



# Differential Alterations in Cortico-Amygdala Circuitry in Mice with Impaired Fear Extinction

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## Abstract

129S1/SvImJ (S1) mice exhibit selective impairments in fear extinction, though the mechanisms underlying these impairments are not fully understood. The medial prefrontal cortex (mPFC) consists of the prelimbic cortex (PL) and infralimbic cortex (IL), which are known to be involved in fear conditioning and extinction, respectively. The PL and IL project to the basolateral amygdala (BLA) that also plays an important role in both mechanisms. In the present study, we utilized optogenetic and electrophysiological approaches to measure inhibitory/excitatory ratios (I/E ratios) in mPFC-BLA circuits of S1 and control C57BL/6 (B6) mice following fear conditioning and extinction. As suggested previously, PL inputs to the BLA became more excitatory after fear conditioning in B6 mice. S1 mice also exhibited strengthened PL-BLA circuit following fear conditioning. Interestingly, fear extinction restored PL-BLA circuit strength to levels comparable to the baseline in B6 mice. However, PL-BLA circuit strength remained abnormally high even after extinction in S1 mice. The IL-BLA circuit became more inhibitory in B6 mice after fear extinction, whereas extinction failed to change the excitability of the IL-BLA circuit in S1 mice. These data suggest that the fear extinction impairments observed in S1 mice may be due to constantly decreased I/E balance in the PL-BLA circuit and lack of changes in I/E balance in the IL-BLA circuit. This further suggests that investigation of both pathways is instrumental in developing more effective therapeutics for psychopathologies that involve impairments in fear extinction, such as chronic pain and posttraumatic stress disorder.

**Keywords** Fear conditioning · Fear extinction · 129S1/SvImJ mice · Prelimbic cortex · Infralimbic cortex · Basolateral amygdala

## Introduction

Auditory fear conditioning is frequently used to assess the formation and expression of fear memories. For rodents, the process of auditory fear conditioning involves pairing a neutral tone (conditioned stimulus, CS) with an aversive stimulus such as an electrical foot shock (unconditioned stimulus, US) by presenting subjects with the CS and US simultaneously or with a short delay. After auditory fear conditioning, subjects exhibit conditioned fear responses such as freezing upon exposure to a CS that is a formerly neutral tone [1, 2]. Association of the CS with the US could be interrupted by fear extinction, which involves repeated exposure to the same CS but this time without a US pairing. After fear extinction, subjects show diminished fear responses to the CS [2, 3].

Failure to extinguish learned fear is one of the major hallmarks of anxiety disorders such as posttraumatic stress disorder (PTSD) [4, 5]. Animal models have been used to study the mechanisms underlying these impairments in fear extinction or the persistence of fear memories. We and others have reported that 129S1/SvImJ (S1) mice exhibit impaired fear extinction following auditory or contextual fear conditioning [6–9]. Although dietary zinc restriction or systemic treatment with an  $\alpha$ 2-adrenoreceptor antagonist restores fear extinction in S1 mice, the etiology of their extinction impairments and associated brain circuit disruptions remain poorly understood [7, 10].

To investigate the mechanisms underlying impaired extinction in S1 mice, we previously studied and reported the abnormal expression of an immediate early gene, *c-Fos*, throughout the brains of S1 mice before and after contextual fear conditioning and extinction. We found that S1 mice had elevated levels of *c-Fos*-positive cells in the prelimbic cortex (PL) of the medial prefrontal cortex (mPFC) compared with control C57BL/6 (B6) mice following contextual fear conditioning and extinction [9]. Moreover, it has been reported that fear extinction retrieval induces less *c-Fos*-positive cells in the

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infralimbic cortex (IL) of the mPFC and more Zif268 (another immediate early gene) positive cells in the PL in S1 mice compared with control mice [7, 10].

The mPFC and basolateral amygdala (BLA) have been thoroughly studied for their roles in both fear memory expression and inhibition [11–13]. The mPFC consists of the PL and IL that are involved in fear conditioning and extinction, respectively [14–17]. It has also been reported that inactivating the PL during fear conditioning does not inhibit fear learning, whereas inactivation of the PL during fear recall disrupts fear memory expression. This suggests that the PL is necessary for fear expression but not fear learning [11, 18]. Furthermore, lesioning or otherwise inactivating the IL does not interrupt extinction learning but does prevent extinction memory retrieval [13, 15, 18]. Thus, the PL and IL of the rodent seem to be involved in fear memory expression and inhibition, respectively. Additionally, studies have proposed that reduced PL activity is also required for fear extinction [14, 17].

The BLA consists of two classes of neurons: fear neurons and extinction neurons. They are classified depending on CS-evoked responses they make after fear conditioning and extinction. Following auditory fear conditioning to a neutral tone, fear neurons in the BLA increase their firing rates to the tone (CS), and extinction neurons show this response upon fear extinction [12, 19]. Inactivation of the BLA disrupts both fear and extinction memory retrieval, indicating that the BLA modulates both fear expression and inhibition [18, 20, 21]. Neuroanatomical data suggest similar interactions between the BLA and neurocircuitry of fear extinction. For instance, both the PL and IL project to the BLA and these circuits have further been proposed to mediate fear conditioning and extinction, respectively [22–26].

Arruda-Carvalho and Clem showed that fear conditioning strengthened the PL-BLA circuit but did not affect the IL-BLA circuit in B6 mice, a mouse strain that exhibits good fear extinction [22]. It was also reported that fear extinction weakened mPFC-BLA circuits in B6 mice when IL of the mPFC was targeted [24]. However, changes in PL-BLA circuit dynamics upon fear extinction were not investigated in B6 mice. Moreover, whether PL-BLA and/or IL-BLA circuits are altered in fear extinction-impaired animal models has not yet been studied.

In the present study, we explored the strengths of PL-BLA and IL-BLA circuits in fear extinction-impaired S1 and control B6 mice, both before and after fear conditioning and extinction, using optogenetic and electrophysiological techniques. We also examined whether paired-pulse ratio (PPR) and AMPAR/NMDAR (A/N) ratios in PL-BLA and IL-BLA circuits in S1 and B6 mice were altered following fear conditioning and extinction. Our data suggest that both PL-BLA and IL-BLA circuits are altered after impaired fear extinction. The study results provide a neurocircuitry framework for further

investigation of the mechanisms underlying impaired fear extinction in related disorders such as chronic pain and PTSD.

