

Trace Fear Conditioning Differentially Modulates Intrinsic Excitability of Medial Prefrontal Cortex–Basolateral Complex of Amygdala Projection Neurons in Infralimbic and Prelimbic Cortices

Chenghui Song,¹ Vanessa L. Ehlers,¹ and James R. Moyer, Jr.^{1,2}

Departments of ¹Psychology and ²Biological Sciences, University of Wisconsin–Milwaukee, Milwaukee, Wisconsin 53201

Neuronal activity in medial prefrontal cortex (mPFC) is critical for the formation of trace fear memory, yet the cellular mechanisms underlying these memories remain unclear. One possibility involves the modulation of intrinsic excitability within mPFC neurons that project to the basolateral complex of amygdala (BLA). The current study used a combination of retrograde labeling and *in vitro* whole-cell patch-clamp recordings to examine the effect of trace fear conditioning on the intrinsic excitability of layer 5 mPFC–BLA projection neurons in adult rats. Trace fear conditioning significantly enhanced the intrinsic excitability of regular spiking infralimbic (IL) projection neurons, as evidenced by an increase in the number of action potentials after current injection. These changes were also associated with a reduction in spike threshold and an increase in *h* current. In contrast, trace fear conditioning reduced the excitability of regular spiking prelimbic (PL) projection neurons, through a learning-related decrease of input resistance. Interestingly, the amount of conditioned freezing was (1) positively correlated with excitability of IL–BLA projection neurons after conditioning and (2) negatively correlated with excitability of PL–BLA projection neurons after extinction. Trace fear conditioning also significantly enhanced the excitability of burst spiking PL–BLA projection neurons. In both regions, conditioning-induced plasticity was learning specific (observed in conditioned but not in pseudoconditioned rats), flexible (reversed by extinction), and transient (lasted < 10 d). Together, these data suggest that intrinsic plasticity within mPFC–BLA projection neurons occurs in a subregion- and cell-type-specific manner during acquisition, consolidation, and extinction of trace fear conditioning.

Key words: amygdala; associative learning; consolidation; extinction; medial prefrontal cortex; memory

Significance Statement

Frontal lobe-related function is vital for a variety of important behaviors, some of which decline during aging. This study involves a novel combination of electrophysiological recordings from fluorescently labeled mPFC-to-amygdala projection neurons in rats with acquisition and extinction of trace fear conditioning to determine how specific neurons change during behavior. This is the first study to demonstrate that trace fear conditioning significantly alters the intrinsic excitability of mPFC-to-amygdala projection neurons in a subregion- and cell-type-specific manner, which is also transient and reversed by extinction. These data are of broad interest to the neuroscientific community, and the results will inspire additional studies investigating the cellular mechanisms underlying circuit-specific changes within the brain as a result of associative learning and memory.

Introduction

Trace fear conditioning is an excellent paradigm for studying learning- and memory-related changes in higher-order brain re-

gions such as the medial prefrontal cortex (mPFC) and the hippocampus (Gilmartin and Helmstetter, 2010; Song et al., 2012). During trace conditioning, the conditioned stimulus (CS) and unconditioned stimulus (US) are separated by a stimulus-free

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Correspondence should be addressed to Dr. James R. Moyer, Jr., Department of Psychology, P.O. Box 413, University of Wisconsin–Milwaukee, Milwaukee, WI 53201. E-mail: jrmoyer@uwm.edu.

C. Song's present address: Department of Neuroscience, The Scripps Research Institute, Jupiter, FL 33458.

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trace interval. Acquisition of trace conditioning is a declarative task that requires the conscious awareness of the CS–US contingency. In contrast, acquisition of a delay paradigm (where the CS and the US temporally overlap or are contiguous) is a nondeclarative task that does not require conscious awareness of the CS–US contingency (Clark and Squire, 1998; Knight et al., 2006; Weike et al., 2007). Thus, studying the cellular mechanisms underlying trace fear conditioning may lay the foundation for understanding not only the control of emotional memory but also other types of declarative memory.

The enhancement of intrinsic excitability (intrinsic plasticity) may underlie some types of associative learning (Moyer et al., 1996; Kaczorowski et al., 2012; Song et al., 2012; Sehgal et al., 2013, 2014). Previous studies suggest that the firing activity in both infralimbic (IL) and prelimbic (PL) subregions of mPFC is critical for acquisition of trace fear conditioning (Han et al., 2003; Runyan et al., 2004; Blum et al., 2006; Gilmartin and Helmstetter, 2010; Gilmartin et al., 2013). Although previous studies suggest that delay fear conditioning suppresses the intrinsic excitability of IL neurons (Santini et al., 2008; Santini and Porter, 2010), little is known regarding how trace fear conditioning affects the intrinsic excitability of mPFC neurons. Thus, the current study was performed to investigate how trace fear conditioning affects the intrinsic excitability of mPFC neurons.

Interestingly, the mPFC has reciprocal projections to a variety of cortical and subcortical brain regions including the basolateral complex of amygdala (BLA; Hurley et al., 1991; Vertes, 2004; Gabbott et al., 2005). Each type of projection neuron within mPFC displays distinct morphological and electrophysiological properties (Mason and Larkman, 1990; DeFelipe and Farinas, 1992; Larkman et al., 1992; Hattox and Nelson, 2007; Dembrow et al., 2010). Thus, learning may change the physiological properties of mPFC neurons in a circuit-specific manner. For example, olfactory fear conditioning excites those IL neurons that receive monosynaptic inputs from the BLA (Laviolette et al., 2005) as well as those neurons that project to the nucleus accumbens, but not the neurons that project to the contralateral mPFC (McGinty and Grace, 2008). In addition, our recent study also suggests that the mPFC neurons display distinct electrophysiological properties in a layer- and subregion-specific manner (Moyer and Song, 2014). Thus, the current study investigated how trace fear conditioning affects the intrinsic excitability of mPFC–BLA projection neurons by injecting a retrograde tracer into the basolateral nucleus of amygdala, which is an area critical for fear conditioning and extinction (LeDoux, 2000). The data suggest that trace fear conditioning transiently changes the in-

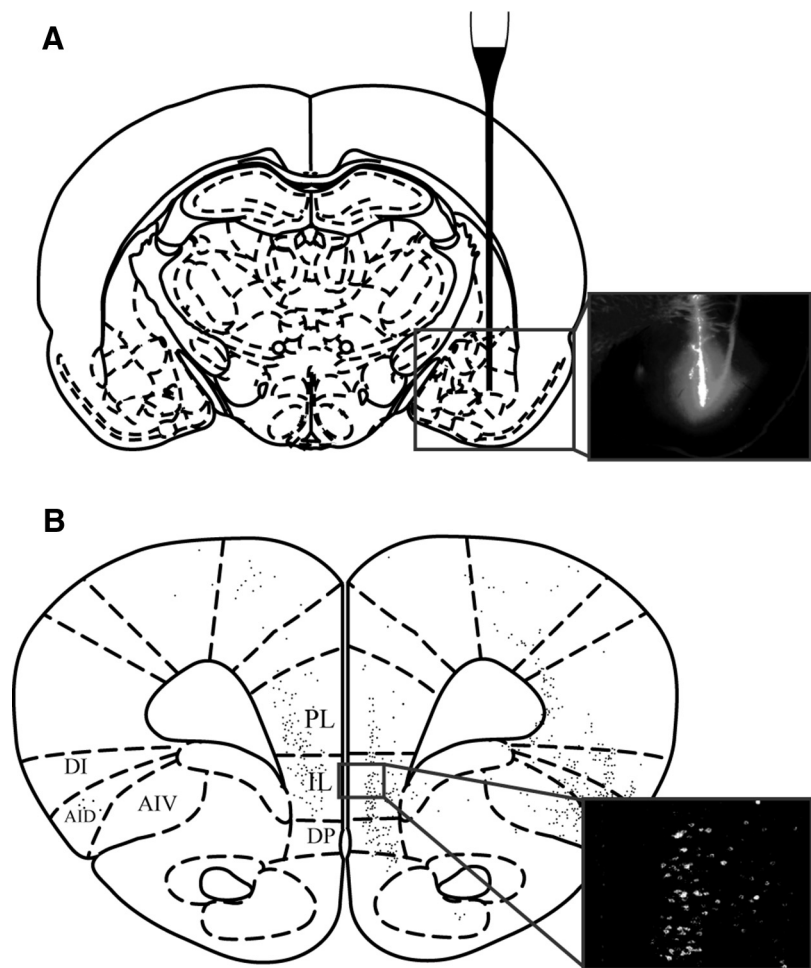


Figure 1. Unilateral infusion of a retrograde tracer into BLA was used to characterize the electrophysiological properties of mPFC–BLA projection neurons. **A**, Schematic diagram of a rat coronal section [illustration modified from Paxinos and Watson (1998), reprinted with permission] showing that a glass pipette was used for the unilateral infusion of red fluorescent microspheres (Retrobeads) into the BLA. Inset, Representative fluorescent image showing that red Retrobeads were injected into BLA. **B**, Coronal section showing the distribution of fluorescently labeled cortico–BLA projection neurons [illustration modified from Paxinos and Watson (1998), reprinted with permission]. Each mark represents one neuron. In the ipsilateral hemisphere, intense distributions of corticoamygdala projection neurons were found in both mPFC and insular cortices. In the contralateral hemisphere, the corticoamygdala projection neurons were primarily distributed within the mPFC. Inset, Representative fluorescent image showing the fluorescently labeled neurons in the IL subregion. AID, Agranular insular cortex, dorsal part; AIV, agranular insular cortex, ventral part; DI, dysgranular insular cortex; DP, dorsal peduncular cortex; IL, infralimbic cortex; PL, prelimbic cortex.

trinsic excitability of layer 5 mPFC–BLA projection neurons in a subregion- and cell-type-specific manner.

Materials and Methods

Subjects. Subjects were 49 adult male F344 rats (5.0 ± 0.1 months old). Rats were maintained in an AAALAC (Association for Assessment and Accreditation of Laboratory Animal Care) accredited facility on a 14 h light/10 h dark cycle and housed individually with *ad libitum* access to food and water. Procedures were conducted in accordance with the University of Wisconsin–Milwaukee Animal Care and Use Committee and NIH guidelines.

