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Blockade of amygdala metabotropic glutamate receptor subtype 1 impairs fear extinction

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Abstract

The metabotropic glutamate receptor subtype 1 (mGluR1) is thought to be crucial for several forms of memory, but its role in memory extinction has not been determined. Here, we examined a role of mGluR1 in the extinction of conditioned fear using microinjection of an mGluR1 antagonist, CPCCOEt, into the lateral amygdala (LA), a critical structure for fear conditioning and extinction. Intra-LA injection of 3 μg CPCCOEt impaired extinction that was initiated 48 h after the conditioning, but not that initiated 2 h after the conditioning, indicating that the effectiveness of CPCCOEt depends upon the length of time since fear conditioning. The CPCCOEt injection failed to alter an mGluR1-like receptor (mGluR5)-dependent acquisition of fear memory, further supporting the specificity of the injected CPCCOEt on mGluR1. Together, our results suggest that amygdala mGluR1 plays a critical role in the extinction of learned fear, but not in the acquisition of fear memory.

Keywords: Extinction; Conditioning; mGluR1; CPCCOEt; Amygdala; Consolidation

Fear extinction has been a good experimental model for the active modulation of fear memory and for clinical applications such as exposure-based psychotherapy. For example, it has recently been shown that agents that augment extinction in rodent models successfully enhance the treatment of specific phobias in humans [1]. Lesioning and pharmacological studies have identified two critical structures for extinction, the basolateral amygdala and prefrontal cortex [2–4]. In the basolateral amygdala, extinction of learned fear has been shown to be dependent on activity of NMDA receptors, L-type calcium channels, CB1 endocannabinoid receptors and BDNF signaling [4–10].

Group I mGluRs including mGluR1 and 5 have been implicated in synaptic plasticity and learning and memory [11,12]. Although mGluR5 has been shown to be critical for the acquisition of fear memory and long-term potentiation at thalamic input synapses onto the LA, a potential cellular substrate for fear memory [13], the involvement of mGluRs in fear extinction has not been documented. Interestingly, previous studies have suggested that mGluR1 might be involved in fear extinction: (1) mGluR1 has been implicated in endocannabinoid signaling which is critical for extinction [14], and (2) mGluR1 activity has been shown to be required for synaptic depotentiation at thalamic input synapses onto the LA, a putative cellular mechanism for fear extinction [15]. In addition, mGluR1 appears to be functionally expressed in the amygdaloid complex including the LA [14,16,17]. These previous findings, therefore, have led us to test the hypothesis that the activation of mGluR1 is a crucial step for the induction of fear extinction.

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Materials and methods

Male Sprague–Dawley rats weighing between 350 and 380 g were used. The rats were housed individually in plastic cages and maintained on an inverse 12/12 h light/dark cycle (light off at 09:00; training was done in the dark portion). The rats were anesthetized with sodium pentobarbital (50 mg/kg, i.p.). When fully anesthetized, they were mounted on a stereotaxic apparatus (David Kopf Instruments, Tujunga, CA), and 26 gauge stainless steel cannulas (model C315G; Plastic Products, Roanoke, VA) were implanted bilaterally into the LA (AP; −2.9 mm, ML; ±5.2 mm and DV; −7.0 mm from bregma). A 32 gauge dummy cannula was inserted into each guide cannula to prevent clogging. Two jewelry screws were implanted in the skull serving as anchors, and the whole assembly was affixed onto the skull with dental cement. The rats were given at least 1 week to recover before the experiments began.

