Dynamic Regulation of the Synaptic Plasticity in the Prefronto-Amygdala Pathway Following Fear Extinction in Rats[*]

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Fear conditioning, in which a neutral conditioned stimulus (CS) is contingently paired with an aversive unconditioned stimulus (US) and acquires capacity to elicit conditioned response (CR), has been studied extensively to elucidate neural substrate of associative memory. It has been shown that the critical change for the fear memory is stored as a form of modified synaptic response in the lateral nucleus (LA) of the amygdala. On the other hand, extinction, in which the fear CR is reduced as a result of repeated exposure to the CS without the US, has been known to depend on the infralimbic cortex (IL) of the medial prefrontal cortex. However, little is known about the interaction between the IL and LA over the course of extinction process. In the current study, we investigated the synaptic changes in the IL-LA pathway by measuring evoked field potentials (EFPs) before and after fear extinction. Following fear conditioning in which the rats were presented with five parings of the CS and footshock US, they were subjected to two extinction sessions composed of 10 CS-only trials each. In addition, they received a retention test composed of three CS-only presentations. To measure synaptic plasticity, evoked field potentials (EFPs) were recorded in the lateral nucleus of the amygdala (LA) by stimulating the IL, six hours after every session. The recorded EFP was significantly increased after the first extinction but not after the subsequent sessions. These data indicate that dynamic regulation of IL-LA synaptic efficacy underlies the suppression of conditioned fear response following extinction and suggest that consolidation of memory extinction involves changes in other synapses than IL-LA pathway.

Key words : fear, conditioning, extinction, medial prefrontal cortex, evoked field potential

Introduction

Fear conditioning has been a popular model system for investigating the neural substrate of associative learning (Fanselow & LeDoux, 1999; LeDoux, 2000). In a typical fear conditioning, a neutral conditioned stimulus (CS) is paired with an aversive unconditioned stimulus (US) and acquires capacity to elicit the conditioned response (CR), a wide array of coordinated behavioral and cognitive responses. Mounting evidences suggest that the formation of fear memory is dependent on synaptic plasticity within the amygdala, especially the lateral nucleus (LA) which interfaces thalamic and cortical sensory projections into the amygdala (Blair, Schafe, Bauer, Rodrigues, & LeDoux, 2001). Specifically, neuronal response to the CS increases significantly following fear conditioning (Pare & Collins, 2000; Quirk, Repa, & LeDoux, 1995) and synaptic efficacy between the auditory thalamus and the LA is potentiated when measured both in vivo (Rogan, Staubli, & LeDoux, 1997)and in vitro (McKernan & Shinnick-Gallagher, 1997). In addition, inactivation of the LA disrupts intracellular signaling process necessary for synaptic long-term potentiation and interferes with the acquisition of the fear CR (Rodrigues, Schafe, & LeDoux, 2004). Therefore, it is reasonable to assume that the crucial component of fear memory is stored in the LA in the form of modified neuronal response.

The acquired fear CR is not permanent, however, and could be reduced by extinction, a behavioral procedure composed of repeated presentation of the CS without the US (Pavlov, 1927). The medial prefrontal cortex (mPFC) and its interaction with the amygdala have been implicated in extinction. Lesions of the mPFC before the acquisition prevents the extinction of the fear CR (Morgan, Romanski, & LeDoux, 1993). In addition, neuronal activity is significantly increased in the mPFC following extinction and stimulation of the same structure facilitates extinction process (Milad & Quirk, 2002). More specifically, the infralimbic subregion of the mPFC (IL) is believed to inhibit amygdala activity to the fearful stimulus under safe conditions, which results in suppression of the fear CR (Lebron, Milad, & Quirk, 2004). In addition, CS-evoked firing rates of LA neurons are reduced following repeated presentation of the CS without the US (Repa et al., 2001). Therefore, it has been proposed that combined actions of the increased neuronal response in the IL and its inhibitory projections onto the amygdala may provide neural mechanisms for decreased cellular response in the thalamo-amygdala pathway following the extinction procedure (Park & Choi, 2010).