Retrobeads infusion. All rats received a unilateral pressure infusion of a red fluorescent retrograde tracer (Retrobeads, Lumafuor) into the basolateral nucleus of the amygdala (relative to bregma: -3 mm anteroposterior, ± 5 mm mediolateral, -8.3 mm dorsoventral; Fig. 1A), with a stereotaxic apparatus and under deep anesthetization. The infusion was made with glass pipettes (20 – 40 μ m) pulled from borosilicate glass (VWR micropipettes) using a Sutter Instrument P97 puller. The pipette was connected to a 2 μ l syringe (Hamilton) driven by an infusion pump (model 975; Harvard Apparatus). The infusion lasted 5–10 min, and the

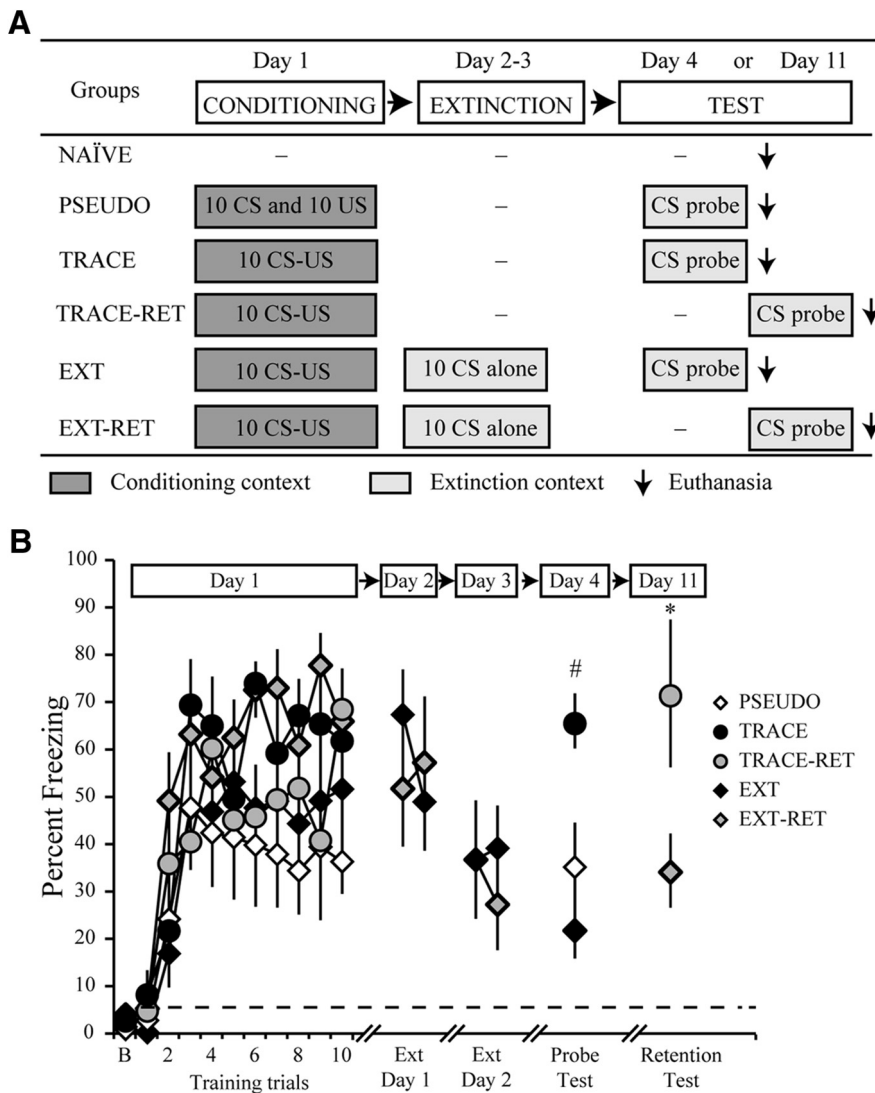


Figure 2. Experimental design and behavioral performance during acquisition and extinction of trace fear conditioning. **A**, Experimental design. Rats received one 10-trial session of trace fear conditioning or pseudoconditioning on day 1. EXT and EXT-RET rats received extinction on days 2–3 in a novel context. TRACE, PSEUDO, and EXT rats received a brief CS-alone probe test on day 4. TRACE-RET and EXT-RET rats received the probe test on day 11 in the extinction context. Immediately after the last behavior test, brain slices were prepared for electrophysiological studies. NAIVE rats remained in their home cages throughout the experiment. **B**, Behavioral fear responses of the rats in the various groups during conditioning, extinction (first 2 trials), and testing. During conditioning, all rats displayed a rapid increase in freezing levels during the first three trials and then maintained elevated freezing levels. A repeated-measures ANOVA of percentage freezing revealed a significant main effect of training trials ($F_{(9,306)} = 19.5, p < 0.01$; Greenhouse–Geisser corrected) and a significant effect of group ($F_{(4,34)} = 3.2, p < 0.05$) but no significant group \times training trial interaction ($F_{(36,306)} = 1.1, p = 0.34$; Greenhouse–Geisser corrected). During the probe test on day 4, all rats displayed comparable levels ($F_{(2,26)} = 1.51, p = 0.24$) of freezing during baseline (dashed line indicates the average baseline freezing of all groups). In contrast, TRACE rats froze significantly more than both PSEUDO and EXT rats during the trace interval after the offset of the CS ($F_{(2,24)} = 10.6, p < 0.01$). On day 11, TRACE-RET rats froze significantly more than EXT-RET rats during the trace interval ($F_{(1,10)} = 5.37, p < 0.05$).

pipette was withdrawn 10 min after infusion. A total volume of 0.1–0.3 μ l of red Retrobeads was infused into BLA.

Fear conditioning chambers. Trace fear conditioning was conducted in Plexiglas and stainless steel chambers (30.5 \times 25.4 \times 30.5 cm; Coulbourn Instruments), each located within a separate sound-attenuating box. Each chamber was rectangular and had a standard grid floor consisting of 26 parallel steel rods (5 mm diameter and 6 mm spacing). The floor was connected to a precision adjustable shock generator (Coulbourn Instruments) for delivery of a scrambled footshock US. Within the sound-attenuating box, a ventilation fan produced a constant background noise of \sim 58 dB (measured by a sound-level meter, A scale; model 33-2050; Realistic). Each chamber

was illuminated by a miniature incandescent white lamp (28 V, type 1819; illumination 1.1 lux) and was wiped with a 5% ammonium hydroxide solution before each training session. During training, the room lights were left on (illumination, 20.9 lux) for the entire session.

Extinction and CS testing chambers. Two additional Plexiglas chambers served as a novel context for the auditory cue tests. These chambers were located within their own separate sound-attenuating boxes within the same room. The test chambers were physically distinct from the training chambers in that they were octagonal (instead of rectangular), their floors were black-painted Plexiglas (instead of stainless steel grid bars), and they were illuminated with infrared light (instead of incandescent lamps). In addition, their floors had an array of holes drilled into them. Before placing rats into the testing chambers, fresh bedding was added to the bottom tray beneath the floor, and the chamber walls were wiped with 2% acetic acid to provide a background odor distinctive from that used during training. The room lights were turned off (illumination, 0.2 lux) for the entire testing session.

Fear conditioning and extinction. After a minimum of 7 d of recovery from surgery, rats were handled for at least 1 week before they were randomly assigned to naive (NAIVE, $n = 10$), pseudoconditioned (PSEUDO, $n = 8$), trace fear conditioned (TRACE, $n = 9$), trace fear conditioned-retention (TRACE-RET, $n = 3$), trace fear conditioned-extinction (EXT, $n = 10$), or trace fear conditioned extinction-retention (EXT-RET, $n = 9$) groups as summarized in Figure 2A. On day 1, TRACE, TRACE-RET, EXT, and EXT-RET rats received one 10-trial session of auditory trace fear conditioning using a 15 s CS (80 dB white noise), followed by a 30 s trace interval (stimulus-free period) and a 1 s footshock US (1.0 mA). A long (5.2 min \pm 20%) intertrial interval (ITI) was used to maximize the CS and minimize background context (i.e., training chamber) conditioning (Detert et al., 2008). PSEUDO rats received the same number of CS and US presentations, but they were explicitly unpaired. Naive rats remained in their home cages throughout the experiment. On days 2–3, rats in the TRACE, TRACE-RET, and PSEUDO groups remained in their home cages, whereas EXT and EXT-RET rats received two consecutive extinction sessions (10 CS-alone presentations; 1 session per day) in a novel context. These sessions were identical to conditioning except no US was presented. After

each extinction session, rats were returned to their home cages 2 min after the last extinction trial.

Behavioral testing. Twenty-four hours after extinction (day 4), PSEUDO, TRACE, and EXT rats received a brief CS test session in the extinction context. TRACE-RET and EXT-RET rats received the CS test on day 11. During this behavioral test, rats were first allowed to explore the new chamber for 2 min (baseline), followed by two 15 s CS presentations (2.9 min ITI), and they were removed 2 min after the second CS presentation. To assess memory, the amount of time spent freezing during the baseline, the CS, and the trace interval (defined as the first 30 s after CS offset) was measured. Consistent with our previous report (De-

tert et al., 2008), the freezing levels during the CS were less than during the trace interval, and therefore the amount of time spent freezing during the trace interval was used as the dependent measure of fear (Fig. 2B). During the probe test, the percentage freezing during the trace interval after each CS presentation was averaged and used as the measurement of fear memory.

Analysis of behavioral data. A remote CCTV video camera (model WV-BP334; Panasonic), mounted to the top of each behavioral chamber, was used to record the activity of each rat during training and testing. The video data were fed to a PC running FreezeFrame 2.04 (Actimetrics Software, Coulbourn Instruments). Data were analyzed using FreezeView 2.04 (Actimetrics Software) where a 1 s bout of immobility was scored as freezing. Freezing was defined as the absence of all movement except that required for respiration (Blanchard and Blanchard, 1969).

PFC slice preparation for electrophysiological recordings. Within 30 min after the CS probe tests (Fig. 2A), rats were anesthetized and decapitated by an individual blind to the training condition. The brains were quickly removed and placed into ice-cold artificial CSF (aCSF; in mM): 124 NaCl, 2.8 KCl, 1.25 NaH₂PO₄, 2 CaCl₂, 2 MgSO₄, 26 NaHCO₃, and 20 glucose (pH 7.5, bubbled with 95% O₂/5% CO₂). Coronal slices (300 μm) containing prefrontal cortex (anteroposterior, +3.2–2.2) were cut in ice-cold aCSF using a vibrating tissue slicer (VT1200; Leica). The slices were placed into individual wells of our custom slice incubation chamber (Moyer and Brown, 1998) where they remained in oxygenated aCSF at 32–36°C until use. The rest of the brain was then blocked, and coronal slices that contained the amygdala were prepared to confirm infusion location. The injection site was immediately verified by placing the slice on the stage of an upright microscope (Olympus BX51WI) equipped with fluorescence (see Fig. 1A for a representative image). As illustrated in Figure 3, all injection sites were within the BLA (including the basal, lateral, and accessory basal nuclei) and intercalated cell masses.

For whole-cell recordings (WCRs), electrodes (5–8 MΩ) were prepared from thin-walled capillary glass and filled with the following solution (in mM): 110 K-gluconate, 20 KCl, 10 di-Tris-P-creatine, 10 HEPES, 2 MgCl₂, 2 Na₂ATP, 0.3 Na₂GTP, and 0.2% biocytin, with pH 7.3 and an osmolarity of 290 mOsmol. All chemicals were obtained from Sigma or Thermo Fisher Scientific unless noted.

