이학석사학위논문

C57BL/6J 생쥐에서의 불안과 조건화된 공포에 대한 행동 분석

Behavioral analysis of basal anxiety and conditioned fear in the mouse strain C57BL/6J

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뇌인지과학과

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Abstract

Behavioral analysis of basal anxiety and conditioned fear in the mouse strain C57BL/6J

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Fear conditioning has been used to study pathogenic mechanisms underlying anxiety disorders. Several studies have shown that humans with anxiety disorders exhibit strong fear responses during the acquisition of conditioned fear. However, there have been no studies investigating whether basal anxiety within the normal range is related to conditioned fear. We hypothesized that individual differences in conditioned fear are correlated to the basal anxiety level of each individual. To test this hypothesis, we measured the basal anxiety of mice by using the elevated-plus maze (EPM) and open field test (OFT) and correlated these data with contextual freezing during contextual

fear conditioning (CFC). Strong correlation was found between the basal anxiety level measured in the OFT and contextual freezing in the CFC. Baseline freezing was also strongly correlated with the freezing level during the retrieval phase of CFC. However, the basal anxiety level measured in the EPM was correlated neither with conditioned fear nor with baseline freezing in the CFC. These results suggest that both basal anxiety in the OFT and baseline freezing are related to contextually conditioned fear.

Key words: Basal anxiety, Conditioned fear, Individual differences, Baseline freezing, Open field test, Elevated-plus maze, Contextual fear conditioning

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Introduction

Fear and anxiety

Fear and anxiety are both negative emotions in dangerous situations, but in neurobiological research, fear and anxiety have distinct definitions. Epstein first suggested that fear and anxiety are distinct. By Epstein's definition, fear is an emotional response to specific threatening cues that can be manifested by avoidance or escape. He defined anxiety in three different ways. First, anxiety is an unresolved fear experienced when avoidance to threats is disrupted. Second, anxiety occurs when an individual overestimates the potential of cues in a dangerous situation. Third, anxiety occurs when an individual's expectancy does not match the environment (Epstein 1972). Öhman posited a similar definition of anxiety. By Öhman's definition, fear occurs when an individual can control environmental threats, while anxiety occurs when there are no effective ways to control a dangerous situation. By these definitions, anxiety is helplessness resulting from an unresolved fear (Öhman 2008).

The most common definitions of fear and anxiety in neurobiological research depend on a specificity of cues for threats. Fear is commonly defined as an aversive response to a specific threat stimulus. The physiological states resulting from fear are fight, flight, or freezing. Anxiety, in contrast, is defined as a prolonged aversive

response to ambiguous threat stimulus, and it sparks hyperviligence (Grillon 2008, Sylvers et al 2011).

Even after conceptually dissociating anxiety from fear, there have been definitional confusions in studies about anxiety. The confusion results from confusing two different types of anxiety: anxiety within normal range (basal anxiety) and pathological anxiety. Basal anxiety is commonly tested in normal laboratory mice, and is different for each mouse strain (Bouwknecht & Paylor 2002). Basal anxiety is evaluated to test basal cognitive functions before performing another behavioral tasks (Crawley 2008), or to test the effects of anxiolytic drugs (Crawley 1981). For this purpose, anxiety is tested with basal anxiety tasks including elevated plus maze, open field test, light/dark box, and cued/contextual fear conditioning paradigms. In contrast, pathological anxiety such as post-traumatic stress disorders is modeled by complex fear conditioning paradigms. Symptoms of anxiety disorders are defined as abnormalities in differentiation of contexts or in extinction of conditioned fear. Discriminative fear conditioning or extinction training measures pathological anxiety (Amano et al 2010, Duvarci et al 2009).

Animal models of fear and anxiety

Fear and anxiety have been extensively studied in animal models. The behavioral paradigms in neurobiological research have

been designed according to the definitions of fear and anxiety. Fear and anxiety are subdivided according to whether they are conditioned of not.

