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Bidirectional modulation of fear extinction by mediodorsal thalamic firing in mice

시상 동측 내측 핵에서 공포기억소멸 조절 기전 연구

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이 석 찬

A thesis of the Degree of Doctor of Philosophy

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Bidirectional modulation of fear extinction by mediodorsal thalamic firing in mice

by

Sukchan Lee

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ABSTRACT

Fear extinction is induced by repeated exposures to conditioned stimuli in the absence of previously associated unconditioned aversive stimuli. However, the underlying neural mechanism remains unclear. Here, we provide evidence for bidirectional modulation of fear extinction by the MD and present a neural mechanism of the thalamic control of extinction. For the behavior analysis, phospholipaseC-β4 (PLCβ4)$^{-/-}$ and wild-type mice were fear-conditioned with tones (CS) and mild foot shocks (US). 24hrs after conditioning, extinction learning was performed. The extinction was recalled 24hr after the conditioning. Three weeks after shPLCβ4 or non-target lentiviral vector infection into the mediodorsal thalamus (MD), fear extinction, elevated-pus maze and open field test were done. To quantify the PLCβ4 expression level in the lentivirus-infected MD, tissue processing and immunostaining were performed. For in vitro patch recording, coronal slices containing MD or isolated single neurons of MD were prepared and stimulated. Signals were digitized and analyzed. For micro-infusion of drugs into MD, a dummy cannula inserted into a guide cannula system was implanted. The drugs were delivered through an injector cannula at least 7 days after the cannula implantation. Single-unit activities of MD in vivo were obtained during exposure to the tone of extinction. For electrical stimulation of MD during extinction tone, mice implanted with bipolar stimulating electrodes received trains of square pulses paired with CSs or unpaired for control, either at 100Hz (tonic) stimulation for 100ms or at 416Hz (burst) stimulation for 12ms. Mice with a knockout or MD–specific knockdown of PLCβ4, a signaling enzyme coupled to group I metabotropic glutamate receptors, showed normal acquisition and expression of auditory-conditioned fear, but were severely impaired in extinction. Mutant MD

neurons in slices showed enhanced burst firing accompanied by increased T-type Ca^{2+} currents, whereas tonic firing was normal. Infusion of T-type Ca^{2+} channel blockers into the MD rescued the impaired extinction in the mutants, and, further, facilitated fear extinction in the wild-type. Tetrode recordings in freely moving mice revealed that during extinction the single-spike (tonic) frequency of MD neurons increased in the wild-type but was static in the mutant and the burst firing frequency of MD neurons did not change in the wild-type but increased in the mutant. Furthermore, tonic-evoking microstimulations of the MD, contemporaneous with the extinction tones, rescued fear extinction in the mutant and facilitate it in the wild-type. In contrast, burst-evoking microstimulation suppressed extinction in the wild-type, mimicking the mutation. These results suggest that the firing mode of the MD is critical in modulation of fear extinction and that T-type Ca^{2+} channels exert an inhibitory effect on this process.

Keywords : Fear extinction, mediodorsal thalamic nucleus, phospholipaseC- 4, T-type Ca2+ channel, tonic and burst firing

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CONTENTS

LIST OF FIGURES AND TABLE

Figures

Table

LIST OF ABBREVIATIONS

MD mediodorsal thalamic nucleus

BLA basol ateral amygdala

CS conditioned stimulus

HVCC high voltage-activated Ca2+ current

Ih hyperpolarization-activated cation current

IL infralimbic

LVCC low voltage-activated T-type Ca2+ current

mGluR1 metabotropic glutamate receptor type 1

mPFC medial prefrontal cortex

PBS phosphate-buffered saline

PL prelimbic cortex

PLCβ4 phospholipaseC-β4

US unconditioned stimulus

INTRODUCTION

The conditioned fear response is extinguished by repeated exposure to a neutral conditioned stimulus (CS) in the absence of the noxious unconditioned stimulus (US). This process, termed fear extinction, is the basis of the general therapeutic process used to treat human fear disorders (Foa, 2006; Hofmann, 2008; Myers and Davis, 2007; Quirk and Mueller, 2008). Although both the cortical and subcortical limbic systems have received considerable attention, the neural mechanism of fear extinction is not yet fully understood. The mediodorsal thalamic nucleus (MD), a part of the basolateral limbic system, has strong interconnections with the medial prefrontal cortex (mPFC) as well as with the amygdala, and these regions together form a tight triangular circuitry (Jones, 2007; Ray and Price, 1992; Ray and Price, 1993). The two brain regions connected to the MD are known to be a key in the formation and extinction of conditioned fear memory (Fanselow and Poulos, 2005; Pare et al., 2004). In humans, damage to the MD may lead to complex disorders of PFC-dependent cognitive functions, including learning deficits, memory loss, attention problems, or emotional changes (Van der Werf et al., 2000; Zoppelt et al., 2003). In previous lesion studies performed in animals, the MD was suggested to be linked to the PFC or the amygdala when functioning to facilitate associative learning (Hunt and Aggleton, 1998; Mitchell and Dalrymple-Alford, 2005; Mitchell and Gaffan, 2008). Anatomical and lesion studies have suggested that activation of the MD in the cortico-thalamo-cortical loop is required if attention is to be devoted to a particular task, and for encoding new information into memory (Ray and Price, 1992; Ray and Price, 1993; Winocur, 1990). Therefore, the MD is thought to function as a bridge in the limbic circuits of the learning and memory

processes. Nevertheless, the contribution of the MD to fear conditioning and extinction remains controversial (Garcia et al., 2006; Herry and Garcia, 2002; Hugues and Garcia, 2007; Inoue et al., 2004; Li et al., 2004) .

In response to input signals, thalamocortical neurons demonstrate dual firing patterns determined by membrane status: a burst of high-frequency action potentials upon hyperpolarization or tonic firing of singular action potentials when depolarization occurs (Llinas and Jahnsen, 1982; Sherman, 2001). Hyperpolarized membrane potentials lead to activation of T-type Ca2+ channels, yielding low-threshold Ca2+ currents which in turn trigger burst firing. Glutamatergic corticothalamic inputs activate metabotropic glutamate receptor type 1 (mGluR1), and thus induce membrane depolarization in thalamocortical neurons, leading to a shift in thalamic firing from the burst to the tonic mode (McCormick and von Krosigk, 1992; Rivadulla et al., 2002). In the corticothalamic postsynapses, mGluR1 is physiologically coupled to phospholipase C- 4 (PLCb4), and both proteins are abundantly expressed in the thalamocortical nucleus, including the MD (Nakamura et al., 2004). Inactivation of mGluR1-PLCb4 in the mouse led to enhanced burst firing of the ventrobasal thalamus and decreased pain responses, presumably because of failure in the relay of persistent pain signals to the cortex (Cheong et al., 2008; Miyata et al., 2003). In addition, inactivation of PLCb4 in the ventrobasal thalamus caused development of spontaneous absence epilepsy, accompanied by increased thalamic burst firing (Cheong et al., 2009). Changes in thalamic firing patterns have also been implicated in sleep and arousal, sensory signaling, and consciousness level (McCormick and Bal, 1997; Sherman, 2001; Steriade and Llinas, 1988). However, little is known about the physiological functions of thalamic firing modes with respect to other aspects of cognitive or emotional processing.

Here, we examined the possible role of the MD and the dual firing modes thereof in

the fear extinction process, employing mice in which the mGluR1-PLCb4 signaling pathway was compromised. Using a combination of tools, including genetics, pharmacology, physiology, and microstimulation in vivo, we reveal that the MD, controlled by the mGluR1-PLCb4 signaling pathway, modulates fear extinction bidirectionally by means of the dual firing modes; specifically, burst firing suppresses and tonic firing facilitates fear extinction.

MATERIALS AND METHODS

1. Animals

Adult male PLCβ4$^{-/-}$ and wild-type littermate mice (12-16 weeks of age) of the B6x129 F1 background were obtained by mating of parental strains C57BL/6J (N20-23) PLCβ4$^{+/-}$ and 129s4/svJ (N20-23) PLCβ4$^{+/-}$ (Kim et al., 1997). Mice were housed with free access to food and water, under controlled temperature conditions, and with a 12 h: 12 h light: dark cycle. Animal care and experimental procedures followed the guidelines of the Institutional Animal Care and Use Committee of the Korea Institute of Science and Technology.

2. Fear conditioning, locomotor activity, and anxiety testing

Mice were placed into a standard operant chamber (Coulbourn Instruments, USA) located within a sound-attenuating box (Coulbourn Instruments, USA) and, after 3 min, were fear-conditioned by three presentations (at an interval of 120 sec) of tones (3 kHz; 30 sec; 90 dB) that coterminated with footshocks (1 sec; 0.7 mA, 0.5 mA, or 0.3 mA). Mice were returned to their home cages after 60 sec. Twenty-four hours after conditioning, mice received extinction learning composed of 20 identical tones (at intervals of 5 sec), without shocks, delivered within an acrylic black circular box (diameter 20 cm) located inside a further sound-attenuating box that was not the box used for conditioning. In the former box, the extent of free movement in the 10 min before extinction learning commenced was analyzed using EthoVision software (Noldus Information Technology, USA). Twenty-four hours after extinction learning concluded, mice were subjected to an additional tone in an identical extinction learning context; this was the extinction recall test. Animal behavior was recorded using a video camera

to score freezing (lack of movement except for respiration); data were manually transcribed to a computer-acceptable format by a colleague blinded to experimental grouping. To determine the optimal electric shock strength in various experiments, we measured freezing level in the fear extinction test using footshocks of 0.7 mA, 0.5 mA, or 0.3 mA, during conditioning. After conditioning employing 0.7 mA, wild-type mice showed a very slow reduction in freezing level upon fear extinction learning. A shock of 0.5 mA was optimal for conduct of fear extinction tests in animals subjected to gene knockdown, drug treatment, and in vivo recording experiments. Use of a 0.3 mA footshock allowed us to rule out a ceiling effect on the freezing response. Also, we determined that 0.3 mA was the optimal shock strength when the dose-dependent effects of tonic-evoking MD and burst-evoking MD microstimulation in fear extinction were to be evaluated. To evaluate sensitivity to footshock, mice were placed into the conditioning chamber and subjected to unsignaled footshocks of increasing amplitude at 100 msec intervals. Commencing at 0.1 mA, footshock strength was increased in 0.1 mA increments until four response thresholds were reached; these were noticing (an orienting head movement), flinching (hind paws briefly raised off the bars), jumping (all paws simultaneously off the bars), and vocalizing. To test locomotor activity and anxiety level, mice were placed in the central region (a square of 20×20 cm) of an open field box (40×40×50 cm), and the extent of spontaneous movement was analyzed using EthoVision software, over 1 h. Two days after conduct of the open-field test, mice were place in a plus-maze (with two opposite open arms [each 45×5 cm] and two opposite closed arms [each 45×5 cm], with 15 cm-high walls) elevated to a height of 30 cm above floor level. The numbers of entries into individual arms were scored over 5 min. Analysis of variance (ANOVA) and Student's t-test was used to assess statistical significance.

3. Lentiviral vectors

Lentiviruses were created by co-transfecting HEK293T cells with three plasmids: (*i*) a construct expressing the heterologous envelope protein, VSV-G; (*ii*) a packaging-defective helper construct expressing the *gag-pol* gene; and, (*iii*) a transfer vector harboring a PLC 4-specific shRNA sequence. Cells were transfected using Lipofectamine-Plus, as instructed by the manufacturer (Invitrogen, USA). Forty-eight hours after transfection, virus-containing culture supernatants were collected, clarified by passage through a 0.45-μm pore-sized membrane filter (Nalgene, USA), and immediately stored at -70ºC. Viral titers were determined using a p24 ELISA kit (Perkin-Elmer Life Science, USA) or by Western blotting employing a monoclonal anti-p24 antibody obtained from the AIDS Research and Reference Reagent Program. The titers of preparations were routinely ~10^6–10^7 transduction units (TU)/ml prior to concentration. Infectious lentivirus particles were concentrated by ultracentrifugation onto a 20% (w/v) sucrose cushion (2 h at 50,000 x *g*) at 4ºC.

4. Injection of lentivirus-borne shPLCβ4 into the MD in vivo

Wild-type mice were anesthetized using 2% (v/v) Avertin (tribromoethyl alcohol/ tertiary amyl alcohol; Aldrich). One microliter amounts of high-titer lentiviral preparations (10^7 TU/ml) were bilaterally injected into the MD (1.7 mm posterior to the bregma, 0.3 mm lateral to the midline, and 3.3 mm below the brain surface) using a 25 μl Hamilton syringe connected to a microinjection pump (the WPI sp100i instrument, USA) at a rate of 0.1 μl per min. To permit diffusion of lentiviral vectors into brain tissue, the needle was held in place for 10 min after completion of injection. Mice were given bilateral injections of either shPLCβ4 (TRCN0000076919: 5'-GCCTCTTCAA

AGTAGATGAA T-3') or non-target control shRNA (non-human and non-mouse shRNA: 5'-CAACAAGATGAAGAGCACCAA-3'). Three weeks after lentiviral vector injection, fear extinction tests, evaluation of elevated-plus maze and open field performance, and spontaneous EEG recording of the MD, were performed.

5. Tissue processing and immunohistochemistry to detect PLCβ4 expression in the MDs of wild-type and PLCβ4$^{-/-}$ mice

Animals were transcardially perfused with phosphate-buffered saline (PBS) followed by 4% (w/v) paraformaldehyde in 0.1 M phosphate buffer (PB, pH 7.4), under urethane anesthesia (1.5 g/kg, i.p.). Brains were removed and post-fixed in the same fixative for 4 h. Brain tissues were cryoprotected by infiltration with 30% (w/v) sucrose overnight. Thereafter, the entire brain was frozen and sectioned, using a cryostat, into 30 m-thick slices; consecutive sections were placed into wells of six-well plates containing PBS. Every sixth section in a series embracing the entire MD area from selected animals was used for immunofluorescence staining. Sections were first incubated with 3% (v/v) bovine serum albumin in PBS, for 30 min at room temperature. Sections were next incubated with a rabbit anti-PLCβ-4 IgG (1:100; Santa Cruz Biochemicals, USA) primary antibody in PBS containing 0.3% (v/v) Triton X-100, overnight at room temperature. Sections were next washed three times, for 10 min each time, with PBS, and incubated sequentially with biotinylated goat anti-rabbit IgG (Vector) and the ABC complex (Vector), diluted 1:200 in the same solution as employed for dilution of the primary antibody. Between incubations, tissues were washed with PBS three times for 10 min each time. Stained sections were visualized by application of 3,3'-diaminobenzidine (DAB) in 0.1 M Tris buffer, and were mounted on gelatin-coated slides. Images were captured and analyzed using an Olympus DP72 digital camera and

DP2-BSW microscopic digital camera software. All Figures were prepared using Adobe Photoshop 7.0 (San Jose, CA). Image manipulation was restricted to threshold and brightness adjustments, applied to an entire image.

6. Double immunofluorescent staining of the MD of PLCβ4-knockdown mice

Morphological and protein expression changes induced by shPLCβ4 of/in MD PLCβ4-positive neurons were evaluated by double immunofluorescence staining using both rabbit anti-PLCβ-4 IgG (1:100; Santa Cruz Biochemicals, USA) and mouse anti-Neuronal Nuclei (NeuN) IgG (1:100; Chemicon, USA). Brain tissues were incubated in a mixture of antibodies overnight at room temperature. After washing three times, for 10 minutes each time, in PBS, sections were incubated in a mixture of FITC- and Cy3-conjugated secondary antibody (1:200; Amersham, USA) for 1 h at room temperature. Sections were next mounted in Vectashield mounting medium with or without DAPI (Vector). Images were captured and analyzed using an Olympus DP72 digital camera and DP2-BSW microscopic digital camera software (Japan). Figures were prepared using Adobe Photoshop 7.0. Image manipulation was restricted to threshold and brightness adjustments applied to the entire image. For quantification of PLCβ4 immunofluorescence in RNAi-mediated PLCβ4 knockdown mice, PLCβ4 immunofluorescent images (10 sections / animal) were captured within a particular region (500×500 μm). Images were sampled from at least five different points within each MD section. Thereafter, the numbers of PLCβ4- positive cells within sampled images were visually counted. All immunoreactive cells were scored, regardless of labeling intensity. Cell counts were performed by two investigators blinded to tissue classification. The average proportion of PLCβ4-positive cells (*C*) in the MD of knockdown mice was estimated and is represented as: $C = 100 \times (\Sigma Qi / Ni)/K$, where

Qi is the count of PLCβ4-positive cells in the MD of shPLCβ4 viral vector-injected animals; *Ni* the number of PLCβ4-positive cells in mice injected with the control shRNA viral vector; and *K* the sample number. All data from quantitative measurements were analyzed using one-way ANOVA, to determine statistical significance. Bonferroni's test was used when post-hoc comparisons were performed. A *P*-value below 0.05 was considered to be statistically significant.

7. Slice patch recording

Two or three coronal slices (300 μM in thickness), containing the MD (P21-P28), were prepared as described previously (Kim et al., 2001). Slices were incubated for 1 h at room temperature in artificial cerebrospinal fluid (ACSF) containing (in mM): 124 NaCl, 26 $NaHCO_3$, 1.25 NaH_2PO_4, 5 $MgSO_4$, 2.4 $CaCl_2$, 3 KCl, and 10 glucose, at pH 7.4, under aeration with 95% O_2 - 5% CO_2 (both v/v). To facilitate recording, slices were transferred to an electrophysiological recording chamber (RC-26G, Warner Instruments, USA) continuously superperfused with ASCF solution (flow rate: 2 ml/min). Patch pipettes (4-6 MΩ in resistance) were filled with a solution containing (in mM): 140 K-gluconate, 10 KCl, 1 $MgCl_2$, 10 HEPES, 0.02 EGTA, 4 Mg-ATP, and 0.4 Na_2-GTP; with the pH adjusted to 7.35 using KOH (278-285 mOsmol). Signals were acquired by sampling at 10 KHz and analyzed using a Digidata 1322A (Molecular Devices, USA) and a Multiclamp 700A amplifier (Molecular Devices, USA), employing pCLAMP 10.0 software (Molecular Devices, USA) and Minianalysis (Synaptosoft, USA). All data from cells showing an access resistance of over 30MΩ were discarded. ANOVA and Student's t-test were used to assess statistical significance.

