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On- and off-line activities in the basolateral amygdala

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Abstract

On- and off-line activities in the basolateral amygdala

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Pavlovian fear conditioning involves repetitive pairing of neutral conditioned stimuli (CS) and noxious unconditioned stimuli (US). It is reported that the basolateral amygdala (BLA) and its neural networks are critical for fear conditioning and extinction in the previous studies using this model. Drawbacks of previous studies are as follows: Researchers have focused on isolating a specific neural subpopulations that shows meaningful changes during conditioning and extinction, which may produce biased

representation of the whole population, and to deal only with CS-evoked responses (on-line responses) but not with spontaneous responses after CS presentation (off-line responses).

In the chapter 1, I recorded activities from single neurons in the basal nucleus of the amygdala (BA) during conditioning and multiple sessions of extinction. First, I probed changes in neural activities of the whole population using Gaussian-process factor analysis (GPFA). When compared with baseline response (recorded during habituation), activity patterns were increasingly different as conditioning and extinction training proceeded. Then I tracked down activities of single neurons and found that distinct subpopulations of BA neurons were recruited during conditioning and three different sessions of extinction, suggesting that the changes in activity patterns of the whole population are, at least partially, due to session-specific recruitment of new subpopulations.

In the second chapter, I examined off-line activities in the lateral nucleus of the amygdala (LA), the medial prefrontal cortex and the hippocampus after fear memory retrieval (i.e., after CS presentation in conditioned animals). The previous results from my laboratory implied the existence of off-line activities which may be involved in recurrent and spontaneous retrieval of fear memory. Consistently, oscillations of neural activities at low frequencies in the three brain regions became stronger during spontaneous freezing behaviors. Furthermore, the

enhanced oscillations at low frequencies became synchronized during spontaneous freezing behaviors, suggesting stronger connectivity between the three regions.

In conclusion, I studied on- and off-line activities in the BLA. First, I found that the basal nucleus changed its on-line activity patterns as conditioning and three different sessions of extinction proceeded. The changes in activity patterns may be due to recruitment of new subpopulations of BA neurons. Second, I found that neural networks between the LA, the prefrontal cortex and the hippocampus became stronger during off-line freezing behavior. Together, my study provides a broader understanding of the way by which the amygdala processes emotional information and will be useful for the treatment of fear related mental disorders.

Key words: basolateral amygdala, basal amygdala, lateral amygdala, fear conditioning, fear extinction, on-line activity, off-line activity

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Background and Purpose

1. Background

1.1. Pavlovian fear conditioning

1.1.1. Features of Pavlovian fear conditioning

When you walk through the woods if you hear a roar somewhere. In this situation, how do you feel? Average person may feel fear. Study of fear is very important because it is closely related to our survival by inducing defensive behavior to threat. Pavlovian fear conditioning is well-verified animal model for study of fear. Study of fear has been extensively conducted by using this model. A neutral conditioned stimulus (CS), which is an auditory cue mainly, is repeatedly presented with an aversive unconditioned stimulus (US) such as electrical foot shock (Fig. 1). During this process, animals learn quickly that the CS is a predictive signal of a noxious stimulus, then they exhibit defensive behavior (e.g. freezing, avoidance) and physiological alterations in heart rate, blood pressure (Kapp et al., 1979; Davis, 1992). These responses also appear when CS is presented alone (Fig. 1).

Fear memory is stable and acquired quickly. Presentation of even only one CS paired with noxious stimulus is enough to fear conditioning (Fanselow, 1994). Furthermore, once fear memory is built, it is long-lasting even throughout life.

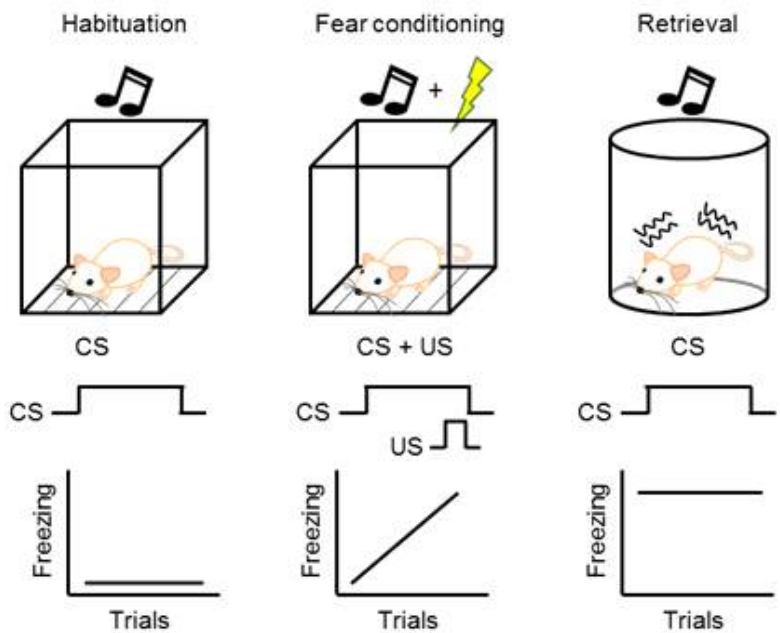

Figure 1. Pavlovian fear conditioning. Rats do not exhibit freezing response to CS in habituation. During fear conditioning, rats learn quickly that CS is predictive cue for US. After fear conditioning, in fear memory retrieval, only CS presentation elicits freezing behavior.

1.1.2. Neural systems underlying fear conditioning

The neural mechanism has been studied extensively via Pavlovian fear conditioning model in various brain regions. Among them, the amygdala has been studied intensively for emotion of fear across species. It was studied that who was damaged the amygdala could not acquire CS-US association (LaBar et al., 1995; Phelps and LeDoux, 2005).

Then, how the amygdala works during fear conditioning? The rodent amygdala which consists of several nuclei anatomically and functionally, including the lateral (LA), basal (BA) and central (CE) (Brodal, 1947; McDonald, 1982). During fear conditioning, these sub regions of the amygdala have distinct role respectively. First, convergence of CS and US occurs in the LA which accepted sensory inputs from the thalamus and cortex. In this process, synaptic plasticity is induced (McKernan and Shinnick-Gallagher, 1997; Quirk et al., 1997). LA neurons have interconnections with CE neurons through direct and indirect pathway via the BA. The CE also connects with the hypothalamus and brainstem which control the responses from conditioned fear such as freezing and autonomic and hormonal responses (Maren and Fanswlow, 1996; Onaka, 2000; Lukkes et al, 2009).

In addition to this process, each sub region has been studied for fear conditioning. First, it was studied that the LA was required to association between CS and US (Nader et al.,

2001). Also, CS-evoked single neurons activities of the LA increases after fear conditioning but disappears after extinction according to freezing level (Quirk et al., 1997; An et al., 2011). Neurons in the CE also have CS-evoked activities which increase after fear conditioning and during retrieval but not after fear extinction like the LA neurons (Duvarci et al., 2011). It was studied that CE-lesioned rats did not exhibit freezing response to CS (Choi and Brown, 2003). In addition, the BA was also studied for fear conditioning. In case of the BA, its activities present state of fear. For evidence of this, activities of BA neurons switch according to fear level of substrate (Herry et al., 2008).

In other brain regions, the medial prefrontal cortex and the hippocampus are mainly involved in fear conditioning. The medial prefrontal cortex (mPFC) consists of two sub regions, including the prelimbic cortex (PL) in dorsal part of the mPFC and the infralimbic cortex (IL) in ventral part. It is also distinct functionally. The PL is related in fear conditioning but the IL is related in fear extinction. During fear memory retrieval, PL-lesioned rats do not express of fear response toward CS, thus activity of the PL is necessary for expression of fear (Corcoran and Quirk, 2007; Sierra-Mercado et al., 2011). In the study of neural oscillations, 4 Hz oscillations were strong during freezing behavior in the PL and also BLA (Karalis et al., 2016). Coherence between PL and BLA even enhanced at the same

band oscillations with freezing. The hippocampus is also involved in fear conditioning. In this case in particular, neutral conditioned stimulus is context (Phillips and LeDoux, 1992; Antoniadis and McDonal, 2000). Inactivation of the hippocampus impairs contextual fear memory (Saxe et al., 2006). In the study with amygdala, improved synchronization of theta activities between the dorsal hippocampal CA1 and the LA was found during fear memory retrieval (Seidenbecher et al., 2003).

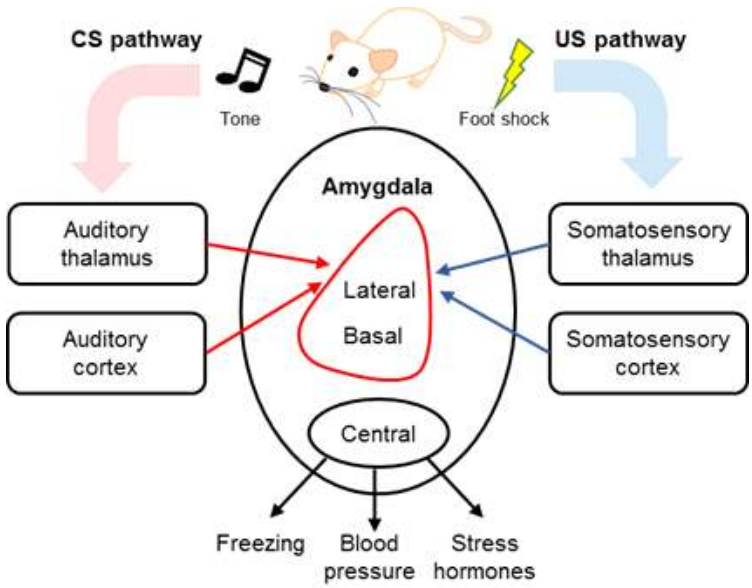

Figure 2. Neural circuits during fear conditioning. The basolateral amygdala receives sensory information of CS and US from the thalamic and the cortical areas. The central amygdala sends outputs to the brainstem which controls behavioral and autonomic responses to the CS.

1.2. Fear extinction

1.2.1. Features of fear extinction

When repeated CS alone in absence of aversive stimuli are presented after fear conditioning, the CS does not elicit fear response no longer (Fig. 2). This phenomenon is termed as fear extinction and has been studied by animal model of exposure therapy.

Different from fear conditioning, fear extinction occurs gradually and is required numerous CS presentations whit no noxious events. In addition, fear extinction arises context-dependent. When CS is presented in different context where fear extinction occurred in previous, extinguished fear memory recovers (Bouton and King, 1983; Bouton, 2004; Maren and Quirk, 2004). This phenomenon is known as fear renewal. Thus, in the same context where fear extinction is conducted, extinction memory retrieves. Furthermore, extinction memory is weaker than fear memory. For this evidence, fear response can spontaneously reappear in several weeks after extensive fear extinction (Quirk, 2002; Rescorla, 2004).

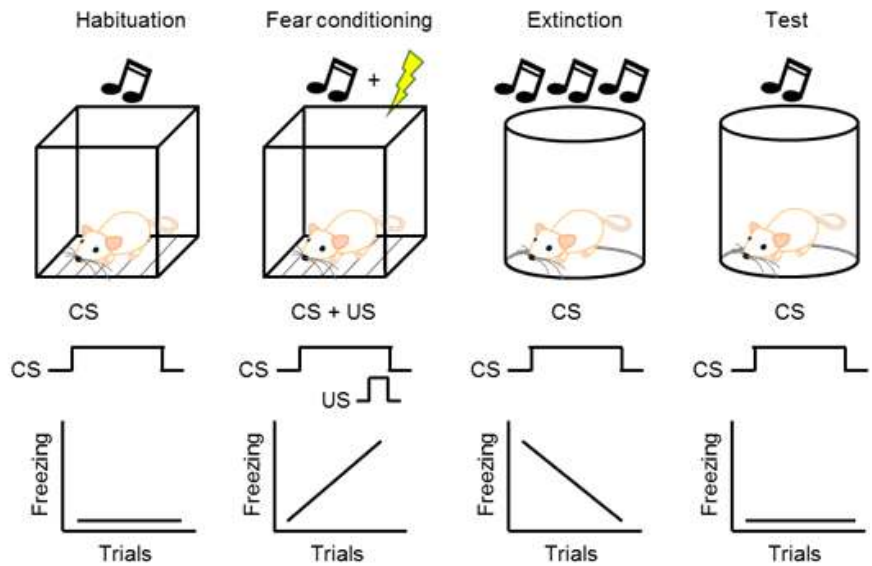

Figure 3. Fear extinction. After fear conditioning, rats exhibit freezing behavior toward CS. When CS is repeatedly presented, during fear extinction, freezing behavior diminishes again.

1.2.2. Neuronal activities underlying fear extinction

The BLA is also known that it is involved in fear extinction. Activities of BLA neurons increases after fear conditioning and depotention occurs after fear extinction in the electrophysiological studies (Rogan et al., 1997; Kim et al. 2007).

Then, are separated populations participated in fear extinction in the BLA? Especially, BLA neurons exhibit various activities for fear extinction. In the previous studies, three populations are observed in the BLA (Duvarci and Pare, 2014). One population have tone-evoked activity after fear conditioning but their activities disappears after fear extinction. This population is known as 'fear neurons' (Herry, et al., 2008; Amano et al., 2011; An et al., 2012). In contrast of this population, activities of 'extinction neurons' selectively increase after fear extinction when low fear states, but do not increase after fear conditioning (Herry et al., 2008; Amano et al., 2011). Activities of these two populations change contrastively according to fear states. It is suggested that fear neurons and extinction neurons have distinct pathway respectively. Indeed, they have differential connections with the mPFC and the hippocampus (Krettek an Price, 1977; Herry et al., 2008). Fear neurons project to the mPFC strongly but receive ventral hippocampal input weakly. In contrast, extinction neurons have strong reciprocal connectivity with the mPFC. These each circuits of fear- and extinction neurons

probably modulate the transitional states between high and low fear states by control the balance of circuit activity (Herry et al., 2010). Furthermore, in recent study, fear- and extinction neurons connect with distinct sub regions of the mPFC, including the PL and the IL. The PL supports expression of fear and the IL is involved in extinction (Sotres-Bayon and Quirk, 2010) Consistent with the previous findings, it was observed that fear neurons projected to the PL and extinction neurons had projections to the IL (Senn et al., 2014). In long-term behavioral paradigm that consists of fear conditioning and multiple extinction, activities of fear neurons and extinction neurons were also recorded (An et al., 2017). CS-evoked responses of both populations decreased after extensive extinction in this study. The last population is 'extinction-resistant neurons'. CS-evoked response of this population increases after fear conditioning and the activity persists even after fear extinction unlike fear neurons (Herry et al., 2008; An et al., 2012; Duvarci and Pare, 2014). It is probably related in the maintenance of association between CS and US after fear extinction. In contrast to fear- and extinction neurons, connection of extinction-resistant neurons with other regions remains unknown.