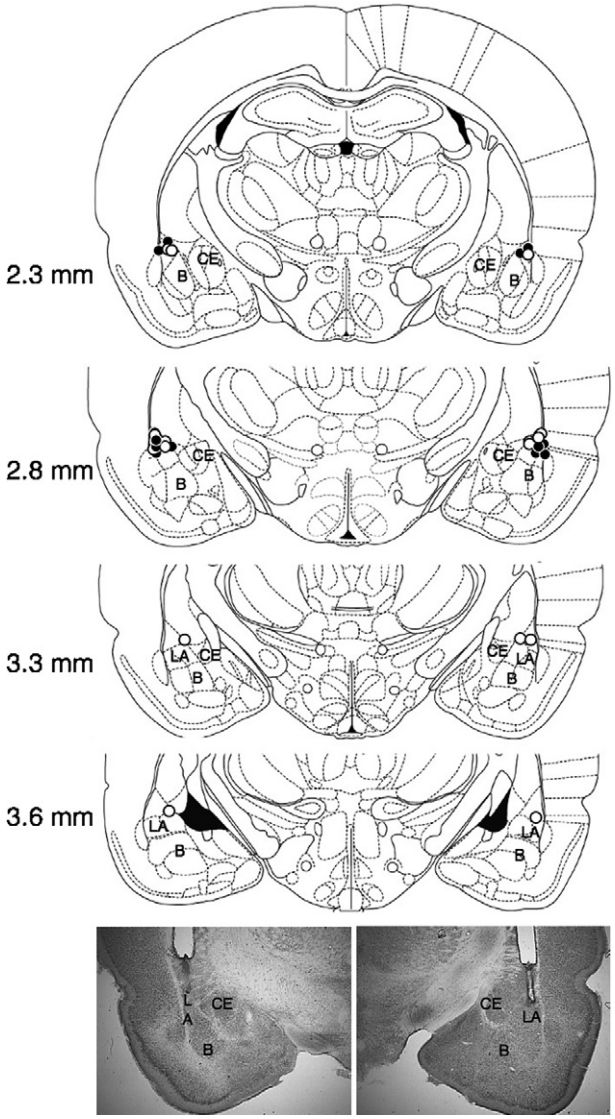

Fig. 1. Location of cannula tips in the LA for the vehicle- and CPCCOEt-injected groups which received extinction training in the experiments shown in Fig. 2A. Top, schematic representation of the LA at four different rostrocaudal planes. The numbers represent the posterior coordinate from bregma. Injector placements in the LA are represented by the symbols (○, vehicle-injected; ●, CPCCOEt-injected). Bottom, photomicrographs of representative cannula placements in the LA. Histology drawings were adapted form Paxinos and Watson [25]. LA, lateral nucleus; B, basal nucleus; CE, central nucleus.

To verify the intra-LA placement of the injector cannula tips, the rats were anesthetized following completion of the experiments with urethane (1 g/kg, i.p.) and transcardially perfused with 0.9% saline solution, followed by 10% buffered formalin. The brains were removed and post-fixed during overnight. Coronal sections (70 μm thick) were cut using a vibratome (Campden Instruments, Loughborough, UK), stained with cresyl-violet and examined under a light microscope.

CPCCOEt (Tocris, UK), an antagonist for mGluR1, was dissolved in 50% DMSO/saline. 30 min before behavioral training, CPCCOEt (0.5 μl in volume) was administered bilaterally into the lateral amygdala via a 33 gauge injector cannula (C315I; Plastic Products) attached to a 10 μl Hamilton syringe at a rate of 0.25 μl/min. Following the drug infusion, the injector cannulas were left in place for an additional minute to diffuse the drug away from the cannula tip. The dummy cannulas were then replaced. And the rats were returned to their home cages.

For fear conditioning, the rats were placed into a conditioning chamber (San Diego Instruments, CA, USA), and then, a tone (30 s, 2.8 kHz, 85 dB) coterminating with a footshock (0.5 s, 1.0 mA) was presented three times with an averaged interval of 100 s between each presentation. The rats were returned to their home cage 60 s after the last shock. A Plexiglas chamber that differed from the conditioning chamber was used for both the extinction training and a retention test. During extinction training, the rats were exposed to 15 trials of a 30 s tone without any footshock, and the retention of extinguished fear was assessed by exposing the rats to 2 trials of a 30 s tone on the following day. In all cases, presentation of the first tone began 4 min after placement of the rats in the chamber.

Freezing, defined as the cessation of all movements other than respiration, was used as the measure of conditioned fear [18], and was quantified by trained observers that were blind to the experimental groups. The results comparing single data points between groups were analyzed with an unpaired *t*-test (for only two treatment groups) or one-way ANOVA with subsequent Newman–Keuls *post hoc* comparison (for more than two treatment groups). The results comparing multiple data points between two groups were then analyzed with two-way ANOVA. Differences were considered significant if $p < 0.05$ (Fig. 1).