8. Single-cell patch recording

Mediodorsal thalamic slices were prepared from various mouse brain regions (P19-P21) as described above. After incubation in ACSF solution (under aeration with 95% O2 - 5% CO2; both v/v) for 1 h at room temperature, slices were transferred to HEPES-buffered incubation solution containing (in mM): 150 NaCl, 4 KCl, 2 CaCl2, 2 MgCl2, 10 HEPES, 10 glucose; adjusted to pH 7.4 with 1 M NaOH, and also with 2 mg/ml protease (type XIV; Sigma) and 1mg/ml BSA (Sigma, USA). Slices were held under pure oxygen for 7 min at 35°C. All solutions were oxygenated. Rinsed slices were next immersed in a medium containing 1 mg/ml trypsin inhibitor (Sigma, USA) for 7 min at room temperature. The MDs were punched out and transferred to tubes containing 0.06 ml amounts of incubation solution. After gentle trituration, cells were harvested and plated on 0.006% (w/v) poly-D-lysine (Sigma, USA)-coated glass coverslips. To measure isolated Ca2+ currents, voltage clamp-mode patch recordings were performed. Recordings were obtained using a borosilicate glass electrode (7-8 MΩ in resistance) filled with an intracellular pipette solution containing (in mM): 130 CsCl, 10 HEPES, 5 TEA-Cl, 10 EGTA, 4 MgCl2, 4 Mg-ATP, and 0.3 Na2-GTP; adjusted to pH 7.3 with 1 M CsOH (278-285 Osmol). The extracellular solution (in mM) was 100 NaCl, 5 TEA-Cl, 5 BaCl2H2O, 20 HEPES, 2 MgCl2, 5 4-AP, 10 glucose, and 0.001 TTX; adjusted to pH 7.4 with 1 M NaOH (320-330 mOsmol; containing sucrose). Data recorded from any cell with an access resistance over 20 MΩ were discarded. The junction potential for voltage clamping of a dissociated single cell was measured (-2.4 mV), but was not corrected. Signals were digitized and analyzed using a Digidata 1322A instrument (Molecular Devices, USA) and an Axopatch 200B amplifier (Molecular Devices) at a rate of 2 kHz employing pCLAMP 9.2 software (Molecular Devices, USA). Student's t-test was used to assess statistical significance.

9. Drug delivery

Mice were implanted with 26-gauge guide cannulae (Plastics One, Inc. , USA) placed unilaterally into the MD region (1.7 mm posterior to the bregma, 0.3 mm lateral to the midline, and 3.3 mm below the brain surface) using a stereotaxic device (Kopf Instruments) under 2 % (v/v) Avertin anesthesia. A 33-gauge dummy cannula (Plastic One, Inc.) was inserted into each guide cannula, to prevent clogging. Mice were allowed to recover for at least 7 days. Mibefradil and nifedipine (Sigma, USA) were dissolved in physiological saline or 10% (v/v) dimethylsulfoxide in saline, respectively, and 0.5 l amounts were infused (0.25 l/min) through the 33-gauge injector cannulae. Infusion positions were confirmed by post-mortem histology. ANOVA and Student's t-test were used to assess the statistical significance of the data obtained.

10. In vivo single-unit recording under urethane anesthesia

On the day after conditioning, each mouse was anesthetized with urethane (1.5 g/kg, i.p.) and placed in a stereotaxic apparatus (Kopf Instruments). A hole was drilled into the skull above the region wherein the MD nuclei are located (1.7 mm posterior to the bregma and 0.3 mm lateral to the midline). A tungsten microelectrode (5-12 MΩ in resistance; A.M. Systems, USA), featuring a tip coated with 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (Sigma, USA), was inserted into the MD region (3.0-3.5 mm below the brain surface), until single-unit activity was detected. We monitored the anesthesia level by evaluation of physiological parameters, and using the forelimb pinch and eyelid response, before commencement and after completion of each recording. Baseline firing data were acquired over 15 min and recordings were next immediately obtained, during which period 20 CS were applied (at intervals of 5 sec) to obtain the sampled images. Signals were amplified 500-fold and bandpass-filtered at

300-3,000 Hz, using an amplifier (Cyberamp 402, Molecular Devices, USA) digitized to reflect a 10-kHz sampling rate, and stored in computer memory (Digidata, Molecular Devices, USA). A T-type current-dependent burst spike was defined as a cluster of two or more action potentials featuring a first interspike interval of 6 msec with progressive prolongation of the successive interspike intervals, and with silent periods 100 msec in duration both before and after a burst (Fanselow et al., 2001). Neurons responsive to the CS challenge ("responsive") were defined as those exhibiting a significant CS-evoked change in firing rate ($P < 0.05$) from baseline, as determined by evaluation of recordings conducted in the 15 min prior to CS. Non-responsive neurons ("non-response") showed no significant change in firing rates under the same conditions ($P > 0.05$). Recording positions were identified upon post-mortem histology. Spike numbers and interspike intervals were quantified using Minianalysis software (Synaptosoft, USA). ANOVA and Student's t-test were used to assess statistical significance.

11. Single unit recording and analysis in freely moving mice

Mice was anesthetized with 2 % (v/v) Avertin and placed in a stereotaxic apparatus (Kopf Instruments). Using a stereotaxic instrument, 4 tetrodes mounted on a movable microdrive (Neuralynx, USA) were chronically implanted just above the MD region (right hemisphere; 1.7 mm posterior to the bregma, 0.3 mm lateral to the midline, and 3.3 mm below the brain surface1.7 mm posterior to the bregma, 0.3 mm lateral to the midline, and 3mm below the brain surface). Each tetrode was constructed using four 12.5 micron nichrome wires (H.P. Reid, USA) and each wire tip was gold-plated (Sifco Process, Independence, USA) to obtain a final impedance of -500 kΩ (tested at 1 kHz). An additional micro-wire was used for the ground. After seven days of recovery,

screening was performed to find signals from single neurons. Once signals from single neurons were found neural signals were recorded using Neuralynx Digital system (Digital Lynx 32 Interface, Bozeman, Montana 59715, USA, 600 - 6000 low and high cut filters respectively). Neural signals were stored in the personal computer and subjected to further cluster-cutting off-line using Spikes Sort 3D software (Neuralynx, USA). Further analyses were performed using a Neuroexplorer software (Nex technologies, USA). Each cluster-cut unit was confirmed to be a signal from a single neuron by verifying that no spike counts existed under the first 1ms in the inter-spike interval histogram. Moreover, if more than one cell were recorded from one animal, cross-correlation analysis was performed to confirm that each cluster of spikes was from different neurons. A T-type current-dependent burst spike was defined as a cluster of two or more action potentials featuring a first inter-spike interval of 4 msec with progressive prolongation of the successive inter-spike intervals, and with silent periods 100 msec in duration both before and after a burst(Fanselow et al., 2001). Data was sorted into 1 sec bin and z-scores were calculated for the baseline and tone trials during habituation and extinction learning. Recording sites were identified upon post-mortem histology. ANOVA and Student's t-test were used to assess statistical significance.

12. EEG recording

A parylene-coated tungsten electrode (A-M Systems, USA) was implanted unilaterally within the MD region (1.7 mm posterior to the bregma, 0.3 mm lateral to the midline, and 3.3 mm below the brain surface) and a grounding electrode was implanted in the occipital region of the skull, using a stereotaxic device (Kopf Instruments), under 2% (v/v) Avertin anesthesia. Mice were allowed to recover for at least 7 days. Only SWDs with minimum voltage amplitudes of twice baseline EEG, and

a minimum duration of 0.5 sec, were included in the analysis (Kim et al., 2001). The recording positions were identified by post-mortem histology (Supplementary Fig. 9).

13. Electrical stimulation of the MD

All mice were implanted with bipolar stimulating electrodes (0.125 mm in diameter, Plastics One, Inc., USA) placed unilaterally within the MD region (1.7 mm posterior to the bregma, 0.3 mm lateral to the midline, and 3.3 mm below the brain surface), using a stereotaxic device (Kopf Instruments, USA) under 2% (v/v) Avertin anesthesia. Mice were allowed to recover for at least 7 days. Twenty-four hours after conditioning (described above), animals received either 100-msec trains of square pulses (100 A; 100 sec stimulus duration; 100 Hz) or 12-msec trains of square pulses (100 A; 100 sec stimulus duration; 416 Hz) using a current stimulator (Model 2100, A-M Systems, Inc.). Trains were delivered every 1 sec or 3 sec, for 30 sec, either in the intertone intervals (for the unpaired stimulation group) or paired with tones (in the paired stimulation group). Tones were delivered on 10 occasions, with an intertone interval of 1-2 min, during extinction learning. In the non-stimulation group, only tones were delivered. Stimulation positions were confirmed by post-mortem histology. ANOVA and Student's t-test were used to assess statistical significance.

RESULTS

Impaired extinction of conditioned fear memory in PLCβ4$^{-/-}$ mice

Wild-type and PLCβ4 knock-out (PLCβ4$^{-/-}$) mice were exposed to three trials of tone (CS), each of which was co-terminated with an electric foot shock (US; 0.7 mA, 0.5 mA, or 0.3 mA). After conditioning employing 0.7 mA, wild-type mice showed a very slow reduction in freezing level upon fear extinction learning (Fig. 1). A shock of 0.5 mA was optimal for conduct of fear extinction tests. During conditioning, both groups of mice showed similar levels of freezing (Fig. 2a, $P = 0.44$). Twenty-four hours or 48 h after conditioning, the freezing levels of the two groups remained similar (Fig. 2b, 2c). Together, the results show that acquisition and expression of auditory-conditioned fear memory were similar in the two groups; these data are consistent with those of a previous report showing that auditory conditioned fear memory was normal in PLCβ4$^{-/-}$ mice (Jiang et al., 1996).

Next, newly fear-conditioned mice were exposed to repeated CS without US 24 h after conditioning (i.e., extinction learning). Wild-type mice showed progressively decreased levels of freezing (Fig. 3, $F_{(19, 209)} = 32.19$, $P < 0.0001$). In contrast, PLCβ4$^{-/-}$ animals maintained persistently high levels of freezing under the same conditions (Fig. 3, $F_{(19, 95)} = 1.47$, $P > 0.1$). Next, 24 h after extinction learning was complete, mice were re-exposed to CS without US (i.e., extinction recall). Wild-type mice showed 54 ± 7.9 % freezing. However, PLCβ4$^{-/-}$ animals showed no reduction in the freezing response compared with that seen during extinction learning ($P = 0.32$). The two groups also did not differ in responses to foot shocks of varying intensities (Fig. 4a, $P > 0.1$) or in total locomotion distance during a 10 min period (Fig. 4b, $P = 0.11$), and showed little

freezing behavior in a novel chamber (data not shown), indicating that the higher freezing response of PLCβ4$^{-/-}$ mice was not the result of an elevated sensitivity to electric shock or decreased locomotor activity in the extinction chamber. To eliminate the presence of a ceiling effect on the freezing response, milder footshock conditioning (0.3 mA) was applied, and extinction learning was examined the next day. The results were similar to those seen when stronger conditioning (above) was employed; PLCβ4$^{-/-}$ animals showed normal conditioning but impaired fear extinction (Fig. 5).

Impaired fear extinction is caused by a defect in the mGluR1-PLCβ4 signaling pathway of the MD

PLCβ4 is abundantly expressed in the MD (Fig. 6). To test whether the impaired extinction phenotype of PLCβ4$^{-/-}$ mice was attributable to a PLCβ4 defect in the MD, either a lentivirus encoding a sequence active to knock down PLCβ4 (shPLCβ4), or a non-target lentivirus (control shRNA), was injected into the MD of wild-type mice (Fig. 7a, b). Three weeks after injection, the two groups showed no difference in the level of freezing seen during conditioning. However, during extinction learning, the shPLCβ4 group showed a significantly higher freezing level than did the control shRNA group (Fig. 8; for group effect: $F_{(1, 14)} = 25.44$, $P < 0.001$; for group × trial interaction: $F_{(29, 171)} = 2.97$, $P < 0.001$). Similar data were obtained when the extinction recall test was conducted (Fig. 8, $P < 0.01$). These results indicate that the MD contributes to the impaired extinction phenotype of PLCβ4$^{-/-}$ mice. The animals in which PLCβ4 knock-down was localized to the MD did not show changes in anxiety level or locomotion, when tested in the open field test (Fig. 9) and the elevated-plus maze test (Fig. 10), respectively. Furthermore, these mice did not exhibit spontaneous absence seizures,

which are typically characterized by the appearance of spike-wave discharges (SWDs) on electroencephalography (3~7 Hz), unlike what was seen when PLCβ4 knockdown was localized to the ventrobasal thalamic nucleus, as reported previously (Cheong et al., 2009) (Fig. 11). To retrospectively quantify the extent of gene silencing, we sacrificed the mice after completion of behavioral tests and measured PLCβ4 expression in MD neurons employing immunohistology. The proportion of PLCβ4-positive neurons in the MD was significantly reduced in shPLCβ4-injected animals compared to control mice (the level fell to 6.2 ± 2.8 % of control; Fig. 7c, $P < 0.001$), indicating that the lentivirus expressing shPLCβ4 significantly reduced PLCβ4 expression in MD neurons.

In the thalamus, PLCβ4 forms a signaling complex with mGluR1 (Miyata et al., 2003; Nakamura et al., 2004), which is expressed mainly in corticothalamic postsynapses (Liu et al., 1998). If mGluR1 were coupled to PLCβ4 during the process of fear extinction, inhibition of mGluR1 in the MD would affect fear extinction, in a manner similar to that of the PLCβ4$^{-/-}$ mutation. To test this possibility, we injected CPCCOEt, a selective mGluR1 antagonist, into the MD of wild-type mice. Wild-type animals in which cannulae had been implanted into the MD were fear-conditioned, and, 24 h later, were injected with CPCCOEt or vehicle. Thirty minutes after injection, mice were assessed in terms of fear extinction learning. As shown in Fig. 12, CPCCOEt–injected animals were significantly impaired in terms of fear extinction, maintaining higher freezing levels across all CS trials, compared to vehicle-injected mice (for group effect, $F_{(1, 20)} = 30.93$; $P < 0.001$; for group × trial interaction, $F_{(19, 380)} = 3.914$; $P < 0.001$). In extinction recall tests performed on the next day, the CPCCOEt-injected group showed significantly higher freezing levels than did vehicle-treated animals ($P < 0.01$). These data show that an active MD mGluR-PLCβ4 signaling pathway is required if fear extinction is to occur efficiently.

Firing properties and voltage-activated Ca^{2+} currents of MD neurons in PLCβ4$^{-/-}$ mice

In an effort to discover the cellular mechanisms of impaired fear extinction in PLCβ4$^{-/-}$ mice, we examined the intrinsic firing properties of MD neurons in brain slices using patch-clamp recording techniques. Input of stepwise hyperpolarizing current into MD neurons, at a holding membrane potential of -60 mV, induced typical thalamic burst firings, and these in turn evoked low-threshold burst spikes crested with a series of action potentials (Fig. 13a). The number of spikes in a burst was positively correlated, in both wild-type and mutant animals, with the magnitude of voltage change created by the hyperpolarizing current. Notably, however, PLCβ4$^{-/-}$ MD neurons showed a significantly increased number of spikes within a burst; the bursts were thus stronger than those of wild-type animals (Fig. 13c, $P < 0.001$). Stepwise depolarizing current inputs into MD neurons at a resting membrane potential of -60 mV evoked a number of action potentials (i.e.; tonic firing) (Fig. 13b). Unlike what was seen upon burst firing, no significant difference in tonic firing pattern between the wild-type and PLCβ4$^{-/-}$ MD neurons was evident (Fig. 13d). Low-threshold burst spikes are mediated by T-type Ca^{2+} currents triggered by the hyperpolarization-activated cation current, I_h (McCormick and Pape, 1990). To test the contribution of I_h to the stronger bursts of PLCβ4$^{-/-}$ MD neurons, I_h magnitude was quantified by measuring the voltage difference between the peak hyperpolarizing sag and the steady state membrane potential, before rebound to the holding potential (McCormick and Pape, 1990). PLCβ4$^{-/-}$ MD neurons showed a significant increase in I_h current (Fig. 13e, $P < 0.05$, 2.56 ± 0.33 versus 4.13 ± 0.5). Next, to understand the ionic mechanism responsible for the altered firing patterns of PLCβ4$^{-/-}$ MD neurons, we measured low voltage-activated

T-type Ca^{2+} currents (LVCCs), and high voltage-activated Ca^{2+} currents (HVCCs), in MD neurons acutely dissociated from wild-type or PLCβ4$^{-/-}$ mice. Membranes were voltage clamped at -60 mV and stepwise hyperpolarizing voltages (from -100 mV to -40 mV) were applied, followed by a depolarizing (-40 mV) potential change (Fig. 14a and 14b). In terms of total calcium current density, PLCβ4$^{-/-}$ neurons showed a significantly increased LVCC but a normal HVCC, compared with wild-type neurons (Fig. 14c, $P < 0.05$). Upon normalized stepwise current activation, PLCβ4$^{-/-}$ neurons also demonstrated a significant rise in LVCC (Fig. 14d, $P < 0.05$), but the HVCC did not differ from that of wild-type animals (Fig. 14e). These results suggest that increases in I_h and LVCC likely explain the rise in T-type channel-mediated burst firing in PLCβ4$^{-/-}$ MD neurons.

Inhibitory role played by T-type Ca^{2+} channels of the MD in fear extinction

The above observations on T-type Ca^{2+} channel-mediated cell properties caused us to hypothesize that a reduction of T-type current in the PLCβ4$^{-/-}$ MD might rescue the phenotype of impaired fear extinction. We tested this hypothesis by blocking T-type Ca^{2+} channel activities in the MD prior to evaluation of fear extinction. The MD of conditioned PLCβ4$^{-/-}$ mice was injected with a general T-type Ca^{2+} channel inhibitor, mibefradil, (Martin et al., 2000) 1 h before fear extinction learning (Fig. 15a). As CS were repeatedly delivered, mibefradil-injected PLCβ4$^{-/-}$ mice showed gradually reduced freezing levels ($F_{(19, 157)} = 17.19$, $P < 0.001$), whereas vehicle-injected PLCβ4$^{-/-}$ mice continued to show higher freezing levels than did the mibefradil-treated group (for group effect, $F_{(1, 13)} = 20.11$, $P < 0.001$; for group × trial interaction, $F_{(19, 247)}$

= 3.61, P < 0.001). On the next day, when the extinction recall test was performed, the mibefradil-treated group showed significantly lower freezing levels than did the vehicle group (P < 0.0001), indicating that mibefradil rescued the impaired extinction phenotype of PLCβ4$^{-/-}$ mice. More importantly, a similar mibefradil treatment facilitated the extinction process in wild-type mice. In Fig. 15b, mibefradil-injected wild-type mice showed a significantly faster reduction of freezing behavior upon extinction learning than did vehicle-injected wild-type animals (for group effect, $F_{(1, 17)}$ = 19.55, P < 0.001; for group × trial interaction, $F_{(19, 323)}$ = 2.48, P < 0.005). Next day, when the extinction recall test was run, the mibefradil group still exhibited somewhat reduced freezing behavior compared to the vehicle group (P < 0.05).