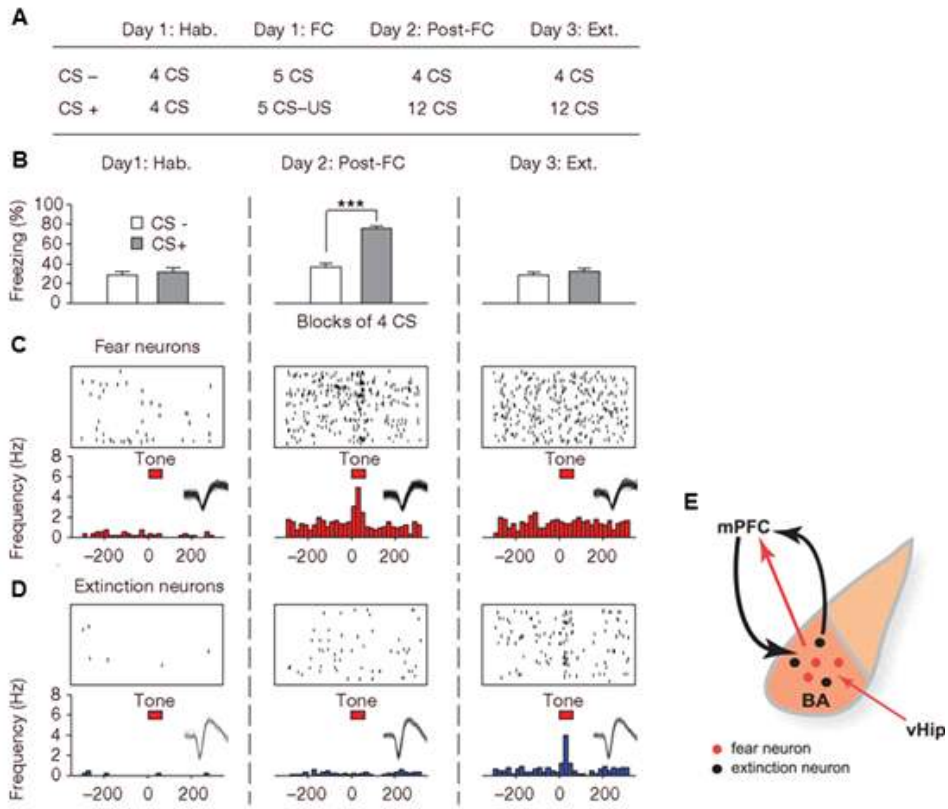

Figure 4. Fear neurons and extinction neurons. (A) Procedure of fear conditioning and extinction. (B) After fear conditioning, freezing behavior increases and diminishes again after fear extinction. (C) Fear neurons activities appear after fear conditioning but disappear after extinction (D) and activities of extinction neurons are vice versa. (E) Fear neurons and extinction neurons connect differentially with the medial prefrontal cortex and the ventral hippocampus. (Herry, et al., 2008)

2. Purpose

Fear and its underlying neural circuits have been extensively studied by using Pavlovian fear conditioning. The amygdala, a main region of fear circuits, is known to be critical for both fear conditioning and extinction. However, there have been some limitations in previous studies. First, the previous studies focused on the three types neurons only according to emotional learning. Also, it is unclear what roles the BA play in fear extinction since previous findings from my laboratory indicate that 'extinction neurons' in the BA, which appear after a single session of fear extinction, disappear when the subjects undergo multiple sessions of fear extinction. Second, most of previous studies have focused on on-line neural responses during CS presentation. I questioned whether the activities occurred during CS (on-line state) would re-appear during off-line states and what kinds of behaviors are related with those off-line activities. Indeed, the previous results from my lab has provided the hints about the existence of off-line activities after CS presentation.

In the chapter 1, I obtained activities of single neurons in the basal amygdala during fear conditioning and multi-sessions of extinction spanning three days. In the chapter 2, I determined whether off-line activities would occur in the lateral amygdala and what kinds of behaviors would be associated with those activities.

Through my studies, I have found that each session of multiple extinction has its extinction neurons, and that the BA activity patterns are increasingly different from the baseline as conditioning and subsequent extinction training proceeds. Furthermore, I found off-line activities of LA neurons which are correlated with freezing behaviors as if conditioned rats repetitively recall their traumatic experience. My study will provide in-depth understanding of roles of the BLA in online and offline processing of emotional information.

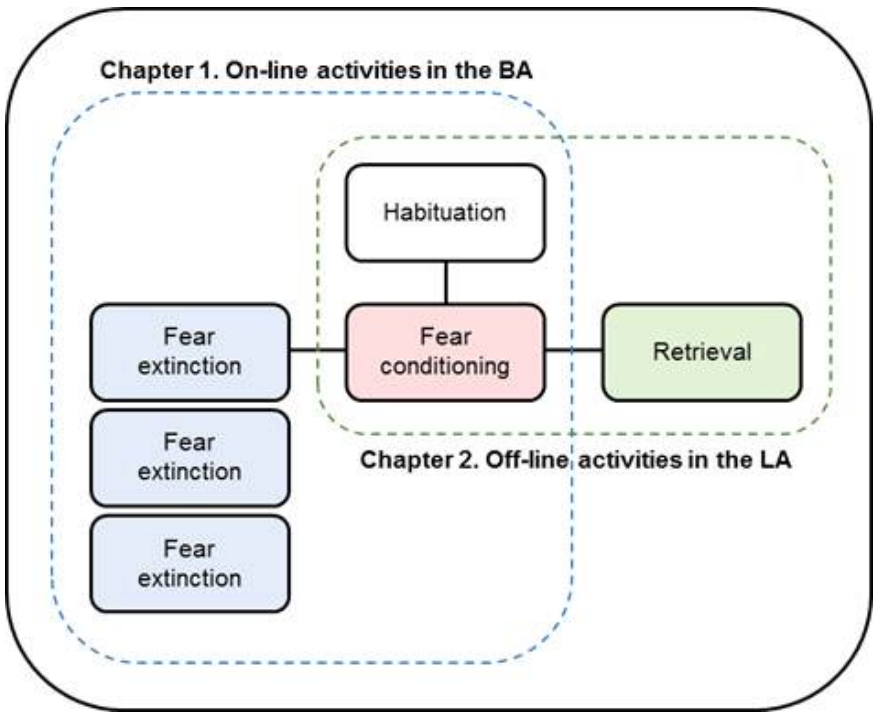

Figure 5. Schematic diagram of this study. In the Chapter 1, on-line activities were examined in BA neurons during fear conditioning and multiple extinction. In the chapter 2, off-line activities were found in the LA after fear memory retrieval.

Chapter 1.

On-line activities in the basal amygdala during fear conditioning and multiple extinction

Abstract

The amygdala is known to be critical for information processing of emotion, and to consist of several sub-nuclei, each of which has distinct structure and function. Among them, the basal nucleus of the amygdala (BA) is proposed to encode valence of emotional stimuli and also to be critical for fear extinction. 'Fear and extinction neurons' in the BA have been shown to encode high and low fear states during fear extinction, respectively, but it is unknown whether there exist other types of neurons and how they react during conditioning and extinction. In addition, due to the technical difficulties to maintain stable recordings of single unit for a long time, there have been a few studies in which activities of single neurons are monitored longitudinally during fear and extinction learning. In the present study, I stably recorded single unit activities in the BA of rats during fear conditioning and subsequent multiple extinction spanning 72 hours. Consistent with previous reports, I found fear and extinction neurons which showed CS-evoked excitation only after conditioning and the first session of extinction, respectively. I also found 'extinction-resistant neurons' which exhibited persistent CS-evoked excitation during conditioning and subsequent multiple sessions of extinction. Furthermore, another question was BA role in multiple extinction. In the previous study, activities of extinction neurons appeared in only

first session of multiple extinction but disappear in second and third extinction. Thus, I analyzed neuronal activity in the BA during another extinction session. As the results, I detected two independent populations of BA neurons which responded after the second or third session of extinction, which may encode a low fear state in each session, respectively. Another striking population of BA neurons showed exhibited CS-evoked inhibition only after the first session of extinction, resembling extinction neurons, and other population showed persistent CS-evoked inhibition during conditioning and subsequent multiple sessions of extinction, resembling extinction-resistant neurons. My findings indicate that diverse ensembles activities in the BA encode fear conditioning and/or each session of multiple extinction, suggesting more complex encoding of emotional states in the BA during extinction than previously thought.

Key words: basal amygdala, fear conditioning, fear extinction

Introduction

The amygdala is known as a critical brain region involved in emotion, especially fear (LeDoux, 2000; LeDoux, 2003; Phelphs ans LeDoux, 2005; Davis, 1992). It is studied well for many years by Pavlovian fear conditioning that is association between a neutral conditioned stimuli (CS) and an aversive unconditioned stimuli (US) (LeDoux, 2000). It could elicit fear response to even only CS presentation. On the contrary to this, fear response reduces when the CS is presented alone repeatedly, which is termed as fear extinction. Using these behavior paradigms, the amygdala, especially basolateral structure of the amygdala (BLA), has been studied about fear behavior (Fanselow and LeDoux, 1999; Fendt and Fanselow, 1999; Collins and Pare, 2000; Goosens and Maren, 2001; Goosens et al, 2003; Gründemann and Maren, 2001). In the previous studies, by inactivating the BLA, it was determined that the BLA was required for acquisition of fear and expression of fear response during fear conditioning and extinction (LeDoux et al., 1990; Garcia et al., 1999; Anglada-Figueroa and Quirk, 2005; Sierra-Mercado et al., 2011). Furthermore, it was studied that the BLA was involved in fear memory consolidation (Vazdarjanova and McGaugh, 1999; McGaugh, 2002; McGaugh et al., 2002; Berlau et al., 2006). In the previous electrophysiological studies, CS-evoked potential in amygdala neurons increased after fear conditioning and depotentiated after extinction (Rogan et al., 1997; Kim et al.,

2007). As another evidence for the BLA in fear behavior, increase and/or decrease of BLA single neurons CS-evoked responses was observed after fear-related learning (Quirk et al., 1995; Herry et al., 2008; An et al., 2012; An et al., 2017).

In the BLA, neurons of lateral part are thought to be homogeneous probably according to the previous single-unit recording study. An et al., 2011 explored activities of lateral amygdala (LA) neurons during fear conditioning and multiple sessions of extinction. In this study, also performed fear conditioning and reconditioning after multiple extinction trainings, they found CS-evoked ensemble activities of LA neurons increased after different two conditioning sessions when state of fear was high. On the other hand, only one neuron exhibited increase of activity after fear extinction training, which was low fear state. According to this study, LA neurons seems to be mainly encoding about fear (An et al., 2011).

Unlike LA neurons, in the previous studies, it was demonstrated to various populations in the basal amygdala (BA), which changed their activity according to high and low fear states. 'Fear neurons' exhibit CS-evoked activity after fear conditioning but disappear after extinction training and in case of 'extinction neurons' are vice versa (Herry et al., 2008). Another type of BA population is 'extinction-resistant neurons' that exhibit CS-evoked response during fear conditioning and maintain their activity at the end of extinction

(Amano et al., 2011; Duvarci and Pare, 2014). However, the previous recording studies in the BA progressed single extinction training (Duvarci and Pare 2014), therefore, I wondered how diverse BA neuronal activities changes according to additional extinction training. Indeed, the previous results in my laboratory, fear and extinction neurons recorded during fear conditioning and multiple extinction trainings (An et al., 2017). In this study, activities of extinction neurons appear after first extinction but did not show after second and third sessions of extinction any more. Thus, I questioned about BA roles during multiple extinction and another population which encodes other session of extinction.

In this study, I used in vivo electrophysiological recording to examine long-term activities in BA neurons. First of all, I looked into change of characters in BA population according to each sessions of fear conditioning and tree multiple extinction trainings. Then, I generally analyzed activities in single neuron level according to emotional states. Using the fixed-microwire single-unit recording, I found not only three types of BLA neurons (fear neurons, extinction neurons and extinction-resistant neurons) as the previous reports but also new populations, which had different activities according to change the emotional states. During fear memory retrieval, I observed three populations, which exhibited firing in this session including a neuronal group had character of fear neurons. In other session, first extinction

memory retrieval, two populations were observed that exhibited CS-evoked response in this window including population which had character of extinction neurons. I also analyzed activities whether existence of new populations encode second and/or third extinction. Surprisingly, novel populations were observed in respective extinction sessions although these sessions were in same emotional states as low fear. I also found inhibited neurons which had activities which were suppressed to CS. These results could suggest that various ensemble activities in the BA encode emotional states related in fear. Furthermore, at least during multiple extinction, they also seemed to be recruited as session-specific.

Materials and Methods

Animals Naive male Sprague-Dawley rats were double-housed for 3-4 days before all experiments and provided with foods and water ad libitum on a 12 h light/dark cycle (lights off at 21:00) until surgery. When weight of rats became 290-310 g, rats had surgery and recovered for a week in a single-housed cage. After recovery, rats started behavior trainings. All procedures were approved by the Institute of Laboratory Animal Resources of Seoul National University.

In vivo electrophysiological surgery and recording 8 weeks old rats were anesthetized with sodium pentobarbital (50 mg/kg, i.p.) and maintained with isoflurane (1-1.5%) in O2. Rats were placed in stereotaxic apparatus horizontally. (All stereotaxic coordinates were described relative to bregma) One electrode consisted of eight microwires (50 μm outer diameter, impedance 0.3-1 MΩ at 1 kHz; California Fine Wire) which were insulated nichrome and bilaterally implanted to BA (AP 2.85 mm, ML 5.10 mm and DV 8.8 mm) very slowly. At the end of insertion, electrode fixed using dental cement (Vertex-dental, Zeist, Netherlands). After surgery, analgesia (Metacam, Boehringer Ingelheim, Germany) and antibiotics were injected into the rats. After recovery, in vivo electrophysiological recording for

individual neuronal activities were executed and analyzed using a Plexon MAP system, as previously described (An et al. 2012).

Behavioral Procedures I used two different behavior contexts (context A and B) for fear conditioning and multiple extinction. Habituation and fear conditioning were implemented at context A that was a Plexiglas rectangular box with 70% ethanol and white light. Context B was a cylinder like Plexiglas chamber with 1% acetic acid and red light used for extinction trainings and retention. When rats were moved to experimental chamber, they were carried in box to context A and in tray to context B.

Recovered rats after surgery were handled for 10–20 minutes twice a day for two days. On day 1, handled rats habituated twice to experiments apparatus. First, they freely moved around in context A for 10 minutes. After 8 hours later, they were placed on context A again and heard 4 CS. The CS was presented for 30 seconds which was a series of twenty-seven 7.5 kHz pure tone pips (200 ms duration repeated at 0.9 Hz, 80 dB sound pressure level) (Herry et al, 2008; An et al., 2012; Repa, 2001). Next day, rats were given to 5 CS in context A and basal CS-evoked activity of the BA was recorded simultaneously (Pre-FC). And then 5 minutes later, fear conditioning was performed by pairing the CS with a foot shock (0.6 mA, 1 s, 5 CS/US pairings, inter-trial interval: 80–120 s) which was co-initiated with last pip onset of the CS. After 8

hours later from fear conditioning, extinction training which consisted of 20 non-reinforced CS went along in context B (Post-FC). On day 3, Post-EX1 and Post-EX2 were performed in context B with 8 hours interval. In case of additional extinction trainings, they were carried out 15 non-reinforced CS for subjects. On the closing day, like Pre-FC session, 5 non-reinforced CS were presented in Post-EX3. Trained experiment measured freezing manually when rats had no movement while CS sounded. Total freezing time was normalized as duration of CS (Kim et al., 2010).

Single-unit spike sorting and analysis Single-unit sorting was performed using Offline Sorter (OFS, Plexon). All waveforms were plotted in a principal component space and clusters consisting of similar waveforms were first defined automatically and then verified manually. If a cluster of waveforms in the principal component space distinguished from other group and if it exhibited a clear refractory period (> 1 ms), the group of waveforms was considered to be generated from single neurons. Each sorted single unit was graded using parameters, J3 and the Davies-Bouldin validity metric (DB), and high J3 and low DB, represented well-separated unit cluster. Sorted neurons with a low grade of these parameters were discarded. Next, long-term stability of single unit was confirmed using Wavetracker (Plexon), in which the principal component space-cylinders of a

unit recorded from different sessions were plotted. A straight degree of cylinder means the clusters of a unit have a similar principal component composition and that same set of single units was recorded during the entire training session. The linear correlation values (r) between the template waveforms represented that same neurons were recorded stably across entire behavioral sessions. I proceeded to further analysis only stable units ($r>0.92$).