Results

In order to determine whether mGluR1 is involved in fear extinction, CPCCOEt, an antagonist for mGluR1, was microinjected into the LA 30 min before the first trial of extinction training that started 48 h after fear conditioning, and freezing was monitored on the following day to assess long-term extinction. Retention control rats were injected with either vehicle or CPCCOEt and placed in the extinction chambers for an equivalent period of time, but were not exposed to any tones. Rats infused with CPCCOEt or vehicle into the LA before extinction training exhibited similar freezing during the first block of extinction training (unpaired *t*-test, $p > 0.05$ for all the pairs shown in Fig. 2A–C), indicating that the fear memory was acquired and expressed to the same extent between the two groups. As shown in Fig. 2A, the microinjection of 3 μg CPCCOEt impaired extinction within the training session (short-term extinction) as compared with vehicle-injected controls (two-way ANOVA; for drug, $F(1,98) = 8.44$, $p = 0.01$; for drug × trial interaction, $F(7,98) = 1.34$, $p = 0.24$). The same injections also impaired long-term extinction as compared with vehicle-injected groups and the two retention controls. One-way

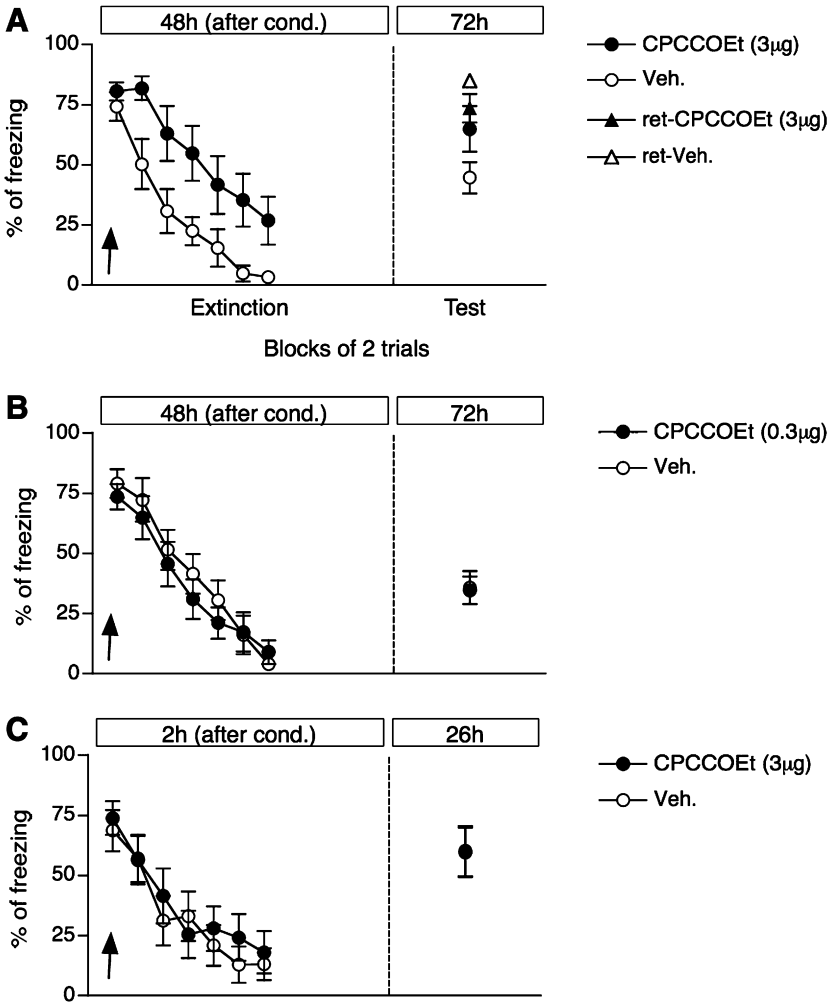

Fig. 2. Infusion of CPCCOEt, a blocker for mGluR1, impaired fear extinction. (A) Infusion of 3 μg CPCCOEt impaired both short-term and long-term extinction when extinction training was initiated 48 h after conditioning. Ret-CPCCOEt and ret-veh. represent retention controls for CPCCOEt- and vehicle-injected groups, respectively (see more details in the text). (B) Infusion of 0.3 μg CPCCOEt failed to impair extinction when extinction training was initiated 48 h after conditioning. (C) Infusion of 3 μg CPCCOEt failed to impair extinction when extinction training was initiated 2 h after conditioning. CPCCOEt or vehicle was injected 30 min before the first trial of extinction training. Long-term extinction was assessed by exposing subjects to two trials of tone presentation with an interval of 100 s 24 h after the end of extinction training. The data were analyzed in blocks of two trials (trial 15 of extinction not shown). The arrows indicate infusion and the error bars indicate SEM.