Mibefradil is also known to inhibit L-type Ca^{2+} channels, although at a lower efficiency compared to the drug effect on T-type channels (Martin et al., 2000). To rule out the possibility that the extinction-promoting effects of mibefradil were attributable to blocking of L-type Ca^{2+} channels, conditioned wild-type mice were injected with nifedipine, a specific L-type Ca^{2+} channel inhibitor, that does not affect T-type channels. MD injections were given 1 h before extinction learning commenced. Nifedipine treatment had no significant effect on fear extinction (Fig. 16).

Finally, we examined whether T-type Ca^{2+} channels might be involved in consolidation of fear extinction, by injecting mibefradil into the MD immediately after extinction learning had concluded. The next day, when the extinction recall test was performed, no significant difference in freezing level between the mibefradil-treated and vehicle groups was apparent (Fig. 17). These data show that T-type Ca^{2+} channels are not essential for consolidation of fear extinction memory, but rather only for acquisition. The results suggest that an increase in the activity of T-type Ca^{2+} channels in the MD

influences the impaired extinction phenotype of PLCβ4$^{-/-}$ mice, and that such channels thus play an inhibitory role in fear extinction.

Changes in the dual firing modes (burst and tonic) of the MD during acquisition of fear extinction under urethane anesthesia

Previously, using rats under mild urethane anesthesia, neural activities in response to fear-conditioned stimuli were detected in the brain in response to sensory stimuli (Apergis-Schoute et al., 2005), (Cotillon and Edeline, 2000; McGinty and Grace, 2008). However, no study has previously examined the behavioral consequences of such treatment given to mice under urethane anesthesia. To test whether mice could learn fear extinction under urethane anesthesia, animals were divided, 24 h after conditioning, into three groups: one test and two controls. The test group of mice (the anesthesia-extinction group) was anesthetized with urethane (1.5 g/kg, i.p.), and exposed 2 h later to 20 CS without US. One control group (the anesthesia group) was similarly anesthetized but was not exposed to the extinction protocol. The other control group (the awake-extinction group) was exposed to the extinction process in the conventional manner, without anesthesia (Fig. 18). Twenty-four hours after urethane injection, most mice (24 of 27) had fully recovered from anesthesia, and showed normal grooming and rearing behaviors. These animals were subjected to the extinction recall test. On this test, mice in the anesthesia-extinction group showed less freezing when the CS tone was delivered compared to those in the anesthesia group without extinction (group effect, $F_{(1, 22)} = 35.4$, $P < 0.001$, each tone $P < 0.01$; *post-hoc*). Interestingly, the extent of reduction in the freezing response of the anesthesia-extinction group was comparable to that in the awake-extinction group (for group effect, $F_{(1, 20)} = 1.64$, $P = 0.21$). These results show that fear extinction can occur in the mouse under urethane anesthesia.

To examine the firing properties of MD neurons during extinction learning, we recorded single-unit activities from the MD of conditioned animals exposed to extinction conditions (Fig. 19, i.e., CS without US), under mild urethane anesthesia. One group of wild-type mice was conditioned three times with CS-US co-terminated stimuli (paired wild-type) and another group of wild-type animals was exposed to three CS that were not paired with US (unpaired wild-type); in the latter group, auditory-conditioned fear memory was not induced (Fig. 20). Upon recording 24 h after conditioning, paired wild-type mice showed significantly more CS-responsive MD neurons than did unpaired wild-type animals (Table 1, 77 *vs*. 21% of recorded neurons, χ^2 =16.37, $P < 0.0001$). Paired PLCβ4$^{-/-}$ mice also showed numbers of CS-responsive MD neurons (70% of recorded neurons) comparable to those of paired wild-type animals (Table 1, χ^2 =0.28, $P = 0.6$), indicating that fear-associated stimuli induced vigorous responses in MD neurons. Therefore, we compared CS-evoked firing patterns between paired wild-type and PLCβ4$^{-/-}$ MD neurons.

First, single unit activities were examined to distinguish tonic firing from low-threshold burst firing, according to the criteria described in Methods. Analysis of baseline firing properties revealed the existence of two kinds of cells, neurons firing both in the burst and tonic modes (dual-mode neurons, Fig. 19b) and neurons firing only in the tonic mode (Fig. 19c). In the following analysis, we first treat the two neuron populations separately; data on dual-mode neurons are presented in Fig. 23, and results from tonic mode neurons appear in Fig. 26.

In dual-mode neurons, the baseline frequencies of both burst firing (Fig. 23a) and tonic firing (Fig. 23b) events were significantly higher in PLCβ4$^{-/-}$ neurons than in wild-type cells ($P < 0.05$ for each comparison). The number of spikes in a burst event

was also significantly greater in mutant than in wild-type cells (Fig. 24; $P < 0.05$), a finding consistent with the patch-clamp data in Fig. 13c. The combined results of these two changes caused the total number of spikes associated with burst events to be significantly higher in PLCβ4$^{-/-}$ neurons than in wild-type cells (Fig. 25; $P < 0.05$). In response to extinction tones, the number of burst events increased from baseline levels in animals of both genotypes (Fig. 23c left; $P < 0.05$ in the paired t-test). However, the total number of burst events was still significantly higher in PLCβ4$^{-/-}$ neurons than in wild-type cells (Fig. 23a; $F_{(1, 21)} = 5.29$, $P = 0.032$). However, in contrast to these burst firing properties, a dramatic difference was evident in CS-evoked tonic firing responses between the two genotypes (Fig. 23c right; $P < 0.001$). Whereas wild-type neurons showed a significant increase in tonic firing over baseline ($P < 0.005$), PLCβ4$^{-/-}$ neurons showed the opposite response, thus a significant fall in tonic firing ($P < 0.05$).

In neurons firing only in the tonic mode, the baseline firing frequency was similar in PLCβ4$^{-/-}$ and wild-type MDs (Fig. 26a). However, when exposed to the extinction tone, the wild-type and PLCβ4$^{-/-}$ MD neurons showed reciprocal responses, that is, increased firing of wild-type but decreased firing of mutant cells (Fig. 26a; group effect, $F_{(1, 21)} = 12.59$, $P = 0.002$; Fig. 26b; $P < 0.01$). This finding was similar to what was observed in the study of dual-mode cells described above. When tonic firings from all responsive neurons were combined and analyzed, wild-type neurons showed a significant increase in tonic firing frequency above baseline throughout the whole period of extinction (Fig. 27, $F_{(5, 85)} = 12.76$, $P < 0.001$), whereas PLCβ4$^{-/-}$ neurons showed a persistent reduction in firing frequency, to below baseline, under the same conditions (Fig. 27, $F_{(5, 105)} = 3.07$, $P < 0.05$).

These results revealed a positive correlation between the increased frequency of

tonic firing of MD neurons during extinction tone exposure and the development of fear extinction in animals. Also, the results raise the possibility that stronger bursting may contribute to impaired extinction in PLCβ4$^{-/-}$mice.

Changes in thalamic firing pattern during extinction in freely moving mice

To examine the firing properties of MD neurons during extinction learning, mice implanted with chronic unit-recoding electrodes in the MD were conditioned, and 24 h later, were assessed in extinction learning. 19 neurons from 15 wild-type mice and 7 neurons form 7 PLCβ4$^{-/-}$ mice were recorded in the MD (Fig. 28a). Single unit activities were examined to distinguish tonic firing from low-threshold burst firing, according to the criteria described in Methods (Fig 28b, Fig. 29).

The baseline frequency of tonic firing was similar in the wild-type and PLCβ4$^{-/-}$ MDs (Fig. 30a; $P > 0.2$, 11.3 ± 1.6 Hz versus 7.8 ± 1.8 Hz). However, when exposed to the extinction tone, significant difference was noted between the two groups (Fig. 30b, $F_{(1, 24)} = 5.94$, $P < 0.05$). Whereas wild-type neurons showed a significant increase in tonic firing over baseline ($F_{(5, 90)} = 6.39$, $P < 0.0001$), PLCβ4$^{-/-}$ neurons showed no change ($F_{(5, 30)} = 0.62$, $P = 0.68$). When tonic firings from all neurons including the mutant and wild-type were analyzed against the degree of extinction learning, the amount of increase in tonic firing during the extinction learning was positively correlated with the extinction level measured in the extinction recall test done 24-hr later (Fig. 30c, $r^2 = 0.45$, $P < 0.001$). The more increase in tonic firing during extinction learning, the more efficient extinction resulting in less freezing in the extinction recall test.

During the baseline period before extinction tone, 14 out of 19 wild-type neurons showed burst firing, whereas all the 7 mutant-type neurons showed burst firing. Interestingly, the baseline frequencies of the spikes associated with burst events were significantly higher in PLCβ4$^{-/-}$ neurons than in wild-type neurons (fig. 31a; $P < 0.05$, 0.4 ± 0.2 Hz versus 0.019 ± 0.004 Hz), a finding relevant to those in the slice experiments above (Fig. 13). In response to extinction tones (Fig. 31b), a significant increase in burst firing over baseline was evident in the mutant ($F_{(5, 30)} = 3.94$, $P < 0.01$) whereas no such increase was shown in the wild-type. As a result, the frequency of burst firing during the extinction was dramatically higher in PLCβ4$^{-/-}$ neurons than in wild-type neurons (Fig. 31b, $F_{(1, 20)} = 35.35$, $P < 0.001$). In other words, the mutant MD neurons showed enhanced burst firing both in baseline and in extinction.

These results revealed, on one hand, a positive correlation between the increased frequency of tonic firing of MD neurons during extinction learning and the development of fear extinction in animals. Furthermore, they also suggest a possibility that an increased burst firing on top of the impaired tonic firing may have contributed to the impaired extinction phenotype in the PLCβ4$^{-/-}$ mice.

Opposite effects of the two contrasting modes of firing on fear extinction; electrical stimulation of the MD during extinction learning

The positive correlation between increased tonic firing of MD neurons and fear extinction encouraged us to hypothesize that the positive change in tonic firing of the MD might be physiologically linked to the learning process of fear extinction. To explore such a possibility, the MDs of fear-conditioned wild-type and PLCβ4$^{-/-}$ mice

were electrically stimulated to increase tonic firing during fear extinction learning. To this end, 100-ms trains of low-density (100 Hz) current mimicking tonic spikes were delivered within the MD at 1 sec or 3 sec intervals during the 30 seconds of continuous tone (paired-stimulated mice). We confirmed that, in brain slices, such train stimulation induced 100-Hz-tonic spikes in adjacent MD neurons (Fig. 32a). As controls, unpaired-stimulated mice received the same level of stimulation during the inter-tone intervals, and non-stimulated mice received only tones without any electrical stimulation. As shown in Fig. 33, paired-stimulated PLCβ4$^{-/-}$ mice showed a more rapid decrease in freezing level than did the two control PLCβ4$^{-/-}$ animals, throughout the whole period of extinction challenge ($F(2, 24) = 21.91$, $P < 0.001$) Interestingly, a dose-dependent fall in freezing levels was observed in the paired-stimulated group (for the 1 sec train vs. the 3 sec train, $F(1, 17) = 6.71$, $P = 0.019$; for group × trial interaction, $F(9, 159) = 3.02$, $P = 0.002$), whereas unpaired-stimulated PLCβ4$^{-/-}$ mice did not show any difference in freezing level compared to non-stimulated PLCβ4$^{-/-}$ animals ($P > 0.2$). In the extinction recall test performed on the following day, paired-stimulated PLCβ4$^{-/-}$ mice still showed significantly reduced freezing compared to that in the two control groups ($F(3, 36) = 7.1$, $P < 0.001$; post-hoc). These results indicate that tonic-evoking electrical stimulation of the MD, when paired with tones, facilitates extinction learning, and suggest that the decrease of tonic firing in MD neurons during acquisition is responsible for the impaired extinction phenotype of PLCβ4$^{-/-}$ mice. In addition, we observed that tonic-evoking stimulation MD neurons facilitated extinction in wild-type animals (blue symbols, Fig. 33). Thus, paired-stimulated wild-type mice showed a significantly faster reduction of freezing compared to that seen in the first tone trial ($P < 0.001$; post-hoc) and continued to show significantly reduced freezing ($F(2, 24) = 15.97$, $P < 0.001$) in both the extinction learning and extinction recall tests ($F(2, 25) = 7.96$, $P < 0.005$).

These results suggest that an increase of tonic firing in the MD is necessary for acquisition of fear extinction.

To explore a possible causal relationship between an enhanced T-type-dependent burst firing and extinction failure in PLCβ4$^{-/-}$ mice, MDs of fear-conditioned wild-type mice were electrically stimulated with a protocol mimicking burst spikes during extinction learning (Fig. 32b, 416 Hz, 6-msec (square symbols) or 12-msec trains (diamond symbols)). As shown in Fig. 34, wild-type mice stimulated by burst-evoking trains paired with tones showed a significantly slower reduction of freezing levels compared to that of control groups, in the extinction learning phase (for group effect, $F(4, 42) = 23.51$, $P < 0.001$; for paired burst-evoking trains vs. unpaired burst-evoking trains, $t(15) = 3.41$, $P = 0.015$; post-hoc; for group × trial interaction, $F(36, 378) = 1.86$, $P = 0.002$). In addition, extinction recall tests showed a lower reduction in freezing level in the former group than in the other groups ($F(4, 41) = 12.23$, $P < 0.001$; $P < 0.05$ for the two respective comparisons; post-hoc). These results show that burst-evoking stimulation of MD neurons mimicked the PLCβ4$^{-/-}$ mutation, and suggest that burst spikes suppress fear extinction, in contrast to the facilitatory role played by tonic firing.

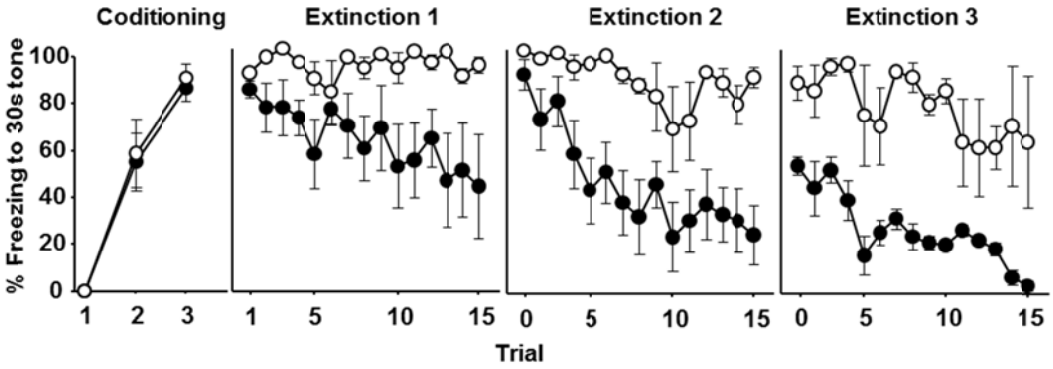


Figure 1. Defective extinction of auditory-conditioned fear memory in high shock-conditioned PLCβ4$^{-/-}$ mice.

Mice were conditioned with 0.7 mA foot-shocks (left). Although in each day of 3 days after conditioning, PLCβ4$^{-/-}$ mice showed impaired extinction learning and poor recall of extinction on the next day, the wild-type showed slow extinction learning. Filled circle indicates wild-type neurons (n = 4) and empty circle indicates PLCβ4$^{-/-}$ neurons (n = 5). mean ± s.e.m

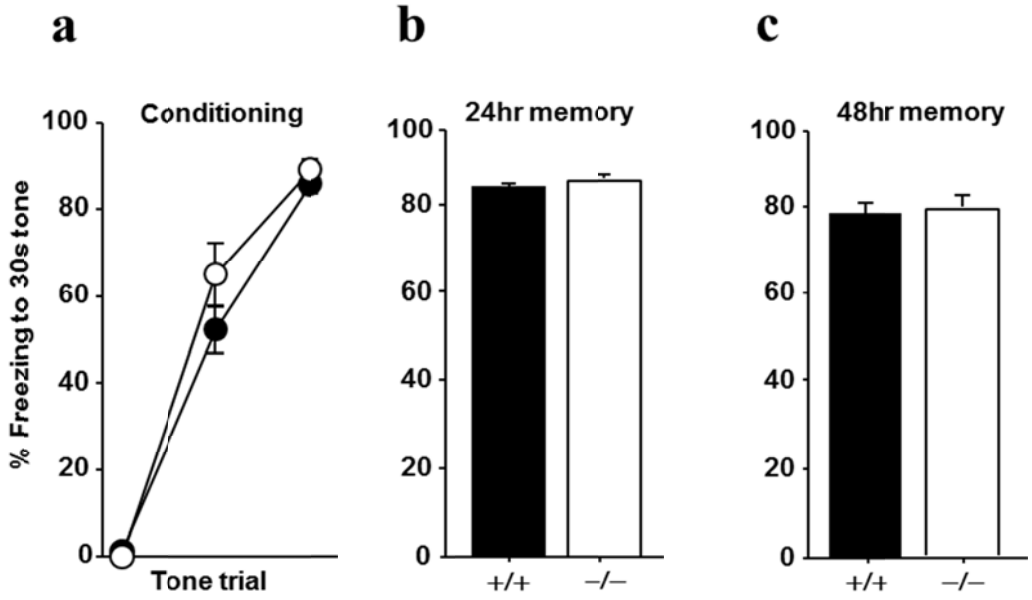


Figure 2. Auditory fear conditioning and memory formation in high shock-conditioned PLCβ4$^{-/-}$ mice.

PLCβ4$^{-/-}$ mice showed normal acquisition of auditory-conditioned fear with 0.5 mA foot-shocks (**a**), and comparable (to wild-type) long-term memory of auditory-conditioned fear 24 hr (**b**) or 48 hr (**c**) after conditioning. n = 6-18 per group. +/+, wild-type mice; –/–, PLCβ4$^{-/-}$ mice. mean ± s.e.m.