To identify transition of neuronal activities of the BA during training, CS-evoked neural activities were normalized using a standard z-score transformation (20ms bin). CS-evoked response was normalized to baseline which was averaged firing rates of 500 ms preceding each pip onset for the CS. Then each basal firing rate of 27 pips came under baseline of one CS as averaged. Thus every CS consisting of 27 pips had own baseline. Tone-evoked responsive neurons were sought by normalized value within 100 ms following CS-onset. If one or more bins within 100 ms from CS-onset were significantly different from the baseline ($p < 0.05$, rank-sum test) during first 5 CS in each session, this neuron was regarded as tone-evoked responsive neuron (Tye et al., 2008; Tye et al., 2010). Among these neurons, excited neurons were significant greater than baseline and, in case of lower, these were considered inhibited neurons. These CS-evoked neurons were analyzed for classified as fear and extinction-related neurons.

To begin with, excited neurons were analyzed. If a neuron exhibited significant z-score in first 5 CS of Post-FC and mean z-value (averaged z-score during 100 ms after CS-onset of first 5 CS) in Post-FC was greater than that of Pre-FC, it was considered fear-related neuron. In case of extinction-related neurons, it had three criteria. First, a unit did not include in fear-related neurons. Second, it exhibited significant excitatory response in extinction retention session, Post-EX1. Lastly, mean z-value in Post-EX1 increased relative to preceding sessions (Pre- and Post-FC). And then, subpopulations were analyzed in each group. Fear-related neurons were divided by existence of significant activity in Pre-FC or not. If fear-related neurons did not exhibit in Pre-FC, they divided again by existence of significant activity in Post-EX1. In case of extinction-related neurons, they just divided by existence of significant activity in Pre-FC session. Inhibited neurons were analyzed using identical manner with fear- and extinction-related neurons of excited neurons but they were not classified as subpopulations because the number of neurons was small.

Histology At the end of the experiments, rats were anesthetized with urethane (1 g/kg, i.p.) and electrolytic lesions were made by passing a current (10 μA, 5-20 s) through recording microwires from which discrete units were identified to identify location of the microwires. Then, animals were transcardially

perfused with 0.9% saline solution and 10% buffered formalin. Brains removed from subjects were post-fixated overnight. Rat brains were sliced with coronal sections (100 μm thick) using a vibroslicer (NVSL; World Precision Instruments, Sarasota, FL) and stained with cresyl violet. At last, they could be observed location of recording microwires under light microscopy.

Statistical analysis I used non-parametric tests in this study. Behavioral results were tested by Friedman test followed by Dunn's test whether between sessions were significant different or not. Neuronal activities were tested by rank-sum test for window of 100ms after CS-onset versus 500ms baseline. Comparison of averaged z-score during 100 ms after CS-onset in each session was tested by Friedman test followed by Dunn's test (An et al., 2012; Duclos et al., 2008). All tests were considered significant difference if p-value <0.05.

Results

Behavioral results of fear conditioning and extensive extinction trainings

Total 41 rats performed fear conditioning and extensive extinction trainings as described in Materials and Methods. Behavioral procedure and results were presented on Fig. 6. Habituated (handled) rats showed no freezing in basal state (Pre-FC). All rats also did not show freezing at first CS in fear conditioning session and their fear responses increased as learning went by. After 8 hours later, rats showed high freezing level at early phase of Post-FC that was a conditioning retrieval session and fear level decreased in late phase. On next day, additional extinction trainings that was presented 15 CS were conducted twice in a day. In early Post-EX1 that was a retention session of first extinction training, rats exhibited lower freezing than early phase of conditioning retention session (Post-FC). As extinction trainings were performed, freezing level diminished than prior session at both early and late phase of extinction. Finally, after three extinction trainings, freezing was very low as basal level (Fig. 6).

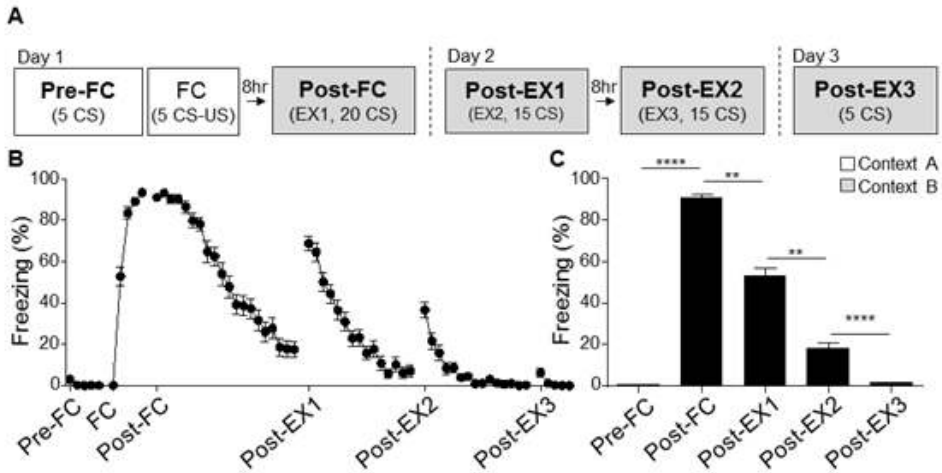

Figure 6. Behavioral procedure and results: fear conditioning and extensive extinction trainings. (A) Behavioral procedure. All behavior process was performed with single unit recording in the BA simultaneously. FC: fear conditioning; EX: extinction. (B) Averaged learning curve of all rats used in this study. (C) Averaged freezing results of first 5CS in each session. Error bars indicate SEM. *p<0.05, ***p=0.0005, ****p<0.00001

CS-evoked activities in the BA neurons

The number of longitudinal and stable recorded neurons in the BA was 204 units and these were analyzed further steps. Recorded neurons who had consistent of waveforms and stable PCA were analyzed (Fig. 7B-D). Recorded sites also were confirmed (Fig. 7A). Among them, 114 neurons (56% of recorded neurons) exhibited significant CS-evoked responses compare to baseline for two types (rank-sum, $p<0.05$). One type was excitatory responsive neurons (84 neurons, 74% of tone-responsive neurons) that were showed greater responses to auditory CS than baseline (Fig. 8A, C) and the other was inhibited group (30 neurons, 26% of tone-responsive neurons) that exhibited lower firing rate than baseline (Fig. 8B, D). Averaged firing rate of excited neurons was 1.21 Hz and it was 1.17 Hz as for inhibited neurons (Fig. 8E). Onset latency was calculated time interval from CS-onset to first significant bin. Averaged latency of excited neurons was 46.06 ms and that of inhibited neurons was 59.93 ms (Fig. 8F).

Before analysis of recorded BA neurons in earnest, I searched characters of theses neurons generally according to emotional states, in particular excited neurons because few inhibited neurons were observed than excited neurons. To investigate characters of activities during fear learning, I employed Gaussian-process factor analysis (GPFA) which was a method for extracting of neural trajectories by unifying the

smoothing and dimensionality reduction operations (Yu et al., 2009). The results of this analysis was presented in Fig. 9. Each axis was components which represented most significant characters of excitatory responsive neuronal population and each line in the graph meant behavioral sessions respectively. Characters of BA neuronal population activities changed according to behavioral sessions even in multiple sessions of extinction. The results provided insight that BLA population changed their activities in different events related in emotional states.

Therefore, I analyzed how activities changed in single neurons level each session. First of all, I examined neuronal activities after fear conditioning and first extinction because emotional states were sharply changed in these sessions. So excitatory responsive neurons were classified bulky according to existence of significant activity during each sessions (precise explains are in Material and Methods). By these criteria, excited BA neurons were divided into two populations largely, fear-related and extinction-related neurons (Fig. 10). A group of excited neurons exhibited significant response during 100ms after CS-onset in Post-FC which was fear conditioning retrieval session (fear-related neuron, n=28). Another group of excited neurons, extinction-related neuron, had z-score value increased in Post-EX 1 compared to previous sessions (extinction-related neurons, n=18) (Fig. 11, 12A). Averaged latency of the

fear-related neurons was 45 ms, and firing rate was 2.13 Hz. In case of extinction-related neurons, averaged latency was 61.04 ms and averaged firing rate was 0.55 Hz (Fig. 12B, C).

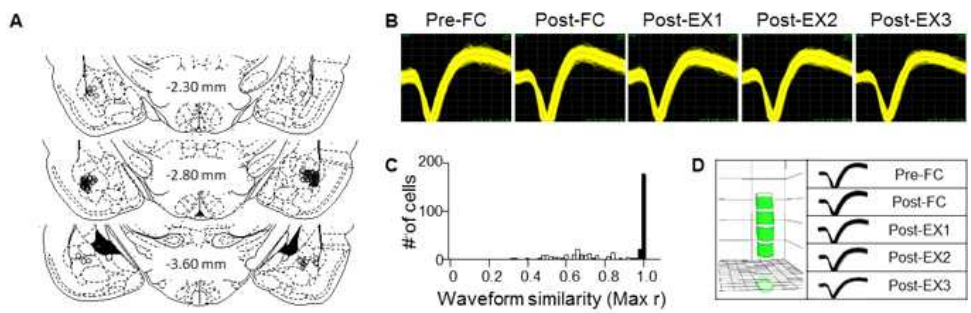

Figure 7. Neuronal information of long-term single unit recording in the BA. (A) Histology of all recorded electrode sites. (B) Representative waveforms of a stable recorded neuron during all behavioral procedure. (C) Quantitative evaluation of waveform similarity across all behavioral sessions. White bars are randomly selected waveforms for control. (D) Verification that long-term single unit recording was stable. Left: Representative principal component space cylinder. A straight cylinder means that same neuron was recorded stably. Right: Waveforms of same neurons with cylinder in each session.

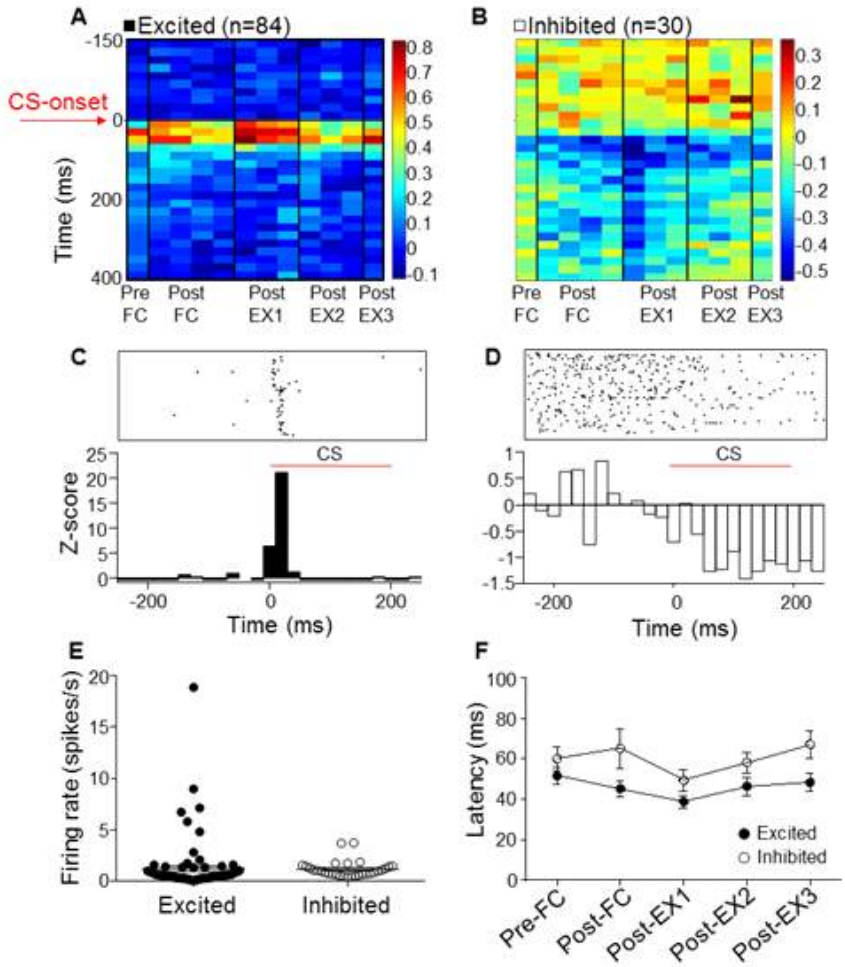
A
Excited (n=84)
-150
CS-onset
0
200
400
Time (ms)
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
-0.1
Pre FC
Post FC
Post EX1
Post EX2
Post EX3
B
Inhibited (n=30)
0.3
0.2
0.1
0
-0.1
-0.2
-0.3
-0.4
-0.5
C
CS
Z-score
25
20
15
10
5
0
-200
0
200
Time (ms)
D
CS
1
0.5
0
-0.5
-1
-1.5
-200
0
200
Time (ms)
E
Firing rate (spikes/s)
20
15
10
5
0
Excited
Inhibited
F
Latency (ms)
100
80
60
40
20
0
Excited
Inhibited
Pre-FC
Post-FC
Post-EX1
Post-EX2
Post-EX3

Figure 8. Recorded excited and inhibied neurons. (A) Averaged z-score of all excited neurons (n=84) were represented as heat plot during all behavioral sessions. In the heat plot, width of a bin reveal duration, averaged 5CS in each session, and height represent time, 20 ms. (B) Heat plot of inhibited neurons (n=30) was drawn same manner with (A). (C) Representative raster plot (top) and PETH (bottom) of a excited neuron and (D) a inhibited neuron. (E) Firing rate of excited and inhibited neurons. (F) Latency of excited and inhibited neurons during all behavioral sessions.

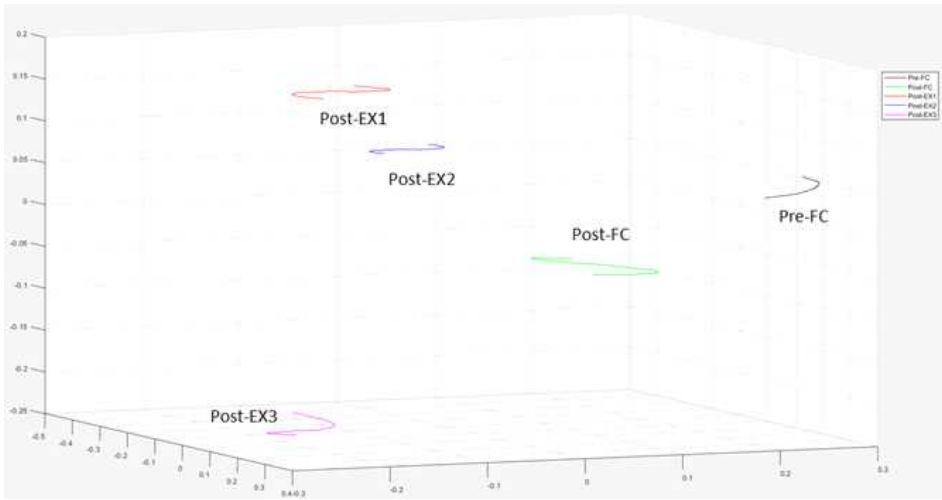

Figure 9. GPFA analysis of excited neuronal population. Each of lines in the three-dimensional space represent population character in each session. All lines appeared in different space.

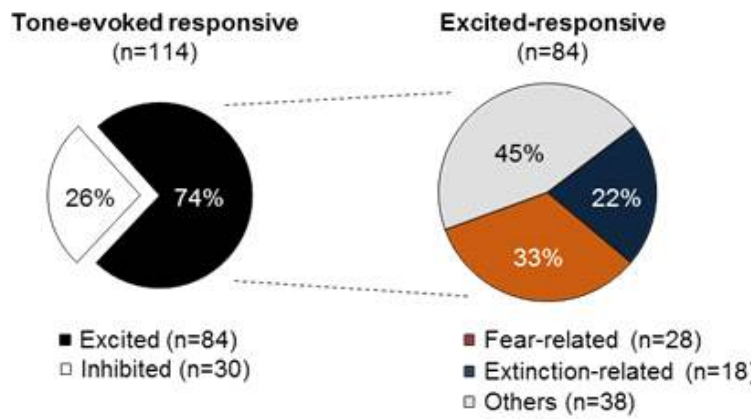

Figure 10. Subpopulations of excited-responsive neurons. Excited-responsive neurons were divided into fear-related neurons (right, n=28 cells) and extinction-related neurons (n=18 cells).