## Materials and Methods

### Subjects

129S1/SvImJ (S1) mice were obtained from the Jackson Laboratory (Bar Harbor, USA) and bred under a sibling-by-sibling mating system as suggested by the Jackson Laboratory. C57BL/6N (B6) mice were acquired from Orient Bio (a branch of Charles River, Gapyeong, Korea). All mice were group-housed (4 per cage) before surgery and singly housed thereafter under standard laboratory conditions in a temperature- ( $23 \pm 1$  °C) and humidity- ( $50 \pm 10\%$ ) controlled vivarium on a 12/12-h light/dark cycle (lights on at 7 a.m.). Food and water were available freely. All experiments were conducted 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 related to animals were performed in accordance with protocols approved by the Institutional Animal Care and Use Committee of Konkuk University, Seoul, Korea.

### Surgery

Three- to four-week-old male S1 and B6 mice were anesthetized with isoflurane throughout the stereotaxic surgery for viral injection to express channelrhodopsin-2 (ChR2) in the PL or IL. To relieve pain and reduce inflammation, Metacam (meloxicam 5 mg/ml, Boehringer Ingelheim) was diluted with saline and injected subcutaneously (2 mg/kg) before surgery. We also applied Optixcare Eye Lube Plus (CLC Medica) to the eyes during surgery to prevent the eyes of subjects from drying. Vector AAV2-CamKII $\alpha$ -ChR2(H134R)-eYFP (0.5  $\mu$ l each, purchased from the Penn Vector Core at the University of Pennsylvania) was injected bilaterally into the PL (in mm: AP +1.9, ML  $\pm$ 0.3, DV  $-$ 2) or IL (in mm: AP +1.7, ML  $\pm$ 0.3, DV  $-$ 2.6) through a glass capillary at a rate of 0.1  $\mu$ l/min. At least 10 min elapsed after completion of the injection before the glass capillary was retracted. After the surgery, mice were placed on a warm plate for approximately 1 h for recovery and then returned to their home cages.

### Auditory Fear Conditioning and Extinction Training

Five weeks after the surgery, mice underwent auditory fear conditioning and extinction training. Following a 5-min acclimation period, mice were exposed to three simultaneous pairings of a tone (conditioned stimulus, CS, 75 dB, 10,000 Hz, 30 s) and a foot shock (unconditioned stimulus, US, 0.6 mA, 2 s) with 20–40-s inter-stimulus intervals (ISIs)

in the hexahedral conditioning context with a width of 18 cm, a depth of 18 cm, and a height of 30 cm (H10-11M-TC, Coulbourn Instruments). Mice remained in the same context for an additional 30 s before being returned to their home cages. Twenty-four hours later, mice were placed in the extinction context (acrylic hexagonal prism with an apothem of 11 cm and a height of 29 cm). After a 2-min acclimation period, a shock-free tone (CS, 75 dB, 10,000 Hz, 30 s) was presented 30 times with 30-s ISIs. After the final tone, mice were placed in the extinction context for an additional 30 s and returned to their home cages. This extinction protocol was repeated for two consecutive days. Freezing responses were observed based on whether mice moved or not (except for respiration) and were scored every 2 s manually.

### Slice Preparation and Electrophysiology

Mice were anesthetized with isoflurane, and coronal brain slices containing the mPFC or amygdala (350  $\mu\text{m}$  thick) were prepared with a vibratome (Leica VT1000 S) and an ice-cold sucrose dissection buffer (in mM: 212 sucrose, 3 KCl, 26  $\text{NaHCO}_3$ , 1.25  $\text{NaH}_2\text{PO}_4$ , 7  $\text{MgCl}_2$ , 10 glucose) bubbled with 95%  $\text{O}_2$ /5%  $\text{CO}_2$  gas. The slices were stored in a chamber filled with artificial cerebrospinal fluid (aCSF; in mM: 1  $\text{NaH}_2\text{PO}_4$ , 26.2  $\text{NaHCO}_3$ , 118 NaCl, 2.5 KCl, 11 glucose, 2  $\text{CaCl}_2$ , 1  $\text{MgCl}_2$ ) bubbled with 95%  $\text{O}_2$ /5%  $\text{CO}_2$  gas at 35 °C for at least 45 min for recovery, and then kept at room temperature. ChR2-eYFP-expressing cell bodies in the mPFC and fibers in the BLA were observed with a fixed-stage upright microscope (Olympus, BX61WI) and WIB green long-pass fluorescence cube which was connected to a power supply (U-RFL-T). Electrophysiological data were acquired from principal neurons in the BLA using Axopatch 200B or MultiClamp 700B (Molecular Devices), filtered at 2 kHz and sampled at 10 kHz, with WinLTP 2.10 (The University of Bristol) or pCLAMP 10 (Molecular Devices). Whole-cell voltage clamp recordings were made with 3–6-M $\Omega$  glass electrodes filled with internal solution (in mM: 115 Cs methanesulfonate, 20 CsCl, 10 HEPES, 2.5  $\text{MgCl}_2$ , 0.6 EGTA, 5 QX-314, 4  $\text{Na}_2\text{-ATP}$ , 0.4  $\text{Na}_2\text{-GTP}$ , and 10 Na-phosphocreatine; pH 7.2–7.4 with CsOH). To generate blue light for optogenetics, we used light-emitting diodes (LEDs) with a 470-nm peak wavelength (M470F3, Thorlabs). The LED driver (LEDD1B, Thorlabs) connected the LEDs to Axopatch 200B or MultiClamp 700B (Molecular Devices). The illumination was made to the BLA-containing slices every 20 s with a 1–2-ms duration through a patch cable (M79L01, Thorlabs). The current of the LEDs was  $\sim 1.2$  A. To acquire an excitatory postsynaptic current/inhibitory postsynaptic current (EPSC/IPSC) biphasic response, we recorded at a holding potential of  $-15$  mV which is between the reversal potentials of EPSCs (0 mV) and IPSCs ( $-42$  mV) in normal aCSF and measured the peak amplitudes of the negative response (EPSC) and positive response

(IPSC). To obtain glutamate-mediated paired-pulse ratio (PPR) and AMPAR/NMDAR (A/N) ratios, we added a GABA<sub>A</sub> receptor antagonist, picrotoxin (PTX; 50  $\mu\text{M}$ , Tocris), to the aCSF. When PTX was used,  $\text{CaCl}_2$  and  $\text{MgCl}_2$  in the aCSF were increased to 4 mM to prevent spontaneous bursting as reported previously [22]. For PPR analysis, 2 EPSCs were evoked by a paired-pulse photostimulation with a 50-ms ISI, then the amplitude of the second response was divided by the amplitude of the first response. To analyze A/N ratios, AMPAR- and NMDAR-mediated currents were recorded. AMPAR- and NMDAR-mediated evoked EPSCs (eEPSCs) were recorded at a holding potential of  $-70$  mV and  $+40$  mV, respectively. The mean peak amplitudes recorded at a holding potential of  $-70$  mV was divided by the mean amplitudes of eEPSCs 50 ms after photostimulation recorded at a holding potential of  $+40$  mV. All recordings were obtained at 30–32 °C, and series and membrane resistances were continuously monitored. Liquid junction potential was not corrected. Recordings with a  $>20\%$  change in the series and membrane resistances were discarded from the analysis.

