, high-frequency stimulation; LTP, long-term potentiation; LFS; low-frequency stimulation; LTD, long-term depression.

during fear conditioning also implies that fear conditioning *per se* is more stressful in S1 mice than in B6 mice. After fear extinction, mLFS-induced LTD was disrupted in both B6 and S1 mice. The impairment of mLFS-induced LTD in B6 mice could be caused by repetitive and/or longer duration of stress exposure because fear extinction is also a stressful event. Another candidate mechanism is the endocannabinoid signaling pathway, which has been proposed as a key regulator of mLFS-induced LTD. This pathway may mediate the extinction of fear memory since B6 mice-based CB1 knockout mice showed impaired fear extinction but no impairment of fear memory formation (Marsicano et al., 2002; Kamprath et al., 2006; Li et al., 2018). Therefore, the impaired mLFS-LTD after fear extinction in B6 mice may be caused by the occlusion of CB1 and its related signaling pathway by fear extinction. Our data confirmed that LFS- and mLFS-induced LTD are two different forms of LTD and emotional learning, which may be related to NMDAR-dependent LFS-LTD.

Our present research suggests that prolonged fear of the CS in S1 mice may be related to the abnormally increased ability to induce LTP and decreased ability to induce LTD at the BLA to CEm synapses after fear extinction. Future studies need to examine the underlying mechanisms and molecular mediators that are involved in the alterations in

synaptic plasticity at the BLA to CEm synapses in S1 mice. Furthermore, as our study focused on synaptic plasticity 24 h after fear conditioning and extinction, researchers should also explore the recruitment of the BLA to CEm synapses and the differences between B6 and S1 mice during fear conditioning and extinction by using *in vivo* electrophysiology or calcium imaging to assess real-time activity of the synapses.

Our present research is limited to male juvenile mice in terms of age and sex. We investigated how fear conditioning and extinction alter synaptic plasticity at the BLA to CEm synapses of 4–6 week, male B6 and S1 mice. It has been reported that both juvenile humans (12–17 years of age) and juvenile mice (P28-55) show reduced fear extinction (Pattwell et al., 2012; Ishii et al., 2019; Bisby et al., 2021). In addition, adult S1 mice (3–6 months) also exhibit impaired fear extinction (Temme et al., 2014), raising a possibility that the current observations are shared with adult mice. Nevertheless it would be interesting to investigate how the levels of fear conditioning, extinction, and synaptic plasticity in the amygdala change as developmental transition happens. Moreover, it has been known that PTSD is more prevalent in women than in men (Velasco et al., 2019; Fonkoue et al., 2020). Female B6 mice show more freezing to the CS after auditory fear conditioning and slower fear extinction learning compared to male B6 mice (Chen et al., 2014; Clark et al.,

2019). Female B6 mice also exhibit greater LTP induction in the LA of the amygdala (Chen et al., 2014). It was reported that female S1 mice also show disrupted fear extinction, however, male and female S1 mice have not been compared directly (Cazares et al., 2019). Future studies investigating synaptic plasticity at the BLA to CEm circuit of female mice may shed light on the sex differences in fear memory formation and erasure.

In conclusion, this study examined the effect of fear conditioning and extinction on synaptic plasticity at the BLA to CEm synapses of B6 and S1 mice and its alteration in S1 mice. We demonstrated that fear conditioning facilitates the induction of LTP, whereas fear extinction decreases the conditioning-enhanced ability to induce LTP in the BLA to CEm circuitry. In the case of S1 mice exhibiting relatively stronger fear conditioning and impaired fear extinction, intact LTP induction and inhibited LFS-LTD induction were observed after conditioning at the BLA to CEm synapses, suggesting that the circuit is altered to get more excitable than B6 mice. This enhanced ability to excite synaptic activity persists even after fear extinction as intact LTP induction and reduced LTD induction were observed at the BLA to CEm synapses of S1 mice. Our observations suggest that the synaptic plasticity between the BLA and the CEm is quite plastic depending on the experience and synaptic alterations that are accompanied by behavioral changes. Our circuit-level findings in the S1 mice may contribute to understanding the mechanisms underlying exaggerated fear conditioning and impaired fear extinction found in patients with PTSD and help in finding effective therapeutics for PTSD.

CRediT authorship contribution statement

Kwanghoon Park: Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Data curation. **Hoyong Park:** Writing – review & editing, Validation, Investigation, Formal analysis, Data curation. **ChiHye Chung:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Project administration, Investigation, Funding acquisition, Formal analysis, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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