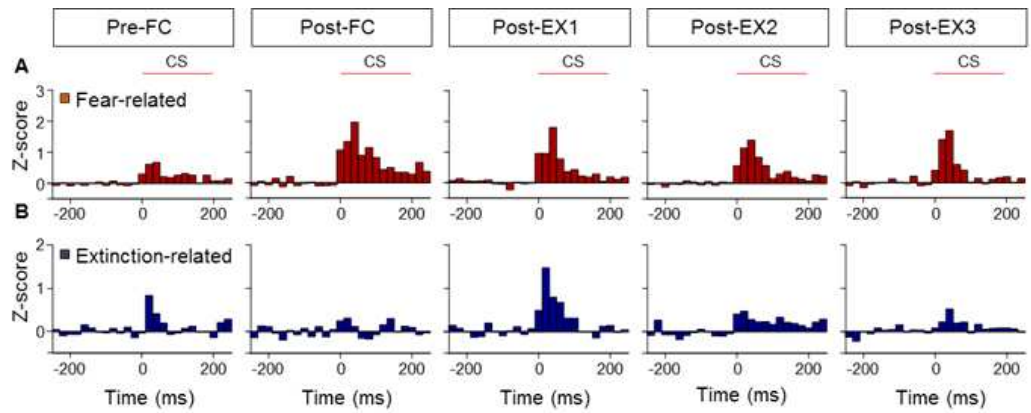

Figure 11. Activities of fear- and extinction-related neurons. (A-B) Z-score PETHs of (A) fear-related neurons (n=28, 33% of excited-responsive) and (B) extinction-related neurons (n=18, 22% of excited-responsive).

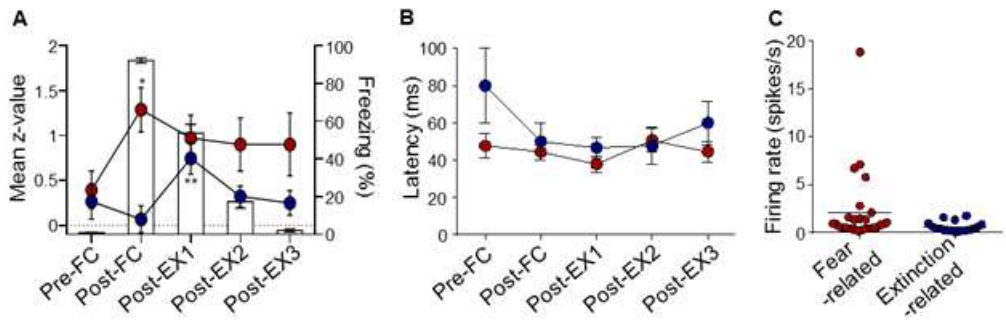

Figure 12. Chacters of fear- and extinction-related neurons. (A) Averaged freezing responses and CS-evoked activities after 100 ms onset of fear- and extinction-related neurons. Fear-related neurons; *p<0.005 for Post-FC vs. the other groups except Post-EX1. Extinction-related neurons; **p<0.01 for Post-EX1 vs. the other groups. (B) Latency of fear- and extinction-related neurons. (C) Firing rate of fear- and extinction-related neurons.

Fear-related neurons

While fear conditioning and extensive extinction trainings proceeded, I observed that 28 neurons (33% of excited-responsive neurons) expressed significant responses in early phase of fear conditioning retrieval session (Post-FC) and their z-score values increased in this session compare to basal responses (Pre-FC). Fear-related neurons were classified into two subpopulation groups according to significant response before fear conditioning (Fig. 13). If a neuron displayed significant z-score value in Pre-FC, it was classified as 'fear-enhanced neuron' (Figure 14A, D). Fear-enhanced neurons exhibited CS-evoked responses in Pre-FC and its responses strengthened after fear conditioning. However, their strengthened CS-evoked activities decreased up to basal level again by only one of extinction training and these responses were maintained as subsequent extinction trainings went along. On the contrary to fear-enhanced group, 'fear-generated neurons' did not exhibit significant CS-evoked activities before fear conditioning. However, their responses to CS that was associated with aversive foot shock newly appeared after fear conditioning. Fear-generated neurons were classified again into two subpopulations, 'fear-transient neurons' and 'fear-persistent neurons', according to significant response during Post-EX1. If a neuron did not display any significant CS-evoked activity during initial 5 CS in Post-EX1, then it was called fear-transient

neuron (Fig. 14B, E). In this case, these neurons were identical to ‘fear neurons’ in the previous studies (Herry et al., 2008; An et al., 2017). Fear-transient neurons had no response to CS before fear conditioning but their activities appeared after fear conditioning. Since then, they lost their CS-evoked responses after first extinction training as previous reports. Although extensive extinction proceeded, fear-transient neurons did not show any responses to CS never again. Unlike this population, fear-persistent neurons had significant z-score value during 100 ms after CS-onset in Post-EX1 (Fig. 14C, F). Significant CS-evoked responses did not show during Pre-FC, but their activities to CS appeared after fear conditioning like fear-transient neurons. However, this group exhibited significant activity after first extinction training on the contrary to fear-transient neurons. In case of these neurons, their CS-evoked responses remained as additional sessions of extinction were progressed.

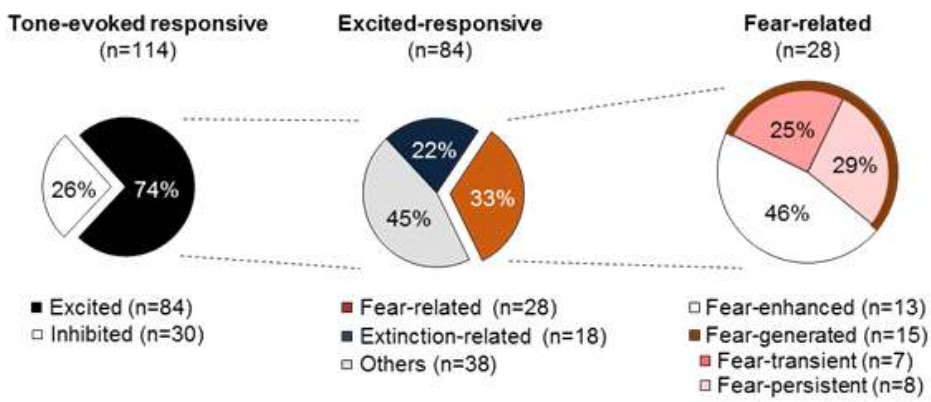

Figure 13. Subpopulations of fear-related neurons. Fear-related neurons consisted of fear-enhanced neurons (right, n=13 cells) and fear-generated neurons (n=15 cells). Fear-generated neurons were divided into fear-transient neurons (n=7 cells) and fear-persistent neurons (n=8).

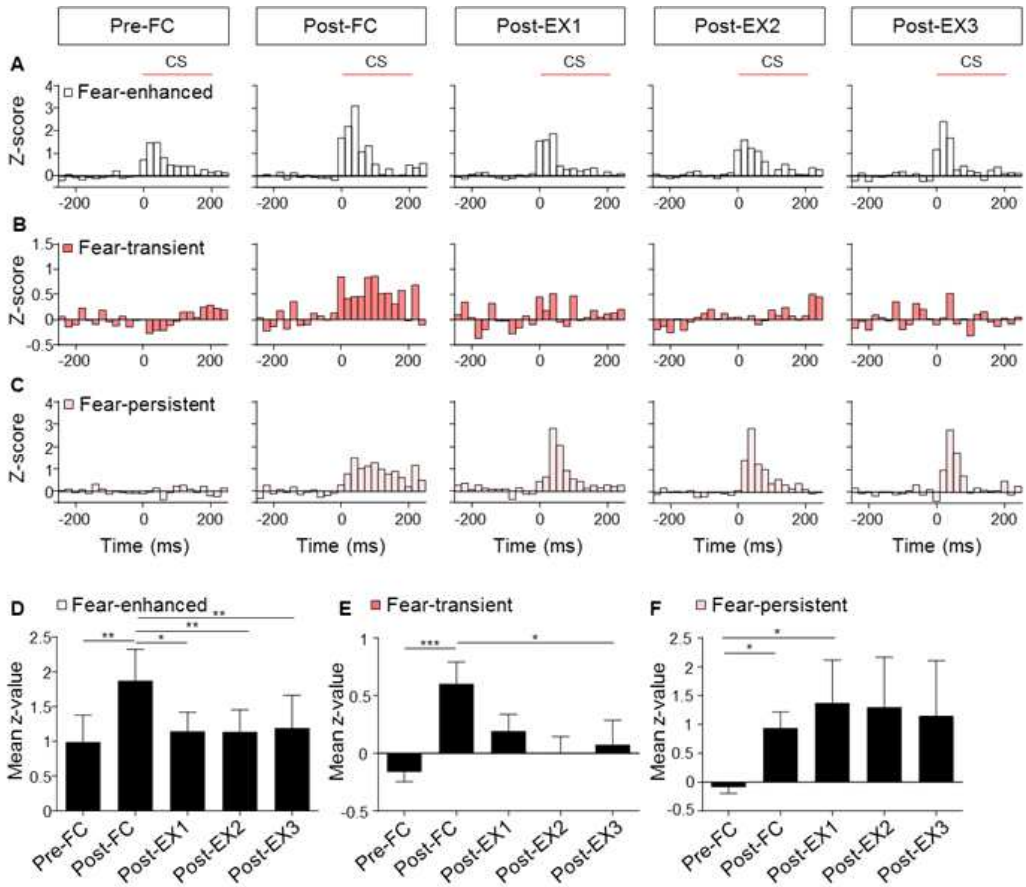

Figure 14. Activities of fear-related neurons subpopulations. (A-C) Z-score PETHs of (A) fear-enhanced neurons (n=13, 46% of fear-related), (B) fear-transient neurons (n=7, 25% of fear-related) and (C) fear-persistent neurons (n=8, 29% of fear-related) in each session. (D-F) Averaged z-scores during 100 ms after CS-onset in each session of (D) fear-enhanced group; *p<0.05, **p<0.01, (E) fear-transient group; *p=0.018, ***p=0.0004 and (F) fear-persistent group; *p<0.05.

Extinction-related neurons

After fear conditioning, rats underwent multiple sessions of extinction in different context with non-reinforced CS. As additional extinction trainings went by, rats exhibited lower freezing than preceding sessions after fear conditioning (Fig. 6). I tracked responses of BA neurons related to extinction during this behavioral paradigm.

I found 18 extinction-related neurons (22% of excited-responsive neurons) under three criteria (see Methods). Similar to fear-related neurons, extinction-related neurons were classified into two subpopulations according to significant CS-evoked activity in previous sessions of first extinction retention session (Fig. 15). In extinction-related neurons, if a neuron had significant response to CS in preceding sessions of Post-EX1, then it called 'extinction-enhanced neurons' (Fig. 16A, C). Their CS-evoked activities showed in basal state, but it reduced in high fear state. Activities of extinction-enhanced neurons increased after first extinction training but decreased again in subsequent extinction trainings. In contrast to extinction-enhanced neurons, 'extinction-provoked neurons' did not exhibit any response before and after fear conditioning (Fig. 16B, D). However this population displayed significant CS-evoked response after first extinction training consistent with 'extinction neurons' in the previous results (Herry et al., 2008; An et al., 2017). As additional extinction went along, their activities

diminished and disappeared similar to extinction-enhanced neurons. In conclusion, two populations of extinction-related neurons showed common character that their CS-evoked responses went down during multiple sessions of extinction.

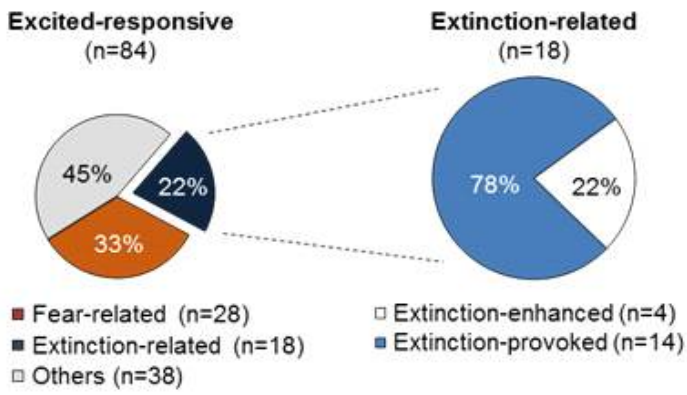

Figure 15. Subpopulations of extinction-related neurons. Extinction related neurons consisted of extinction-enhanced neurons (right, n=4 cells) and extinction-provoked neurons (n=14 cells).

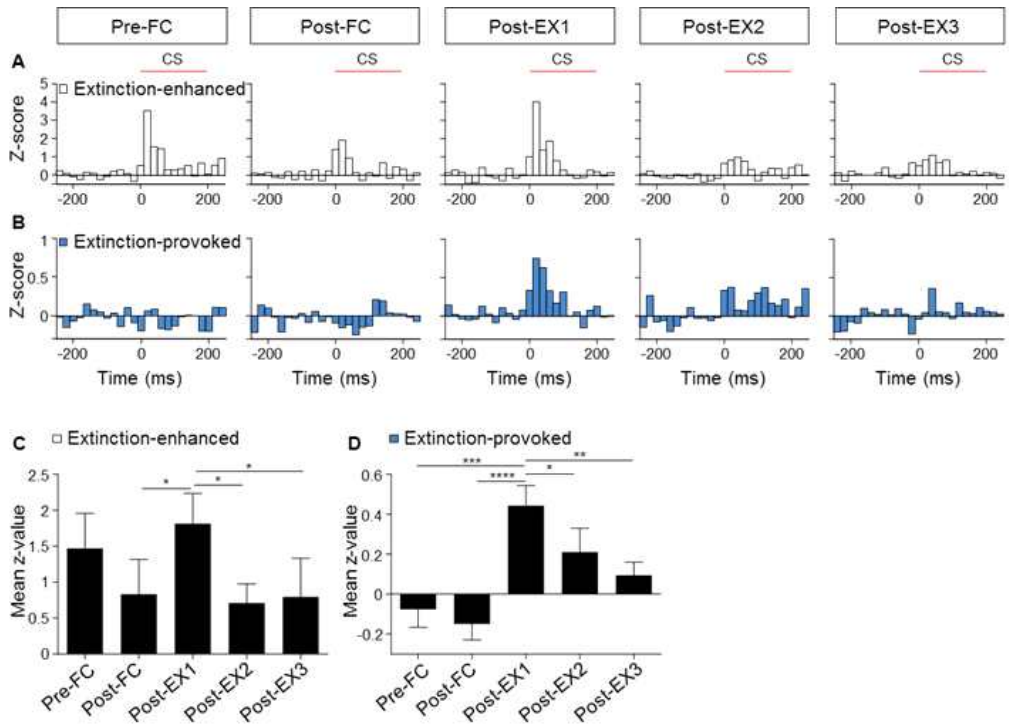

Figure 16. Activities of extinction-related neurons subpopulations. (A-B) Z-score PETHs of (A) extinction-enhanced neurons (n=4, 22% of extinction-related) and (B) extinction-provoked neurons (n=14, 78% of extinction-related) in each sessions. (C-D) Averaged z-scores during 100 ms after CS-onset in each session of (C) extinction-enhanced group; *p<0.05 and (D) extinction-provoked group; *p=0.0121, **p=0.0041, ***p=0.0001, ****p<0.0001.