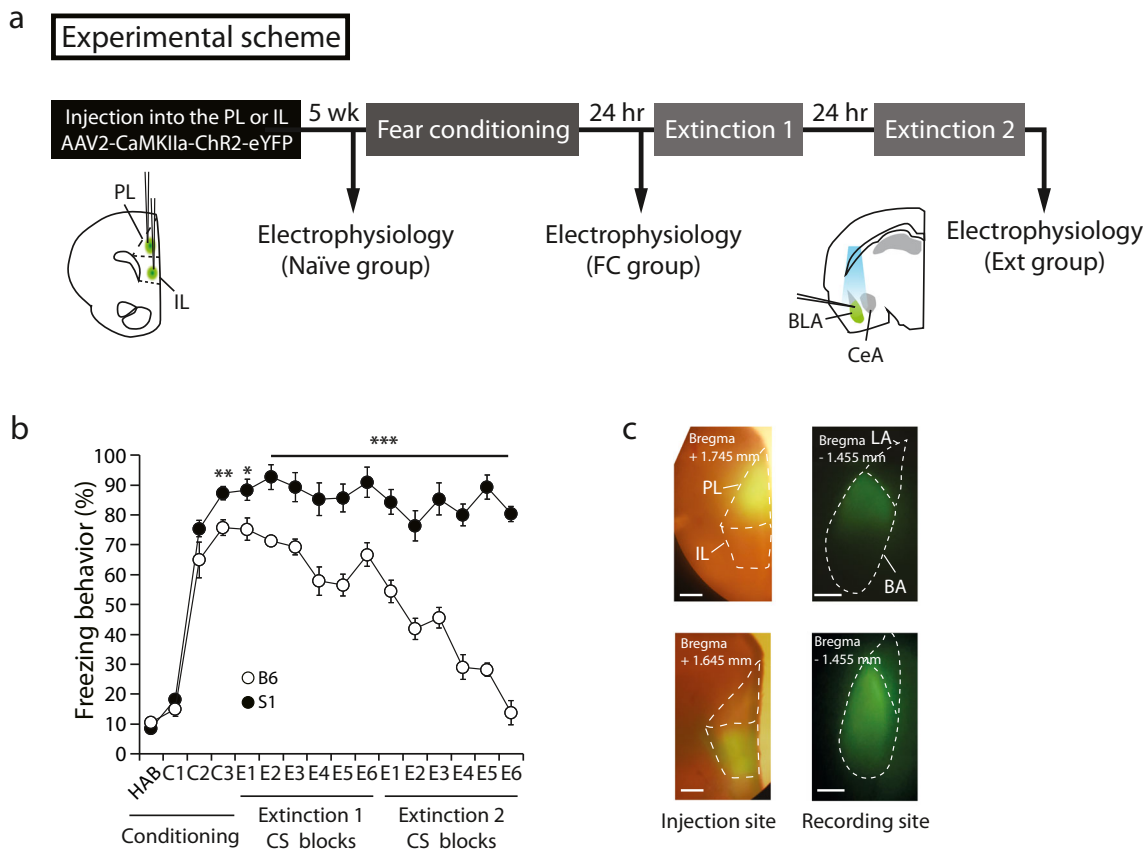
### Statistical Analyses

All data are given as means  $\pm$  SEM. Statistical comparisons were performed with GraphPad Prism 8.0.0 and Microsoft Excel. The fear conditioning and extinction performance of each strain were compared by one-way analysis of variance (ANOVA). Multiple *t*-tests were used to compare the two strains of mice for each behavioral manipulation. Two-way ANOVA was used to analyze strain by condition interactions, and comparisons within each strain of mouse were made by Dunnett's multiple comparisons test after two-way ANOVA. *p* values lower than 0.05 were considered as statistically significant.

## Results

### Specific PL-BLA and IL-BLA Circuit Investigation After Fear Conditioning and Extinction

To measure the strengths of PL-BLA and IL-BLA circuits in a fear extinction-impaired animal model, we measured inhibitory/excitatory ratios (I/E ratios) in these circuits in B6 and S1 mice, both before and after fear conditioning and extinction training (Fig. 1a). To selectively stimulate BLA-projecting PL or IL neuronal terminals, we expressed channelrhodopsin-2 (ChR2) in the PL or IL and recorded blue light-evoked responses in the BLA. As PL and IL axons projecting to the BLA have been known to release glutamate as a neurotransmitter, we aimed to express ChR2 in excitatory neurons in the PL and IL [22, 24, 25, 27]. To do so, we expressed ChR2 in neurons expressing  $\text{Ca}^{2+}$ /calmodulin



**Fig. 1** Experimental scheme of PL-BLA and IL-BLA circuit analyses in B6 and S1 mice. **a** C57BL/6 (B6) and 129S1 (S1) mice were injected with an adeno-associated virus vector expressing channelrhodopsin-2-eYFP into the prelimbic (PL) or infralimbic (IL) cortices and divided into three groups: naïve, fear conditioning (FC), and fear extinction (Ext). Coronal brain slices were prepared 24 h after each behavioral condition for electrophysiological experiments. **b** S1 mice ( $n = 9$ ) exhibited general

auditory fear conditioning and significantly more freezing behavior in the third fear conditioning training than did B6 mice ( $n = 10$ ) ( $p < 0.01$ ). S1 mice ( $n = 4$ ) exhibited impaired fear extinction with consistently higher levels of freezing behavior throughout the extinction training sessions than did B6 mice ( $n = 5$ ). **(c)** ChR2-eYFP was expressed in the PL and IL neurons and BLA-innervating PL and IL terminals. Scale bars: 500  $\mu\text{m}$ . \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$

(CaM)-dependent protein kinase II (CaMKII) since it has been reported that CaMKII is expressed in excitatory neurons specifically [28–30]. Therefore, we injected an adeno-associated virus 2 (AAV2) vector-carrying CaMKII $\alpha$  promoter and ChR2-eYFP, which has been widely used to express ChR2 in mPFC excitatory neurons, into the PL or IL of B6 and S1 mice [15, 22–24, 31]. Five weeks later, animals of each strain were divided into three groups: naïve mice (Naïve), mice 24 h after the fear conditioning (FC), and mice 24 h after the fear extinction (Ext) (Fig. 1a).