Electrophysiological recordings. PFC slices were transferred to a submerged recording chamber mounted on a fluorescence-equipped Olympus BX51WI upright microscope where they were continuously perfused with oxygenated aCSF at a rate of 2 ml/min and maintained at 32–36°C using an in-line temperature controller (Warner Instruments). The fluorescently labeled mPFC neurons were visualized using a Texas Red epifluorescent filter set. A Hamamatsu CCD camera (Hamamatsu Camera Ltd.) was used to visualize and photograph the neuron and the brain slice for verification of the recording location. Although both layer 2/3 (L2/3) and L5 neurons project to the amygdala (Fig. 1B), we restricted our studies to L5 neurons because they are the major output neurons (Groh et al., 2010) and have electrophysiological properties that are distinctly different from L2/3 neurons (Boudewijns et al., 2013; Moyer and Song, 2014). Neurons with somata that were ≥400 μm from the pial surface were defined as L5 neurons (Gabbott and Bacon, 1996; Perez-Cruz et al., 2007) and were recorded and analyzed. L6 was recognized as distinct from L5 because it contains a high density of fibers (Gaillard and Sauve, 1995). After fluorescently labeled neurons were identified, the microscope was switched to infrared-differential interference contrast mode to guide whole-cell recording from identified projection neurons. Data were collected from those neurons whose resting membrane potential (RMP) was more negative than –50 mV. Series resistance was fully compensated and always monitored to ensure the stability of recording con-

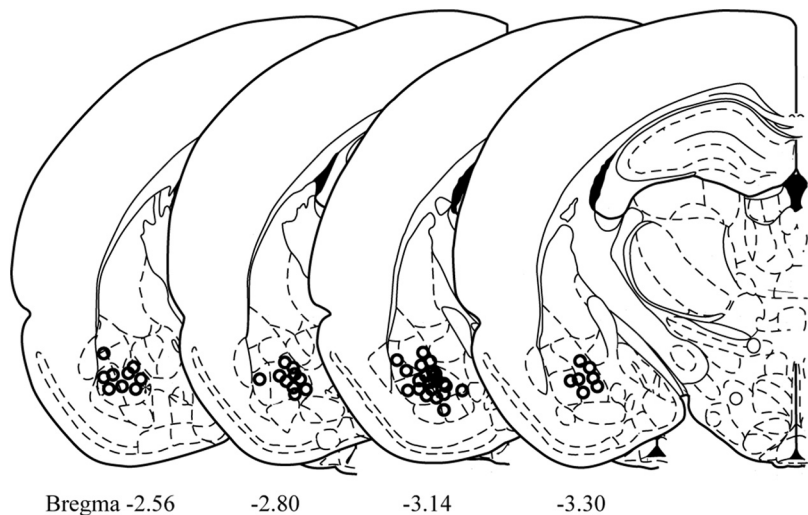


Figure 3. Retrobeads infusions targeted BLA. Schematic drawings of coronal sections of rat brain showing the infusion locations within the amygdala [illustration modified from Paxinos and Watson (1998), reprinted with permission]. Retrobeads were infused into either hemisphere and counterbalanced between rats, but all infusion locations are shown in the left hemisphere for simplicity because no significant differences were observed between hemispheres.

ditions. Cells were only accepted for analysis if the initial series resistance was ≤30 MΩ and did not change by >30% throughout the recording period. The distribution of all recorded neurons included in this study is shown in Figure 4, A (regular spiking) and B (burst spiking).

Intrinsic properties of mPFC–BLA projection neurons were studied under current-clamp conditions according to the following protocols. (1) *I–V* relationships were obtained from a series of 500 ms current injections (ranging from –300 to 50 pA) and by plotting the plateau voltage deflection against current amplitude. Neuronal input resistance (R_N) was determined from the slope of the linear fit of the portion of the *V–I* plot where the voltage sweeps did not exhibit sags or active conductance (Fig. 4E,G). The sag ratio during hyperpolarizing membrane responses was expressed as $[(1 - \Delta V_{ss}/\Delta V_{max}) \times 100\%]$, where $\Delta V_{ss} = MP - V_{ss}$ and $\Delta V_{max} = MP - V_{max}$ (MP is the membrane potential before the current step, V_{ss} is the steady-state potential at the end of the current step, and V_{max} is the peak amplitude during the first 150 ms of the current step). For each neuron, the sag ratio was calculated from current injections of –300, –250, and –200 pA and averaged. (2) Action potential (AP) properties, including $I_{threshold}$, were studied with an ascending series of 500 ms depolarizing pulses. At rheobase, neurons could be classified as either regular spikers or burst spikers (Fig. 4). Neurons that generated a single action potential at threshold or suprathreshold current injections were classified as regular spiking (RS) neurons (Moyer et al., 2002). Neurons that generated two or more action potentials riding atop the $I_{threshold}$ depolarizing current in an all-or-none fashion were classified as burst spiking (BS) neurons (Connors et al., 1982; Moyer et al., 2002). For BS neurons, AP properties were studied from the first spike. AP threshold (AP_{thresh}) was defined as the voltage when dV/dt first exceeded 28 mV/ms (Kaczorowski et al., 2012). The AP amplitude (AP_{amp}) was measured relative to the AP_{thresh} . Action potential width (AP_{width}) was measured as the width at half of the AP amplitude. (3) The postburst afterhyperpolarization (AHP) was studied after a 50 Hz burst of 10 spikes, each of which was evoked by a 2 ms suprathreshold current injection (three times, at 20 s intervals). After the last action potential, the postburst AHP was measured at both the peak amplitude within the first 150 ms (mAHP) and at 1 s (sAHP). (4) Neuronal excitability was assessed by counting the number of spikes evoked in response to a series of 1 s depolarizing steps (range, 50–450 pA; 50 pA increments, 20 s ITI; Fig. 4F,H).

All recordings were obtained in current-clamp mode (holding potential –67 mV; mean holding current, -62 ± 4 pA) using a HEKA EPC10 amplifier system (HEKA). Data were transferred to a PC computer using an ITC-16 digital-to-analog converter (HEKA Instruments). The signals

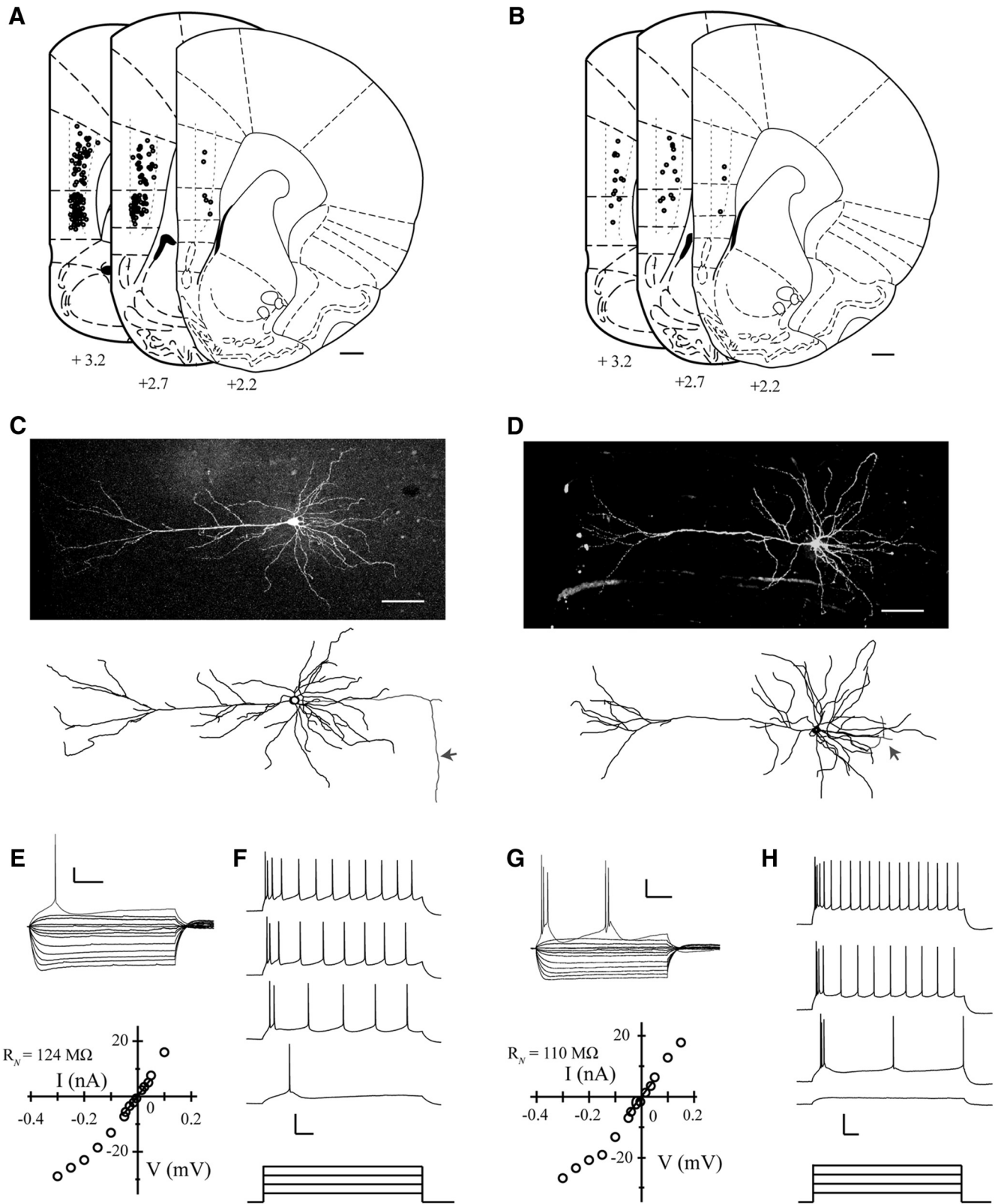


Figure 4. Two classes of mPFC–BLA projection neurons. **A, B**, Schematic diagrams of rat coronal sections (Paxinos and Watson, 1998) illustrating the distribution of all regular spiking (**A**) and burst spiking (**B**) mPFC–BLA projection neurons obtained in this study. Neurons were recorded from either side, but all were labeled in one hemisphere for simplicity as no differences were observed. **C, D**, Confocal images show representative examples of regular spiking (**C**) and burst spiking (**D**) mPFC–BLA projection neurons along with their 3D reconstructions. Arrows point to the axons of each cell. **E, G**, Voltage responses to a series of current injections and the accompanying V – I plot used to measure neuronal input resistance (R_N). **F, H**, Neuronal excitability was studied by injecting a series of depolarizing currents and counting the number of action potentials evoked. Scale bars: **A, B**, 500 μ m; **C, D**, 100 μ m. Calibration: **E, G**, 20 mV, 100 ms; **F, H**, 40 mV/100 pA, 100 ms.

were filtered at 2.9 kHz and digitized at 20 kHz using Patchmaster software (HEKA). Data were analyzed off-line using Patchmaster and Igor Pro software (version 4.03; Wavemetrics). Voltages were not corrected for the liquid–liquid junction potential (approximately +13 mV; Moyer and Brown, 2007). Recordings were made from mPFC neurons located both ipsilateral and contralateral to the BLA injection site. Physiological data were combined because no significant differences were observed.