Session-specific neurons during multiple extinction

Results of extinction-related neurons also could not be found activity during second and/or third session of extinction. However, on Fig. 9, GPFA analysis implied that BA neurons had different characters in all each sessions although the characters might not include neuronal activity. Thus, I re-analyzed these neurons with each session of multiple extinction equally criteria which was different from previous ones. In this analysis, I looked into BA neurons which had significant activity in each session of extinction not focusing on activity of first extinction. As the results, I found that different neuronal populations also appeared in each session of extinction respectively (Fig. 17). Post-EX1 group exhibited same activities with extinction-provoked neurons (Fig. 17A, D). Surprisingly, neuronal populations which had activities in another session of extinction. Post-EX2 neurons exhibited responses during only after second extinction (Fig. 17B, E) and Post-EX3 neurons showed in after third extinction (Fig. 17C, F). According to these results, each session of extinction were encoded by different populations respectively. It provided suggestion that BA neurons were recruited as session-specific during multiple extinction.

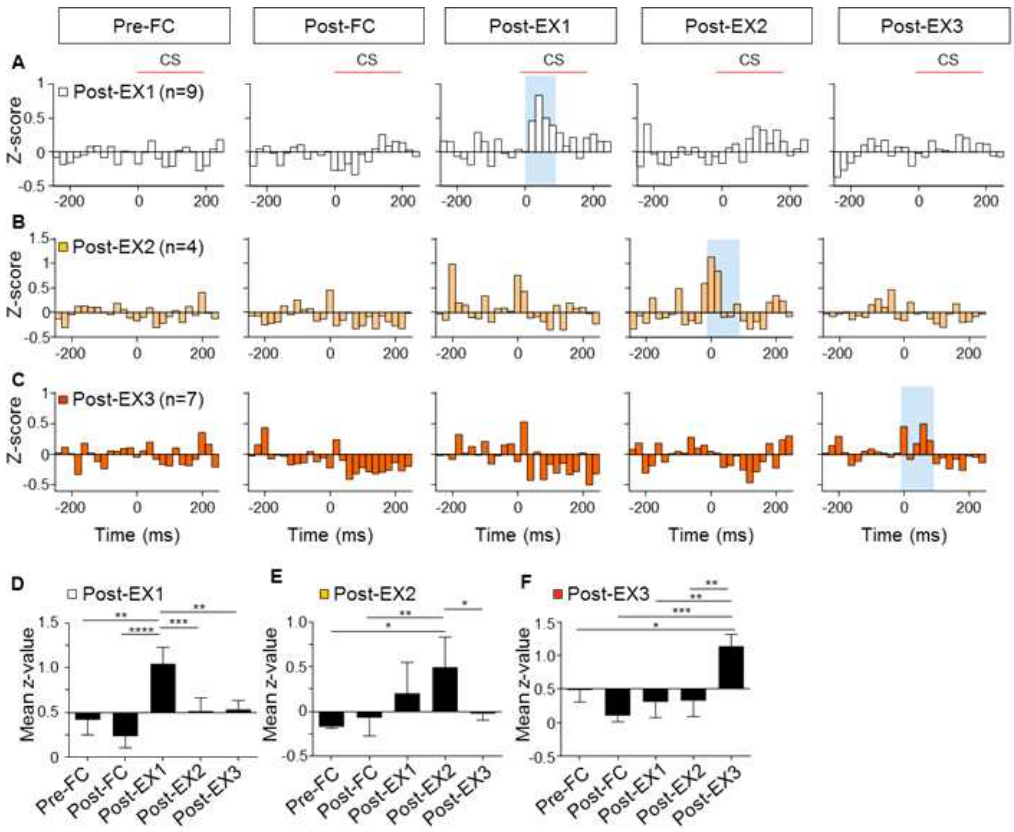

Figure 17. Session-specific neurons during multiple extinction. (A-C) Z-score PETHs in each session of (A) Post-EX1 neuorns (n=9 cells) which has significant activation in Pos-EX1 session and (B) Post-EX2 (n=4 cells) and (C) Post-EX3 (n=7 cells). (D-F) Averaged z-scores during 100 ms after CS-onset in each session of (D) Post-EX1; **p<0.005, ***p=0.0006, ****p<0.0001, (E) Post-EX2; *p<0.05, **p=0.007 and (F) Post-EX3; *p<0.05, **p<0.005, ***p=0.0001.

Inhibited neurons

I found another population that had inhibitory response to auditory stimulus in the BA. Inhibited-responsive neurons were divided into 'inhibited-fear neurons' and 'inhibited-extinction neurons' using same manners with fear- and extinction-related neurons in excitatory responsive neurons (Fig. 18). Inhibited-fear group showed maximum activities after fear conditioning like fear-related neurons (Fig. 18A, C). Since extensive extinction proceeded, their CS-evoked activities decreased gradually but did not return to baseline level. This character was similar to extinction-resistant neurons which were excitatory responsive neurons in the previous studies (Herry et al., 2008; Duvarci and Pare, 2014). In case of inhibited-extinction group, their activities were quite similar to extinction-provoked neurons (Fig. 18B, D). Their responses showed after first extinction but it nearly disappeared during subsequent extinction trainings.

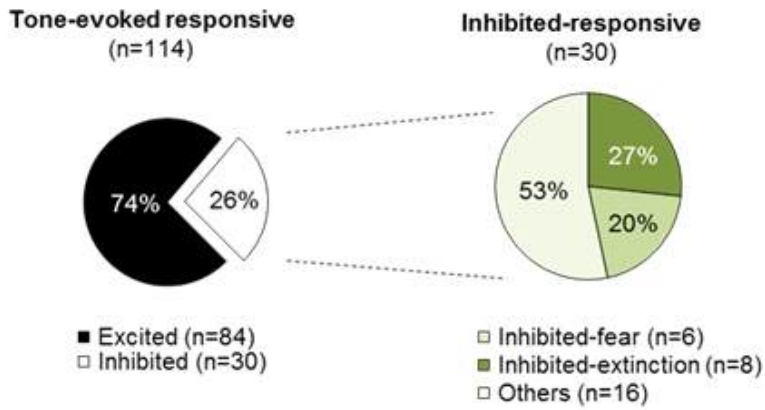

Figure 18. Subpopulations of inhibited-responsive neurons. Inhibited- responsive neurons were divided into inhibited-fear neurons (right, n=6 cells) and inhibited-extinction neurons (n=8 cells).

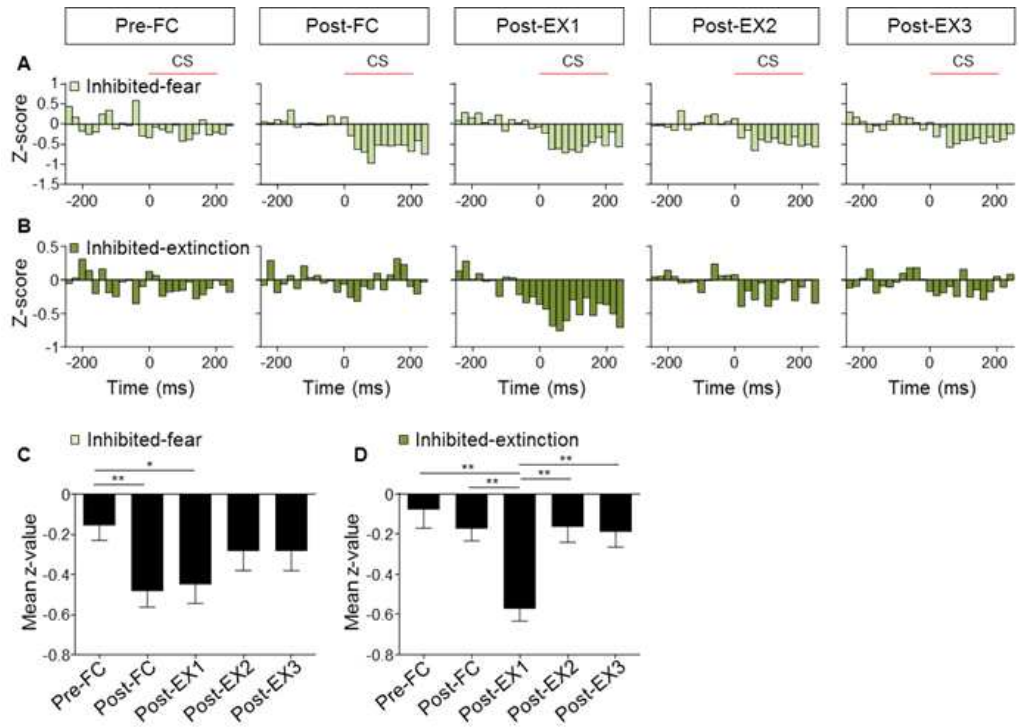

Figure 19. Activities of inhibited-responsive neurons subpopulations. (A-B) Z-score PETHs in each session of (A) inhibited-fear neurons (n=6, 20% of inhibited-responsive), (B) inhibited-extinction neurons (n=8, 27% of inhibited-responsive). (C-D) Averaged z-scores during 100 ms after CS-onset in each session of (C) inhibited-fear neurons; *p=0.0106, **p=0.0062 and (D) inhibited-extinction neurons; **p<0.01.

Discussion

In this study, I observed diverse activities in the BA during fear conditioning and multiple extinction. First of all, I earned the insight of characters change of BA population according to emotional behavioral sessions through the analysis of all recorded excited neuronal population (Fig. 9). The analysis outcome consistent with the previous results. In the previous study, BLA population vector changed between habituation and fear conditioning to different direction (Grewe et al., 2017). It also changed after extinction but the population vector went to another space instead of going back to habituation level. However, the study did not observe population activity according to each sessions of extinction and single unit level activity during each sessions of behavioral process. Then I analyzed change of activities in single neuron level in accordance with emotional states in Post-FC and Post-EX2 which were changed fear level remarkably. During these sessions, various fear- and extinction-related neurons were found. According to these results, BA neurons encode emotional states as diverse ensemble activities.

However, extinction-related neurons did not exhibited CS-evoked activities after second extinction. Then, I re-analyzed BA neurons with non-biased condition to sessions.

As the results, I found that different neuronal populations also appeared in each session of extinction respectively. These results conflict with the previous results of my laboratory used same behavioral procedures. In the study, extinction neurons of the BA exhibited CS-evoked responses after only first extinction session but did not show on another multiple sessions of extinction (An et al., 2017). However, this study observed change of activities in extinction neurons purely which had same responses change with Post-EX1 in Fig. 17A. In the view point of results of the previous study, it is make sense that inhibition mechanism to extinction disappears at late phase of extinction. On the contrary to the previous study, I found that another populations also appeared in respective extinction sessions which correspond to inhibition mechanism. Because inhibition mechanism to extinction has the perspective that enhanced new activity implicates in signaling of safety and suppression of fear responses (Maren and Quirk, 2004; Ehrlich et al., 2009). Changed characters of BA population in each behavioral session even multiple extinction like Fig. 9 is caused by session-specific recruitment of different neurons in BA probably. These results provide that diverse BA ensembles encode emotional states related in fear even identical states. In this view point, it can be possible that BA neurons encode event per se maybe regardless of emotional states.

Also I observed another population represented inhibitory

response to conditioned auditory stimulus. Among inhibitory responsive neurons, inhibited-fear and inhibited-extinction neurons showed activities like fear-related and extinction-related neurons in excited-responsive neurons. Probably, cause of inhibited responses was effect of intra-amygdala connections. In the previous study, it is known that interneurons inhibited other glutamatergic neurons during fear recall and extinction learning. When fear state was high, cholecystokinin (CCK) interneurons inhibited extinction neurons. Also, different kinds of interneurons, parvalbumin (PV), inhibited fear neurons during extinction (Duvarci and Pare, 2014). However in this case, inhibited neurons were fear and extinction neurons respectively, but observed inhibited neurons in this study did not exhibit excitatory response. Furthermore, inhibited-extinction group exhibited large response in Post-EX1 significantly. Similarly to this, inhibited activity in the basal amygdala reported previously during fear extinction learning (Amano et al, 2011). In this study, extinction cells revealed inhibited activities in early extinction training and responses changed excitedly in the late of training. However, in this study, inhibited neurons not showed the characters of that neurons. In other words, neurons that have inhibited response not alter their activities following the training and it means that inhibited neurons observed in this study are another new population of the BA.

Throughout this experiments, single neuron level, BA

neurons reflect emotional states as diverse ensemble activities. In addition, activities of different neuronal population in the BA appear in even identical emotional states, thus it can be also suggest that BA neurons encode the events probably at least during multiple extinction.

Chapter 2.

Off-line activities in the lateral amygdala after fear retrieval

Abstract

Pavlovian fear conditioning induces reflexive freezing behavior toward conditioned stimuli (CS) as called on-line state. Using this model, neural activity and mechanism has been intensively studied in the amygdala when rats are provided threatful stimuli. According to the previous studies, lateral amygdala (LA) in particular, CS-induced single neuron activity and synchronization with other brain regions enhanced with freezing behavior after fear conditioning. Then, is it possible that these activities also appear during spontaneous freezing behavior in absence of CS which is termed as off-line state? Different from studies about on-line activity, off-line activity in the LA has not been studied well. However, the previous results from my laboratory provided the hint that off-line activity maybe appear in the lateral amygdala after fear memory retrieval. Thus, I wondered that provoked neural response during specific behavior or stimulus in on-line period also re-appears when the same emotional behavior occur during off-line state in practice. To solve this question, I examined various activities of the LA and its networks in off-line state, first. Then, I found that power of low frequencies oscillations were strong in all recorded regions and neural synchronization between LA and other regions also increased during freezing behavior in off-line state after fear memory retrieval. When rats were resting, especially

sleeping, neuronal firing patterns that resembled activity of replay were also observed in the LA. These results could provide extended understanding in aspects of LA function.

Key words: lateral amygdala, fear conditioning, fear retrieval, off-line activity

Introduction

Study of fear is very important and necessary for us because it can be provided treatment of post-traumatic stress disorder (PTSD) and other fear-related disorders. Fear conditioning which is the association between a neutral conditioned stimuli (CS) and an aversive unconditioned stimuli (US) is a very useful behavioral model for study of fear (LeDoux, 2000). This association is stored in the lateral amygdala (LA) which is a main target of sensory afferents from the thalamus and cortex (Rogan et al., 1997; Maren and Quirk, 2004; Johansen et al., 2011). In a large number of previous studies, the LA is pivotal role in fear acquisition and retrieval regardless of species (Adolphs et al., 1995; Pare and Duvarci, 2012; Fanselow and Gale, 2003). It was also studied that LA neuronal activity increased after fear conditioning (Quirk et al., 1995; Maren and Quirk, 2004; An et al., 2012). Furthermore, LA neurons exhibit theta (3-12 Hz) activities during emotional arousal and consolidation of fear memory (Pare et al., 2002; Pelletier and Pare, 2004; Maratos et al., 2009; Likhtik and Gordon, 2011). Specially, in the previous study, the rectification index of LA increased after fear conditioning was also enhanced after fear memory retrieval and it maintained during few times after retrieval. Consistent with this, AMPAR-mediated EPSCs became more sensitive to NASPM, a polyamine derivative that use-dependently blocks calcium-permeable AMPARs

(CP-AMPARs), until 5 minutes after fear memory retrieval in the same study (Hong et al,, 2013). These results provided insight of existence activity even after fear memory retrieval.