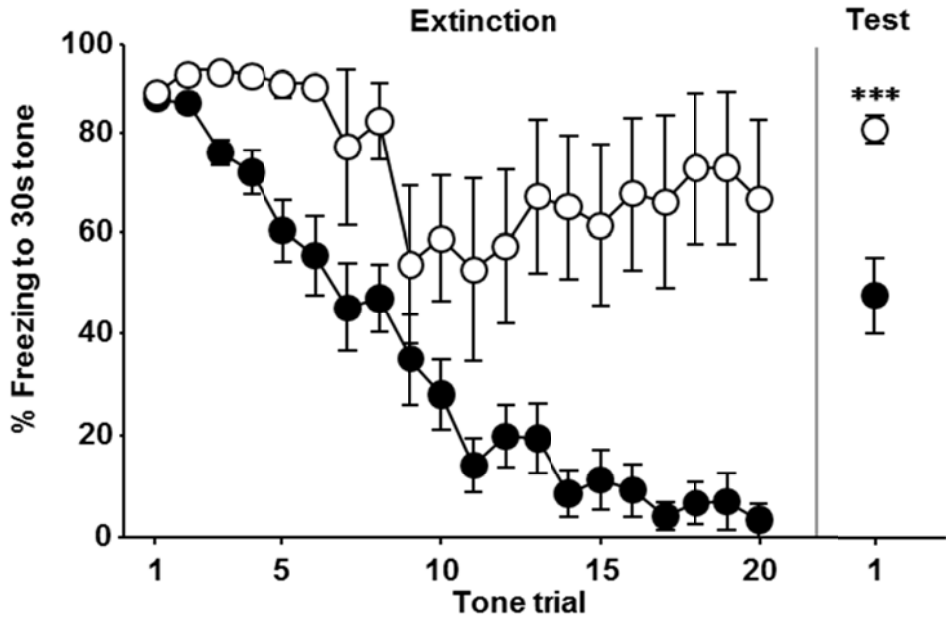


Figure 3. Defective extinction of auditory-conditioned fear memory in PLCβ4$^{-/-}$ mice.

PLCβ4$^{-/-}$ mice were impaired in extinction learning of auditory fear memory 24 hr after conditioning fear with 0.5 mA foot-shocks, and next day, showed higher levels of freezing in extinction recall test. n = 6-18 per group. Filled circle, wild-type mice; empty circle, PLCβ4$^{-/-}$ mice. mean ± s.e.m. ***, $P < 0.001$.

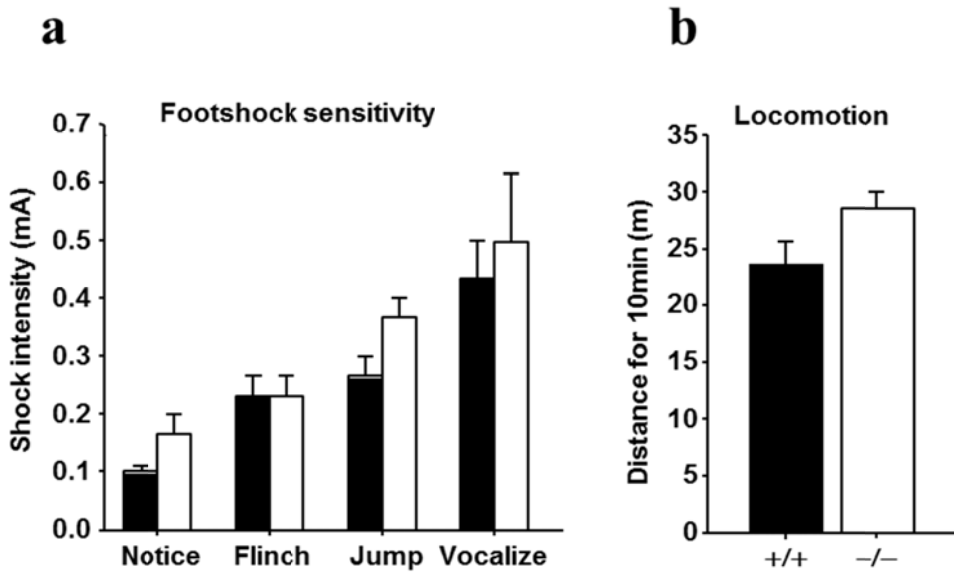


Figure 4. Shock sensitivity and locomotion in PLCβ4$^{-/-}$ mice.

The two groups displayed similar levels of footshock sensitivity (**a**), locomotor activity in the fear extinction chamber without the tone (**b**). n = 6-18 per group. +/+, wild-type mice; –/–, PLCβ4$^{-/-}$ mice. mean ± s.e.m.

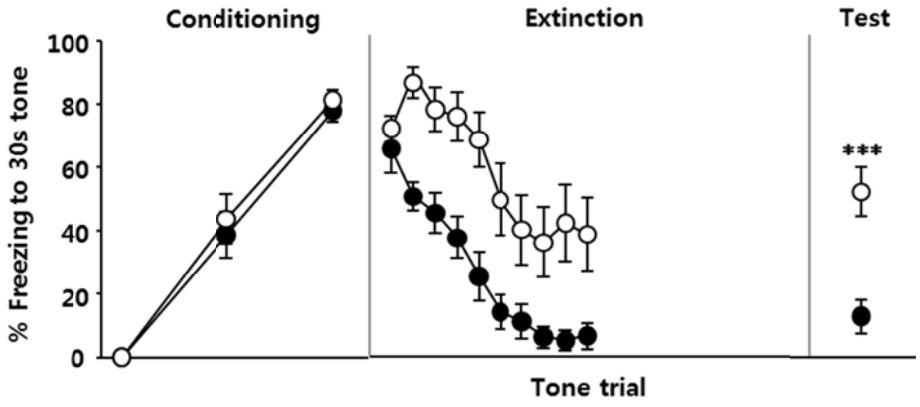


Figure 5. Defective extinction of auditory-conditioned fear memory in low shock-conditioned PLCβ4$^{-/-}$ mice.

Mice were conditioned with 0.3 mA foot-shocks (left). Twenty-four hours thereafter, PLCβ4$^{-/-}$ mice showed impaired extinction learning (middle, $F_{(1, 16)} = 8.54$, $P < 0.01$), and poor recall of extinction on the next day (right, $P < 0.001$). Filled circle indicates wild-type (n = 9) and empty circle indicates PLCβ4$^{-/-}$ (n = 12). mean ± s.e.m. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

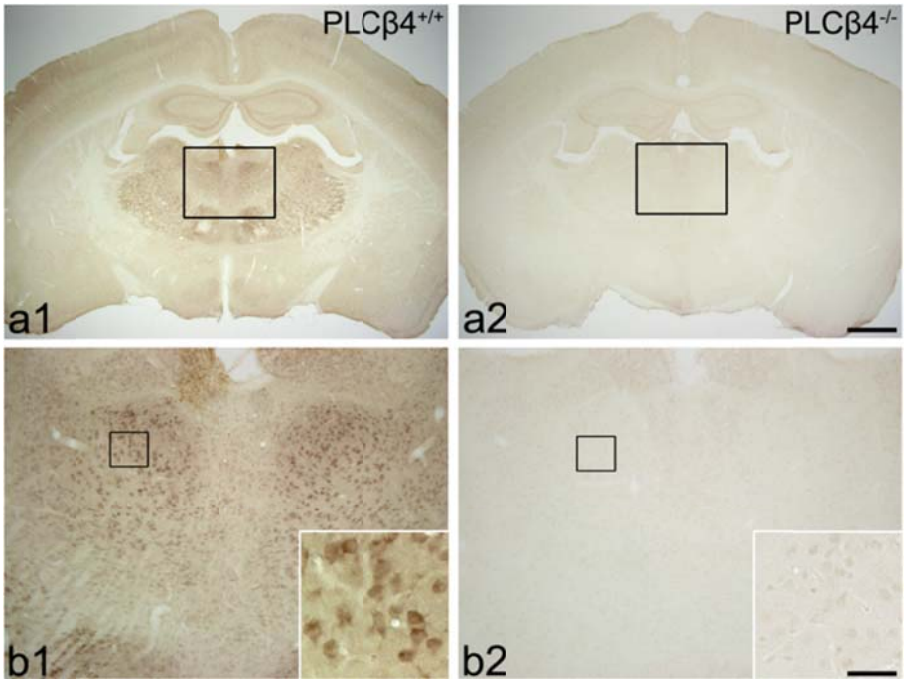
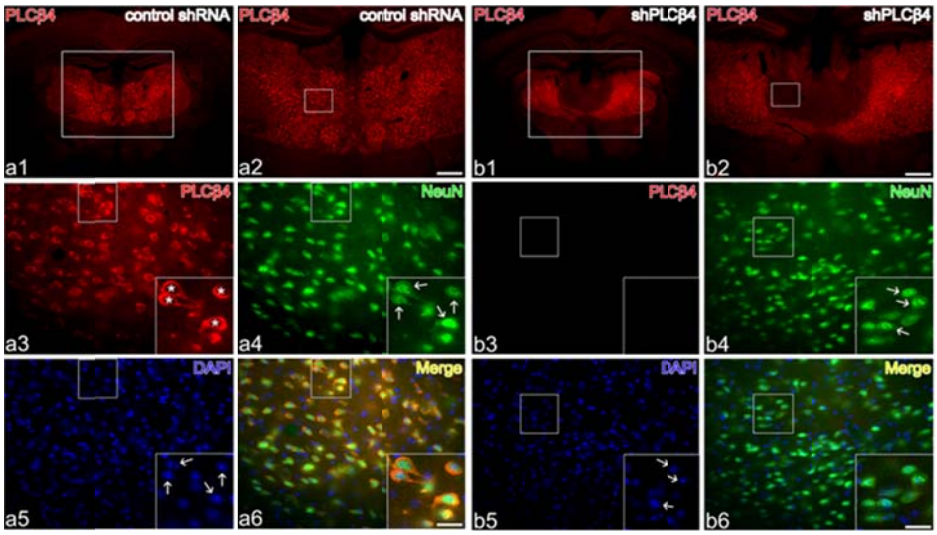


Figure 6. PLCβ4 expression in MD thalamic area of wild-type and PLCβ4$^{-/-}$ mice.

Specific immunohistochemical staining for PLCβ4 is primarily observed in the entire thalamus except reticular thalamic neurons (nRT) of wild-type mice. **F**ew background staining for PLCβ4 is detected in both the wild-type (**a1**) and PLCβ4$^{-/-}$ mouse (**a2**). Rectangles in a1 and a2 indicate regions of b1 and b2 respectively. PLCβ4-positive neurons in MD thalamic areas are mainly detected in wild-type mice (**b1**), compare to PLCβ4$^{-/-}$ mice (**b2**). Rectangles in **b**1 and b2 indicate high magnification of right bottom. Scale bars are 560 um (a1 and a2), 100 um (b1 and b2) and 25 um (high magnification in b1 and b2).



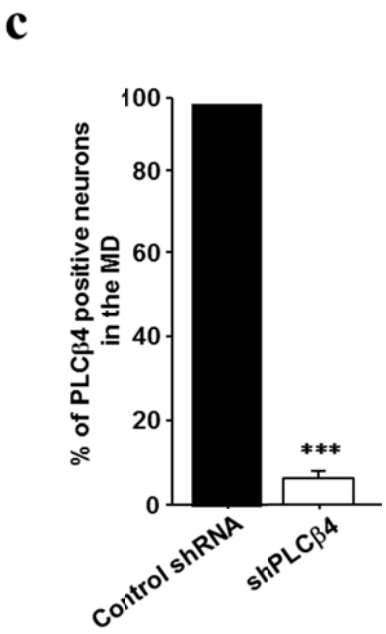


Figure 7. Effect of PLCβ4 knockdown on the PLCβ4 expression in the MD using lentivirus.

Double immunofluorescence staining for PLCβ4 expression within NeuN-positive

neurons in both control shRNA-injected MDs (a1-a6) and in shPLCβ4 lentiviral vector-injected MDs (b1-b6). The rectangles in a1 and b1 indicate subregions of a2 and b2, respectively, and the rectangles in a2 and b2 delineate subregions of a3-a6 and b3-b6, respectively. Asterisks in the high-magnification area of a3 indicate PLCβ4- positive neurons in the control shRNA group. Rectangles in a3-a6 and b3-b6 show high-magnification regions of the right area of each panel. **c**, The numbers of PLCβ4-positive neurons in the MDs of the shPLCβ4 group were significantly reduced compared with those in control shRNA animals. Scale bar: a1 and b1, 560 μm; a2 and b2, 280 μm; a6 and b6, 25 μm; rectangles within a6 and b6, 12.5 μm. Data are means ± SEMs. **, $P < 0.01$; ***, $P < 0.001$.

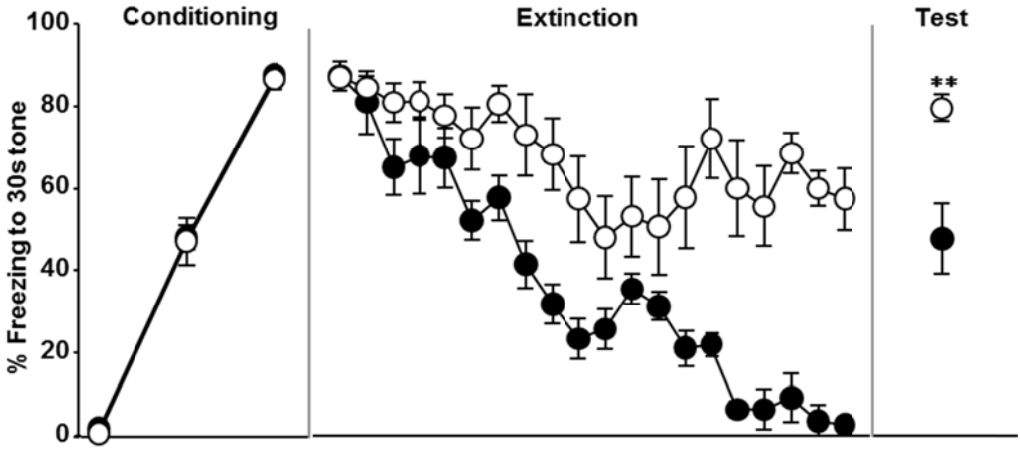


Figure 8. Effect of PLCβ4 knockdown in the MD on fear extinction using lentivirus.

shPLCβ4 animals (empty circles, n = 9) showed normal acquisition of auditory-conditioned fear, but higher levels of freezing in extinction learning and recall tests, compared with control wild-type mice (filled circles, n= 7). Data are means ± SEMs. **, $P < 0.01$; ***, $P < 0.001$.

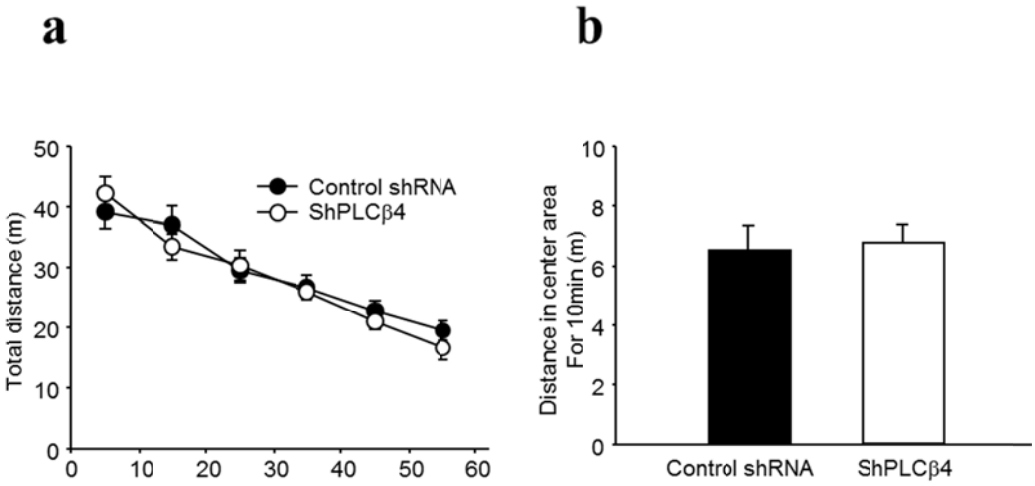


Figure 9. Effect of PLCβ4 knockdown in the MD on open field test using lentivirus.

There was no significant difference in total distance of spontaneous locomotion in an open field for 1hr (**a**) and in distance of locomotion in the center area for first 10min (**b**). n = 6 per group. mean ± s.e.m.

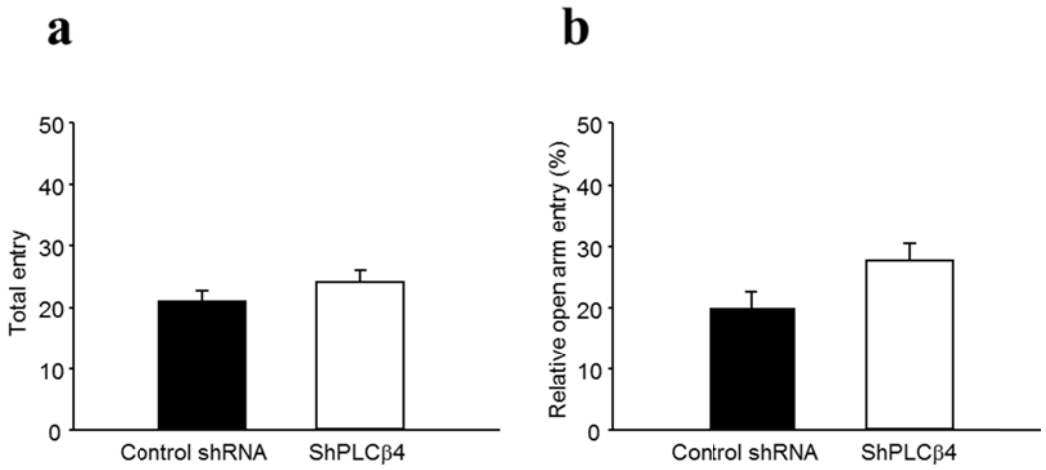


Figure 10. Effect of PLCβ4 knockdown in the MD on elevated pus maze test using lentivirus.

There was no significant difference in number of total entry into the open arms and closed arms (**a**) and in relative open arm entry to total arm entries (**b**). n = 6 per group. mean ± s.e.m.

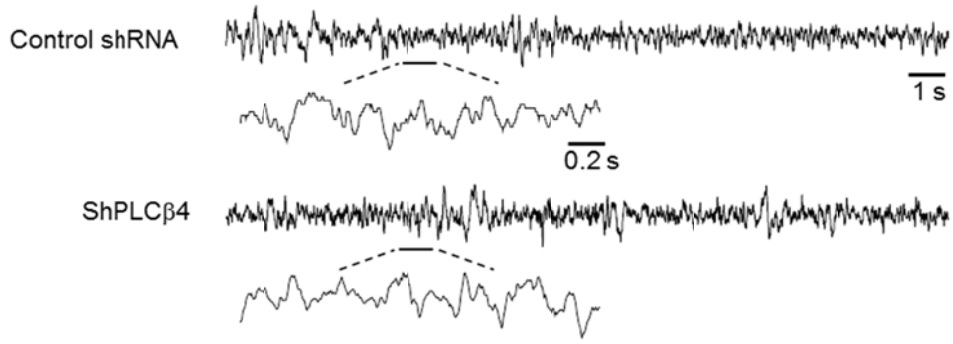


Figure 11. Effect of PLCβ4 knockdown in the MD on spontaneous spike-wave discharges using lentivirus.