As reported previously, S1 mice exhibited impaired fear extinction following intact auditory fear conditioning (Fig. 1b). Both B6 and S1 mice exhibited successful fear conditioning, with a significant effect of conditioning trial on freezing time ( $F(2, 27) = 3.35$ ,  $p < 0.001$  for B6 mice,  $F(2, 24) = 3.40$ ,  $p < 0.001$  for S1 mice). However, S1 mice exhibited impaired fear extinction, while B6 mice exhibited good fear extinction with a significant effect of extinction block on decreased freezing time ( $F(11, 48) = 1.99$ ,  $p < 0.001$  for B6 mice,  $F(11, 36) = 2.07$ ,  $p > 0.3$  for S1 mice).

Amygdala-containing slices were obtained after each behavioral assay, and terminal fibers innervated by the PL or IL were imaged using ChR2-eYFP fluorescence in the BLA. Blue light (470 nm)-evoked responses were measured from principal pyramidal neurons in the BLA of B6 and S1 mice before and after fear conditioning and fear extinction for the experiments below (Fig. 1c).

### The PL-BLA Circuit Was Constantly Strengthened in S1 Mice After Fear Extinction

Decreased I/E ratios in the PL-BLA circuit of B6 mice following fear conditioning were previously reported [22]. We measured I/E ratios in the PL-BLA in order to investigate the changes in the strength of the PL-BLA circuit in B6 mice after successful fear extinction and in S1 mice following normal fear conditioning and impaired fear extinction. We obtained inhibitory postsynaptic currents (IPSCs) and excitatory postsynaptic currents (EPSCs) from the same neuron at the same time by acquiring an EPSC/IPSC biphasic response, as

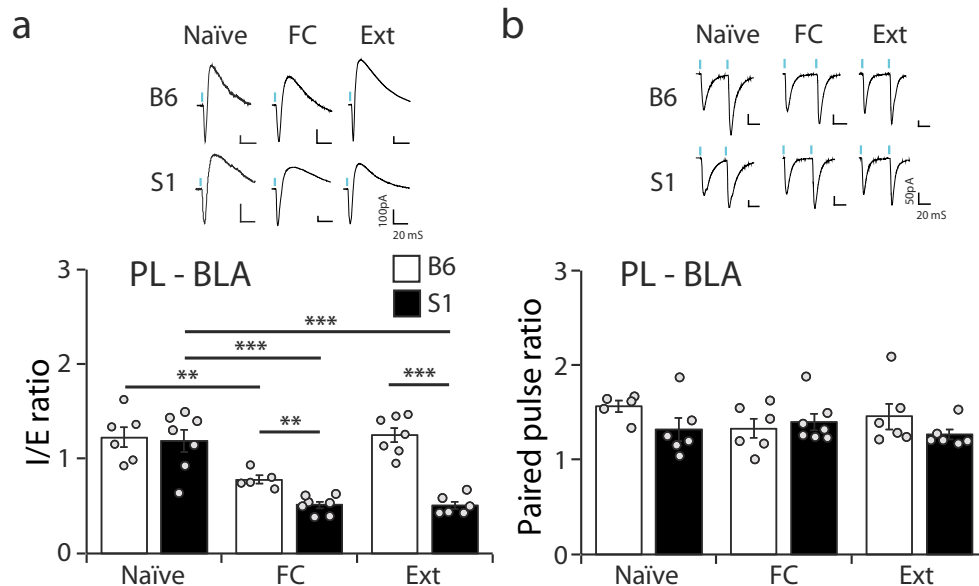
suggested previously, at a holding potential of  $-15$  mV which is between the reversal potentials of EPSCs ( $0$  mV) and IPSCs ( $-42$  mV) [22, 32]. There was a significant strain-by-group interaction in the I/E ratios in the PL-BLA circuit ( $F(2,32) = 10.11, p < 0.001$ ). Naïve B6 and S1 mice had comparable I/E ratios in the PL-BLA circuit (I/E ratios: B6 =  $1.23 \pm 0.11$ ; S1 =  $1.19 \pm 0.12, p > 0.8$ ). Following fear conditioning, I/E ratios in the PL-BLA circuit in both B6 and S1 mice decreased compared with each strain-matched naïve group ( $p < 0.01$ ). S1 mice showed significantly decreased I/E ratios than those of B6 mice (I/E ratios: B6 =  $0.78 \pm 0.04$ ; S1 =  $0.51 \pm 0.04, p < 0.01$ ). After fear extinction, I/E ratios in B6 mice increased back to basal levels and were equivalent to those in B6 naïve mice, while S1 mice exhibited persistently reduced I/E ratios in the PL-BLA ( $p > 0.9$  for B6 mice,  $p < 0.001$  for S1 mice). I/E ratios in S1 mice were also significantly decreased than those in B6 mice (I/E ratios: B6 =  $1.24 \pm 0.08$ ; S1 =  $0.50 \pm 0.04, p < 0.001$ ) (Fig. 2a).

To examine whether the changes in I/E ratios in the PL-BLA circuit were due to presynaptic modifications in the glutamate release probability, we measured paired-pulse ratio (PPR) in the PL-BLA circuit of S1 and B6 mice. No strain-by-group PPR interaction was found in the PL-BLA circuit ( $F(2,30) = 1.50, p > 0.2$ ). Furthermore, there were no PPR differences between the strains or among the three behavioral conditions (Naïve, FC, and Ext) within each strain (PPR (Naïve): B6 =  $1.57 \pm 0.07$ ; S1 =  $1.32 \pm 0.12$ , (FC): B6 =  $1.33$

$\pm 0.10$ ; S1 =  $1.40 \pm 0.09$ , (Ext): B6 =  $1.46 \pm 0.14$ ; S1 =  $1.26 \pm 0.06$ ) (Fig. 2b). Taken together, these results demonstrate that the PL-BLA circuit was strengthened after the fear conditioning in both B6 and S1 mice, and S1 mice persistently exhibited enhanced excitability of the PL-BLA circuit even after the extinction. However, the PL-BLA circuit with elevated excitability exhibited no residual alterations in presynaptic release probability in B6 and S1 mice.