The prelimbic cortex (PL), the dorsomedial part of the prefrontal cortex, is also involved in fear expression and fear memory retrieval (Burgos-Robles et al., 2009; Courtin, J et al., 2014; Sierra-Mercado et al., 2010; Stern et al., 2013; Kim et al., 2013). Also, in the human study, fear-evoking stimuli led to strengthened theta activities in the anterior midcingulate cortex (AMC), which is a homolog region of the PL of rats (Mueller et al., 2014). Meanwhile, the PL is known that it has projection to BLA (Mcdonald et al., 1996; Vertes, 2003). Along with these evidences, neuronal co-firing between amygdala and prefrontal cortex during resistance to extinction behavior was observed (Livneh and Paz, 2012). Furthermore, in many previous studies, it was studied that synchrony between basolateral amygdala (BLA) and PL in the theta frequency increased during and after fear behavior (Popa et al., 2010; Likhtik et al., 2014; Karalis et al., 2016; Bocchio et al., 2017; Taub et al., 2018).

Another brain region that is closely linked to the amygdala is the hippocampus (HPC). They have reciprocal connection (Aggleton, 1986; Pikkarainen et al., 1999; Pitkanen et al., 2000) and this connectivity is functionally related in emotion (Richardson et al., 2004; Richter-Levin, 2004; Smith et al., 2006; Terada et al., 2013). In particular among the amygdala

subnuclei, the LA and HPC are critically involved in the formation and retention of fear memory (Holt and Maren, 1999; LeDoux, 2003; Sanders et al., 2003). In the previous studies, synchronization of theta activities between LA and CA1 area of the HPC was observed during fear memory retrieval (Seidenbecher et al., 2003; Narayanan et al., 2007; Bienvenu et al., 2012).

However, these previous studies observed neural activities in on-line periods when were directly presentation of CS. So I wondered whether neural activity during on-line periods for fear memory maintains continuously during off-line state when is absence of CS period. Indeed, the previous study immediately observed off-line activity after fear conditioning (Popa et al., 2010). In this study, it was revealed that theta coherence between amygdala, medial prefrontal cortex and hippocampus was modified during paradoxical sleep after fear conditioning.

Although Karalis et al., 2016 also observed BLA and PL activities in the absence of CS during fear memory retrieval, this study was not free to CS perfectly. Because neural activities were estimated between CS in this study. Therefore, neural activity of fear memory in off-line state remains unknown precisely. In this study, I observed off-line activities for fear memory in the LA, PL and CA1 during entirely absence of CS. In addition, I measured activities according to several behavioral

aspects then I found interesting LA neuronal firing patterns during resting/sleep state of rats after fear retrieval.

Materials and methods

Animals Naive male Sprague-Dawley rats were double-housed for 3-4 days before all experiments and provided with foods and water ad libitum on a 12 h light/dark cycle (lights off at 21:00) until surgery. When weight of rats became 290 g, rats had surgery and recovered for a week in a single-housed cage. After recovery, rats started behavior trainings. All procedures were approved by the Institute of Laboratory Animal Resources of Seoul National University.

In vivo electrophysiological surgery and recording Anesthetized rats, 8 weeks olds, were fixed in stereotaxic apparatus horizontally with sodium pentobarbital (50 mg/kg, i.p.) and maintained with isoflurane (1-1.5%) in O2. These rats were implanted ipsilaterally with electrodes targeting the LA (2.85 mm posterior, 5.10 mm lateral, 8.1 mm deep from the bregma) and PL (2.8 mm anterior, 1.23 mm lateral from the bregma and 2.6-2.8 mm deep from surface of the brain) and dorsal hippocampal CA1 area (3.25 mm posterior, 2.5 mm lateral, 2.9 mm deep from the bregma) very slowly. The electrodes divided into three parts for implant the three regions respectively. The electrodes embedded in the PL and CA1 consisted of two individually insulated stainless steel microwires (0.15 mm outer

diameter; Plastics One, Roanoke, VA, USA) and that of LA included ten insulated nichrome microwires (50 μm outer diameter; impedance 0.3-1 MΩ at 1 kHz; California Fine Wire, Grover Beach, CA, USA) in addition to other regions one. Implanted electrodes fixed by dental cement (Vertex-dental, Zeist, Netherlands). After surgery, rats injected analgesia (Metacam, Boehringer Ingelheim, Germany) and antibiotics. After recovery for one week, neural activities were recorded using Plexon MAP system (Plexon, Dallas, TX, USA). Recorded electrical neural responses were processed by a differential amplifier and band-pass filtered at 0.7 and 200 Hz with a 1 kHz sampling rate.

Behavioral procedure Different behavioral contexts (context A and B) were used in this study. Habituation:on- and on-line session and retrieval:on- and off-line session were performed in the context A that was a cylinder like Plexiglas chamber with 1% acetic acid and red light and rats were transferred to this context by tray. Context B was a rectangular box performed fear conditioning with 70% ethanol, white light and box carrier when rats moved to this chamber.

On day 1, handled rats underwent acclimation periods for the contexts. In this case, rats moved freely in the context A and B. Next day, habituation sessions were performed. First, 3 CS were presented to rats in the context A during

habituation:on-line session and habituation:off-line session was started for 30 minutes right after last CS pip. The CS was composed of a series of twenty-seven 7.5 kHz pure tone pips (200 ms duration repeated at 0.9 Hz, 80 dB sound pressure level) for 30 seconds. After 10 minutes to end of habituation:off-line session, fear conditioning was conducted in the context B by pairing the CS with a foot shock (0.6 mA, 1 s, 5 CS/US pairings; inter-trial interval: 80-120 s). On third day, rats underwent retrieval sessions that were same protocol with habituation sessions.

Freezing was measured manually during CS presentation. I also recorded neural activities in the LA and PL, CA1 and behavioral aspects simultaneously when habituation and retrieval sessions were conducted. Behavior aspects were divided into 4 conditions as follows. When rats had no movement with crouching in a state of tension and were out of a breath was freezing. Resting was also immobilization but it was presented relaxing posture of rats like laying down flat and sleeping. When rats raised their front paw and looked around for exploration or avoidance probably was rearing. Grooming was stroking their cheeks or body.

Data analysis Local field potential activities were transformed by an A/D interface and analyzed via NeuroExplorer (Nex Technologies, Madison, AL, USA) and MATLAB (MathWorks,

Natick, MA, USA). Recorded field potential analyzed only which had clear and less noise. Power spectral density and coherence analysis was performed in a range of 0-20 Hz frequency. Power spectrum was normalized so that the sum of all the spectrum values equals to the mean squared value of the rate histogram. Coherence analysis was performed as follows. If both the reference and the target variables have the same digitizing frequency, the fast fourier transforms (FFTs) of variable values were calculated and then resampled to the specified frequency steps. If the reference is a timestamped variable or two continuous variables have different digitizing rates, the values of continuous variables were averaged within the specified bins and then FFTs of these averages were calculated.

Single-units were sorted via Offline Sorter (OFS, Plexon, Dallas, TX). Recorded all waveforms were plotted in a principal component space and clustered between similar waveforms. Single-unit cluster was graded using two parameters. High J3 and low Davies-Bouldin validity metric (DB) were represented well separation. Among sorted neurons, low graded neurons were not used. Long-term stability of single-unit from habituation to retrieval was conducted via Wavetracker (Plexon). Straight cylinder means that single-unit was recorded in similar principal component composition during habituation and retrieval. In this study, single-unit who represented well straight cylinder only used.

Histology Last order of the experiments, rats were anesthetized with urethane first (1 g/kg, i.p.), and electrolytic lesions were made by passing a current (10 μA, 5–20 s) through recording microwires in the LA, PL and CA1 for identifying the recording site each regions. Then, animals were transcardially perfused with 0.9% saline solution and 10% buffered formalin. Brains removed from subjects were post-fixated overnight. Rat brains were sliced with coronal sections (100 μm thick) using a vibroslicer (NVSL; World Precision Instruments, Sarasota, FL) and stained with cresyl violet. At last, they could be observed location of recording microwires under light microscopy.

Statistical analysis I used non-parametric tests in this study. Behavioral results of freezing between habituation:on-line and retrieval:on-line were tested by Wilcoxon matched-pairs singed rank test were significant different or not. Area under curve in coherence between freezing and other behaviors were tested also Wilcoxon matched-pairs signed rank test.

Results

Behavioral process and results for off-line activities

I built behavioral procedure for measuring of off-line activities as described in Materials and Methods (Fig. 20A). Total 6 rats performed the behavioral procedure and local field potential in the LA, PL and CA1 was recorded simultaneously. Then, I obtained their behavioral results and neural activities. Before fear conditioning, all rats did not show freezing to CS during habituation:on-line session. After habituation:on-line session, rats underwent off-line state for 30 minutes when was that rats were set fee to stimuli completely. During fear conditioning, freezing of rats increased. Next day, during fear memory retrieval session (retrieval:on-line), freezing was maintained at a high level (Fig. 20B, C). Then, rats also underwent retrieval:off-line session for 30 minutes. I also recorded neural activities in the LA, PL and CA1 on behaving rats simultaneously. Recorded sites were presented on Fig. 20D. In addition, I classified behavioral aspects as 4 conditions, freezing, resting, rearing, grooming. Then, I observed neural activities according to 4 behavioral conditions.

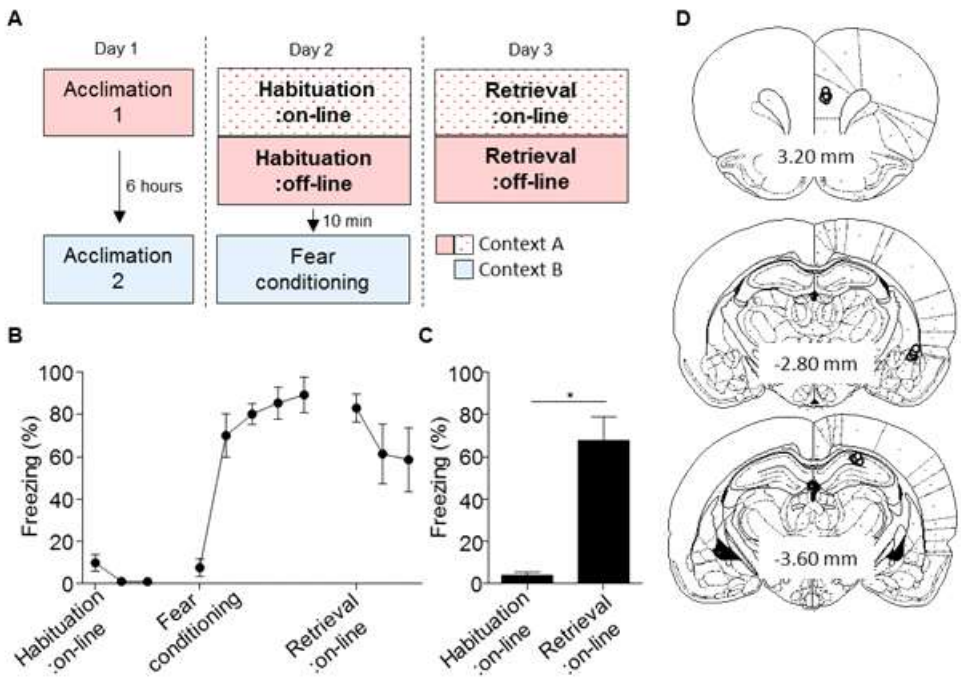

Figure 20. Behavioral procedure and results. (A) Behavioral procedure (B) Freezing level during behavioral procedure. (C) Averaged freezing level to three CS during each on-line session. *p<0.5 (D) Sites of recorded electrodes in the PL (top), LA (middle), CA1 (bottom).

Low frequencies oscillations during on- and off-line states

First of all, I looked into spectrograms on whole sessions during on- and off-line states for searching distinct signals. Then, I found a striking signal during retrieval:on-line session. When CS was presented in this session, low frequencies (1.5-4 Hz) oscillations were observed in the LA and also the PL, CA1 with freezing behavior (Fig. 21D-F). However, low frequencies oscillations did not show during habituation:on-line session even if CS was presented in common with retrieval:on-line session (Fig. 21A-C). Therefore, the signal was not representation of CS per se. It probably stands for freezing behavior like the previous studies (Seidenbecher et al., 2003; Karalis et al., 2016). So then, is it maintained during retrieval:off-line states that low frequencies oscillations appeared at freezing behavior during retrieval:on-line session?

I also observed same signal in retrieval:off-line session during freezing behavior (Fig. 22D-F). However, low frequencies signal did not appear in habituation:off-line session in spite of occurrence of freezing behavior. Thus, the signal that I found represented freezing behavior after fear conditioning and it was sustained even if rats were not in situation of CS presentation. Furthermore, different signal was even observed during off-line states. When rats were resting, very strong signals appeared at about 2-10 Hz oscillations (Fig. 22A-C). However, unlike low frequencies oscillations, it was not related to fear memory

because it was observed in both habituation:off-line and retrieval:off-line sessions during resting state of rats.

Like the preceding, I found low frequencies oscillations at freezing behavior after fear conditioning and wide band frequencies oscillations at resting behavior regardless of fear conditioning. Then, I analyzed these signals more precisely by power spectral density (PSD) as 4 behavioral conditions in the PL, CA1 and LA. First, on-line periods results were presented (Fig. 23). During habituation:on-line session, level of PSD was not difference according to 4 behavioral conditions (Fig. 23A-C). In the case of retrieval:on-line session, rats did not exhibit various behavioral aspects (Fig. 23D-F). Rats almost exhibited freezing or rearing for avoidance probably because threatful stimuli were presented during retrieval:on-line session. Thus, I compared neural activities between freezing and rearing behaviors. In consequence, difference of activities between freezing and rearing in retrieval:on-line session was observed. When rats showed freezing, PSD was higher than during rearing behavior at low frequencies band in the LA and also two regions.

Off-line activities were also analyzed (Fig. 24). During habituation:off-line session, it was no difference of PSD during freezing between other behaviors (Fig. 24A-C). However, PSD level of freezing behavior in retrieval:off-line session was higher than that of rearing and grooming but that of resting was also higher as much

as of freezing in the LA, PL and CA1 (Fig. 24D-F). Indeed, PSD of resting was also higher in habituation:off-line session. It was probably involved in that wide frequencies oscillations was observed on spectrograms during resting behavior in both off-line sessions.

In summary, I found low frequencies oscillations were strong in all regions, the LA, PL and CA1 during freezing behavior in retrieval:on-line session after fear conditioning and it appeared continuously when rats exhibited freezing in retrieval:off-line session. Then, I wondered whether communications of LA networks also were enhanced at low frequencies oscillations in off-line state during freezing behavior as previous studies of on-line state (Seidenbecher et al., 2003; Karalis et al., 2016).