While evidence supporting that the activation

of the mPFC is correlated with extinction memory is strong, little is known about the nature of synaptic response change in the sensory pathway into the amygdala following extinction. To address this issue, we monitored synaptic efficacy in the IL-LA pathway following the initial acquisition of auditory fear conditioning and subsequent extinction sessions by measuring extracellular population response (McKernan & Shinnick-Gallagher, 1997; Rogan et al., 1997).

Materials and Methods

Subjects　Male Sprague-Dawley rats (Orient Bio Inc., Gyunggi-do, Korea) weighing 200 - 450g were used. They were individually housed in standard plastic cages (22.5 cm height × 22 cm length × 30.5 cm wide) located in a room with an inversed 12-hr light-dark cycle (light off: 09:00, light on: 21:00). Food and water were offered ad libitum. All experiments were conducted during the dark phase of the cycle.

Surgery　The rats were anesthetized with sodium pentobarbital (60 mg/kg, i.p.). Additional doses were used when necessary. Anesthetized rats were mounted on a stereotaxic instrument (David Kopf Instruments, Tujunga, CA, USA), and their skull was aligned in a way that bregma and lamda were on the same horizontal plan. The scalp was incised, and the skull was drilled for electrodes and screws. Bipolar stimulating electrodes (Plastics One, Roanoke, VA, USA) insulated with polyimide except for the tip (tip diameter, 0.125 mm) was unilaterally placed in the left IL (AP: 2.9 mm, ML: 0.5 mm, DV: 5.0 mm from bregma). For the field potential recording, a sharp tungsten electrode (A-M systems, Carlsborg, WA, USA) insulated with epoxy (impedance; 4 - 5 MΩ) was placed in the left LA (AP: -3.2 mm, ML: 5.3 mm, DV: 6.0 mm from bregma) and a reference electrode was implanted in the motor cortical area. All rats were allowed a minimum of seven days for recovery before the behavioral sessions began.

Recording the neural activity　Evoked field potentials (EFPs) were measured in the IL-LA pathway. The rats were anesthetized with sodium pentobarbital. The EFPs were amplified (× 1000), filtered (10 Hz - 1 kHz), digitized (10 kHz) and saved to computer for off-line analysis using LabVIEW (National Instruments, Austin, TX, USA). The EFPs were displayed on an oscilloscope for real-time monitoring. Recorded signals were analyzed by a custom-made program (LABVIEW, National Instrument) to measure the peak-to-valley amplitude (potential difference between the first positive peak and negative valley) (Kwon &

Choi, 2009). Before the recording sessions began, the current intensity was determined by stimulating IL with low-frequency monophasic test pulses (100 μs at every 30 s). The intensity was gradually increased from 0.2 - 1.2 mA and the size of the EFPs were measured in LA until the maximum current intensity was determined, which was defined as the current amount that no longer increases the EFP amplitude with additional current. Half of the maximum current was used to stimulate the IL for EFP recording in the LA throughout the experiment.

Behavioral apparatus   The conditioning chamber (30 cm × 34cm × 42 cm) was made of Plexiglas and placed in a sound attenuating cubicle (58 cm × 58 cm × 68 cm). Illumination was provided by an overhead LED light. A small speaker (8 cm × 4 cm, 8 Ω) was attached on the wall of the chamber and a video camera was mounted inside the cubicle. There were two distinct contexts for context shift. For context A, the floor consist of 16 stainless steel rods (0.5 cm in diameter, 1.5 cm apart) connected to a shock generator (Coulbourn Instruments, Whitehall, PA, USA) to produce footshock and the illumination was provided by LED light through red-colored filter. For context B, the floor was covered with a soft plastic mat and the illumination was blue. The CS was a tone (2 kHz, 80 dB, 30 s) and the

US was mild footshock (0.5 mA, 0.7 ms). After the each session, the chamber was cleaned with 70% ethanol.

Experimental Procedures   Behavioral procedures were performed for five consecutive days as illustrated in Fig. 1. On day 1, all rats were placed in context A and after 3 min, received three CS-only presentations (Habituation) separated by an averaged inter-trial interval (ITI) of 180 s (randomly varying between 120 - 210 s). On day 2, they received delayed fear conditioning consisted of five pairings of the CS and co-terminating US with the ITI of 180 s (Conditioning). On day 3 and 4, the rats were divided into two groups. The extinction group (EXT) received 10 CS presentations in context B and no-extinction group (NE) simply sit in the chamber without any CS for the same duration of time. On day 5, all rats were tested for response strength by presenting 3 CS-alone trials (Retention). After each behavioral session, all rats were sent back to the home cage and allowed to stay without disturbance. Six hours later, the recording session began. The rats were anesthetized with sodium phentobarbital and the EFPs were measured by stimulating the IL through the stimulating electrode at every 30 s for 20 min.