### Fear Extinction Weakened IL Inputs to the BLA in B6 Mice but Not in S1 Mice

To examine the changes in inhibitory/excitatory balance in the IL-BLA circuit upon fear conditioning and extinction in S1 mice, we measured I/E ratios in the IL-BLA circuit as described for the PL-BLA circuit above. There was a significant strain-by-group interaction in I/E ratios in the IL-BLA circuit ( $F(2, 31) = 6.55, p < 0.01$ ). Naïve B6 and S1 mice displayed similar I/E ratios in the IL-BLA circuit (I/E ratios: B6 =  $1.13 \pm 0.18$ ; S1 =  $1.24 \pm 0.10, p > 0.5$ ). Following fear conditioning, I/E ratios in both B6 and S1 mice were comparable to those in the strain-matched naïve group animals ( $p > 0.8$  for B6 mice,  $p > 0.7$  for S1 mice). Furthermore, there were no significant differences in I/E ratios between B6 and S1 mice (I/E ratios: B6 =  $1.23 \pm 0.07$ ; S1 =  $1.12 \pm 0.09, p > 0.5$ ). After fear extinction, however, B6 mice showed increased I/E ratios compared with naïve B6 mice, while S1 mice exhibited comparable I/E



**Fig. 2** S1 mice exhibit a prolonged enhancement of PL inputs to the BLA after fear extinction with no changes in presynaptic release probability. **a** Naïve 129S1 (S1) ( $n = 7$ ) and C57BL/6 (B6) mice ( $n = 6$ ) had comparable IPSC/EPSC ratios (I/E ratios) in their PL-BLA circuit ( $p > 0.9$ ). After the fear conditioning, both B6 ( $n = 5$ ) and S1 mice ( $n = 7$ ) exhibited decreased I/E ratios in their PL-BLA circuitry compared with same-strain naïve animals ( $p < 0.001$  for B6 mice,  $p < 0.01$  for S1 mice). There was significant difference in the I/E ratios of B6 and S1 mice ( $p < 0.01$ ). After

the fear extinction, I/E ratios in the PL-BLA circuit of B6 mice ( $n = 7$ ) returned to basal levels, while S1 mice ( $n = 6$ ) exhibited persistently decreased I/E ratios compared with same-strain naïve animals ( $p > 0.9$  for B6 mice,  $p < 0.001$  for S1 mice). S1 mice also exhibited significantly lower I/E ratios than B6 mice ( $p < 0.001$ ). **b** There were no differences in PPR neither among the three behavioral conditions nor between the mouse strains ( $n = 5-7$ ).  $**p < 0.01$ ,  $***p < 0.001$

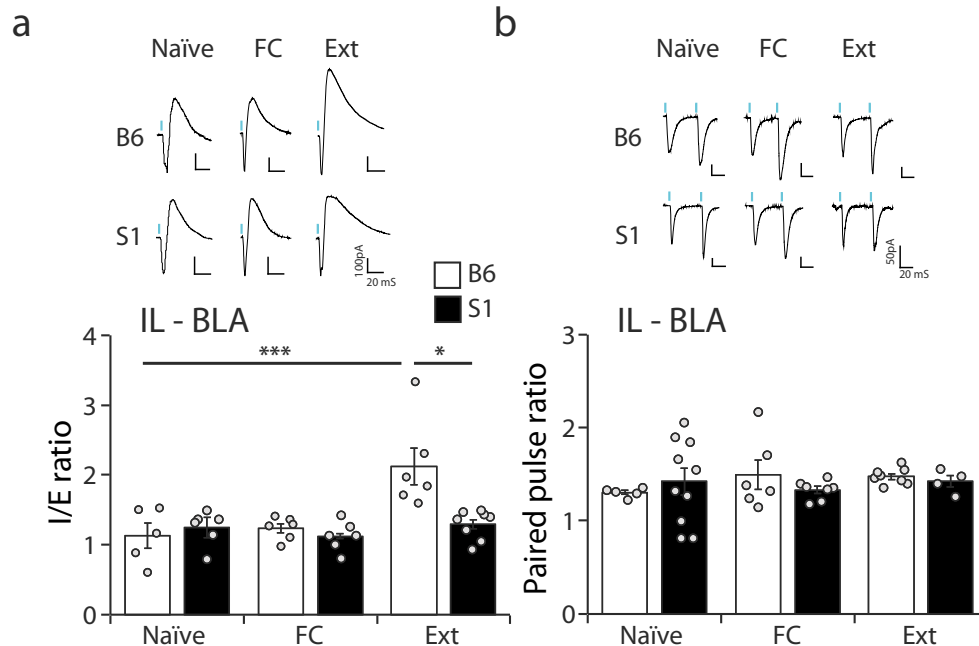
ratios to naïve S1 mice ( $p < 0.001$  for B6 mice,  $p > 0.9$  for S1 mice). Moreover, B6 mice showed significantly higher I/E ratios than S1 mice (I/E ratios: B6 =  $2.12 \pm 0.26$ ; S1 =  $1.29 \pm 0.07$ ,  $p < 0.05$ ) (Fig. 3a).

To investigate whether the changes in I/E ratios in the IL-BLA circuit were due to presynaptic modifications in glutamate release probability, we further measured PPR in the IL-BLA circuit. There was no significant strain-by-group PPR interaction in the IL-BLA circuit ( $F(2, 34) = 0.83$ ,  $p > 0.4$ ). In addition, no significant PPR differences in the IL-BLA circuit were found between B6 and S1 mice or within each strain among the three behavioral conditions (PPR (Naïve): B6 =  $1.30 \pm 0.02$ ; S1 =  $1.42 \pm 0.14$ , (FC): B6 =  $1.49 \pm 0.16$ ; S1 =  $1.33 \pm 0.04$ , (Ext): B6 =  $1.47 \pm 0.03$ ; S1 =  $1.42 \pm 0.06$ ) (Fig. 3b). Given these results, the strength of the IL-BLA circuit did not appear to change upon fear conditioning in neither B6 nor S1 mice. Furthermore, fear extinction weakened the IL-BLA circuit in B6 mice but not S1 mice without changing the presynaptic release probability.