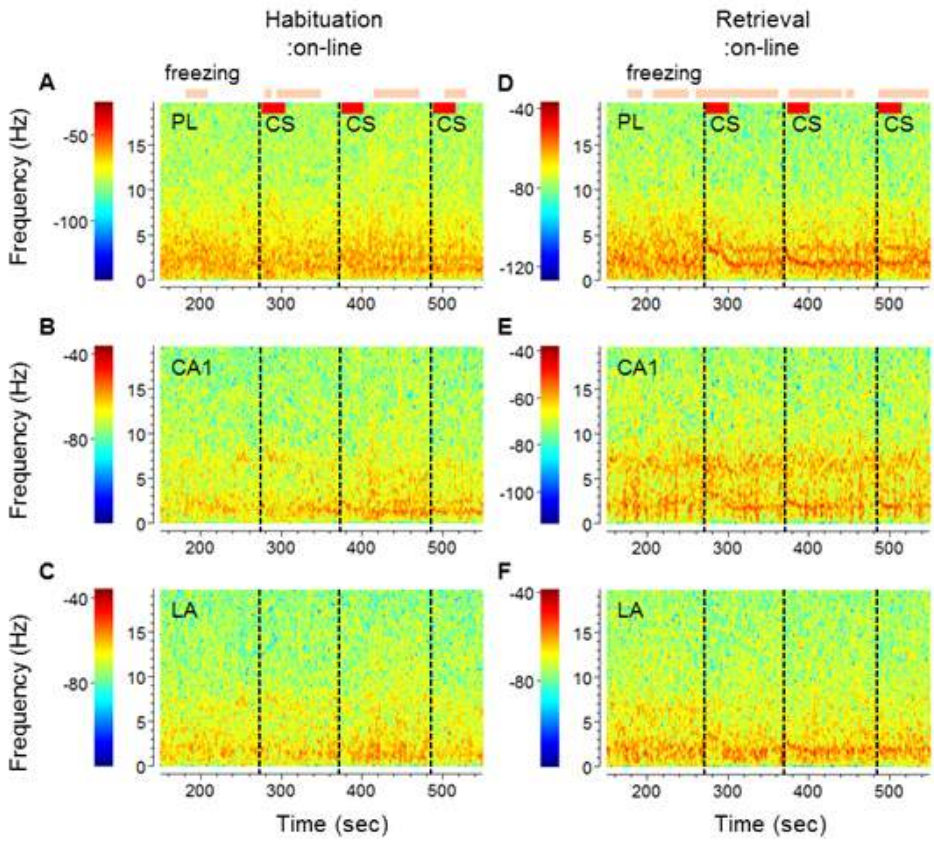

Figure 21. Spectrograms during on-line state. (A-C) Spectrograms during habituation:on-line session in (A) the PL, (B) the CA1 and (C) the LA. (D-F) Spectrograms during retrieval:on-line sessions in (D) the PL, (E) the CA1 and (F) the LA. Duration of freezing (pink) and CS (red) were represented by bars.

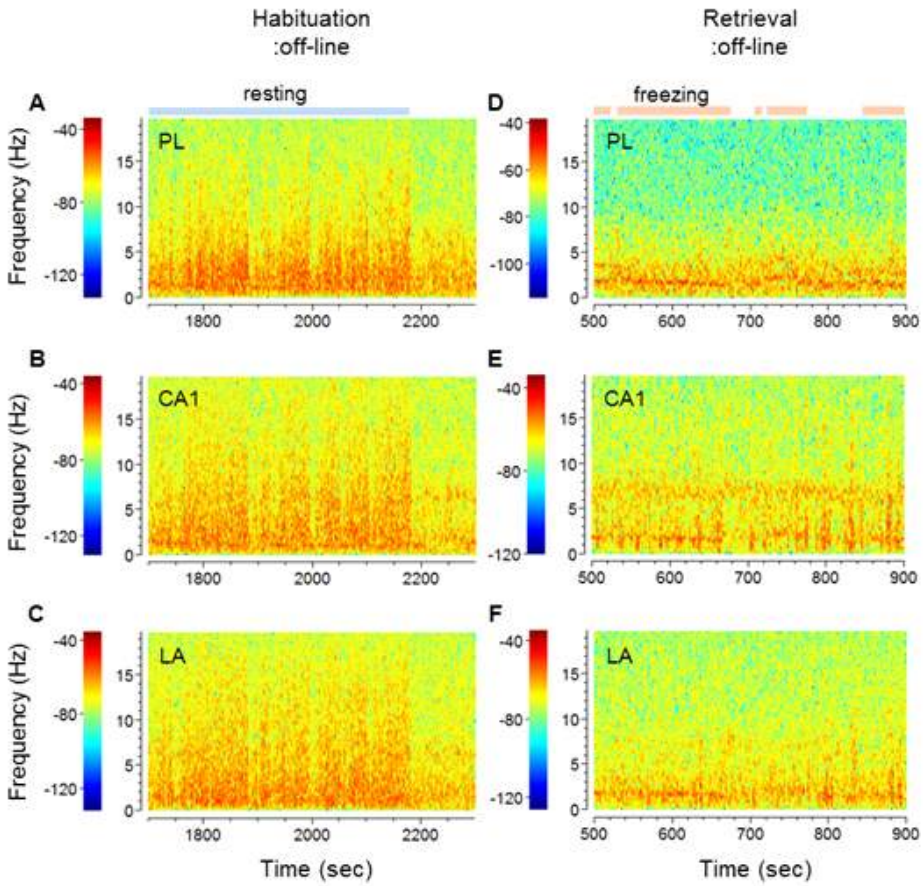

Figure 22. Spectrograms during off-line state. (A-C) Spectrograms during habituation:off-line session in (A) the PL, (B) the CA1 and (C) the LA. (D-F) Spectrograms during retrieval:off-line sessions in (D) the PL, (E) the CA1 and (F) the LA. Duration of freezing (pink) and resting (blue) were represented by bars.

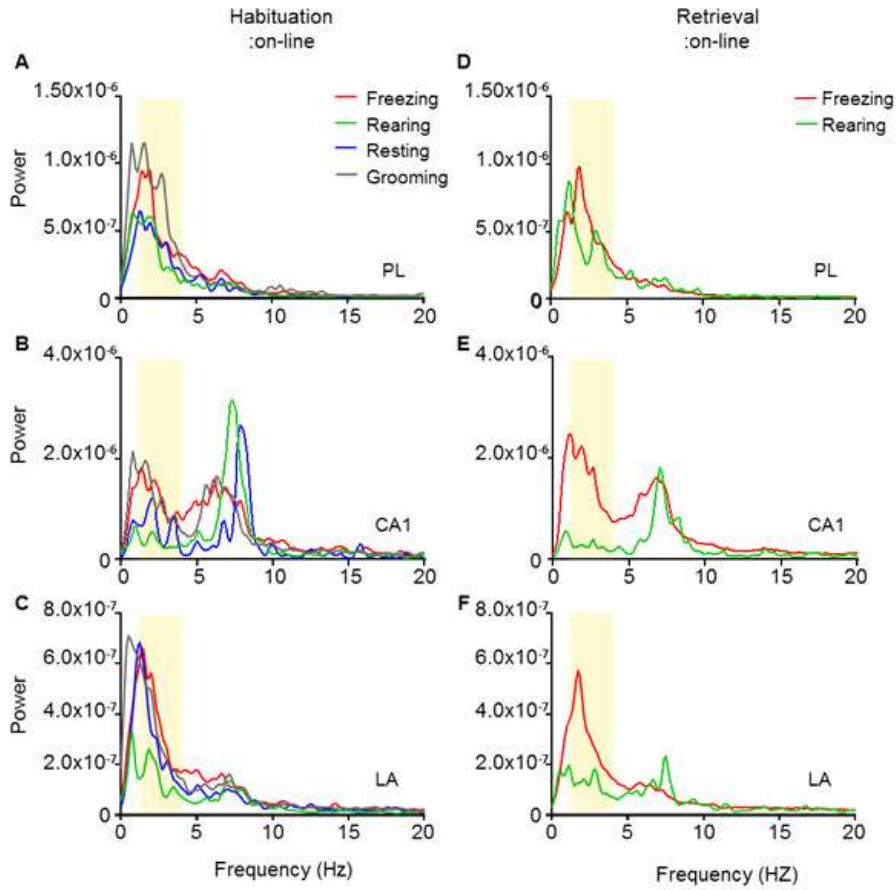

Figure 23. Power spectral density during on-line state. (A-C) Power spectral density during habituation:on-line session in (A) the PL, (B) the CA1 and (C) the LA. (D-F) Power spectral density during retrieval:on-line sessions in (D) the PL, (E) the CA1 and (F) the LA as behavioral conditions. Shaded area presented low frequencies oscillation band.

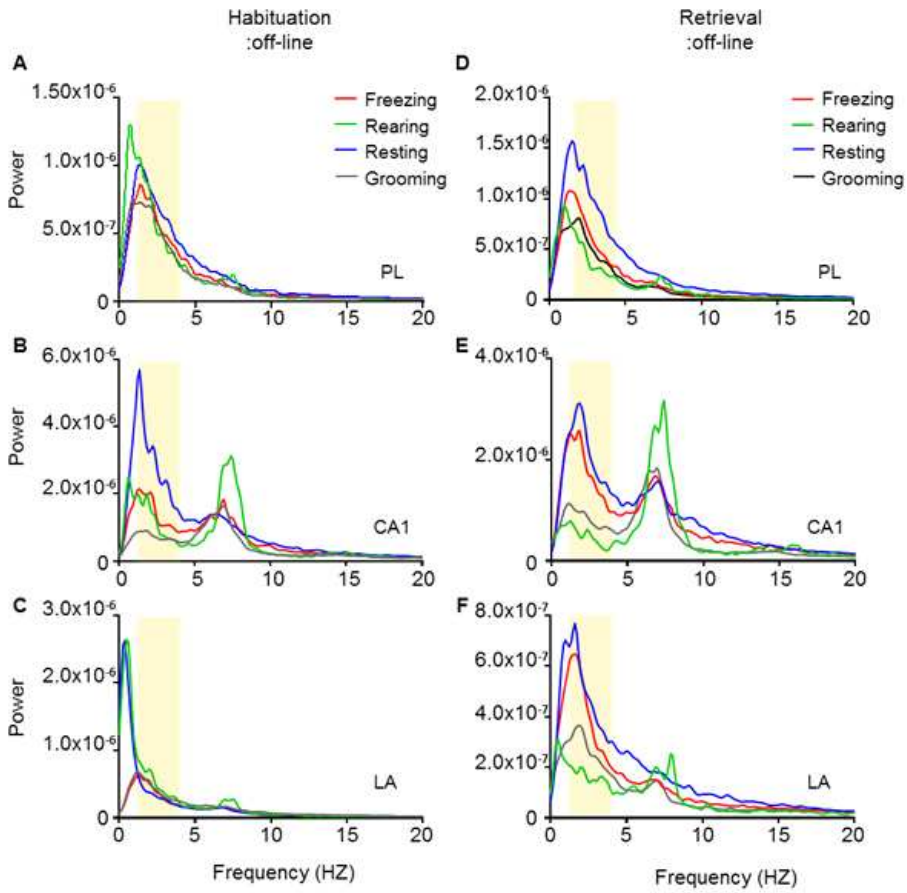

Figure 24. Power spectral density during off-line state. (A-C) Power spectral density during habituation:off-line session in (A) the PL, (B) the CA1 and (C) the LA. (D-F) Power spectral density during retrieval:off-line sessions in (D) the PL, (E) the CA1 and (F) the LA as behavioral conditions. Shaded area presented low frequencies oscillation band.

Coherence at low frequencies oscillations during off-line state

For examine off-line activities of LA networks, I analyzed coherence between LA and PL, and CA1 during freezing and other behaviors. First, coherence was observed in habituation:off-line session (Fig. 25A, C). As a result, coherence of PL-LA and CA1-LA during freezing behavior was not difference from other behavior periods. Averaged of area under curve of low frequencies oscillation was also no difference between freezing and other behaviors at habituation:off-line session (Fig. 25B, D; $p=0.84$ in PL-LA; $p=0.69$ in CA1-LA). However, in retrieval:off-line session, coherence between PL and LA at low frequencies oscillations during freezing was higher than during other behaviors (Fig. 26A). Coherence between CA1 and LA at low band was also observed difference between freezing and other behaviors (Fig. 26C). Averaged area under curve of PL-LA and CA1-LA was not significant but it had higher tendency during freezing than during other behaviors (Fig. 26B, D; $p=0.06$ in PL-LA; $p=0.31$ in CA1-LA). According to the results, I found that communications of LA networks had tendency to be strong at low frequencies oscillations during freezing behavior against other behaviors in retrieval:off-line session as well as that neural power was enhanced in all three regions during same period.

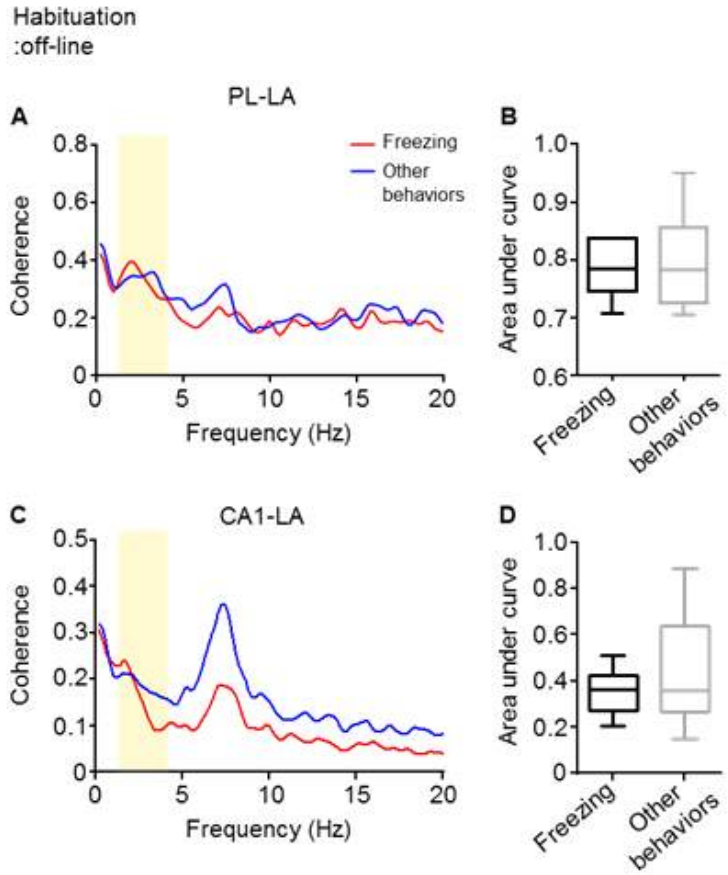

Figure 25. Coherence during habiuation:off-line state. (A) Coherence between PL and LA as freezing vs. other behaviors. (B) Area under coherence curve at 1.5-4 Hz frequencies band of PL-LA. (C) Coherence between CA1 and LA as freezing vs. other behaviors. (D) Area under coherence curve at 1.5-4 Hz frequencies band of CA1-LA.

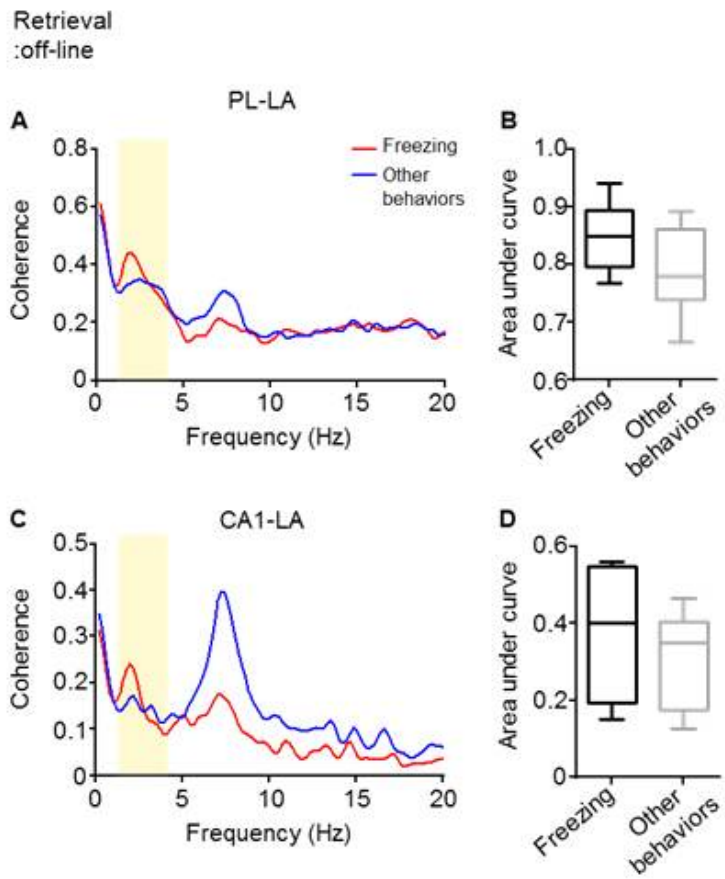

Figure 26. Coherence of retrieval:off-line state. (A) Coherence between PL and LA as freezing vs. other behaviors. (B) Area under coherence curve at 1.5- 4 Hz frequencies band of PL-LA. (C) Coherence between CA1 and LA as freezing vs. other behaviors. (D) Area under coherence curve at 1.5-4 Hz frequencies band of CA1-LA.