Biocytin staining. Biocytin-filled neurons were fixed in formalin for up to 4 weeks and processed for visualization using a streptavidin Alexa Fluor 488 reaction as described previously (Song et al., 2012). Briefly, the slices were incubated in 3% H₂O₂/10% methanol for 45 min and washed with PBS, followed by 0.4% Triton X-100/2% BSA for 45 min. The slices were then incubated with 1:500 streptavidin Alexa Fluor 488 (Invitrogen) for 2 h in the dark and washed with PBS. They were mounted onto slides, coverslipped with Ultra Cruz Mounting Medium (Santa Cruz Biotechnology), and sealed with nail polish. The labeled neurons were visualized and photoimaged using either a fluorescence microscope (BX51WI; Olympus) or a laser-scanning confocal fluorescence microscope (FV-1200; Olympus). Confocal image stacks were used for 3D reconstruction (Fig. 4C,D) using NeuroLucida software (MBF Bioscience). Morphological analyses confirmed that all neurons analyzed were pyramidal neurons. Confocal images and reconstructions of representative RS and BS neurons are shown in Figures 4, C and D, respectively.

Statistical analyses. All statistical analyses were performed using IBM SPSS statistics software (version 22; SPSS). Data were analyzed using parametric statistics with two-tailed Student's *t* test, one-way ANOVA, or repeated-measures ANOVA as appropriate. For significant main effects ($\alpha = 0.05$), a Fisher's PLSD test was used for *post hoc* comparisons. All data are expressed as mean \pm SEM.

Results

We examined the effect of trace fear conditioning and extinction on the intrinsic excitability of mPFC–BLA projection neurons by using a combination of retrograde labeling with whole-cell recording. All rats received a unilateral injection of fluorescently labeled microspheres before behavioral training (except NAIVE rats that received a Retrobeads injection but remained in their home cages). Rats received conditioning or pseudoconditioning on day 1, followed by either extinction training or no treatment (i.e., they remained in their home cages) on days 2 and 3. To correlate both learning- and extinction-related changes in mPFC excitability with behavioral performance, rats in the TRACE, PSEUDO, and EXT groups received a brief CS-alone probe test on day 4. To investigate whether any changes in mPFC excitability persisted over a prolonged period of time, rats in the TRACE-RET and EXT-RET groups received a probe test on day 11 (Fig. 2A). Prefrontal cortical slices were prepared within 1 h after the probe test, and the fluorescently labeled neurons in L5 of both IL and PL subregions were recorded. The duration from the date of surgery to the date of slicing was not significantly different between groups (mean, 30.8 \pm 2.2 d; $F_{(5,43)} = 0.19$, $p = 0.97$).

Analysis of behavioral performance during the trace interval indicated that all rats exhibited a rapid increase in percentage freezing during the first three trials, after which they maintained a high level of freezing throughout the remainder of the training session (Fig. 2B). However, a repeated-measures ANOVA with *post hoc* tests showed that PSEUDO rats froze significantly less than TRACE ($p < 0.05$) or EXT-RET ($p < 0.01$) rats. Although EXT rats froze less than EXT-RET rats ($p < 0.05$), they displayed comparable levels of freezing during the first two trials of extinction, indicating the memory of conditioned fear was comparable between the two groups. When memory was tested on day 4, rats in the TRACE, EXT, and PSEUDO groups displayed comparably low levels of baseline freezing (Fig. 2B, average baseline freeze is shown as a dashed line). In contrast, there was a statistically sig-

nificant difference in percentage freezing during the trace interval immediately after CS offset. *Post hoc* analyses indicated that TRACE rats froze significantly more than both PSEUDO ($p < 0.01$) and EXT ($p < 0.01$) rats, whereas PSEUDO rats displayed comparable levels of freezing with EXT rats. During the long-term retention test on day 11, TRACE-RET rats also froze significantly more than EXT-RET rats ($p < 0.05$), suggesting good memory retention for both trace fear conditioning and extinction.

Trace fear conditioning enhances intrinsic excitability of regular spiking IL-BLA projection neurons

To evaluate the effect of trace fear conditioning on the intrinsic excitability of mPFC–BLA projection neurons, PFC slices were prepared after the memory test, and WCRs were performed on the neurons that were labeled with fluorescent microspheres. Trace fear conditioning significantly increased the intrinsic excitability of regular spiking IL-BLA projection neurons as evidenced by more AP firing in response to depolarizing current steps compared with NAIVE neurons (Fig. 5A,C). A repeated-measures ANOVA revealed significant main effects of group ($F_{(5,103)} = 3.0$, $p < 0.05$) and current intensity ($F_{(1,6,162.1)} = 497.3$, $p < 0.01$; Greenhouse–Geisser corrected). There was also a significant interaction of group by current intensity ($F_{(7,9,93.6)} = 2.4$, $p < 0.05$; Greenhouse–Geisser corrected). Follow-up analyses using a one-way ANOVA revealed a significant group effect when depolarizing current steps were >250 pA (all values, $p < 0.05$). *Post hoc* comparisons confirmed that cells in TRACE rats fired significantly more APs than those in NAIVE, PSEUDO, EXT, or TRACE-RET rats in response to the depolarizing currents ranging between 250 and 400 pA (Fig. 5A). Interestingly, the intrinsic excitability was not enhanced in TRACE-RET rats, although they displayed good memory retention 10 d after conditioning, suggesting the conditioning-induced effect was transient, which is consistent with previous studies in hippocampal neurons after trace eyeblink conditioning (Moyer et al., 1996; Thompson et al., 1996) and odor discrimination learning (Zelcer et al., 2006). The enhancement of intrinsic excitability was also not observed in EXT or EXT-RET rats. In both groups, intrinsic excitability was comparable with that of neurons from naive rats (Fig. 5A,C), suggesting that extinction reversed the effects of trace fear conditioning.

Although PSEUDO rats displayed an increase in trace interval freezing during conditioning, their freezing levels during the probe test were not only similar to rats that underwent extinction but they were also significantly lower than the TRACE rats (Fig. 2B). Not surprisingly, intrinsic excitability in IL-BLA projection neurons from PSEUDO rats was not significantly changed (Fig. 5A,C), suggesting that the enhancement of intrinsic excitability in trace fear conditioned rats was associative and learning specific. Thus, our results indicate that acquisition of trace fear conditioning transiently enhances the intrinsic excitability of IL-BLA projection neurons and extinction reverses the effect of conditioning.

Trace fear conditioning modulates spike threshold and I_h current (I_h) in regular spiking IL-BLA projection neurons

To further explore the underlying mechanisms that may contribute to our observed learning-related enhancement of neuronal excitability, basic membrane properties as well as the postburst AHP were also compared between the various training groups. A planned comparison between neurons from naive and conditioned animals suggests that the spike threshold was significantly hyperpolarized after conditioning ($t_{(40)} = 2.4$, $p < 0.05$; Fig. 6A)

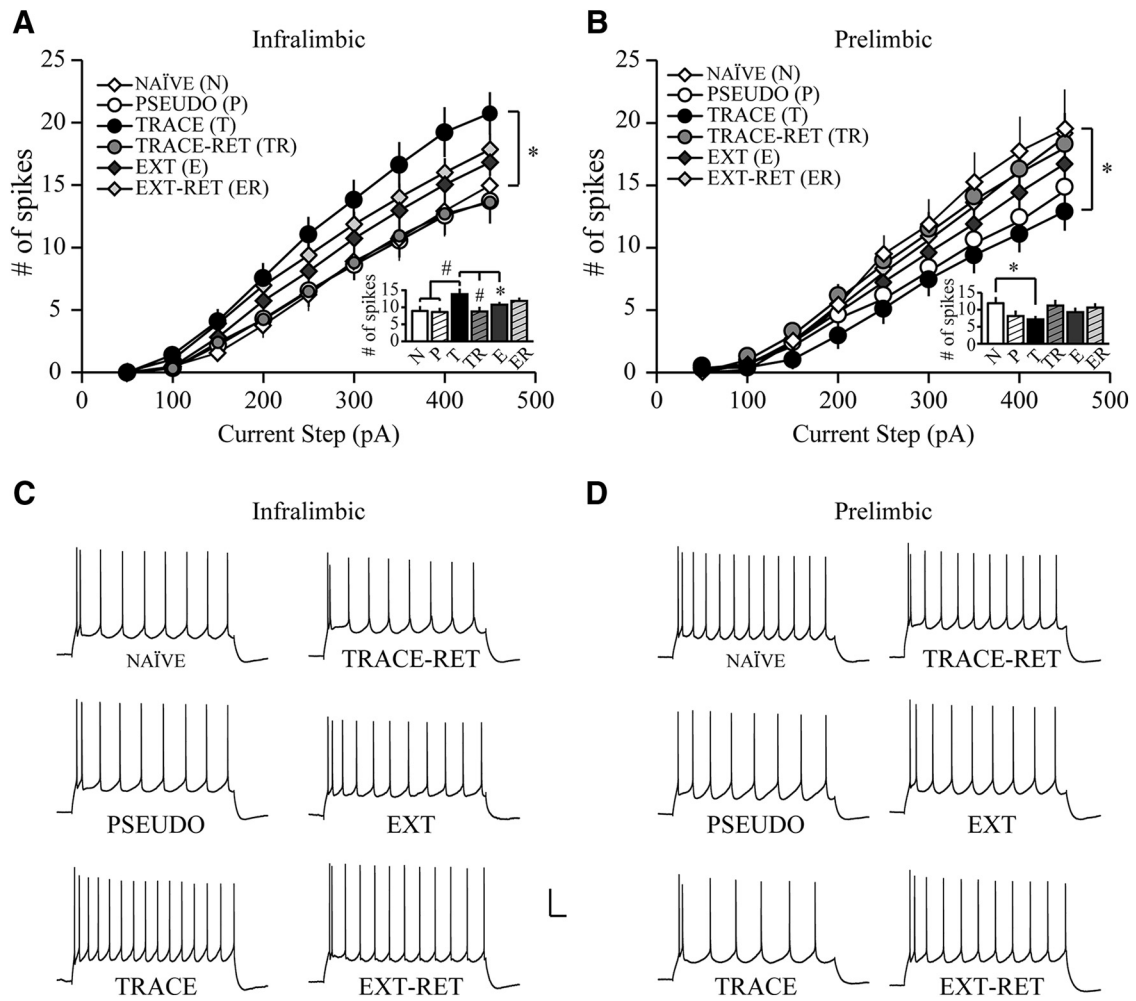


Figure 5. Trace fear conditioning differentially modulates the intrinsic excitability of regular spiking IL- and PL-BLA projection neurons. **A**, Trace fear conditioning significantly enhanced the intrinsic excitability of IL-BLA projection neurons. Neurons from TRACE rats fired significantly more spikes than those from NAIVE rats ($p < 0.05$). **B**, Trace fear conditioning significantly suppressed the intrinsic excitability of PL-BLA projection neurons. Neurons from TRACE rats fired significantly fewer spikes than those from NAIVE rats ($p < 0.05$). In both IL and PL subregions, extinction reversed the conditioning effect such that the intrinsic excitability in EXT neurons was comparable with neurons from other groups and remained stable after extinction retention (EXT-RET). The conditioning-induced plasticity observed in TRACE rats was transient in both IL and PL subregions as the intrinsic excitability returned to naive level after memory retention (TRACE-RET). The insets in **A** and **B** show the number of spikes evoked by a 300 pA current injection in mPFC–BLA projection neurons (statistically different between TRACE and other groups: * $p < 0.05$, # $p < 0.01$). **C, D**, Representative traces showing the number of spikes evoked by a 300 pA current injection. Calibration: 20 mV, 100 ms.