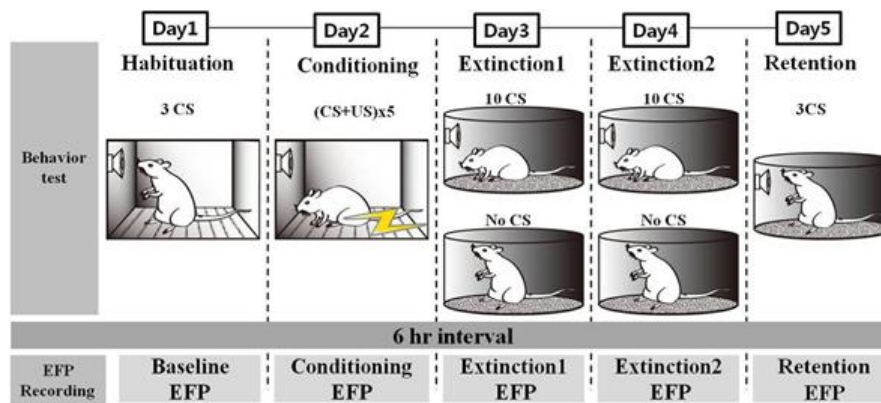**Histology**   At the end of the experiments, all

- 120 -

Figure 1. Diagram showing experimental sessions for 5 days. Refer to the text for further description of the experimental procedure.
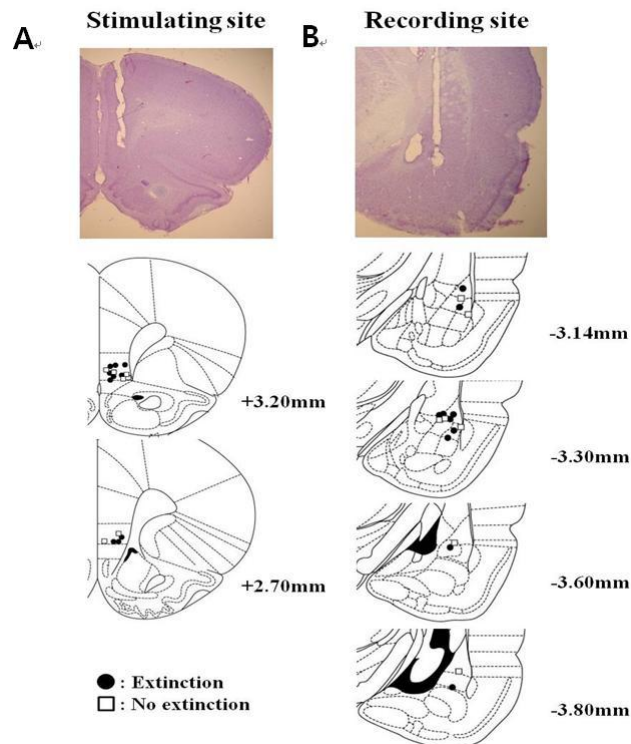

Figure 2. Reconstruction of electrode placements. (A) Stimulating electrode sites in the IL. (B) Recording electrode sites in the LA. Open square: no-extinction group (n = 7), black circle: extinction group (n = 10).

rats were overdosed with sodium pentobarbital (80 mg/kg, i.p.) and recording and stimulation sites were marked by a small lesion (0.1 mA D.C, 10 s). They were perfused with saline (0.9%), followed by formaldehyde (10%) in 0.9% saline. The brains were stored in 30% sucrose in 10% formaldehyde solution. The brains were cut into 50 μm coronal sections with a sliding microtome (Leica SM 2000R, Leica Microsystems, Nussloch, Germany). The brain slices were mounted on gelatin-coated slides, stained with cresyl violet and cover-slipped with Permount. After the staining, the slices were examined under a microscope (x 10) and photographed. Only data from rats with both stimulating and recording electrodes in the targeted area were used for further analyses.