Interesting BLA neuronal firing patterns during off-line state

Meanwhile, unique firing patterns in LA single neurons were even observed when rats were resting in retrieval:off-line session. I also recorded activities of single neurons in the LA with LFP simultaneously so I analyzed neuronal firing patterns. I arranged firing spikes of LA neurons of same rats based on the firing of a certain neuron on perievent raster plot. In perievent raster plots of retrieval:off-line, almost LA neuronal firing patterns were lined up in certain shape (Fig. 28). It implied that LA neurons could activate in sequence such as replay in the hippocampus (Derdikman and Moser, 2010; Olafsdottir et al., 2018). Indeed, resting behavior was sleep when these firing patterns appeared and strong power was also observed at wide band oscillations on spectrograms in the same duration as Fig. 28. The firing patterns also observed in retrieval:on-line session in sequence like retrieval:off-line session (Fig. 27). However, time scale of firing patterns in retrieval:off-line was not faster than that of retrieval:on-line, so it was different from firing patterns of replay. The unique firing patterns were not found in habituation:off-line session during resting behavior or whatever rats did.

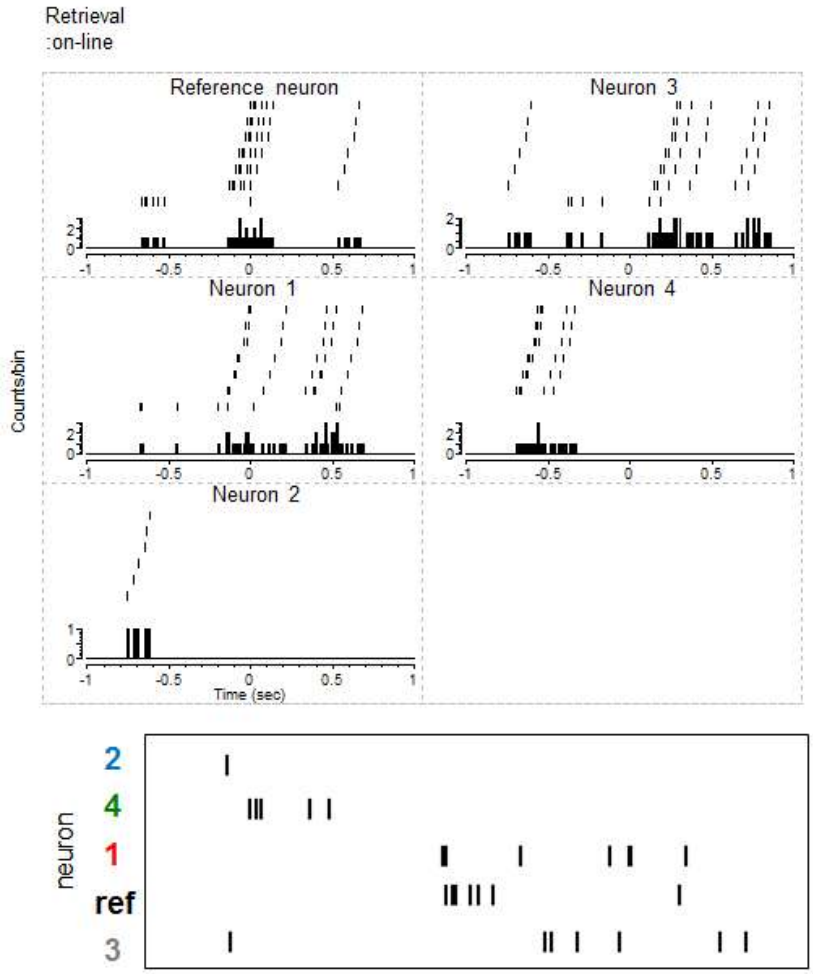

Figure 27. Firing patterns of LA single neurons in retrieval:on−line. During retrieval:on−line session, unique activity patterns of LA neurons were observed. It implies that these neurons co−firing in sequence.

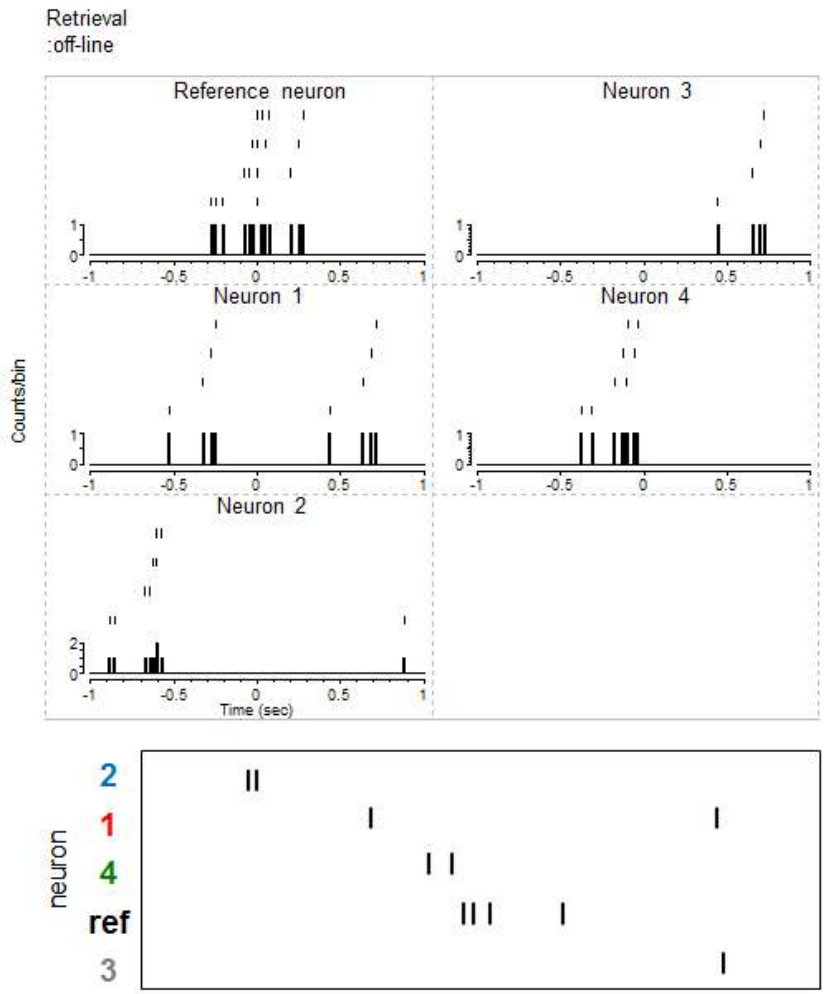

Figure 28. Firing patterns of LA single neurons in retrieval:offline. During retrieval:off-line session, especially sleeping, unique activity patterns of LA neurons re-occurred which were observed in retrieval:on-line session. These neurons co-firing in sequence as patterns of retrieval:on-line state.

Discussion

In this study, I demonstrated various activities in LA networks during off-line state. First, I found that oscillations in low frequencies band appeared during freezing in on- and off-line session after fear conditioning. Also, at this band, increased tendency of coherence between LA and PL, the CA1 was observed during freezing in retrieval:off-line not in habituation:off-line state. Finally, I found curious neuronal firing patterns in retrieval:off-line session when rats fell into a profound sleep probably.

In fact, the specific signals that I found in this study are oscillations in very low frequencies where is generally corresponding high delta frequency range (Buzsaki and Draguhn, 2004). Slow oscillations in this frequency is observed in the amygdala, in particular basolateral (BLA) including lateral and basal nuclei, during slow wave sleep and it is suggested that delta wave in the BLA is related in that of rhinal cortices probably (Collins et al., 1999; Pare et al, 2002). In addition, it is known that delta oscillations occur during synchronization with cortical regions (Karalis et al., 2016) and coordinate the spikes timing of BLA neurons (Pare and Gaudreau, 1996; Ryan et al., 2012). Indeed, in Karalis et al., 2016, it is observed that high delta/low theta (2-6 Hz) oscillations organize firing activities of

mPFC and BLA during freezing behavior in retrieval session. Also, they found that this oscillations trigger freezing behavior and are drove from mPFC to BLA. Thus, it is possible that co-firing between PL and BLA or maybe CA1 is observed during perfectly off-line state at low frequencies oscillations in my study though I did not record activity of PL and CA1 neurons. Also, it will be required that causality analysis of three regions as to know who lead the signals on the superior side during freezing behavior.

It is known that off-line activities of brain regions are involved in cognition mainly sleep state (Poe et al., 2010; Wamsley and Stickgold, 2010; Diekelmann, 2014). In the previous studies, it was studied that memory consolidation is facilitated (Maquet, 2001; Walker and Stickgolf, 2006) and neuronal reactivation of firing patterns is occurred, which is known as replay, during sleep (Peyrache et al., 2009; Lee and Wilson, 2002). It is also demonstrated during state of wakefulness (Foster and Wilson, 2006; Peigneux et al., 2006). Then, neural activity that I observed in the LA as well as other brain regions is possible to represent cognitive state as follows evidences. First, when increased PSD and coherence in the LA and other regions at low frequencies oscillations observed is not during reflexive response of rats. Indeed, this signals are observed in retrieval:on-line session when rats are provided CS induced reflexive freezing behavior. However, off-line activities

during freezing is not implication of the reflexive behavior. It is probably cognitive activities during non-reflexive freezing behavior. In this case, rats freeze because they feel fear when they think about CS, which presented in advance. If so, off-line signal implied freezing have to precede the freezing behavior. The corresponding evidence can be found in the previous study. It is demonstrated that 4 Hz oscillations in the BLA and mPFC predict freezing behavior regardless of CS presentation (Karalis et al, 2016). Therefore, it is possible that off-line activities observed in this study during spontaneous freezing behavior are cognitive signal although precise further analysis is required. Second, neuronal activity patterns in the LA are observed during sleep state. This firing patterns resembled characters of replay in aspects that activity patterns appeared in sequence of neurons and this patterns occurred during sleep. However, it is required more precisely analysis in the future study for evidence of replay phenomenon.

Although more evidence is required, I found diverse off-line activities in LA networks in this study. These results could provide that the LA participates in close cognitive behavior because off-line activities encode non-reflexive behavior. In another evidence for this, replay-like firing patterns in the LA was even observed during sleeping. These results might extend understanding of characters in the amygdala.

Concluding remarks

In this study, I examined neural activities which were not found in the previous studies in the BLA at on- and off-line states. Through a series of experiments, I earned new findings as follows.

In the first chapter, diverse neuronal activities in the BA were studied during on-line state. At first, I gained insight into existence of different activities of BA neurons in each sessions of behavioral process by means of the population analysis. Then, I found diverse excitatory and inhibitory responsive neurons according to emotional states. In particular, new populations appeared in each session of extinction although these sessions were in similar emotional state as low fear level. Therefore, I found that cause of different activities in the population analysis was session-specific recruitment of BA neurons. In the second chapter, on-line activity during freezing behavior was also observed in off-line state with the same behavior. In addition, I found that neural communications of the LA, PL and CA1 strengthened during freezing at off-line state. These results can provide that the LA participates in cognitive behavior beyond reflexive response. Consistent with this, replay-like firing patterns even appeared when animals were sleeping although this firing pattern needs more analysis what it encodes precisely, in the future study.

In conclusion, I found that different BA neurons was recruited during multiple sessions of extinction at on-line state

and the LA and its networks encoded spontaneous behavior at off-line state after fear memory retrieval. These findings provide new perspective and broad understanding of amygdala function.

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국문초록

기저외측핵 편도체의 온라인과 오프라인 신경활성

(On- and off-line activities in the basolateral amygdala)

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이 정 화

공포 조건화 학습은 중성 자극과 유해한 자극을 동시에 반복적으로 제시함으로써 이를 연합하는 학습 모델이다. 이전 연구들에서는 이 모델을 사용하여 기저측 편도체와 그의 신경 네트워크가 공포 조건화 학습 및 소거에 중요한 역할을 담당하고 있음을 밝혔다. 하지만 이전 연구들은 다음과 같은 문제점을 안고 있다. 먼저, 연구자들은 공포 조건화 학습과 소거에 따라 의미 있는 활성 변화를 보이는 특정 뉴런 집단에 초점을 맞추어 연구를 진행해왔다. 하지만 이런 연구들은 기저측 편도체의 전체 뉴런 집단의 특성을 편향되게 해석할 우려가 있다. 또한, 학습된 자극에 대한 뉴런의 활성 (온라인 활성) 에 대해서는 연구가 많이 진행되어왔지만, 자극이 끝난 이후에 일어나는 자발적인 활성 (오프라인 활성) 에 대한 연구는 미미한 수준이다.

제 1장에서는, 기저 편도체의 단일 뉴런의 활성을 공포 조건화 학습 및 소거 모델을 통해 탐구하였다. 먼저 가우시안-처리 지수 분석

을 통해 전체 뉴런 집단의 활성 변화 패턴을 살펴보았다. 그 결과, 공포 조건화 학습과 소거가 진행됨에 따라 기저 편도체 뉴런 집단의 특성이 학습 이전의 상태와 점점 더 다르게 변화하는 것이 관찰되었다. 이 결과를 시작으로 먼저 단일 뉴런 수준에서 각각의 학습이 진행되는 동안 활성 변화를 장기적으로 추적하였고, 이를 통해 공포 조건화 학습과 세 단계의 소거 학습에서 다른 소집단의 뉴런들이 매 학습 마다 활성을 나타냄을 관찰하였다. 이러한 결과를 통해 기저측 편도체의 전체 뉴런의, 적어도 부분적으로는, 활성 패턴이 변화하는 것은 새로운 소집단이 학습 특이적으로 사용되기 때문이라고 제안할 수 있다.

제 2장에서는, 공포 조건화 학습을 진행한 실험 동물에게 조건화 자극을 제시하고, 그 이후에 측면 편도체와 변연전 피질, 등쪽 해마의 오프라인 활성을 탐구하였다. 본 실험실의 이전 연구에서, 아마도 공포 기억의 재귀적 활성과 자발적 인출에 관여할 것이라고 기대되는 오프라인 활성의 존재에 대해 시사한 바 있다. 이와 일관되게, 공포 기억 인출 후 자발적으로 공포 반응이 일어나는 동안 세 개의 뇌 영역 모두에서 낮은 주파수의 신경 활성이 강해지는 것을 관찰하였다. 더불어, 자발적 공포 반응이 일어나는 세 영역의 동시화된 신경 활성이 증가함을 관측하였고 이로써 세 영역의 연결이 강화되는 것을 알 수 있었다.

결론적으로, 이 연구에서는 기저측 편도체의 온라인, 오프라인 활성을 탐구하였다. 첫째로, 기저 편도체의 온라인 활성 패턴이 공포 조건화 학습과 세 단계의 소거 학습에 따라 변화하는 것을 관찰하였다. 이러한 활성 패턴이 나타나는 이유는 다른 소집단의 뉴런들이 참여하기 때문일 것이다. 둘째로, 오프라인에서 공포 반응이 일어나는 동안 측면 편도체와 변연계 피질 그리고 등쪽 해마의 신경 네트워크 활성이 강화되는 것을 밝혔다. 이 연구를 통해 편도체가 감정적 정보를 처리하는 방법에

대한 넓은 이해를 얻을 수 있고, 공포 관련 정신 질환에 대한 치료에 대한 기반을 제시할 수 있을 것이다.