Spontaneous spike-wave discharges (3~7Hz) in EEG were not observed in MD for 10min. n = 6 per group. mean ± s.e.m.

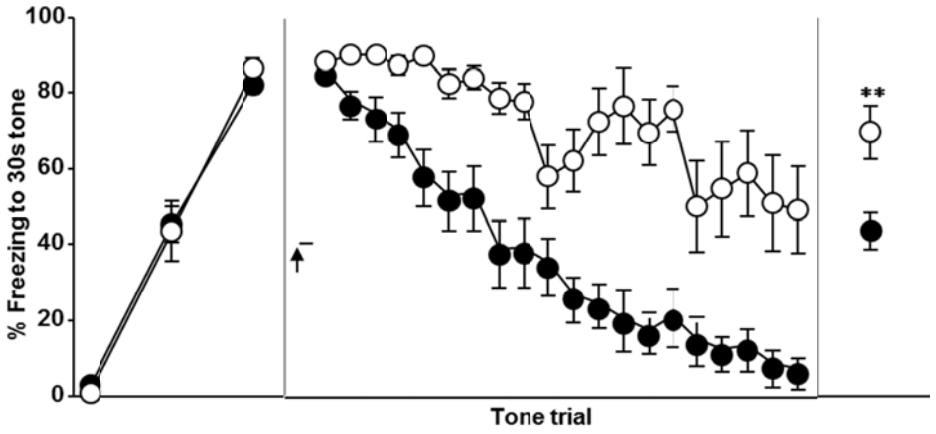


Figure 12. Blockade of the mediodorsal thalamic mGluR1-PLCβ4 pathway inhibits fear extinction.

Intra-MD CPCCOEt (60 mM) infusion (empty circles, n = 8) 30 min before extinction learning induced higher levels of freezing in both the extinction learning and recall tests (conducted on the next day), compared with animals receiving intra-MD vehicle injection (filled circles, n = 14). ↑, injection timepoints. Data are means ± SEMs. **, $P < 0.01$; ***, $P < 0.001$.

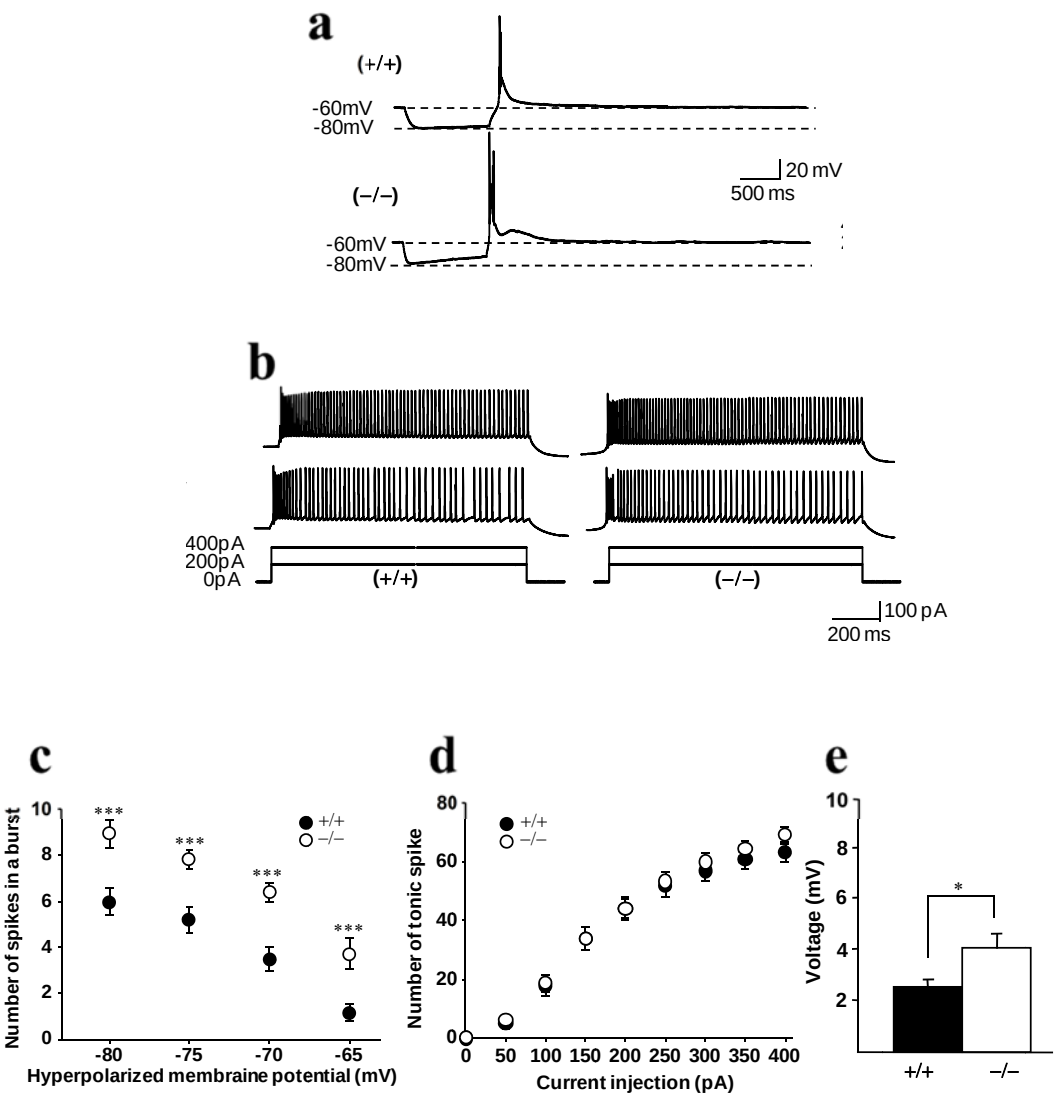


Figure 13. Intrinsic firing property of the MD neurons.

Low-threshold burst spikes induced by stepwise hyperpolarizing current injection (**a**) were significantly more robust (than wild-types) in PLCβ4$^{-/-}$ slice neurons (**c**). Tonic spikes induced by stepwise depolarizing current injection (**b**) were elicited equally in wild-type and PLCβ4$^{-/-}$ slice neurons (**d**). +/+, wild-type mice; –/–, PLCβ4$^{-/-}$ mice. n = 10-19 per group. Means ± SEM. *, $P < 0.05$; ***, $P < 0.001$.

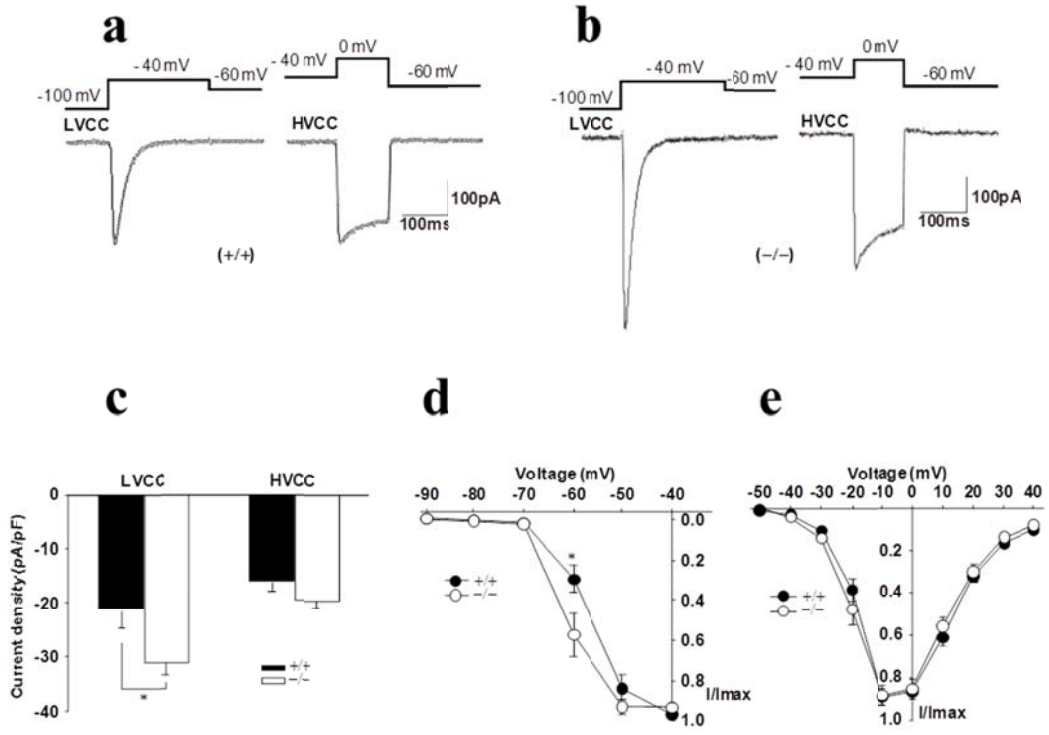


Figure 14. Voltage-activated Ca^{2+} currents in the MD neurons.

Low voltage-activated Ca^{2+} currents **(**LVCC) and high voltage-activated Ca^{2+} currents (HVCC) were measured in acutely isolated neurons of the wild-type (**a**) and PLCβ4$^{-/-}$ MD (**b**). PLCβ4$^{-/-}$ neurons showed increased LVCC but normal HVCC, compared with wild-type neurons, when raw values of LVCC and HVCC (**c**), normalized LVCC (**d**), and normalized HVCC (**e**), were compared. +/+, wild-type mice; –/–, PLCβ4$^{-/-}$ mice. n = 10-19 per group. Means ± SEM. *, $P < 0.05$; ***, $P < 0.001$.

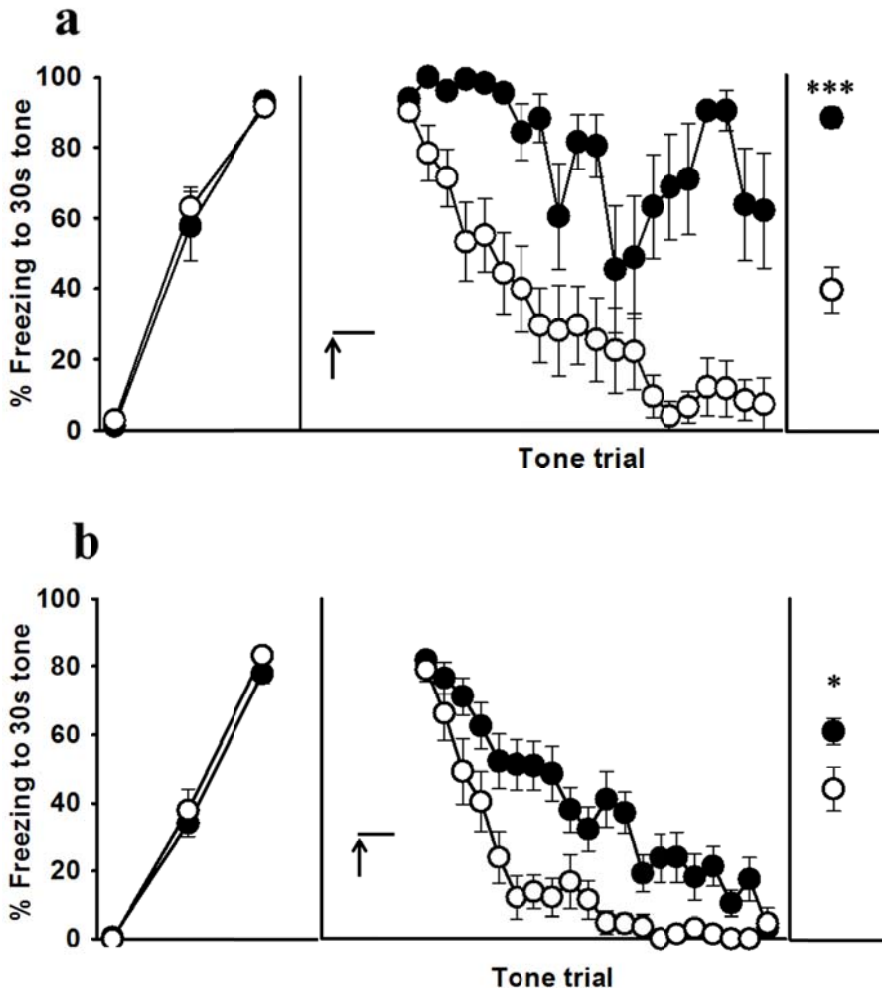


Figure 15. Accelerating effect of an intra-MD infusion of a T-type Ca^{2+} channel blocker on fear extinction learning.

Intra-MD mibefradil (4 mM) infusion 1 h before extinction learning commenced induced rescue of impaired extinction learning in PLCβ4$^{-/-}$ mice (a, empty symbols), and facilitation in wild-type animals (b, empty symbols). On the following day, extinction recall was significantly improved in both groups. ↑: injection timepoints. n =10–13 mice per group. Filled symbols refer to vehicle-injected PLCβ4$^{-/-}$ mice in (a) and wild-type animals in (b). Data are means ± s.e.m.*, $P < 0.05$; ***, $P < 0.001$.

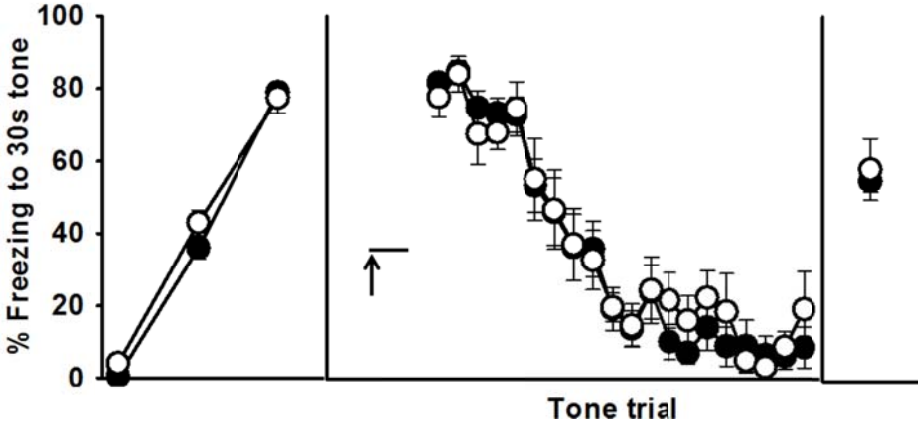


Figure 16. No effect of intra-MD infusion of L-type Ca^{2+} channel blocker on fear extinction learning.

Intra-MD nifedipine infusion (1 mM) did not have any effect in wild-type mice (empty symbol). n =10, 9 per group. ↑, injection time point. Filled symbol indicates vehicle-injected wild-type mice. mean ± s.e.m.

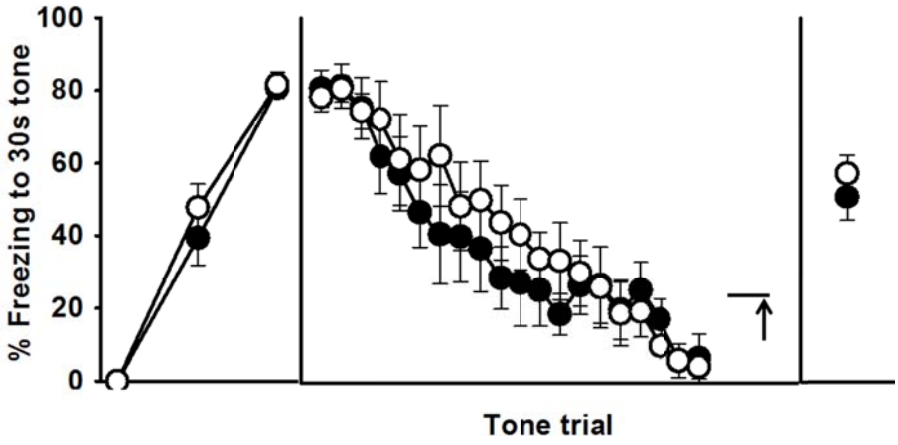


Figure 17. No effect of intra-MD infusion of T-type Ca^{2+} channel blocker on consolidation of extinction.

Intra-MD mibefradil infusion (4 mM) immediately after extinction learning did not have any effect on extinction recall in wild-type mice (empty symbol) on the next day. n = 9 per group. ↑, injection time point. Filled symbol indicates vehicle-injected wild-type mice. mean ± s.e.m.

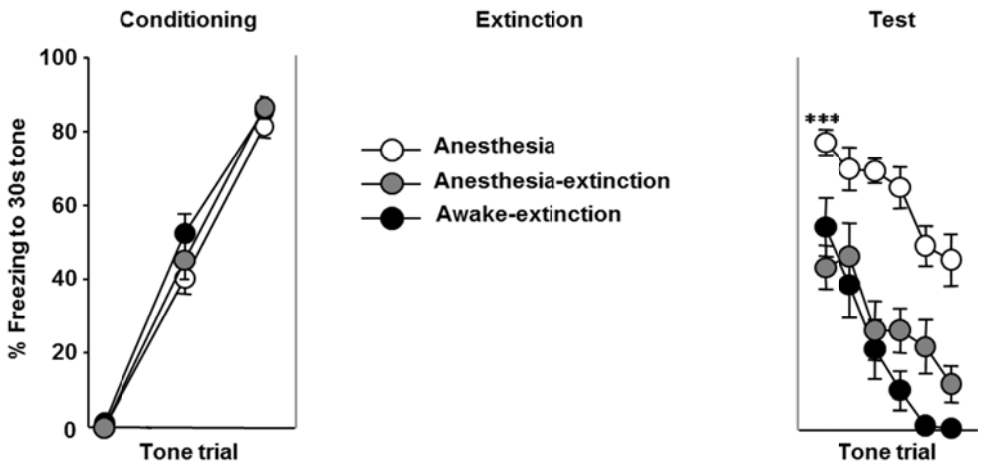


Figure 18. Fear extinction learning under urethane anesthesia.