and that there was a significant correlation between spike threshold and the number of spikes evoked by the depolarizing currents ($r = -0.46, p < 0.01$). The spike threshold returned to naive levels in neurons from the TRACE-RET rats after the 10 d trace memory retention test such that it was significantly different from that of neurons from the TRACE rats ($t_{(32)} = 2.1, p < 0.05$; Figure 6A). Furthermore, trace fear conditioning significantly increased the depolarizing sag ($t_{(38)} = 3.4, p < 0.01$; Fig. 6B, Table 1), suggesting the activation of I_h , which is mediated by hyperpolarization-activated cyclic nucleotide-gated (HCN) channels. In addition, trace fear conditioning enhanced the mAHP (measured at the peak after a burst of 10 APs) but not sAHP (measured at 1 s after the burst of APs; Fig. 6C, Table 1), which is consistent with the activation of I_h (Oswald et al., 2009; Kaczorowski, 2011). That activation of I_h contributed to the enhanced mAHP was also supported by the observation that the mAHP was significantly correlated with the sag ($r = -0.38, p < 0.05$). Other membrane properties (e.g., RMP, input resistance, $I_{threshold}$) were not significantly different (Table 1). Together, these data suggest that trace fear conditioning specifically en-

hances intrinsic excitability of IL-BLA projection neurons through modulating the expression or properties of the ion channels that regulate spike threshold and I_h .

Trace fear conditioning suppresses intrinsic excitability of regular spiking PL-BLA projection neurons

In contrast to IL neurons, acquisition of trace fear conditioning significantly reduced the intrinsic excitability of RS neurons in the PL subregion (Fig. 5B,D). A planned repeated-measures ANOVA between NAIVE and TRACE neurons revealed a significant main effect of group ($F_{(1,22)} = 4.6, p < 0.05$) and current intensity ($F_{(1.2,26.7)} = 70.4, p < 0.01$; Greenhouse–Geisser corrected) but no significant group \times current intensity interaction ($F_{(1.2,26.7)} = 3.4, p = 0.07$; Greenhouse–Geisser corrected). Additional analysis with a one-way ANOVA revealed that trace fear conditioned rats fired significantly fewer spikes in response to depolarizing current injections between 250 and 400 pA (all values, $p < 0.05$). However, the intrinsic neuronal excitability returned to naive levels within 7 d (TRACE-RET rats). Likewise, the effects of condi-

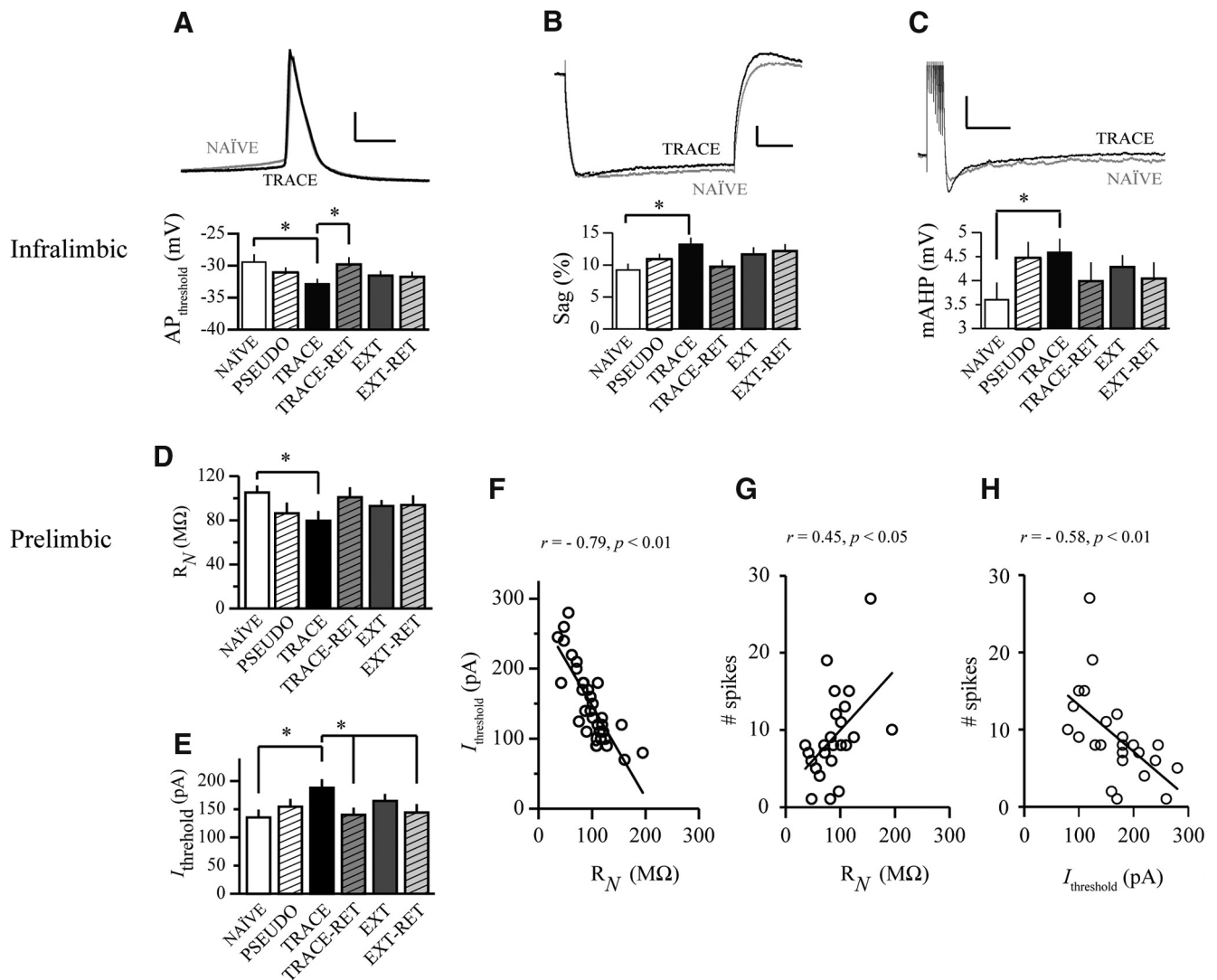


Figure 6. Distinct mechanisms underlie learning-related changes within mPFC–BLA projection neurons after acquisition and extinction of trace fear conditioning. **A–C**, In IL subregion, acquisition of trace fear conditioning significantly reduced the AP threshold (**A**), increased the depolarizing sag (**B**), and enhanced the mAHP (**C**). After memory retention (TRACE-RET), the AP threshold returned to naive level and was significantly higher than that of TRACE neurons ($p < 0.05$). Calibration: **A**, 20 mV, 2 ms; **B**, 5 mV, 100 ms; **C**, 5 mV, 0.5 s. **D, E**, In PL subregion, acquisition of trace fear conditioning significantly reduced the neuronal input resistance or R_N (**D**) and increased the minimal current ($I_{\text{threshold}}$) required to evoke one action potential or spike (**E**). The $I_{\text{threshold}}$ was reduced to naive level after TRACE-RET or EXT-RET and was significantly different from that of TRACE neurons (for all values, $p < 0.05$). All other comparisons were not significantly different from **A–E** ($p > 0.05$). **F–H**, Correlations between $I_{\text{threshold}}$ and R_N (**F**), the number of action potentials and R_N (**G**), and the number of action potentials and $I_{\text{threshold}}$ (**H**) suggest that within PL-BLA projection neurons, the changes in both R_N and $I_{\text{threshold}}$ contribute to the learning-related reduction in intrinsic excitability.

tioning were reversed by extinction (EXT rats), and they remained at naive levels even after a subsequent extinction retention test 7 d later (e.g., EXT-RET rats), suggesting that extinction permanently reversed the effects of conditioning on intrinsic excitability (Fig. 5*B, D*). Thus, the reduction of intrinsic neuronal excitability in PL-BLA projection neurons from trace fear conditioned rats was learning specific, transient, and reversible.

Trace fear conditioning reduces input resistance of regular spiking PL-BLA projection neurons

Trace fear conditioning induced a subtle but significant reduction in the input resistance (R_N) of PL-BLA projection neurons ($t_{(24)} = 2.1$, $p < 0.05$; Fig. 6*D*). Interestingly, this subtle change in R_N was sufficient to significantly reduce the excitability of PL-BLA projection neurons such that neurons from the trace fear conditioned rats required a significantly larger threshold current

injection ($I_{\text{threshold}}$) to evoke a single action potential ($t_{(24)} = 2.7$, $p < 0.05$; Fig. 6*E*). The $I_{\text{threshold}}$ was reduced to naive level after TRACE-RET or EXT-RET and was significantly different from that of TRACE neurons (TRACE-RET: $t_{(26)} = 2.2$, $p < 0.05$; EXT-RET: $t_{(27)} = 2.0$, $p < 0.05$; Fig. 6*E*). The relationship between R_N and $I_{\text{threshold}}$ was supported by the strong correlation between the two measurements ($r = -0.79$, $p < 0.0001$; Fig. 6*F*). Furthermore, the number of spikes evoked by depolarizing current was strongly correlated with both R_N ($p < 0.05$; Fig. 6*G*) and $I_{\text{threshold}}$ ($p < 0.01$; Fig. 6*H*). Moreover, the changes in R_N and $I_{\text{threshold}}$ were only observed in conditioned rats but not in pseudoconditioned rats (see also Table 1 for other membrane properties that were not changed by conditioning), suggesting they were learning specific. Thus, trace fear conditioning suppresses the intrinsic excitability of PL-BLA projection neurons by modulating ion channels that affect R_N and $I_{\text{threshold}}$.