Results

Histological Analysis After excluding the rats with electrode tip placed outside the targeted areas of LA or IL, a total of 17 rats were included in the final analysis. Figure 2 shows a photomicrograph of a coronal brain section and schematic representation of stimulating and recording sites based on the rat brain atlas (Paxinos & Watson, 1998).

Behavioral changes during fear conditioning, extinction and retention

test Figure 3A shows freezing responses across all behavioral sessions. A repeated measures ANOVA revealed significant session effect [$F_{(3, 45)}$ = 13.842, $p < 0.001$], but not group effect [$F_{(1, 15)}$ = 0.234, $p > 0.05$]. There was significant group x session interaction [$F_{(3, 45)}$ = 14.245, $p < 0.05$]. For further analysis of group effect, Bonferroni's post hoc comparison was performed. There was no significant difference in freezing level between the two groups during the conditioning session. However, EXT group showed significantly more CS-elicited freezing compared to the NE group during Extinction 1 ($p < 0.05$). The difference was expected because the NE group did not receive any CS during the extinction session. This difference was markedly reduced in Extinction 2 ($p = 0.131$) due to the reduced freezing level in EXT group reflecting effective extinction. In the retention test session in which both groups received the CS, freezing level was significantly different. ($p < 0.05$) because the level of freezing to the CS in NE group was high.

Dynamic changes in EFPs following a single and multiple extinction sessions To test changes in the synaptic efficacy in the IL-LA pathway during the course of extinction, EFPs were recorded repeatedly after every session (Fig. 3B & C).