### Postsynaptic Changes in Glutamatergic Transmission Occurred in the PL-BLA Circuit in B6 and S1 Mice

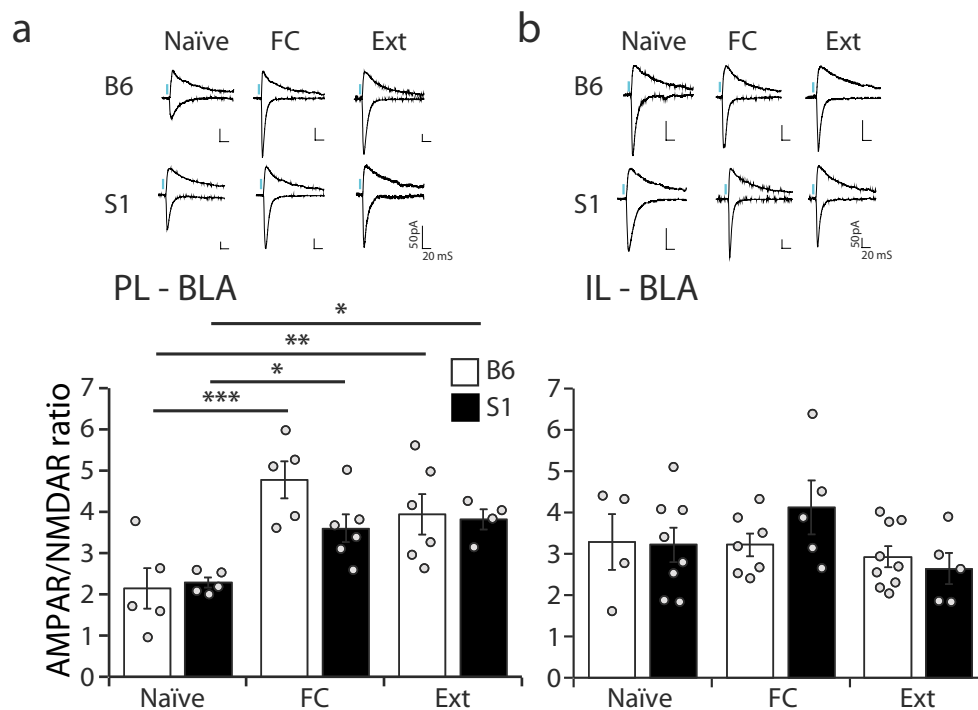
To examine whether I/E alterations in PL-BLA and IL-BLA circuits of B6 and S1 mice are accompanied by postsynaptic changes in glutamatergic transmission, we

assessed AMPAR/NMDAR (A/N) ratios. We injected an AAV vector expressing ChR2-eYFP into the PL or IL of mice and measured light-evoked AMPAR- and NMDAR-mediated currents in the BLA pyramidal neurons. There was no significant strain-by-group interaction in A/N ratios in the PL-BLA circuit ( $F(2, 25) = 1.61$ ,  $p > 0.2$ ). Naïve B6 and S1 mice also displayed similar A/N ratios (B6 =  $2.13 \pm 0.49$ ; S1 =  $2.28 \pm 0.12$ ,  $p > 0.8$ ). After fear conditioning, A/N ratios in the PL-BLA circuit were enhanced in both B6 and S1 mice compared with those in strain-match naïve animals ( $p < 0.001$  for B6 mice,  $p < 0.05$  for S1 mice), with no statistically significant differences between the strains (B6 =  $4.77 \pm 0.44$ ; S1 =  $3.59 \pm 0.33$ ,  $p > 0.1$ ). Following fear extinction, B6 and S1 mice persistently exhibited increased A/N ratios in the PL-BLA circuit compared with strain-matched naïve animals ( $p < 0.01$  for B6 mice,  $p < 0.05$  for S1 mice) without significant differences between the strains (A/N ratios: B6 =  $3.93 \pm 0.49$ ; S1 =  $3.82 \pm 0.24$ ,  $p > 0.8$ ) (Fig. 4a). Regarding A/N ratios in the IL-BLA circuit, there was no significant strain-by-group interaction ( $F(2, 32) = 1.13$ ,  $p > 0.3$ ). Furthermore, no significant differences were detected between or within B6 and S1 mice across the three behavioral conditions (A/N ratios (Naïve): B6 =  $3.28 \pm 0.67$ ; S1 =  $3.21 \pm 0.41$ , (FC): B6 =  $3.21 \pm 0.27$ ; S1 =  $4.12 \pm 0.65$ , (Ext): B6 =  $2.92 \pm 0.25$ ; S1 =  $2.63 \pm 0.38$ ) (Fig. 4b). In summary, these data



**Fig. 3** IL inputs to the BLA are reduced in B6 mice but not altered in S1 mice. **a** Naïve C57BL/6 (B6) ( $n = 5$ ) and 129S1 (S1) mice ( $n = 6$ ) had comparable I/E ratios in their IL-BLA circuits ( $p > 0.5$ ). After the fear conditioning (FC), IL-BLA circuit I/E ratios in both B6 ( $n = 6$ ) and S1 mice ( $n = 6$ ) did not differ from same-strain naïve group ratios ( $p > 0.8$  for B6 mice,  $p > 0.7$  for S1 mice). B6 and S1 mice also exhibited similar I/E ratios ( $p > 0.5$ ). After the fear extinction, I/E ratios in B6 mice ( $n = 6$ ), not

in S1 mice ( $n = 8$ ), increased in IL-BLA circuit compared with same-strain naïve controls ( $p < 0.001$  for B6 mice,  $p > 0.9$  for S1 mice), while I/E ratios in B6 mice were significantly higher than those in S1 mice ( $p < 0.05$ ). **b** No differences were found in PPR among behavioral conditions (Naïve, FC, and Ext) or between mouse strains ( $n = 4-10$ ). \* $p < 0.05$ , \*\*\* $p < 0.01$



**Fig. 4** Postsynaptic changes in glutamatergic transmission occur in PL-BLA circuit but not in IL-BLA circuit both in B6 and S1 mice. **a** Naïve C57BL/6 (B6) ( $n = 5$ ) and 129S1 (S1) mice ( $n = 5$ ) had comparable A/N ratios in PL-BLA circuits ( $p > 0.8$ ). After the fear conditioning (FC), both B6 ( $n = 5$ ) and S1 mice ( $n = 6$ ) exhibited increased A/N ratios in their PL-BLA circuits compared with same-strain naïve group controls ( $p < 0.001$  for B6 mice,  $p < 0.05$  for S1 mice) and there were no significant differences between B6 and S1 mice ( $p > 0.1$ ). After the fear extinction, there

were no significant differences between B6 ( $n = 6$ ) and S1 mice ( $n = 4$ ) among behavioral conditions (Naïve, FC, and Ext), while both B6 and S1 showed elevated A/N ratios compared with same-strain naïve group controls ( $p < 0.01$  for B6 mice,  $p < 0.05$  for S1 mice). **b** In the IL-BLA circuit, no changes were detected between B6 and S1 mice among all conditions (Naïve, FC, and Ext) or between conditions in the same strain ( $n = 4-9$ ).  $*p < 0.05$ ,  $**p < 0.01$ ,  $***p < 0.001$