Table 1. Effect of trace fear conditioning and extinction on membrane properties of regular spiking mPFC–BLA projection neurons

Group	RMP (mV)	R_N (M Ω)	$I_{\text{threshold}}$ (pA)	AP _{thresh} (mV)	AP _{amp} (mV)	AP _{width} (μ s)	mAHP (mV)	sAHP (mV)	Sag (%)
Infralimbic cortex									
NAIVE	-60.7 \pm 1.2 (21)	123 \pm 9 (21)	147 \pm 12 (20)	-29.5 \pm 1.2 (20)*	77 \pm 2 (20)	971 \pm 49 (20)	-4.2 \pm 0.3 (20)*	-0.9 \pm 0.1 (20)	9.2 \pm 1.0 (18)*
PSEUDO	-64.1 \pm 1.3 (18)	104 \pm 8 (18)	154 \pm 14 (18)	-31.2 \pm 0.9 (18)	78 \pm 2 (18)	950 \pm 36 (18)	-4.9 \pm 0.4 (18)	-0.8 \pm 0.1 (18)	10.8 \pm 0.9 (18)
TRACE	-62.5 \pm 1.2 (22)	118 \pm 9 (22)	137 \pm 8 (22)	-32.8 \pm 0.8 (22)	78 \pm 1 (22)	968 \pm 42 (22)	-5.1 \pm 0.3 (22)	-0.7 \pm 0.1 (22)	13.2 \pm 1.0 (22)
TRACE-RET	-61.8 \pm 1.2 (13)	99 \pm 8 (13)	150 \pm 15 (12)	-29.8 \pm 1.1 (12)*	75 \pm 2 (12)	912 \pm 26 (12)	-4.7 \pm 0.4 (13)	-0.7 \pm 0.2 (13)	9.7 \pm 1.1 (13)
EXT	-61.3 \pm 1.1 (23)	107 \pm 6 (23)	147 \pm 10 (23)	-31.5 \pm 0.7 (23)	78 \pm 1 (23)	912 \pm 34 (23)	-4.8 \pm 0.2 (22)	1.0 \pm 0.1 (22)	11.7 \pm 1.1 (22)
EXT-RET	-62.1 \pm 1.0 (24)	108 \pm 6 (24)	144 \pm 12 (24)	-31.8 \pm 0.8 (24)	81 \pm 2 (24)	840 \pm 29 (24)	-4.8 \pm 0.3 (22)	0.9 \pm 0.1 (22)	12.2 \pm 1.1 (21)
Prelimbic cortex									
NAIVE	-61.7 \pm 0.4 (12)	105 \pm 6 (12)*	135 \pm 14 (12)*	-30.6 \pm 1.6 (12)	80 \pm 2 (12)	818 \pm 37 (12)	-4.8 \pm 0.3 (12)	-0.9 \pm 0.1 (12)	11.7 \pm 2.1 (9)
PSEUDO	-62.0 \pm 1.3 (9)	86 \pm 10 (9)	154 \pm 14 (9)	-30.9 \pm 1.9 (9)	82 \pm 1 (9)	780 \pm 23 (9)	-6.0 \pm 0.4 (9)	-1.0 \pm 0.1 (9)	13.2 \pm 2.3 (9)
TRACE	-63.4 \pm 0.9 (14)	80 \pm 11 (14)	188 \pm 15 (14)	-32.2 \pm 0.6 (14)	85 \pm 2 (14)	846 \pm 35 (14)	-4.8 \pm 0.3 (14)	-0.9 \pm 0.1 (14)	12.4 \pm 1.1 (14)
TRACE-RET	-63.0 \pm 1.5 (15)	101 \pm 9 (15)	140 \pm 13 (14)*	-31.5 \pm 0.9 (14)	80 \pm 2 (14)	750 \pm 26 (14)	-4.9 \pm 0.3 (15)	-1.0 \pm 0.1 (13)	9.4 \pm 1.0 (13)
EXT	-61.3 \pm 1.2 (22)	93 \pm 5 (22)	165 \pm 12 (22)*	-31.3 \pm 0.8 (22)	82 \pm 2 (22)	829 \pm 26 (22)	-4.6 \pm 0.3 (22)	-0.6 \pm 0.1 (22)	11.8 \pm 1.0 (22)
EXT-RET	-62.8 \pm 1.5 (15)	94 \pm 9 (15)	144 \pm 15 (15)	-33.5 \pm 0.9 (15)	85 \pm 2 (15)	798 \pm 31 (15)	-4.7 \pm 0.4 (13)	-0.8 \pm 0.1 (13)	11.4 \pm 1.4 (13)

Data are mean \pm SE for the number of cells in parentheses. Rat groups are as follows: NAIVE, rats that did not receive any behavioral training; PSEUDO, rats that received pseudoconditioning on day 1 and tested on day 4; TRACE, rats that received trace fear conditioning on day 1 and tested on day 4; TRACE-RET, rats that received trace fear conditioning on day 1 and tested on day 11; EXT, rats that received trace fear conditioning on day 1, extinction on days 2–3, and tested on day 4; EXT-RET, rats that received conditioning on day 1, extinction on days 2–3, and tested on day 11. AP_{amp} was relative to threshold. The mAHP was measured at the peak of the AHP within 150 ms after a burst of 10 APs. The sAHP was measured at 1 s after the burst of 10 APs. * p < 0.05, statistically different from TRACE.

Correlations between behavioral performance and intrinsic excitability after conditioning and extinction

Previous data demonstrated that the amount of conditioned fear was significantly correlated with the intrinsic excitability of hippocampal CA1 neurons in trace fear conditioned but not pseudoconditioned rats (Song et al., 2012). We therefore examined whether such correlations also exist in mPFC neurons after trace fear conditioning and extinction. Data from IL-BLA projection neurons revealed a significant positive correlation between behavioral performance (percentage freezing) and intrinsic excitability in the TRACE rats (Fig. 7A). In contrast, data from PL-BLA projection neurons revealed a significant negative correlation between behavioral performance and intrinsic excitability in the EXT rats (Fig. 7B). No other significant correlations were observed within IL or PL subregions. Thus, these data suggest that the activity of regular spiking IL-BLA projection neurons is critical for the formation of the trace conditioned fear memory, whereas the activity of regular spiking PL-BLA projection neurons is critical for extinction of this memory.

Trace fear conditioning enhances the intrinsic excitability of burst spiking PL-BLA projection neurons

Neurons that generated two or more action potentials in an all-or-none fashion in response to a just-suprathreshold current injection ($I_{\text{threshold}}$) were classified as BS neurons (Connors et al., 1982; Moyer et al., 2002). BS and RS neurons display different firing patterns, which may be related to differences in the distribution and/or properties of their voltage-gated (especially T- and R-type Ca^{2+}) ion channels (Magee and Carruth, 1999). Our previous data demonstrated that enhanced intrinsic excitability in L2/3 BS neurons may underlie aging-related extinction deficits (Kaczorowski et al., 2012), suggesting that bursting activity enhances conditioned fear. We therefore analyzed BS neurons separately. A total of 30 burst spiking mPFC–BLA projection neurons (9 IL-BLA, 21 PL-BLA) were obtained in this study (Fig. 4B, Table 2). These represent 7 and 19% of the total number of IL- and PL-BLA projection neurons, respectively. Within the IL subregion, no BS neurons were obtained from NAIVE or PSEUDO rats. Interestingly, there seems to be an increase in the percentage of BS neurons after behavioral training, especially after extinction (percentage BS neurons in the IL subregion: TRACE, 8%;

TRACE-RET, 7%; EXT, 15%; EXT-RET, 8%). No further statistics were performed in IL burst spiking neurons because of the small sample size. Interestingly, this observed trend toward an increase in bursting activity after extinction of trace fear conditioning is consistent with previous findings in IL neurons after extinction of delay fear conditioning (Santini et al., 2008).

Within the PL subregion, the intrinsic excitability of BS neurons was significantly enhanced after trace fear conditioning (Fig. 8, Table 2). Since bursting may be an important way for the brain to synchronize activity between brain regions (Buzsáki et al., 2002), this enhanced bursting activity within PL-BLA projection neurons may be critical for the expression of a trace fear memory. Interestingly, this change was opposite to that observed in regular spiking PL-BLA projection neurons, in which intrinsic excitability was reduced after conditioning (Fig. 9A). Moreover, other membrane properties, such as R_N and $I_{\text{threshold}}$, were also changed in burst spiking PL-BLA projection neurons after trace fear conditioning, but opposite to those observed in regular spiking PL-BLA projection neurons (Fig. 9B,C). Together, these data suggest that trace fear conditioning differently modulates the intrinsic excitability of regular spiking and burst spiking PL-BLA projection neurons.

Discussion

With the combination of retrograde labeling and whole-cell patch-clamp recordings, we demonstrate for the first time that trace fear conditioning significantly alters the intrinsic excitability of mPFC–BLA projection neurons in a subregion- and cell-type-specific manner. Interestingly, the conditioning-induced effects were reversed by extinction as well as the passage of time, since they were not evident during the memory retention test given 10 d after conditioning. Thus, these data suggest that the intrinsic plasticity within mPFC–BLA projection neurons is required for the consolidation of, but not for the retrieval of, a trace fear memory.