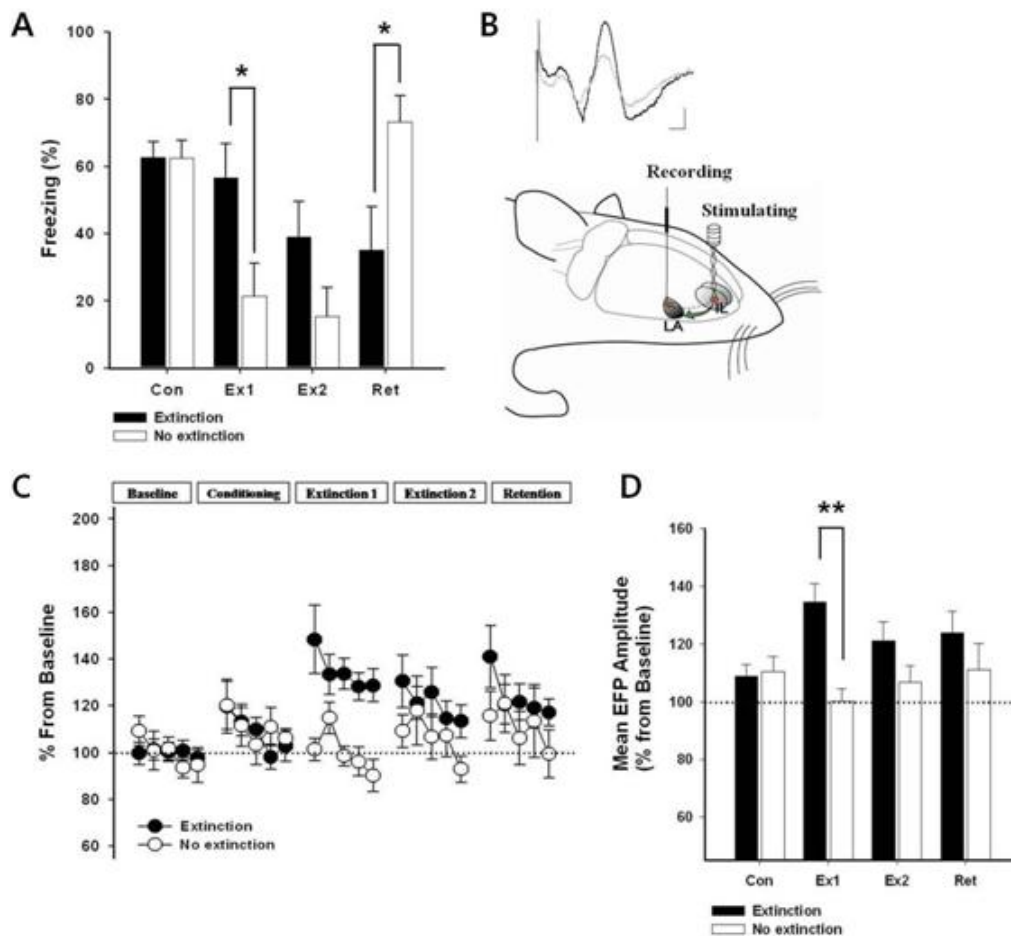The mean EFP amplitude of conditioning

Figure 3. Synaptic changes in the IL-LA pathway following fear extinction.
(A) Average freezing levels during conditioning, extinction and retention test sessions. (B) An example of recorded EFP following conditioning and extinction (top) and a schematic diagram of the electrode locations (bottom). Compared to the baseline, the EFP amplitude increased after the first extinction session (Scale bar = 200 μV, 10 ms). Grey line: After Baseline, Black line: EFP After Extinction 1. (C) Average changes in EFP during baseline, conditioning, extinction and retention sessions. (D) Comparison of average EFP amplitudes in each session. The mean EFP amplitude of EXT group was significantly higher compared to the NE group only after Extintion 1. **: p < 0.01

session was used as the baseline with which the percentage amplitudes of all other sessions were calculated. The mean changes in the EFP

amplitude following conditioning and extinction were analyzed by two-way repeated measures ANOVA. There was no significant session effect

[F (3, 45) = 0.999, p > 0.05] but a significant group effect [F (1, 15) = 5.106, p < 0.05]. In addition, there was significant interaction [F (3, 45) = 3.954, p < 0.05]. For the detailed comparison between the two groups, Bonferroni post-hoc test was performed. There was a significant difference between the NE and EXT group only in Extinction 1 (p < 0.01). None of the comparisons between the two groups in other sessions were significant (Fig. 3D).

Discussion

In the current study, repeated presentations of the CS following fear conditioning led to suppression of the CR, showing a robust time course of behavioral extinction. Concurrently measured EFP between the IL and the LA was increased following the first extinction training. The increased EFP, however, was reduced in the subsequent extinction sessions, returning toward the baseline level. The initial increase in synaptic plasticity between IL-LA indicates that the neural circuit involving the IL-LA synapse is critically engaged during the acquisition of extinction memory. However, the reduced population response in the subsequent extinction sessions suggests that dynamic regulation of the synapse might be necessary for the storage and maintenance of the extinction memory.

Enhanced EFP after extinction Neuronal response within the IL is considered as a representation by which the extinction memory is stored and expressed (Milad & Quirk, 2002). To support, the IL neurons projecting to the LA inhibit the activity of the amygdala neurons (Herry et al., 2008) perhaps via GABAergic interneurons (Ehrlich et al., 2009), resulting in reduced fear response. Consolidation of fear extinction memory needs protein synthesis in the mPFC (Santini, Ge, Ren, Pena de Ortiz, & Quirk, 2004). Therefore, the connection between the IL and the defensive circuit in the amygdala by which a fear response generation is modulated is the key to understanding the extinction process.

In the current study, population potentials measured in the LA evoked by IL stimulation was significantly increased after Extinction 1 but not after Conditioning indicating that the IL-LA synapse is closely linked to post-acquisition regulation of the fear response **(Fig. 3C & D)**. The EFPs were measured only 6 hours after the extinction session and might reflect changes in the neurons within the IL (Quirk, Russo, Barron, & Lebron, 2000) as well as synaptic changes in IL-LA pathway. The increased EFPs may reflect enhanced synaptic efficacy between the IL and inhibitory interneurons in the LA, thus contributing to the suppression of the fear CR. In sum, rapid changes in the EFP amplitude

provide an additional support that increased connection strength between the IL and LA is critical for extinction-mediated suppression of defensive response.

Transient change of the EFP Somewhat surprising from the current study is the finding that the initial increase in the EFP was reduced on the subsequent extinction sessions. In fact, the magnitude of the EFP was not significant from the baseline or post-conditioning session. This dynamic change could be interpreted in several ways.