27 mice were injected with urethane. Two hours later, they were divided into two groups, the anesthesia-extinction (n = 12) and the anesthesia groups (n = 15). In the next step, the anesthesia-extinction (n = 12) and the awake-extinction, i.e., naïve mouse, groups (n = 12) were exposed to CS. On the next day, the mice were subjected to extinction tests and were sacrificed immediately after the tests. The level of freezing in the anesthesia-extinction group was significantly reduced compared to that of the anesthesia group without extinction tone, and was indistinguishable from that of the awake-extinction group ($F_{(2, 33)} = 31.86$, $P < 0.001$; post-hoc). Means ± SEMs are shown. ***, $P < 0.001$.

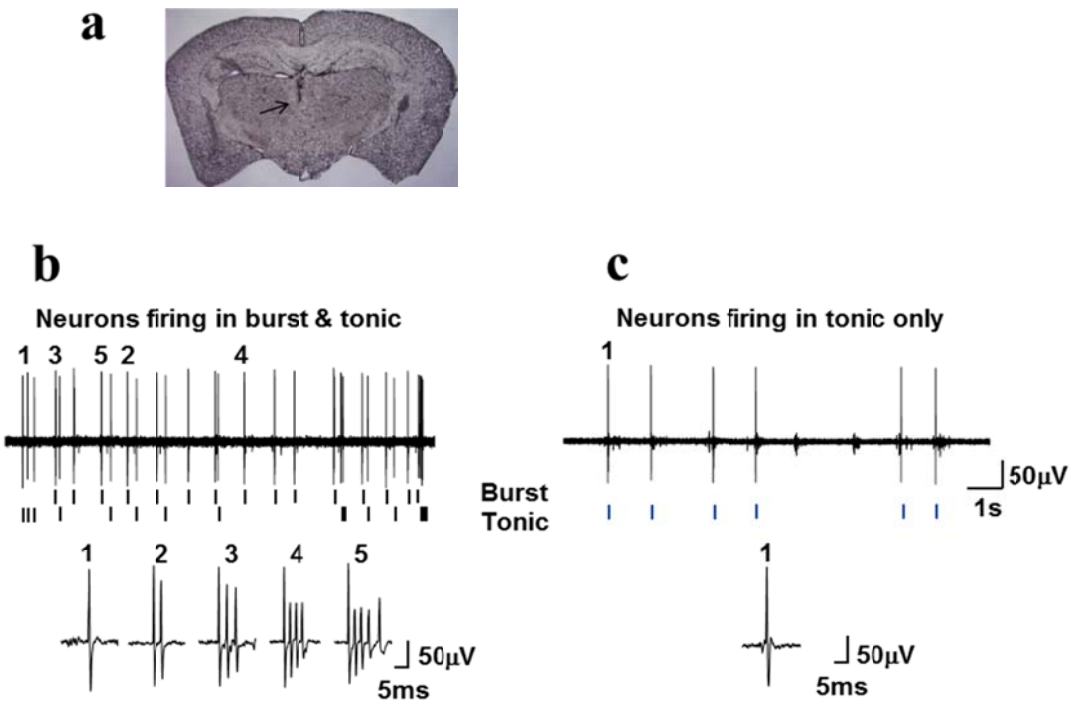


Figure 19. Two types of MD neurons exist; these are dual-mode and tonic mode neurons under urethane anesthesia.

a, coronal brain slice showing the position of the electrode tip in the MD. **b** and **c**, sample traces of single-unit recordings. The left panels show data from neurons firing in the dual mode (**b**, Burst and Tonic), and the right panels are the results from neurons firing only in the tonic mode (**c**, Tonic only). Vertical strokes below traces indicate either burst events (first line) or tonic spikes (second line). Expanded traces of sample burst or tonic spikes are shown at the bottom of the Figure. 1: tonic spike; 2, 3, 4, and 5: burst events consisting of two, three, four, or five spikes, respectively.

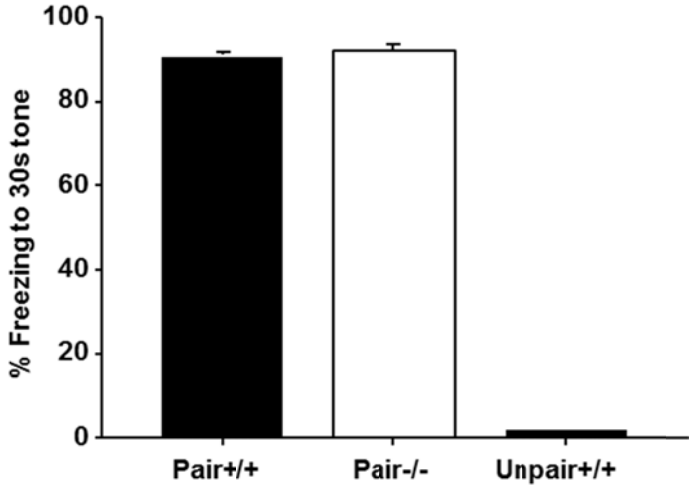


Figure 20. Comparison of fear memory formation after paired fear conditioning and unpaired fear conditioning.

For fear conditioning, wild-type (Pair +/+) and PLCβ4$^{-/-}$ mice (Pair –/–) were exposed to three tones that coterminated with foot-shocks (Fig. 1a), and the other wild-type mice (Unpair +/+) were exposed to the same three tones and three foot-shocks, which were not paired with the tones. Twenty-four hours after conditioning, unpaired +/+ mice showed very low freezing levels, compared with paired groups. n = 7 per group. mean ± s.e.m.

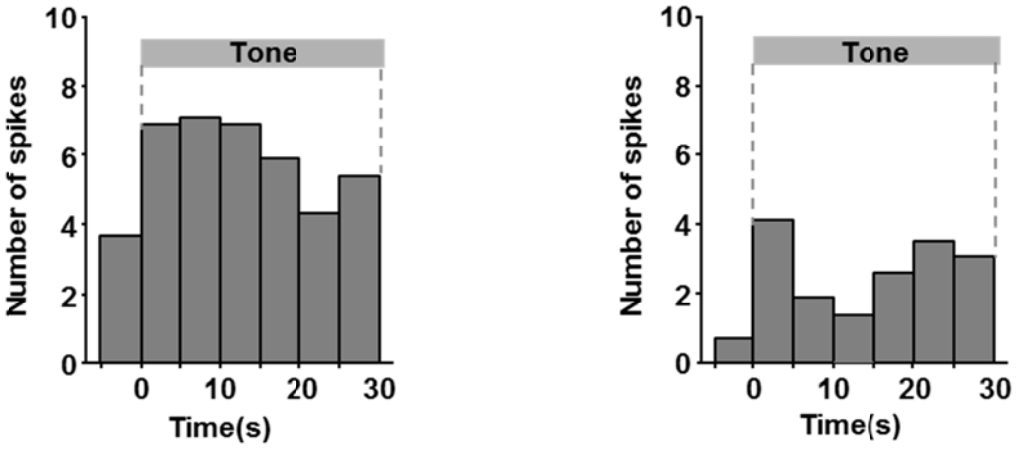


Figure 21. Conditioned tone response of representative neurons in MD.

a and **b**, peristimulation time histogram shows tone-elicited activity of a representative MD neuron firing in the dual mode (**a**) and only in the tonic mode (**b**). Trials 1-10 in extinction learning were averaged and 30s tone was divided into 6 bins (bin size = 5sec). Horizontal lines indicate 30 second tone stimulation.

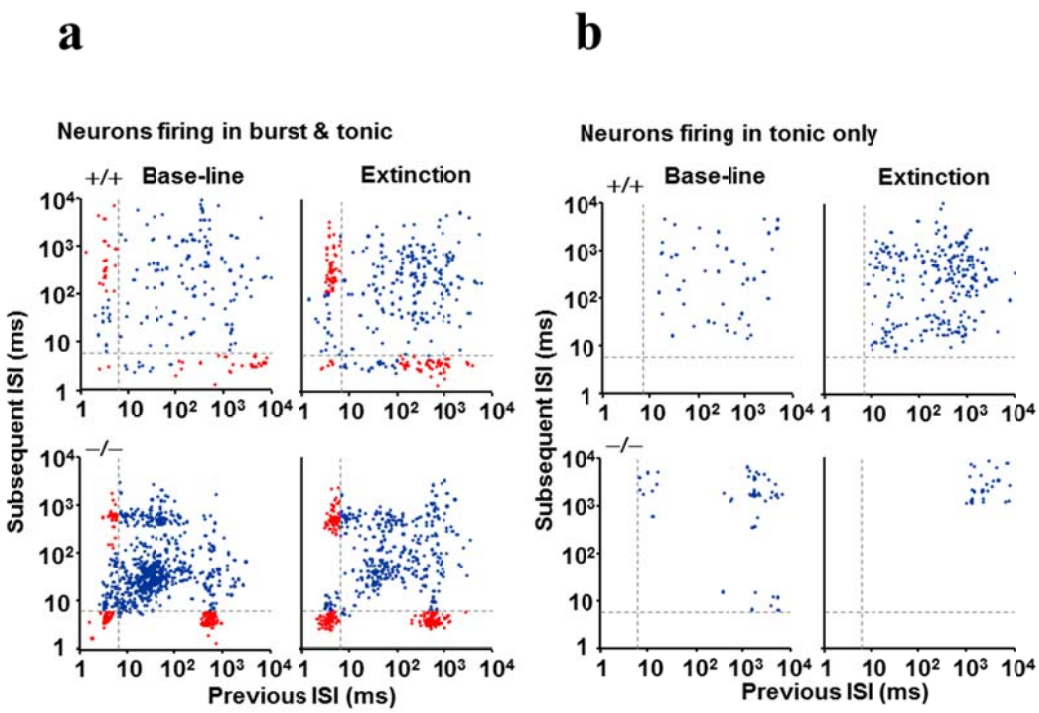


Figure 22. Change of firing patterns during extinction under urethane anesthesia.

Dotted lines indicate 6 msec intervals. All spikes (red spots, burst spikes; blue spots, tonic spikes) were analyzed in terms of interspike interval (ISI) before (X axis) and after (Y axis) stimulation, to obtain a baseline response over 120 sec (Baseline) and an extinction learning response from the 17th to the 20th tones in neurons firing in the dual mode (**a**), and in neurons exhibiting tonic firing only (**b**).

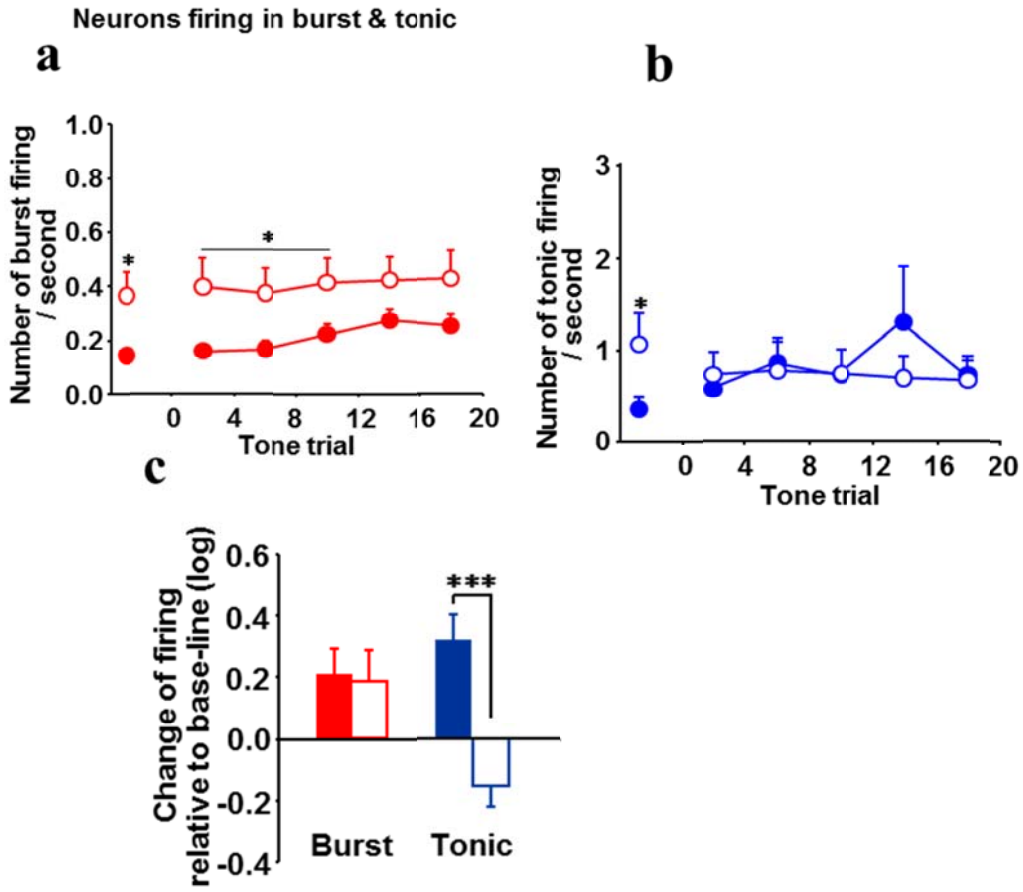


Figure 23. Changes in CS-evoked burst and tonic firing patterns of MD neurons in wild-type and PLCβ4$^{-/-}$ mice when extinction conditions were operative; neurons firing in the dual mode.

Average frequency of burst events (**a**) and tonic spikes (**b**) before CS (Baseline) were significantly higher in PLCβ4$^{-/-}$ mice ($P < 0.05$ for each comparison). Also, during fear extinction, the total number of burst events was higher in PLCβ4$^{-/-}$ animals. During extinction, burst and tonic firing characteristics changed (**c**). Each firing rate was normalized to baseline values and changes are shown on a log scale (**c**). Data are plotted in blocks of four-tone trials (**a** and **b**). Filled circles and filled bars indicate data from wild-type neurons (n = 11), whereas empty circles and empty bars refer to information from PLCb4$^{-/-}$ neurons 9n = 12). Means ± SEMs are shown. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

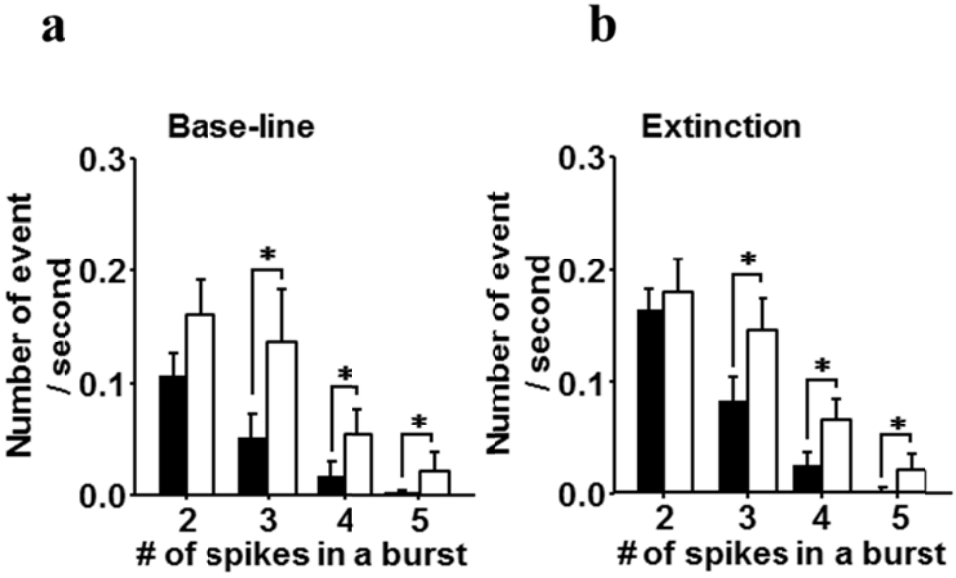


Figure 24. PLCβ4$^{-/-}$ MD neurons produce stronger bursts, i.e., bursts with more intra-burst spikes, compared to the wild type neurons.

The number of spikes per burst event was counted during the resting state before CS stimulation (**a**) and during CS stimulation (**b**). The PLCβ4$^{-/-}$ MD neurons generated significantly more burst events with more than three spikes, under extinction conditions, than did wild-type neurons. filled bar, wild type mice; empty bar, PLCβ4$^{-/-}$ mice. n = 11-12 per group. mean ± s.e.m. *, $P < 0.05$.

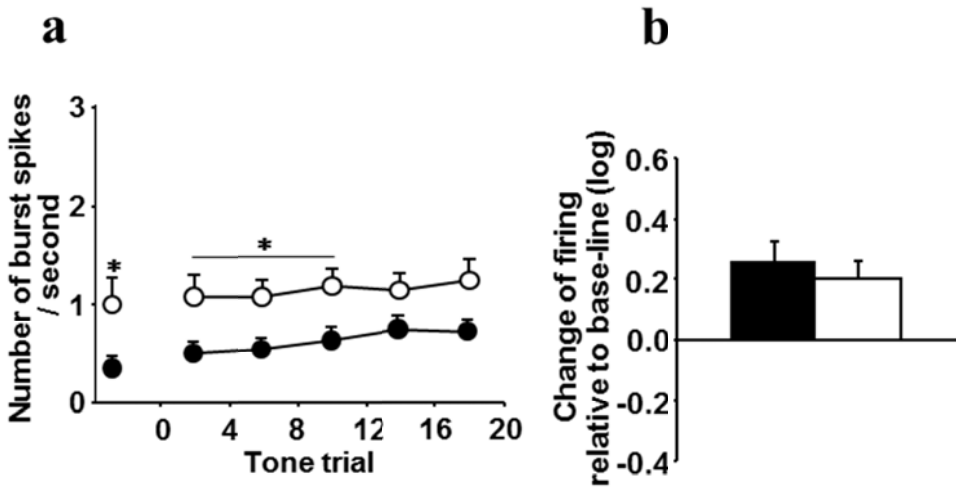


Figure 25. Changes in CS-evoked burst spikes pattern of MD neurons firing in the dual modes during extinction conditions.

a, Average frequency of spikes associated with burst events before the CS (base-line) was significantly higher in PLCβ4$^{-/-}$ mice ($P < 0.05$). During fear extinction, also, total number of burst spikes was significantly higher in PLCβ4$^{-/-}$ mice ($F_{(1, 21)} = 6.37$, $P = 0.02$). **b**, During extinction, burst firing was changed. Filled circle and filled bar indicate wild-type neurons (n = 11) and empty circle, and empty bar indicate PLCβ4$^{-/-}$ neurons (n = 12). mean ± s.e.m. *, $P < 0.05$.