ANOVA indicated a main effect of group (ANOVA: $F(3,32) = 9.8$, $p < 0.0001$) with *post hoc* tests confirming that freezing in the vehicle-injected groups differed significantly from the CPCCOEt-injected groups, vehicle-injected retention controls and CPCCOEt-injected retention controls ($p < 0.05$), and that freezing did not differ between vehicle-injected retention controls and CPCCOEt-injected retention controls ($p > 0.05$). The latter result also indicates that the CPCCOEt injection along with the contextual exposure has no significant effects on the maintenance of consolidated fear memory. We next determined whether the observed effect of CPCCOEt was dose-dependent. As shown in Fig. 2B, the injection of 0.3 μg CPCCOEt showed no significant effects on either short-term or long-term extinction as compared with vehicle-injected controls (short-term extinction, for drug, $F(1,112) = 0.12$, $p = 0.74$, for drug × trial interaction, $F(7,112) = 1.13$, $p = 0.35$; long-term extinction, unpaired t-test, $p = 0.85$).

Recent studies have raised an intriguing possibility that mechanisms underlying the extinction of long-term fear memory (maintained >24 h after fear conditioning) differ from those underlying the extinction of short-term memory (maintained <4 h after fear conditioning) [19–21]. To examine whether CPCCOEt has different effects on the extinction of short-term fear memory, extinction training was initiated 2 h after fear conditioning. As shown in Fig. 2C, the injection of CPCCOEt had no significant effects on short-term extinction as compared with vehicle-injected controls (for drug, $F(1,133) = 0.13$, $p = 0.73$; for drug × trial interaction, $F(7,133) = 0.74$, $p = 0.64$). Unlike the case of the extinction of long-term fear memory (see Fig. 2A), the extinction of short-term fear memory in the

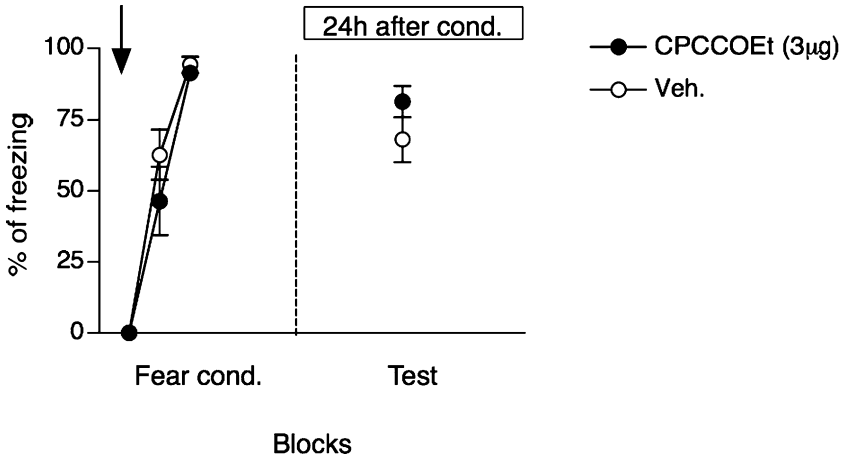

Fig. 3. CPCCOEt failed to alter the acquisition of fear memory. CPCCOEt or vehicle was injected 30 min before the first trial of fear conditioning. The testing sessions (exposing subjects to two trials of a 30 s tone with an interval of 100 s) were performed 24 h after the conditioning. The conditioning and testing data points were a block of one and two trials, respectively. The arrows indicate infusion and the error bars indicate SEM.

two groups failed to be retained 24 h after the extinction training. Therefore, we could not examine effects of CPCCOEt on long-term extinction in this case, though freezing monitored 24 h after the extinction training did not differ significantly between the two groups (unpaired t-test, $p = 0.97$). The failure in retention of the extinguished fear in the present study is consistent with a recent report showing that recent fear is resistant to extinction [22]. In sum, our findings support a model in which the status of memory consolidation determines predominant mechanisms underlying fear extinction. In addition, our findings argue against the possibility that the injected CPCCOEt produces gross reductions in movement or some defects in freezing behaviors as compared with the vehicle-injected rats: (1) the injected CPCCOEt failed to alter the extinction of short-term fear memory (see Fig. 2C), (2) it did not alter freezing during the first block of extinction training (see Fig. 2A–C), and (3) it did not change freezing during the testing session for the retention controls (see Fig. 2A).