Learning-induced intrinsic plasticity within mPFC–BLA projection neurons supports memory consolidation

In both IL and PL subregions, we observed transient changes in intrinsic excitability, which were diminished over time (e.g., TRACE-RET group) or with extinction. These data suggest that the conditioning-induced intrinsic plasticity was re-

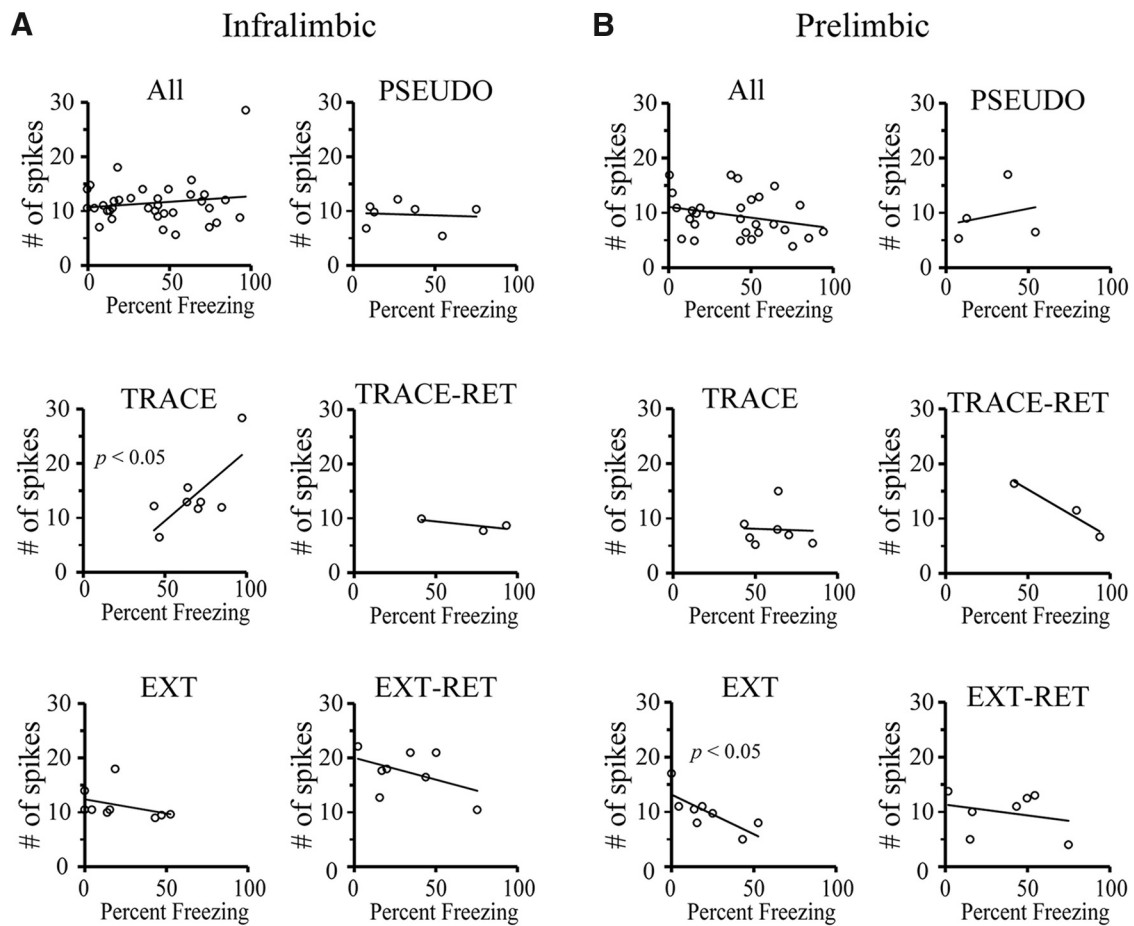


Figure 7. Acquisition and extinction of trace fear conditioning are differentially correlated with intrinsic excitability of regular spiking mPFC–BLA projection neurons. **A**, Within the IL subregion, a significant correlation was observed in neurons from TRACE rats ($r = 0.73$, $p < 0.05$) but not in any other groups or when all groups were combined together (p values for other groups were as follows: All, 0.40; PSEUDO, 0.84; TRACE-RET, 0.45; EXT, 0.33; EXT-RET, 0.25). **B**, Within the PL subregion, a significant correlation was observed in neurons from rats that received extinction (EXT; $r = -0.72$, $p < 0.05$) but not any other groups (p values for other groups were as follows: All, 0.15; PSEUDO, 0.75; TRACE, 0.92; TRACE-RET, 0.16; EXT-RET, 0.57).

Table 2. Effect of trace fear conditioning and extinction on membrane properties of burst-spiking mPFC–BLA projection neurons

Group	RMP (mV)	R_N (M Ω)	$I_{\text{threshold}}$ (pA)	AP_{thresh} (mV)	AP_{amp} (mV)	AP_{width} (ms)	Number of spikes	mAHP (mV)	Sag (%)
Infralimbic cortex									
NAIVE									
PSEUDO									
TRACE	-59.0 ± 3 (2)	84.7 ± 7 (2)	160 ± 40 (2)	-31.7 ± 5.4 (2)	88 ± 4 (2)	0.66 ± 0.04 (2)	8 (1)	-4.9 (1)	12.4 ± 8.3 (2)
TRACE-RET	-62.0 (1)	79.7 (1)	180 (1)	-35.2 (1)	95 (1)	0.60 (1)	12 (1)	-4.3 (1)	5.4 (1)
EXT	-66.3 ± 1.4 (4)	97 ± 15 (4)	133 ± 13 (4)	-34.2 ± 4 (4)	92 ± 9 (4)	0.72 ± 0.07 (4)	17 ± 4 (4)	-2.9 ± 0.4 (4)	3.5 ± 0.7 (4)
EXT-RET	-59.5 ± 5.5 (2)	74 ± 33 (2)	170 ± 90 (2)	-39.1 ± 0.9 (2)	91 ± 3 (2)	0.73 ± 0.02 (2)	15 ± 9 (2)	-5.3 ± 1.7 (2)	16.1 ± 0.3 (2)
Prelimbic cortex									
NAIVE	-66.0 ± 2.1 (5)	110 ± 4 (5)	116 ± 6 (5)	-35 ± 1.7 (5)	90 ± 3 (5)	0.66 ± 0.05 (5)	12 ± 3 (5)*	-4.5 ± 0.8 (5)	8.2 ± 2.6 (4)
PSEUDO	-63.0 (1)	101 (1)	120 (1)	-34.4 (1)	90 (1)	0.86 (1)	14 (1)	-5.0 (1)	9.7 (1)
TRACE	-66.0 ± 1.2 (3)	132 ± 16 (3)	87 ± 9 (3)	-34.6 ± 0.3 (3)	93 ± 2 (3)	0.68 ± 0.04 (3)	21 ± 1 (3)	-5.4 ± 0.5 (3)	8.0 ± 1.4 (3)
TRACE-RET	-64.0 ± 0.9 (4)	85 ± 8 (4)	130 ± 20 (4)	-31.6 ± 0.9 (4)	80 ± 1 (4)	0.78 ± 0.02 (4)	12 ± 2 (4)	-5.4 ± 0.8 (4)	7.8 ± 1.3 (4)
EXT	-60.3 ± 1.8 (3)	110 ± 106 (3)	113 ± 23 (3)	-32.9 ± 1.4 (3)	93 ± 2 (3)	0.60 ± 0.01 (3)	16 ± 4 (3)	-4.8 ± 0.4 (3)	9.4 ± 0.3 (3)
EXT-RET	-66.0 ± 1.2 (5)	112 ± 10 (5)	112 ± 13 (5)	-32.5 ± 2.1 (5)	89 ± 6 (5)	0.70 ± 0.11 (5)	16 ± 3 (5)	-4.3 ± 0.3 (5)	5.3 ± 1.0 (5)

Data are mean \pm SE for the number of cells in parentheses. Rat groups are as follows: NAIVE, rats that did not receive any behavioral training; PSEUDO, rats that received pseudoconditioning on day 1 and tested on day 4; TRACE, rats that received trace fear conditioning on day 1 and tested on day 4; TRACE-RET, rats that received trace fear conditioning on day 1 and tested on day 11; EXT, rats that received trace fear conditioning on day 1, extinction on days 2–3, and tested on day 4; EXT-RET, rats that received conditioning on day 1, extinction on days 2–3, and tested on day 11. AP_{amp} was relative to threshold. The mAHP was measured at the peak of the AHP within 150 ms after a burst of 10 APs. The number of spikes was counted from a voltage response to a 300 pA depolarizing current injection. * $p < 0.05$, statistically different from TRACE.

lated to more of a systems-level consolidation (rather than a rapid synaptic consolidation) involving a gradual reorganization of the brain regions that support the memory (Frankland and Bontempi, 2005). Such transient changes have been observed in hippocampal neurons after trace eyeblink conditioning (Moyer et al., 1996; Thompson et al., 1996) as well as

olfactory discrimination rule learning (Zelcer et al., 2006). In addition to memory consolidation, this learning-induced intrinsic plasticity may also facilitate synaptic plasticity and subsequent learning (i.e., metaplasticity; see Abraham and Bear, 1996; Zelcer et al., 2006; and Sehgal et al., 2013). It is likely that the learning-induced intrinsic plasticity creates a hyperexcit-

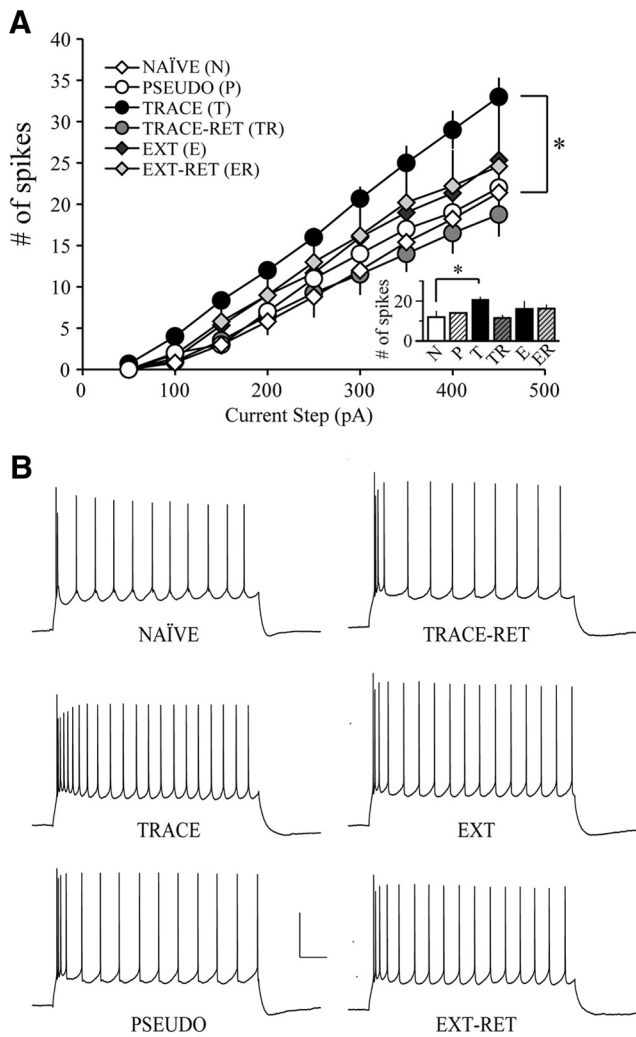


Figure 8. Trace fear conditioning enhances intrinsic excitability of burst spiking PL-BLA projection neurons. **A**, Trace fear conditioning significantly enhanced the intrinsic excitability of burst spiking PL-BLA projection neurons ($F_{(1,6)} = 6.1, p < 0.05$; planned repeated-measures ANOVA). Student's *t* test confirmed that cells in TRACE rats fired significantly more APs than those in NAIVE rats in response to the depolarizing currents ranging between 100 and 300 pA (for all values, $p < 0.05$). Inset, Bar graph showing the average number of spikes evoked by 300 pA depolarizing currents from all groups. The asterisk indicates statistically significant difference between neurons from NAIVE and TRACE rats ($F_{(1,6)} = 7.6, p < 0.05$; planned comparison). **B**, Representative voltage traces illustrating that PL-BLA projection neurons from trace conditioned rats fired more action potentials than neurons from naive rats (calibration: 40 mV, 100 ms).

able or hypoexcitable state to promote memory consolidation (Zhang and Linden, 2003). Furthermore, the transient feature of learning-induced plasticity also suggests that some regulatory process exists for restoring excitability. Although our data suggest that intrinsic plasticity within mPFC is not required for expression of the trace fear memory 10 d later (Figs. 2, 5), pharmacological inactivation of mPFC severely impairs the expression of conditioned fear (Blum et al., 2006; Corcoran and Quirk, 2007), suggesting that another process, such as synaptic plasticity (Song et al., 2012), may exist during long-term memory retrieval.