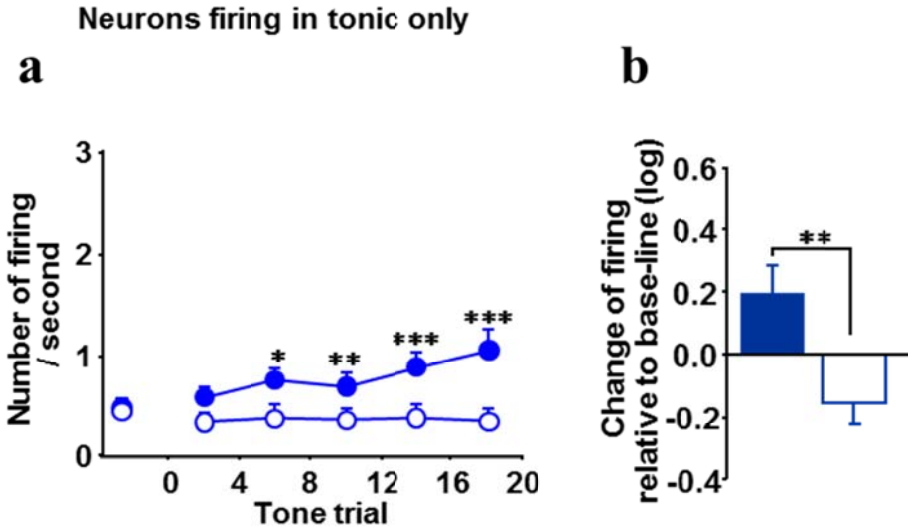


Figure 26. Changes in CS-evoked burst and tonic firing patterns of MD neurons in wild-type and PLCβ4-/- mice when extinction conditions were operative; Neurons firing in the tonic mode only (n = 10, 13 mice per group).

a, The average firing rate of tonic spikes before CS did not differ between animals of different genotype. Data are plotted in blocks of four-tone trials. **b**, During extinction, tonic firing varied reciprocally. Each firing rate was normalized to baseline values and changes are shown on a log scale. Filled circles and filled bars indicate data from wild-type neurons, whereas empty circles and empty bars refer to information from PLCβ4$^{-/-}$ neurons. Means ± SEMs are shown. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

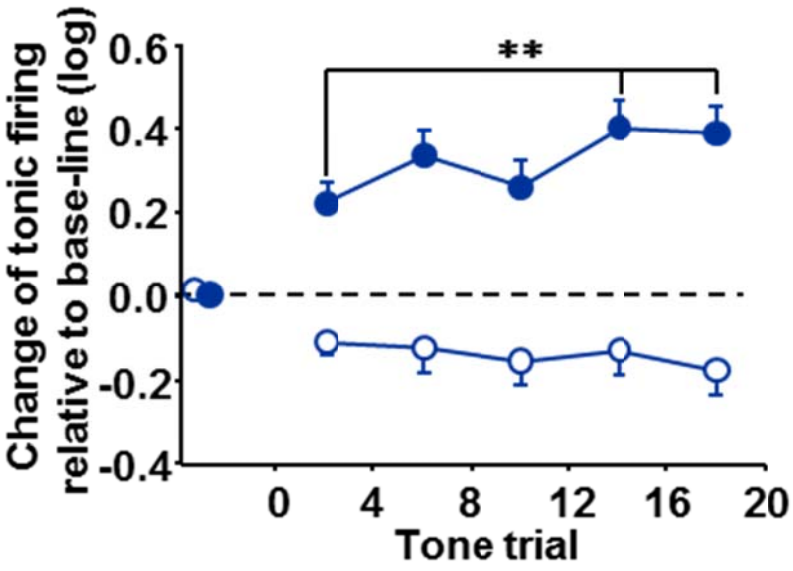


Figure 27. Change of tonic firing relative to base-line in all responsive neurons.

Tonic firing in all responsive neurons was significantly increased in the fourth and fifth CS blocks compared to the first block ($P < 0.01$). Each firing rate was normalized to baseline values and changes are shown on a log scale. Data are plotted in blocks of four-tone trials. Filled circles indicate data from wild-type neurons, whereas empty circles refer to information from PLCβ4$^{-/-}$ neurons. Means ± SEMs are shown. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

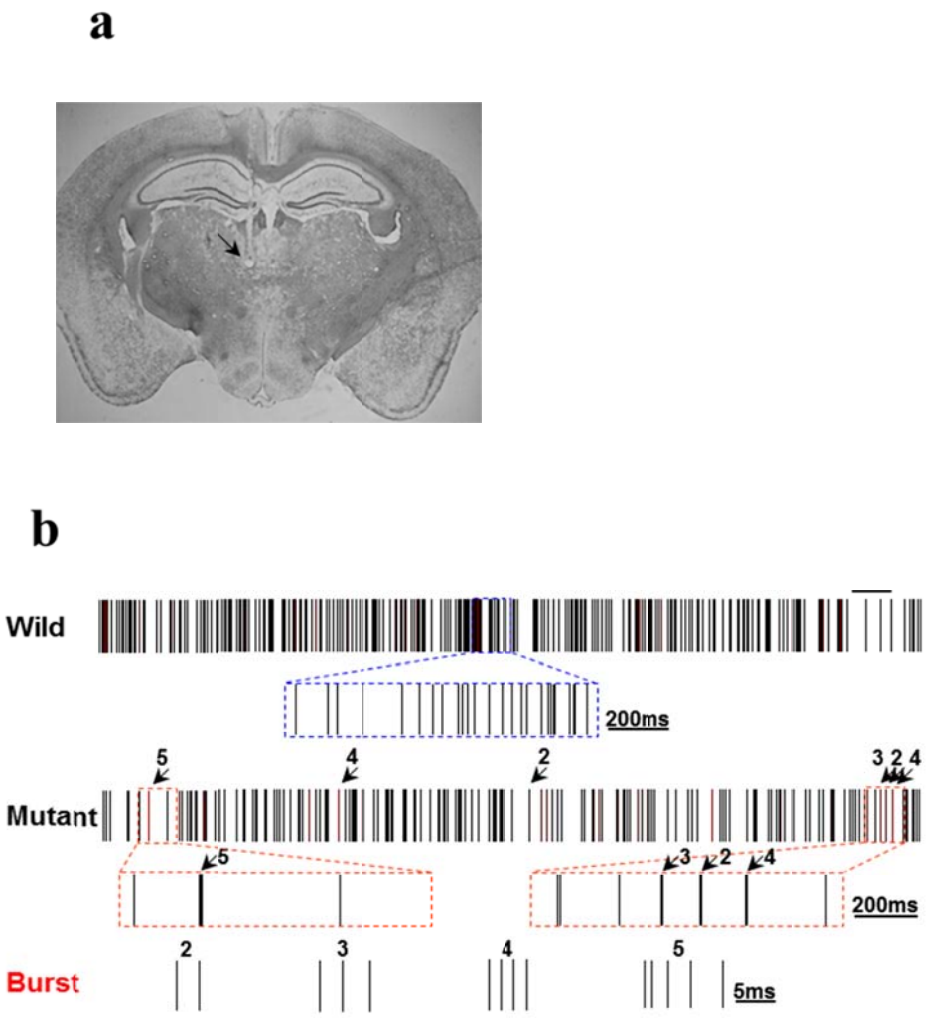


Figure 28. Sample trace of single unit recordings in freely moving wild-type and PLCβ4$^{-/-}$ mice during extinction learning.

a, Coronal brain slice showing the position of the electrode tip in the MD. **b**, Sample traces of single-unit recordings. Expanded traces of sample burst are shown at the bottom of the Figure. 2, 3, 4, and 5: burst events consisting of two, three, four, or five spikes, respectively.

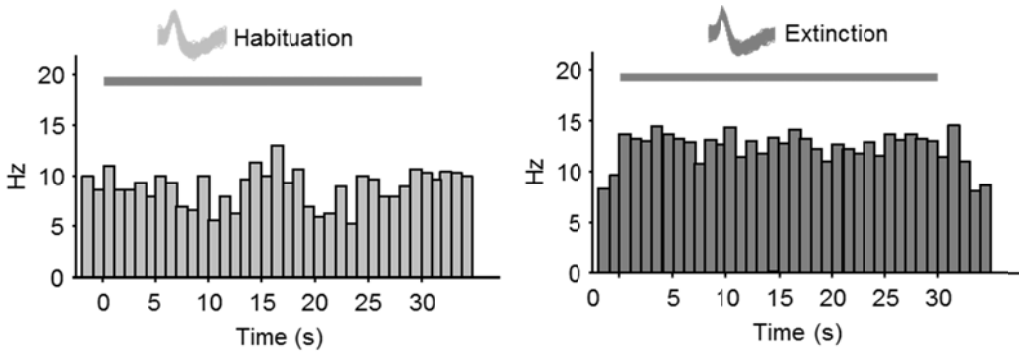


Figure 29. Tone response of representative neuron in the MD. Peristimulation time histogram shows tone-elicited activity of a representative MD neuron firing during habituation tone trials (left) and extinction tone trials (right). Waveforms indicate superimposed spikes recorded during habituation (grey) and extinction learning (dark grey). Horizontal lines indicate 30 second tone stimulation. 30s tone was divided into 30 bins (bin size = 1sec). 3 trials in habituation and 20 trials in extinction learning were averaged.

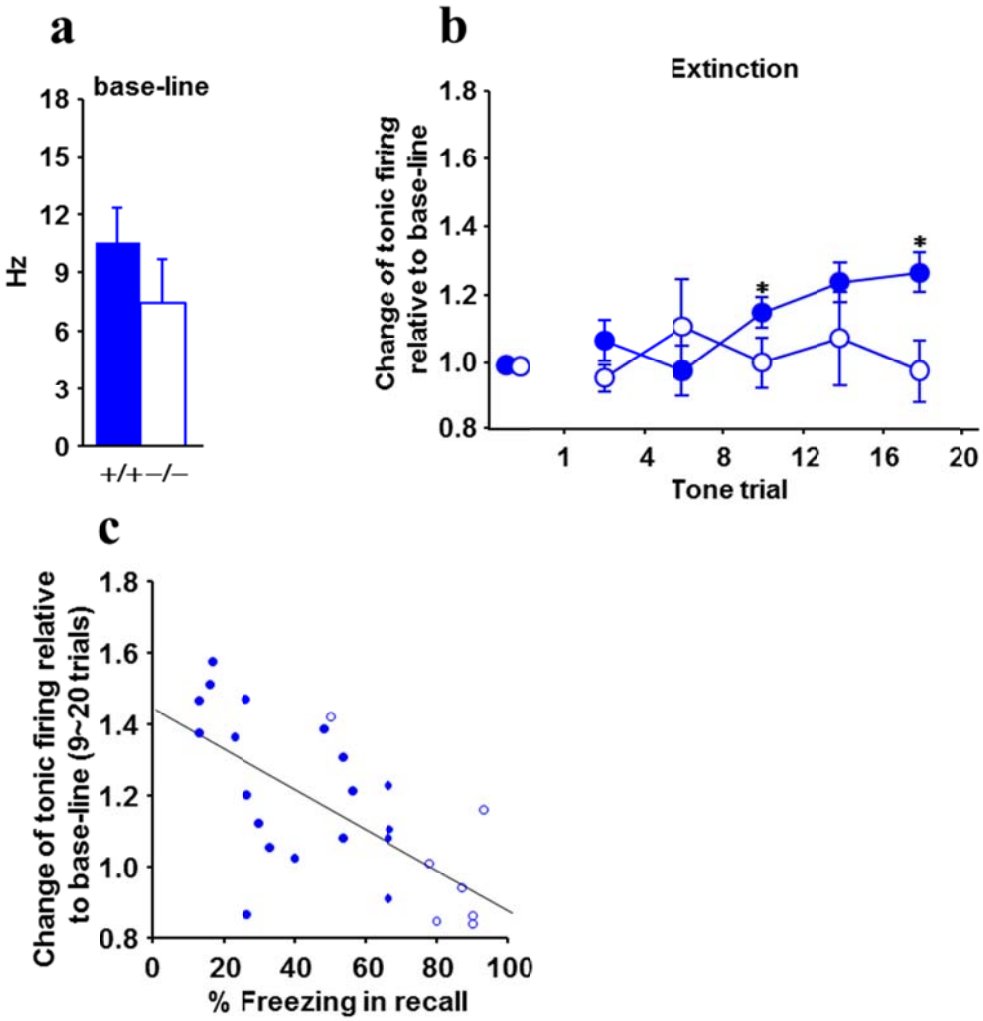


Figure 30. Changes in CS-evoked tonic firing patterns of MD neurons in freely moving wild-type and PLCβ4$^{-/-}$ mice during extinction learning.

a, The average firing rate of tonic spikes before CS did not differ between two groups. **b**, During extinction, tonic firing increased significantly from third block in the wild–type neurons, compared to PLCβ4$^{-/-}$ neurons ($P < 0.05$). Each firing rate was normalized to the baseline value of the neuron, and data are plotted in blocks of four-tone trials. **c**, A significant correlation between increase of tonic firing during the last three blocks of extinction tone trials and decrease of freezing level in extinction recall ($R^2 = 0.45$, $t = -4.38$, $P < 0.0005$). Filled circles and filled bars, wild-type neurons; empty circles and empty bars PLCβ4$^{-/-}$ neurons. Data are means ± s.e.m. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

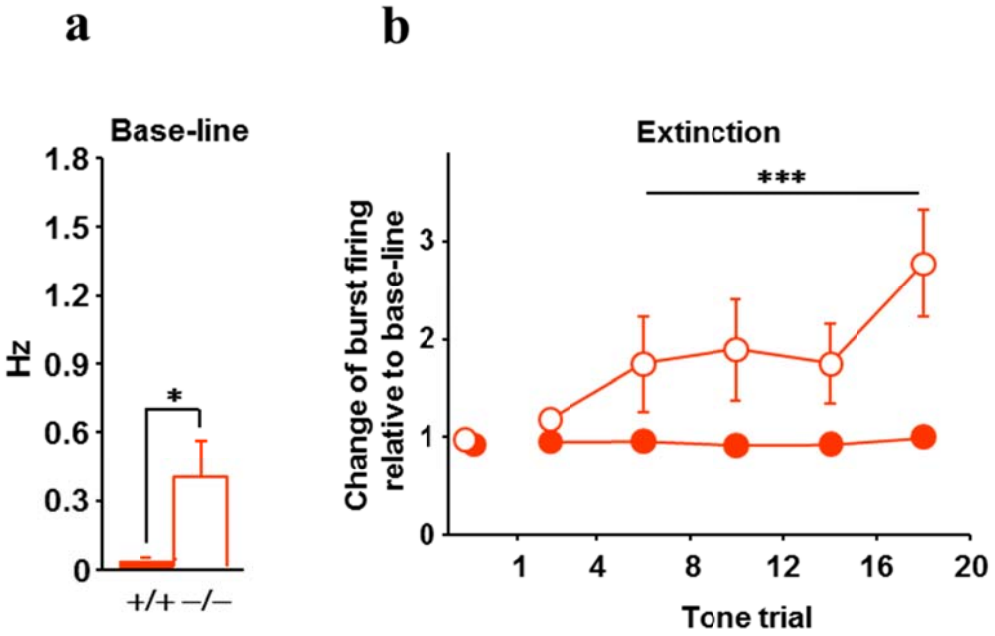


Figure 31. Changes in CS-evoked burst firing patterns of MD neurons in freely moving wild-type and PLCβ4$^{-/-}$ mice during extinction learning.

The average firing rate of burst spikes was significantly higher in PLCβ4$^{-/-}$ mice before CS (**a**) as well as during extinction (**b**). Each firing rate was normalized to the baseline value of the neuron, and data are plotted in blocks of four-tone trials (**b**). Filled circles and filled bars indicate data from wild-type neurons, whereas empty circles and empty bars refer to those from PLCβ4$^{-/-}$ neurons. Data are means ± s.e.m. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

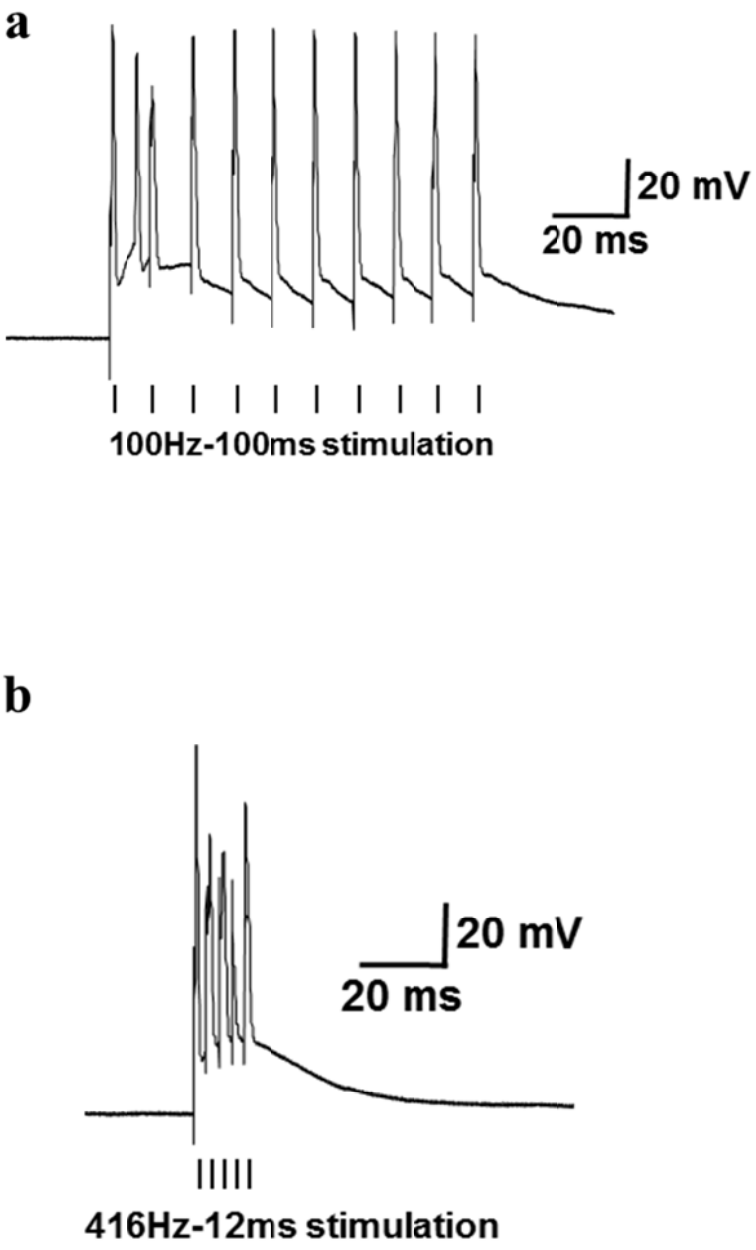


Figure 32. Firing patterns of patch-clamped MD neurons adjacent to the electrical stimulating electrode.

a, Tonic spikes induced by a train of 10 electrical stimuli, delivered at 100 Hz. **b**, Burst spikes induced by a train of five electrical stimuli at 416 Hz.