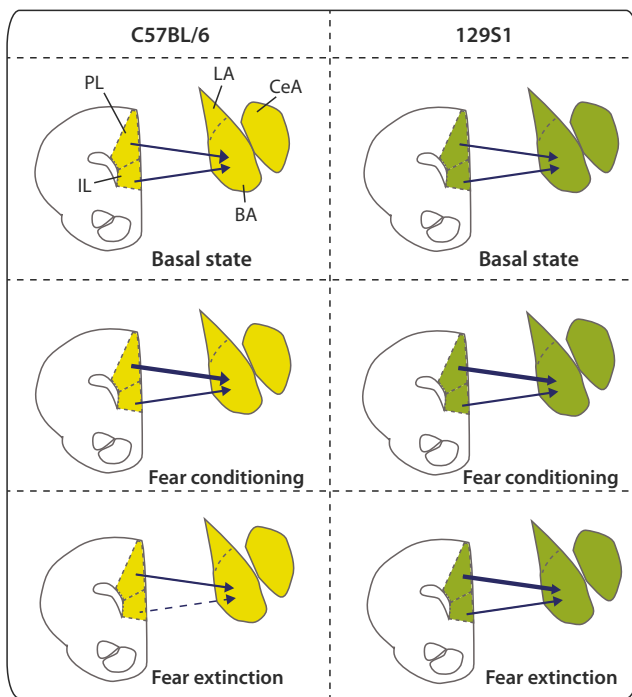
suggest that postsynaptic changes in glutamate receptors in the PL-BLA circuit may be involved in decreasing I/E ratios after the fear conditioning in both B6 and S1 mice and maintaining elevated I/E ratios following the fear extinction in S1 mice. Furthermore, the changes in A/N ratios were preserved even when I/E ratios in the PL-BLA circuit of B6 mice returned to basal levels following the fear extinction (Fig. 4a).

## Discussion

The PL and IL of the mPFC are thought to regulate fear conditioning and extinction behaviors largely through their projections to the BLA [22–26, 33]. Here, we investigated altered excitatory synaptic transmissions in PL-BLA and IL-BLA circuits of fear extinction-impaired S1 mice before and after fear conditioning and extinction training to determine the neural mechanisms underlying the impaired extinction evident in these animals. By combining optogenetic and electrophysiological methods, we were able to stimulate specifically the PL-BLA or IL-BLA circuit and obtain light-evoked responses in the BLA pyramidal neurons. We found that the strengths of

the PL-BLA and IL-BLA circuits of naïve B6 and S1 mice were comparable, and PPR and A/N ratios were also similar. Following the fear conditioning, both B6 and S1 mice exhibited strengthened the PL-BLA circuit with increased A/N ratios, while there were no changes in the IL-BLA circuit in B6 and S1 mice. After the fear extinction, however, the PL-BLA circuit in B6 mice was weakened to the level of that in naïve B6 mice, while S1 mice exhibited persistently strengthened the PL-BLA circuit. B6 mice also exhibited a weakened IL-BLA circuit following the fear extinction, unlike S1 mice. The PL-BLA circuit strengthening and lack of a change in the IL-BLA circuit in S1 mice even after the fear extinction may underlie the impaired fear extinction in S1 mice (Fig. 5).

It has been reported that excitatory neurons in the mPFC activate pyramidal neurons and interneurons in the BLA monosynaptically and the activated interneurons inhibit the pyramidal neurons, which means that the mPFC could activate pyramidal neurons in the BLA directly and inhibit the same neurons indirectly by feedforward inhibition [22, 24, 31, 34]. For B6 mice, fear conditioning strengthens the PL-BLA circuit by enhancing excitatory transmission, while feedforward inhibition is minimally affected [22]. In the present study, we discovered that I/E ratios in the PL-BLA circuit



**Fig. 5** Altered mPFC-amygdala circuitry in animals with impaired fear extinction. Fear conditioning strengthened the PL-BLA circuit of both C57BL/6 (B6) and 129S1 (S1) mice. Following the fear extinction, the strength of the PL-BLA circuit of B6 mice went back to the basal level, while S1 mice presented a constantly strengthened PL-BLA circuit. Fear extinction weakened the IL-BLA circuit of B6 mice, while S1 mice exhibited unaltered strength in the IL-BLA circuit

of B6 mice decreased by fear conditioning and increased back to the basal level following fear extinction (Fig. 2), whereas the reduced I/E ratios in PL-BLA in S1 by fear conditioning remained decreased after extinction. Given the observations in B6 mice, it is more likely that excitatory transmission of PL-BLA remained enhanced after extinction in case of S1 as well, although we cannot completely rule out the unexpected modulations of feedforward inhibition levels in this particular strain of mice. In addition, fear extinction seems to weaken the IL-BLA circuit reducing excitatory transmission with no alterations in feedforward inhibition [24]. Therefore, the strength of mPFC-BLA circuits is rather modulated by alterations in excitatory transmission. Future studies need to investigate whether the changes in I/E ratios are mediated by excitatory or/and inhibitory synaptic alterations in two strains of mice.

The weakening of the PL-BLA circuit in B6 mice following fear extinction (Fig. 2 and 5) supports the notion that reduced PL activity is required for the generation of fear extinction behaviors. Increased activity of the PL has been implicated in fear expression [11, 35–37], and decreased PL activity has been implicated in fear extinction [17, 18, 38]. Fear conditioning induces CS-evoked conditioned PL response which decreases only after successful fear extinction [14]. In fear extinction-impaired S1 mice, PL single-unit

activity increases during extinction retrieval [39]. Microstimulation of the PL during fear extinction further increases the expression of conditioned fear, thereby preventing extinction learning. Inhibition of the PL by muscimol treatment enhances fear extinction learning [17, 38]. Furthermore, in the present study, B6 mice exhibited reduced PL-BLA circuit strength following fear extinction, while S1 mice persistently exhibited strengthened the PL-BLA circuit.

The weakening of the IL-BLA circuit in B6 mice following fear extinction (Fig. 5) may seem contradictory as activating the IL has been suggested to enhance fear extinction and retrieval [16, 40]. The weakened IL-BLA circuit in B6 mice after fear extinction has already been reported by Cho et al., suggesting that fear extinction reduces IL inputs to BLA pyramidal neurons and does not affect IL inputs to BLA interneurons. Fear extinction also does not change BLA interneuron inputs to BLA pyramidal neurons [24]. This fear extinction-induced synaptic plasticity would inhibit BLA pyramidal neurons effectively to suppress fear expression when a CS presentation activates the IL later. To figure out whether this indeed happens, future studies are required to measure activity of IL-BLA circuits during fear extinction retrieval.