Learning-induced intrinsic plasticity in mPFC is subregion and cell-type specific

Trace fear conditioning significantly enhanced the intrinsic excitability of regular spiking IL-BLA projection neurons (Fig.

5A). As illustrated in Table 3, 10% of the neurons were defined as “changed.” Our observation that learning alters the excitability of only a subset of neurons is consistent with prior studies (Moyer et al., 1996; Rumpel et al., 2005; Sehgal et al., 2014) and suggests that not every labeled IL-BLA projection neuron underwent trace fear conditioning-associated changes. In contrast, the excitability of regular spiking PL-BLA projection neurons was suppressed (Fig. 5B), with ~14% of labeled neurons exhibiting a decreased excitability after trace fear conditioning.

Our results suggest that the activity of IL-BLA projection neurons enhances fear memory consolidation (Figs. 5A, 7A), whereas the activity of PL-BLA projection neurons inhibits the expression of conditioned fear (Figs. 5B, 7B). This is consistent with other studies showing that local blockade of NMDARs in the IL subregion decreased both delay and trace conditioned fear (Kwapis et al., 2014b), whereas PL lesions or local blockade of PL NMDARs increased both delay (Morgan and LeDoux, 1995) and trace conditioned fear (Kwapis et al., 2014b). Similarly, our recent data suggest that the intrinsic excitability of IL neurons is enhanced 24 h after delay fear conditioning in adult rats (Song et al., 2015). Previous studies also suggested that delay fear conditioning suppressed the firing activity of PL neurons during the CS test (Garcia et al., 1999). Such suppression may arise from the activation of BLA and IL during memory recall because stimulation of either brain region inhibited PL neuronal activity (Pérez-Jaranay and Vives, 1991; Ji and Neugebauer, 2012; Dilgen et al., 2013). After extinction, such inhibition was reversed and even converted into potentiation (Herry et al., 1999; Herry and Garcia, 2002), thus allowing PL neurons to inhibit fear. Conversely, synaptic strength within the IL-BLA pathway is enhanced after delay fear conditioning and reversed by extinction (Vouimba and Maroun, 2011). Together, these data suggest that our observations are general for fear conditioning and that IL and PL subregions are involved in consolidation and extinction of conditioned fear, but that their roles are opposing.

Although our observations are consistent with other studies, some discrepancies exist (for review, see Kim and Jung, 2006). For instance, Porter and colleagues (Santini et al., 2008) showed that intrinsic excitability of IL neurons was suppressed, whereas the excitability of PL neurons did not change after delay fear conditioning. There are several possible explanations for these discrepancies. First, Porter and colleagues (Santini et al., 2008) recorded from mPFC pyramidal neurons within both L2/3 and L5, whereas the present study recorded from only L5 mPFC–BLA projection neurons. Second, recent studies suggest that the brain circuits underlying acquisition and extinction of delay and trace fear paradigms are different. Although extinction of delay requires the amygdala (Falls et al., 1992; Parsons et al., 2010) and the IL subregion (Burgos-Robles et al., 2007; Sotres-Bayon et al., 2009; Pattwell et al., 2012), extinction of trace fear conditioning does not require the amygdala, but it does require IL, PL, and retrosplenial cortices (Kwapis et al., 2014a,b). Our observation that IL excitability correlates with behavioral performance after conditioning whereas PL excitability correlates with behavioral performance after extinction is consistent with Kwapis et al. (2014b). However, our data do not rule out transient changes to IL excitability that may occur during extinction training after either delay or trace conditioning. Finally, a number of other procedural differences (e.g., animal age, recording temperature, external and internal recording solutions) may account for the

discrepancies between the current study and that of Santini et al. (2008).

We observed that both RS and BS projection neurons are involved in fear memory consolidation. Although the role of BS neurons is obscured by the small sample size compared with that of RS neurons, this effect was robust and consistent with previous studies showing that stimulation of BLA inhibits most mPFC pyramidal neurons (Pérez-Jaranayand Vives, 1991; Dilgen et al., 2013) and excites most mPFC interneurons (Dilgen et al., 2013). These observations are also reminiscent of intrinsic plasticity observed in mPFC after olfactory fear conditioning, which excites those neurons that receive monosynaptic inputs from BLA (Laviolette et al., 2005) as well as those neurons that project to the nucleus accumbens, but not those that project to the contralateral mPFC (McGinty and Grace, 2008). Thus, it is likely that learning-induced intrinsic plasticity is specific to mPFC–BLA projection neurons but not all mPFC neurons, or even all mPFC–BLA projection neurons (Table 3).

Distinct mechanisms underlie trace fear conditioning-induced intrinsic plasticity in IL and PL subregions

In IL-BLA projection neurons, the enhanced intrinsic excitability of RS neurons was associated with a reduction in AP threshold (Fig. 6A) and an enhancement in I_h (Fig. 6B, C). One possible explanation for our observed reduction in spike threshold is an activity-dependent increase in the probability of opening of somatic voltage-gated sodium channels, which has been previously observed in hippocampal neurons (Ganguly et al., 2000; Xu et al., 2005). It is likely that our observed enhancement in I_h as well as the mAHP in infralimbic RS neurons is mediated by HCN channels because the selective HCN channel blocker ZD7288 diminishes both components (Kaczorowski, 2011; Angelo and Margrie, 2011). A learning-specific, temporary enhancement of I_h was also observed in hippocampal CA1 neurons after trace eyeblink conditioning (Moyer et al., 1996), suggesting that modulation of HCN channels may play a role in brain regions involved in acquisition of trace paradigms. Interestingly, such learning-specific modulation of I_h was not observed in hippocampal or mPFC neurons after delay eyeblink or fear conditioning (Disterhoft et al., 1986; Santini et al., 2008). Although it remains unclear how I_h uniquely contributes to different learning paradigms, it may facilitate cortical rhythmic oscillations during slow-wave sleep and potentiate memory consolidation (McCormick and Pape, 1990; Lüthi and McCormick, 1998; Marshall et al., 2006).

Within PL-BLA projection neurons, the effects of trace fear conditioning on input resistance was cell-type specific (R_N was decreased in RS neurons and increased in BS neurons). The activity-dependent modulation of R_N has been observed not only in cerebellar granule cells after high-frequency stimulation of mossy fiber inputs (Armano et al., 2000) but also in amygdala neurons after fear conditioning (Rosenkranz and Grace, 2002). In both cases, the increase in R_N significantly enhanced intrinsic excitability. Within PL-BLA regular spiking projection neurons, however, there was a significant neg-

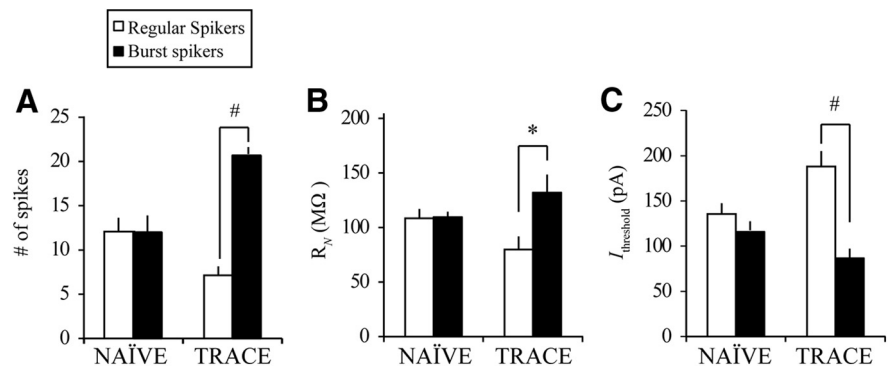


Figure 9. Trace fear conditioning differentially modulates intrinsic properties of RS and BS projection neurons within the PL subregion. Intrinsic excitability (A), R_N (B), and $I_{\text{threshold}}$ (C) were comparable between regular and burst spiking PL-BLA projection neurons in NAIVE rats but were significantly different after trace fear conditioning (* $p < 0.05$; # $p < 0.01$).

Table 3. Trace fear conditioning alters intrinsic excitability in a subset of IL-BLA projection neurons

Group	Number of spikes	Ratio of cells changed
NAIVE	9.0 ± 1.3	0:15
PSEUDO	8.6 ± 1.2	0:18
TRACE	13.9 ± 1.6***	2:20
TRACE-RET	8.8 ± 1.4	0:13
EXT	10.7 ± 0.9	0:22
EXT-RET	11.9 ± 1.0	1:21

Data are the number of action potentials elicited in response to a 300 pA current injection for 1 s. The ratio of cells changed refers to the number of individual neurons (relative to the total number of neurons recorded) whose action potential output at 300 pA was more than 2 SDs beyond the mean of neurons from NAIVE rats. Data from the TRACE group are significantly different from the NAIVE, PSEUDO, TRACE-RET (* $p < 0.01$), and EXT (** $p < 0.05$) groups.

ative correlation between $I_{\text{threshold}}$ and R_N , suggesting that as neuronal input resistance decreased, a concomitant increase in the current required to elicit an action potential was observed (Fig. 6F). These changes, in turn, led to a decrease in the intrinsic excitability of PL regular spiking neurons after trace fear conditioning. This was also supported by (1) a significant positive correlation between intrinsic excitability and R_N (Fig. 6G) and (2) a significant negative correlation between intrinsic excitability and $I_{\text{threshold}}$ (Fig. 6H).

Conclusions

The current data are the first to demonstrate that trace fear conditioning modulates the intrinsic excitability of layer 5 mPFC–BLA projection neurons in a subregion- and cell-type-specific manner. The intrinsic excitability of regular spiking IL-BLA projection neurons was enhanced after acquisition of trace fear conditioning in a learning-specific manner, suggesting that these neurons are involved in the formation of long-term conditioned fear memory. In contrast, the intrinsic excitability of regular spiking PL-BLA projection neurons was reduced after conditioning and was negatively correlated with the amount of conditioned fear after extinction, suggesting that these neurons are involved in the suppression of conditioned fear. Interestingly, the excitability of burst spiking PL-BLA projection neurons was enhanced after conditioning, which was opposite to that observed in regular PL-BLA projection neurons. Together, these data provide new information about how intrinsic changes within IL- and PL-BLA projection neurons are differentially involved in the formation and regulation of a trace fear conditioned memory.

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