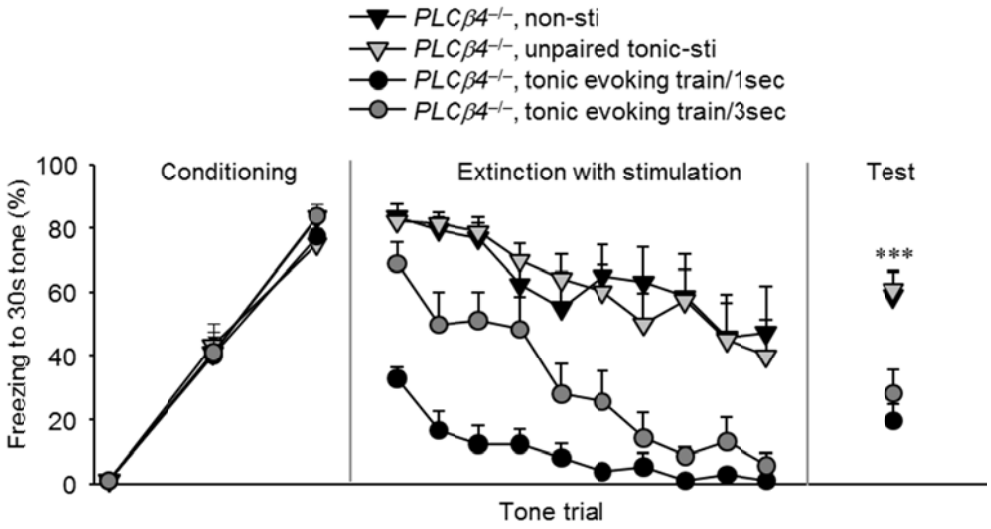


Figure 33. Accelerating effects of MD-electric stimulation, mimicking tonic firing, on fear extinction learning.

Twenty-four hours after conditioning, PLCβ4$^{-/-}$ mice were not stimulated (black triangle symbols), or were electrically stimulated in the MD, in a manner that was not paired with CS (tonic-evoking stimulations every 1 sec: gray triangle symbols; burst-evoking stimulations every 1 sec: dark grey triangle symbols in c); or were paired with CS (tonic-evoking stimulations every 1 sec: black circle symbols; tonic-evoking stimulations every 3 sec, gray circle symbols) during extinction learning. n = 6-12 mice per group. PLCβ4$^{-/-}$, PLCβ4$^{-/-}$ mice. Data are means ± s.e.m. ***, P < 0.001

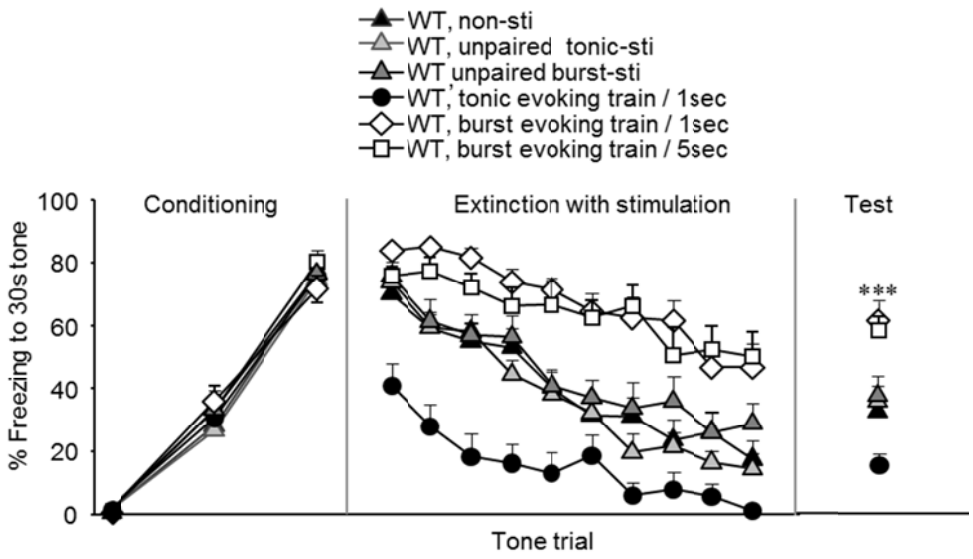


Figure 34. Opposite effects of MD-electric stimulation, mimicking dual firing, on fear extinction learning.

Twenty-four hours after conditioning, wild-type animals were not stimulated (black triangle symbols), or were electrically stimulated in the MD, in a manner that was not paired with CS (tonic-evoking stimulations every 1 sec: gray triangle symbols; burst-evoking stimulations every 1 sec: dark grey triangle symbols); or were paired with CS (tonic-evoking stimulations every 1 sec: black circle symbols; burst-evoking stimulations every 1 sec, diamond symbols; burst-evoking stimulations every 5 sec, rectangular symbols) during extinction learning. n = 8-12 mice per group. . WT, wild-type mice; PLCβ4$^{-/-}$, PLCβ4$^{-/-}$ mice. Data are means ± s.e.m. ***, P < 0.001

Basal firing	Extinction	+/+		–/–
		Unpair	Pair	Pair
Burst & Tonic	Response (n)	2	11	12
	Non-response (n)	6	2	3
Tonic only	Response (n)	3	10	13
	Non-response (n)	12	4	6
Total		23	27	34

Table 1. Summary of CS-evoked responses of MD neurons during extinction conditions.

There were two kinds of neurons, distinguished by baseline firing pattern. Burst & Tonic: neurons firing both in the burst and tonic modes; Tonic only: neurons firing only in the tonic mode. Twenty CS were delivered to mice for fear extinction. Neurons responsive to the CS challenge (response) were defined by those with a significant CS-evoked change of firing rate ($P < 0.05$) from the baseline firing rate that was determined from recording over the 15 min before CS. Non-responsive neurons (non-response) showed no significant change of firing rates under the same conditions ($P > 0.05$).Chi-square test revealed that paired wild-type mice showed significantly more CS-responsive MD neurons than did unpaired wild-type mice (77% vs. 21%, $\chi^2 = 16.37$, $P < 0.0001$), and the paired PLCβ4$^{-/-}$ mice also showed numbers of CS-responsive MD neurons (70%) comparable to those of the paired wild-type mice ($\chi^2 = 0.28$, $P = 0.6$). Unpair, unpaired conditioned mice; Pair, paired conditioned mice.

DISCUSSION

In this study, we present evidence that the MD is critically involved in modulation of fear extinction, through experiments using genetics (a null mutation as well as a localized gene knockdown), pharmacology, electrophysiological recording, and microstimulation. The tonic firing in MD neurons critically contributes to extinction learning as shown in normal and pathological conditions, whereas increased burst firing has suppressive effect on extinction as shown in the mutation.

We have previously shown that a ventrobasal thalamus-specific knockdown of PLCβ4 resulted in spontaneous absence epilepsy (Cheong et al., 2009). In contrast, however, it was suggested that the MD does not contribute to induction of absence epilepsy. For example, in GAERS rats, which show spontaneous absence epilepsy, no SWDs were observed in any limbic structure including the MD, whereas SWDs were abundant in other brain regions including the ventrolateral, posterior, and reticular thalamic nuclei, and the cortex (Vergnes et al., 1990). In addition, creation of electrolytic lesions in the rat MD did not affect the development of drug-induced absence epilepsy (Riban et al., 2004). Consistently, mice with an MD-specific knockdown of PLCβ4 showed no SWD on EEG (Fig. 11). These results suggest that the extinction phenotype of PLCβ4 knockout animals is not simply attributable to seizure activity.

Anatomically, the MD exhibits reciprocal projections with the infralimbic (IL) and prelimbic cortex (PL) of rats (Vertes, 2002); both cortical areas can be defined as subregions of the mPFC and are known to be involved in fear extinction (Sotres-Bayon and Quirk, 2010). Therefore, the MD, influenced by the mGluR1-PLCβ4 signaling, may

be involved in modulation of fear extinction in conjunction with the PL and IL. It is also feasible that during fear extinction MD neurons may communicate with the basolateral amygdala (BLA), the principal site of fear extinction (Fanselow and Poulos, 2005; Pare et al., 2004). Previously, it was shown that MD stimulation induced excitatory responses in the BLA, either directly or indirectly, with involvement of the mPFC (Likhtik et al., 2005; Zhang and Bertram, 2002). In addition, tetanic stimulation of the MD prior to extinction learning can induce long-term potentiation-like changes in the mPFC, which were associated with consolidation of fear extinction (Herry and Garcia, 2002; Hugues and Garcia, 2007). Therefore, the MD could be involved in acquisition and consolidation of fear extinction. Our results clearly show that the MD is involved in control of fear extinction. These results are consistent with the notion that the MD plays a role in the control of emotional circuitry linking the mPFC and the BLA (Hugues and Garcia, 2007; Likhtik et al., 2005; Sotres-Bayon and Quirk, 2010).

Corticothalamic fiber inputs to neurons in the dorsal thalamus are 10-fold more numerous than are thalamocortical inputs (Jones, 2007). Corticothalamic activation modulates both thalamic firing and excitability (Godwin et al., 1996; McCormick and von Krosigk, 1992; Rivadulla et al., 2002). Such properties of the corticothalamic system suggest that the thalamus is not merely a gateway to the cortex but rather an important hub through which the cortex can communicate with other brain regions (Llinas et al., 1998). In the corticothalamic postsynapses of thalamic neurons, mGluR1-PLCβ4 signaling is essential for corticothalamic modulation of thalamic firing (Cheong et al., 2008; McCormick and von Krosigk, 1992; Miyata et al., 2003). The present findings suggest that corticothalamic activation via mGluR1-PLCβ4 signaling facilitates fear extinction by increasing tonic firing in the MD. This interpretation is based on the strong correlation that was revealed between the tonic firing frequency of MD neurons

and the degree of fear extinction among the tested mice, including the wild-type as well as the mutant. In addition, we observed a robust enhancement of burst firing accompanied with augmentation of T-type Ca^{2+} currents in the PLCβ4$^{-/-}$ MD neurons, a finding consistent with the previous report on the PLCβ4 mutant (Cheong et al., 2008). An increase of I_h currents could also have contributed to the enhanced burst level in the mutant neurons. However, the detailed molecular mechanisms involved in this process remain to be further investigated. Nevertheless, our results indicate that the impaired extinction phenotype of the PLCβ4$^{-/-}$ mice must be the combined effect of the two events that occurred in the mutant: decreased tonic firing as well as increased burst firing in the MD neurons.

Our results showed that fear extinction can occur in the mouse under urethane anesthesia (Fig. 18). This observation is in contrast to the extinction deficit of rats under pentobarbital anesthesia which is known to depress the cortex (Park and Choi, 2010). Compared with other anesthetics, urethane has modest effects on GABA, NMDA, and AMPA receptors (Koblin, 2002). In addition, there have been reports showing single unit responses to fear-conditioned stimulus in the frontal cortex and the mPFC under urethane anesthesia (McGinty and Grace, 2008; Pirch et al., 1985), and the mPFC activity is known to be necessary for fear extinction (Park and Choi, 2010; Sotres-Bayon and Quirk, 2010). The successful extinction under urethane anesthesia shown in our results is consistent with this idea.

Earlier studies suggested that the burst firing mode, which was dependent on T-type Ca^{2+} channel activity, was a characteristic feature of thalamic neurons during sleep or absence seizures, whereas the tonic firing mode was associated with wakefulness

(McCormick and Bal, 1997; McCormick and Contreras, 2001). The function of the dual firing mode in the overall thalamic sensory relay process remains controversial(Cheong et al., 2009; Sherman, 2001; Swadlow and Gusev, 2001; Wang et al., 2007), and no causal link between expression of this firing mode and behavior has been reliably shown. We observed stronger bursts, coupled with reduced tonic firing, in MD neurons during the fear extinction phase in PLCβ4$^{-/-}$ mice impaired in extinction (Fig. 23, 26, 30, and 31), and found that tonic-evoking microstimulation facilitated extinction of the fear response, whereas burst-evoking microstimulation suppressed such activity (Fig. 33 and 34). This demonstrates that a causal relationship exists between the firing mode employed and extinction. In other words, the burst effect was inhibitory and the tonic effect acceleratory. We thus describe a functional role for thalamic dual firing in a cognitive process distinct from sensory relay; dual firing is important in emotional regulation. However, it is important not to over-interpret data from microstimulation experiments. Not only target neurons, but also axons ending or coursing near the stimulating electrodes may be activated via antidromic recruitment. Nevertheless, the combined results of our multi-level analysis are consistent with the conclusion that the firing mode of MD neurons is important in modulation of fear extinction.

The midline thalamus, including the MD, is considered to mediate arousal of cortical and subcortical regions; this is required to assure awareness of incoming information (Jones, 2007; Van der Werf et al., 2002). The MD plays a role in the interaction between arousal level and selective attentional performance, allowing focus on only one informational target (thus excluding others) (Portas et al., 1998). In this manner, the MD serves to enhance prefrontal top-down modulation of selective attention (LaBerge, 1997; LaBerge and Buchsbaum, 1990). In addition, MD lesions are known to induce

prefrontal-associated memory impairment in humans, primates, and rats (Hunt and Aggleton, 1998; Mitchell and Gaffan, 2008; Tanibuchi and Goldman-Rakic, 2003; Van der Werf et al., 2000; Zoppelt et al., 2003), suggesting an important role for the MD in cognition (Jones, 2007; Tham et al., 2009; Van der Werf et al., 2002). In the present study, inactivation of mGluR1-PLCβ4 signaling in the MD, with a concomitant enhancement of burst firing, may have compromised the attention level required for learning of fear extinction. Within the triangular circuit of the mPFC, MD, and amygdala, enhancement of MD neuron firing via mGluR1-PLCβ4 signaling, the top-down corticothalamic depolarization-induced increase of tonic firing, may enhance the attention level, thus facilitating fear extinction.

Our data offer a new insight into the neural mechanism of fear extinction, and may help development of improved treatments for anxiety disorders. In this respect, it is notable that some patients with post-traumatic stress disorder (PTSD), a generalized anxiety disorder, show abnormal changes in thalamic activities when the traumatic memory is recalled (Bremner et al., 1999; Geuze et al., 2008; Lanius et al., 2003). Medications controlling the excitability of the MD might help in the treatment of such disorders.

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국문 초록

조건화를 통해 공포를 학습한 경우, 일반적인 상황에서는 전혀 공포 반응을 수반하지 않는 조건 자극만을 제시하는 경우에도 공포 반응이 나타나게 되며, 극단적인 경우에는 무해한 조건화 자극이 주어지기만 해도 비정상적인 수준의 공포를 느끼게 되는 외상후스트레스 장애로 발전하기도 한다. 이러한 공포 학습을 통한 조건화는, 비 조건화 자극 없이 조건 자극을 반복 제시하게 될 경우 중립 자극이 더 이상 공포와 무관함을 학습하게 됨으로써 점차 억제 될 수 있는데, 이를 공포기억소멸(fear extinction)이라 한다. 공포기억소멸에 관여하는 뇌 부위와 분자생물학적 기전에 대해서는 상대적으로 잘 밝혀져 있지 않은 것이 사실이다. 본 논문에서는 기억 조절 기능을 하는 것으로 알려진 시상의 내측동측핵 (mediodorsal thalamic nucleus, MD)이 공포기억소멸을 양방향으로(촉진 또는 억제)조절할 수 있음을 보였으며, 유전공학, 분자생물학 및 전기생리학 기술을 이용해 그 기전을 밝혀냈다. phospholipaseC−β4 (PLCβ4) 녹−아웃이나 MD 특이적 녹−다운 돌연 변이 생쥐는 정상적인 공포 조건화 학습 능력과 공포 조건화 기억을 지니고 있었지만, 공포기억소멸이 되지 않았다. PLCβ4 녹−아웃 돌연 변이 생쥐의 MD 뇌세포에서 T−type 칼슘 이온 통로 특이적 전류 및 T−type 칼슘 이온 통로 특이적 다발 발화가 증가되어 있음을 확인하였다. T−type 칼슘 이온 통로 억제제를 MD에 투여한 경우, PLCβ4 녹−아웃 돌연 변이 생쥐의 억제되었던 공포기억소멸 기능이 정상적으로 회복되는 것을 확인하였다. 뿐만 아니라 정상 생쥐의 MD에 T−type 칼슘 이온 통로 억제제를 투여한 경우에는 공포기억소멸이 더욱 촉진되었다. 공포기억소멸 중, 정상 생쥐는 단발

발화가 증가하는 반면 다발 발화는 거의 보이지 않았다. 이와 반대로 PLCβ4 녹-아웃 돌연 변이 생쥐는 단발 발화의 변화는 없는 반면 다발 발화가 증가하는 패턴을 보였다. PLCβ4 녹-아웃 돌연 변이 생쥐의 MD에 단발 발화를 유발하는 전기 자극을 가한 경우 억제되었던 공포기억소멸이 정상화 되었으며, 자극의 횟수가 많을수록 더욱 빠르게 공포기억이 소멸되었다. 뿐만 아니라 정상 생쥐의 MD에 전기 자극으로 단발 발화를 유도한 경우에도 공포기억소멸이 촉진 되었다. 이와 반대로, 정상 생쥐인 경우에도 MD에 다발 발화를 유발하는 전기 자극을 흘려준 경우에는 돌연 변이 생쥐의 경우와 같이 공포기억소멸이 억제되었다. 이러한 결과들로 미루어 볼 때, 단발 발화는 공포 기억이 정상적으로 소멸되는 과정에 있어서 필수적인 요소이며, 반대로 다발 발화는 공포기억소멸을 방해하는 작용을 한다고 할 수 있다. 즉, 이 결과는 MD의 발화 패턴이 공포기억소멸을 조절하는 데 중요한 역할을 한다는 것을 의미하며, 구체적으로 T-type 칼슘 이온 통로가 이에 큰 기여를 하고 있음을 보여준다. 본 연구 결과는 외상후 스트레스 장애를 비롯 최근 사회적으로 큰 이슈가 되고 있는 다양한 공포 장애를 치료하기 위한 시도에 새로운 가능성을 시사한다.

중심 단어 : 공포 기억 소멸, 시상 동측 내측 핵, phospholipaseC- 4, T-type 의존성 칼슘 이온 통로, 단발 발화, 다발 발화

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