One way of interpreting the dynamic changes in the EFP is that the IL is involved in short-term modification of the fear CR but not necessarily for the maintenance of the suppressed fear response. The extinguished fear CR continued to be low in freezing in the subsequent extinction sessions despite the returned EFP. In fact, several studies suggest that other structures might be involved in storing and expressing extinction memory. For example, rats with mPFC lesion, although more slowly than normal animals, could learn extinction eventually (Lebron et al., 2004). In addition, the hippocampus is essential for fear extinction in conjunction with the mPFC and could modulate fear extinction independently from the interaction (Farinelli, Deschaux, Hugues, Thevenet, & Garcia, 2006). Other

studies suggest that modified neuronal property within the amygdala especially in the basolateral amygdala (BLA) could maintain the level of fear response beyond the initial extinction training (Kim et al., 2007).

Second possibility comes from the fact that the EFPs measured in the LA by IL stimulation involve multiple types of synapses of which plasticity develop with different time course in addition to the IL-LA connection (Rosenkranz & Grace, 2002). A bipolar stimulating electrode has relatively large diameter (~125 um) which could recruit thousands of neurons or more and field potential recordings also involve heterogenous population of neurons. Therefore, an initial change which was expressed as increased EFP in the IL-LA synapse, could have triggered a cascade of other interconnections between IL-LA pathway later, resulting in little net change of EFP as observed in the subsequent sessions (**Fig. 3C & D**). Therefore further study is needed to measure synaptic response in much smaller group of neurons, by employing more elaborate techniques such as extracellular or intracellular single-unit recording.

In sum, extinction-related cellular changes within the IL-LA pathway accompany behavioral suppression of learned fear response, supporting the cortical contribution to the acquisition of extinction memory. In addition, these enhancements were transient, indicating that the

regulation of extinction memory is dynamic and may require changes in broader brain circuit outside the IL-LA synapse.

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공포조건화 소거에 따른
전두엽-편도체 시냅스 강도의 역동적 조절

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공포조건화는 연합기억의 신경생물학적 기반을 설명하기 위해 널리 사용되어온 모델 파라다임으로 소리와 같은 조건자극(CS)과 발바닥 전기자극과 무조건 자극(US)을 짝지워 제시하는 절차를 통해 조건반응(CR)이 출현하게 된다. 이러한 공포조건화의 학습에는 편도체의 외측핵(lateral nucleus: LA)가 관여한다는 사실이 알려져 있다. 반면, 조건화 학습 후 CS만을 반복해서 제시하면 CR이 감소되는 결과를 초래하며 이를 소거(extinction)라고 일컫는다. 이러한 소거에는 전전두 피질의 하위 영역인 변연하영역(infralimbic cortex: IL)이 관여함이 알려져 있다. 본 연구에서는 변연하 영역과 외측핵 간(IL-LA)의 시냅스 가소성이 소거 과정에 따라 변화하는 양상을 유발 장전위 (evoked field potential: EFP) 기록을 통해 규명하였다. 이를 위해 랫트(rat)을 대상으로 수술을 통해 뇌의 IL에는 자극전극을 LA에는 기록전극을 항구적으로 임플랜트 한 후 공포조건화를 실시하였다. 그 이후 10회의 CS로 이루어진 소거 회기를 2회 실시하였고 최종적으로 3회의 CS를 제시하는 검사 회기를 통하여 CR의 변화 양상을 측정하였다. 이와 동시에 매 회기 6시간 후마다 EFP를 기록하여 IL-LA 시냅스 강도를 측정하였다. 그 결과 첫 번째 소거 회기 후에 IL-LA 의 EFP가 학습 전의 기저선에 비해 유의미하게 증가한 것을 발견하였다. 그러나 이러한 증가는 두 번째 소거 회기 후나 검사 회기 후에는 다시 기저선과 유사한 수준으로 감소하였다. 이러한 결과는 소거 관련 IL-LA 시냅스변화가 고정적이지 않고 역동적이며, 뿐만 아니라 소거와 관련된 기억이 최초에는 IL-LA 경로와 관련하여 저장되지만 장기 기억으로 전환되는 과정에서 이외의 다른 시냅스도 아울러 포함할 가능성을 제시한다.

주요어 : 공포, 조건화, 소거학습, 내측 전전두엽, 유발 장전위