A previous study has shown that S1 mice exhibit elevated single-unit firing in the IL compared with B6 mice during fear extinction retrieval, while activated IL has been known to enhance fear extinction retrieval. The authors suggested that more elevated IL of S1 mice is a compensatory effort to mitigate excessively activated PL or amygdala of S1 mice during fear extinction retrieval, which is still not enough to induce fear extinction [23, 39]. In our current study, we found that S1 mice exhibited unaltered IL-BLA circuit strength after fear extinction, while B6 mice exhibited weakened IL-BLA circuit (Fig. 3). This lack of plasticity in the IL-BLA circuit of S1 mice might be one of the reasons why S1 mice require excessive IL activation to inhibit BLA pyramidal neurons to the extent of B6 mice.

The IL has been implicated in the regulation of fear extinction consolidation by others, as inactivation of or lesions in the IL disrupts the extinction of memory retrieval [13, 15, 18]. Furthermore, both activation and inhibition of the IL-BLA circuit do not affect extinction learning, though they do enhance and prevent extinction retrieval, respectively [23]. Our group and others have reported that S1 mice exhibit deficits not only in fear extinction retrieval but also in fear extinction learning with persistently high levels of freezing [6–9]. Given that the IL is specifically involved in fear extinction retrieval, it seems that the impairments in extinction learning in S1 mice may be due to the persistently strengthened PL-BLA circuit which may further play critical roles in the expression of fear memories and disrupt fear inhibition together with the unchanged IL-BLA circuit. We thus suggest that both PL-BLA and IL-BLA circuits may underlie the impairments in fear extinction learning and retrieval in S1 mice. Is there a more



weighted contribution of PL-BLA or IL-BLA? Are both enhanced conditioning signal from the PL and reduced extinction signal from the IL required for extinction impairment? To answer these questions, further behavioral studies involving selective manipulation of each circuit are required. In the present study, we explored the residual alterations in the PL-BLA and IL-BLA circuits 24 h after each behavioral condition because of the limitations of *ex vivo* electrophysiology. Further research using *in vivo* electrophysiology or calcium imaging may provide additional information on how the circuits react during fear conditioning and extinction in real time.

With impaired fear extinction, S1 mice have also been known to show elevated fear responses during fear conditioning and fear memory retrieval compared to B6 mice, which was what we observed in the present study as well. While both B6 mice and S1 mice learn and store fear memory well in fear conditioning, S1 mice present a higher level of freezing behavior to CS during fear conditioning training and retrieval [6, 7, 9]. Here, we found that while both B6 and S1 mice exhibited decreased I/E ratios in the PL-BLA circuit after fear conditioning, I/E ratios in S1 mice were significantly more reduced compared to B6 mice (Fig. 2). As reduced I/E ratios in the PL-BLA circuit have been reported following fear conditioning in B6 mice, we suggest that higher freezing behavior of S1 mice in fear conditioning (Fig. 1) might be related to more decreased I/E ratios in the PL-BLA circuit of S1 mice.

After fear conditioning, both B6 and S1 mice exhibited strengthened PL-BLA circuit with elevated A/N ratios. The levels of increase in A/N ratio upon fear conditioning is not statistically significant between B6 and S1 partially due to the limited sample size ( $p > 0.1$ , Fig. 4). Nonetheless, when compared to basal condition, there is a clear increase in A/N ratio in both strains of mice. The A/N ratios remained high following the fear extinction in S1 mice and B6 mice, while only B6 mice showed good fear extinction with weakened PL-BLA circuit. The constantly elevated A/N ratios in B6 mice even after the fear extinction may further support the idea that fear extinction is a form of new learning rather than the erasure of previously formed fear memories. After successful fear extinction, extinguished fear memories may be recovered spontaneously over time in a process known as spontaneous recovery [2, 41, 42], wherein the CS is presented in a new context (via renewal) [43–45] or after US exposure (via reinstatement) [2, 46]. Therefore, fear extinction has been thought to form new circuits that inhibit previously formed fear circuits [47, 48] and weakened PL-BLA circuit with persistently elevated A/N ratios in B6 mice after the fear extinction in our study may be caused by the formation of new inhibitory circuits (Fig. 2, 4 and 5). However, it remains unclear which circuit changes are responsible for impaired extinction.

For fear conditioning, an electrical foot shock is usually used as an US. Therefore, fear conditioning and extinction have been studied to understand pain-related fear and,

moreover, chronic pain which is a disorder that pain-related fear is a major factor in the development and maintenance [49, 50]. Chronic pain patients show impaired pain-related fear extinction, and exposure-based interventions that promote pain-related extinction have been investigated to treat chronic pain, the mechanisms of which have not been fully understood yet [51–57]. Hence, our current study on altered cortico-amygdala circuits after pain-related fear conditioning, extinction, and impaired extinction could provide a clue to understanding the mechanisms underlying chronic pain.

PTSD is a prevalent anxiety disorder that occasionally develops in individuals experiencing a traumatic event. Patients with PTSD experience fear memories, often spontaneously, and exhibit impaired fear extinction [4, 5, 58]. In humans, fear extinction retrieval increases ventromedial prefrontal cortex (vmPFC) activity, the homolog of the rodent IL [59, 60]. Moreover, PTSD patients exhibit decreased prefrontal blood flow and diminished activation of the vmPFC during recall of the traumatic events [61–63]. The dorsal anterior cingulate cortex (dACC), the human homolog of the rodent PL, is also activated by conditioned and unconditioned fear stimuli [64–67]. As with the rodent PL and IL, the human vmPFC and dACC also project to the BLA [67, 68]. Thus, understanding fear-learning neuroanatomical circuits in rodents may provide avenues for further neuroimaging-based investigation of anxiety circuitry in humans.

Although PTSD is a common disorder, an effective treatment has not been developed yet due to lack of a profound understanding of the etiology of PTSD. In the present study, we investigated how the mPFC-BLA circuits underlying fear conditioning and extinction were particularly altered in S1 mice. Our circuit-level data on these impaired fear extinction processes in mice may contribute to fear extinction, chronic pain, and PTSD research, potentially helping to develop effective circuit-based therapeutics for impaired fear extinction in humans.

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## Compliance with Ethical Standards

**Conflict of Interest** The authors declare that they have no conflict of interest.

**Ethical Approval** All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. All procedures performed in studies involving animals were in accordance with the ethical standards of the institution or practice at which the studies were conducted (Institutional Animal Care and Use Committee of Konkuk University, Seoul, Korea). This article does not contain any studies with human participants performed by any of the authors.

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