We next examined whether the observed effect of CPCCOEt is specific to extinction. For this, we examined the effects of CPCCOEt on the acquisition of fear memory. CPCCOEt (3 μg) was microinjected into the LA 30 min before the first trial of fear conditioning, and freezing was monitored 24 h after the conditioning. The microinjection of CPCCOEt failed to impair fear conditioning as compared with vehicle-injected controls (unpaired t-test, $p = 0.26$) (Fig. 3). Since mGluR1 and 5, which belong to Group 1 mGluRs, show high degree of similarity in both the amino acid sequences and agonist selectivity [23], CPCCOEt, a blocker for mGluR1, might have crossreactivity to mGluR5. The CPCCOEt effect on the extinction of long-term fear memory, therefore, would be due to its blockading of mGluR5 rather than mGluR1. However, no significant effect of the injected CPCCOEt on fear conditioning, which has been shown to require mGluR5 activity [13], rules out this possibility, thereby backing up our conclusion that mGluR1 is the receptor subtype involved in fear extinction.

Discussion

In the present study, we have shown that the microinjection of CPCCOEt, a blocker for mGluR1, into the LA impairs both short-term and long-term extinction in a dose-dependent manner when extinction training is initiated 48 h after fear conditioning. Interestingly, the CPCCOEt injection does not exhibit any significant effects on extinction when extinction training is initiated 2 h after conditioning. Thus, whether extinction requires mGluR1 activity appears to depend on the length of time since fear conditioning. Additionally, the CPCCOEt injection fails to alter acquisition of fear memory, suggesting that mGluR1 activity is linked specifically to mechanisms underlying extinction.

The involvement of mGluR1 in extinction is consistent with previous findings. Azad et al. [14] has provided evidence that mGluR1 activation in inhibitory neurons in the basolateral amygdala induces activation of endocannabinoid signaling which is required for fear extinction. Also, a form of depotentiation at thalamic input synapses onto the LA, one of the major excitatory synapses in the LA, has been proposed to underlie fear extinction, and to depend upon mGluR1 activity [15]. Therefore, mGluR1 has been implicated in both excitatory and inhibitory pathways as a factor of potential importance in fear extinction. It remains to be determined what molecular and cellular mechanisms underlie mGluR1 dependency of fear extinction, and which of the pathways (excitatory vs. inhibitory) is more important for the action of mGluR1.

The most parsimonious explanation for the impairment of long-term extinction in the CPCCOEt-injected groups would be the extension of the attenuated short-term extinction. However, there is, albeit less likely, an

alternative explanation for the impairment of long-term extinction, a phenomenon referred to as state-dependent learning [24]: recall of extinction training experience (long-term extinction) often requires the presence of a drug that has been infused during extinction training, as if subjects learned to associate the drug injection with the extinction training experience. In another words, the impairment of long-term extinction observed in the CPCCOEt-injected groups (when long-term extinction is assessed in the absence of CPCCOEt as in the present study) would be due to a failure in recall of the extinction training experience; that is, the rats would fail to recall the extinction training experience without CPCCOEt injected in testing sessions and so would freeze as strongly as conditioned rats. Together, activation of mGluR1 during short-term extinction appears to be required for long-term extinction, but its underlying mechanisms remain to be elucidated.

Our findings also support a recent view that predominant mechanisms for extinction are altered depending upon the consolidation state of fear memory [19–21]. Recently, Myers et al. [21] have demonstrated that relapse phenomena, such as renewal and reinstatement, are observed only when conditioned fear is extinguished 24–72 h after conditioning, and that the relapse does not exist when extinction training is performed 10 min to 1 h after conditioning. Consistently, Cain et al. [19] have reported that fear extinction initiated immediately following conditioning is insensitive to the L-type voltage-gated calcium channel (L-VGCC) blocker nifedipine, whereas extinction initiated 1 or 3 h after conditioning is impaired. Moreover, Mao et al. [20] have suggested that, under their experimental conditions, conditioning-induced enhancement in the surface level of GluR1 subunits is reversed by extinction training applied 1 h after conditioning, but not 24 h after conditioning. Consistently, in the present study, CPCCOEt has been shown to be effective in attenuating extinction after fear memory is consolidated, but not before. It is not totally clear for now whether memory consolidation is crucial for adopting different extinction mechanisms, but it suffices to say that multiple mechanisms underlie the extinction of conditioned fear.

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