Unconditioned anxiety or basal anxiety is tested by elevated plus maze, open field test, and light/dark box (Sylvers et al 2011). All three experiments use animals' natural tendency to stay in enclosed arms, peripheral areas, and dark environments, respectively. Elevated plus maze consists of two enclosed arms and two open arms that are elevated above the floor. Animals in the elevated plus maze conflict between preferences for enclosed arms and motivation to explore novel environments **(Figure 1a)**. In the open field test , animals conflict between preferences for peripheral areas in the open field and motivation to explore novel environments **(Figure 1b)**. In the light/dark box, animals conflict between staying in the dark box and exploring the bright, novel environment. Based on a finding that dysregulation of a stress hormone called corticotrophin releasing hormone (CRH) is linked to anxiety and mood disorders (Carroll et al 1976), CRH-enhanced startle is also used to induce anxiety (Lee & Davis 1997). These paradigms are widely used to test effects of anxiolytic drugs (Crawley 1981). Still, they are not appropriate for modeling the development of experience-induced anxiety such as post-traumatic stress disorders. For this reason, fear conditioning paradigm has also been used as a model of anxiety.

Fear conditioning is widely used in fear learning as well as a model of pathogenic mechanisms underlying anxiety disorders (Duvarci et al 2009, Indovina et al 2011, Lissek et al 2005, Mahan & Ressler 2011, Mineka & Oehlberg 2008, Ressler et al 2011, Yehuda & LeDoux 2007). In this paradigm, animals are subjected to the pairing of an aversive unconditioned stimulus (US) and a neutral stimulus. Pairing the US with the neutral stimulus modifies neutral stimulus to a conditioned stimulus (CS) that signals the onset of the US and induces fear through anticipation of the aversive US **(Figure 2)**. Cued fear conditioning and contextual fear conditioning predict danger in a different way and activate different defensive responses. A cue is a precise predictor of a discrete threat, whereas the context consists of multimodal stimuli and induces a sustained state of anxiety. Contextual fear conditioning is a behavioral model for inducing sustained aversive responses in humans and rodents (Grillon 2008). Freezing in response to the CS is considered a fear response (Johansen et al 2011).

Conditioned fear is measured as freezing responses (Blanchard & Blanchard 1972, Öhman & Mineka 2001) and fear-potentiated startle (Brown et al 1951, Walker & Davis 1997) in the cued/contextual fear conditioning. Conditioned anxiety is measured by cued/contextual fear conditioning (Grillon 2002, Grillon 2008), light-potentiated startle (Walker & Davis 1997), and shock sensitization (Gewirtz et al 1998). Fear conditioning paradigm can be modified to more complex versions

to test abnormalities of anxious individuals in generalization or extinction of conditioned fear (Amano et al 2010, Duvarci et al 2009). On the other hand, unconditioned fear or innate fear is commonly tested by exposing the animal to the scents of predators (Chan et al 2011).

Neural correlation of fear and anxiety

Neural correlations of fear and anxiety have been studied through different behavioral paradigms depending on distinct definitions of fear and anxiety.

Blanchard and Blanchard found that amygdala lesions disrupted freezing responses when presented with a shock-associated CS (Blanchard & Blanchard 1972). Later studies suggested that the lateral nucleus of the amygdala (LA) is implicated in the acquisition of fear (LeDoux et al 1990), and the central nucleus of the amygdala (CeA) is implicated both in the acquisition and expression of fear (LeDoux et al 1988). A fear conditioning paradigm also showed that the medial prefrontal cortex (mPFC) is necessary in the extinction of fear. Morgan, Romanski, and Ledoux demonstrated that lesions of the mPFC had no effect on fear conditioning but did impair extinction training in the freezing paradigm (Morgan et al 1993).

Walker and Davis found that lesions to the CeA diminished startle responses in fear conditioning paradigms, whereas there was no

change in the startle response in the light-enhanced paradigm. Conversely, lesions to the bed nucleus of the stria terminalis (BSNT) failed to diminish startle responses in the fear conditioning paradigm, but diminished startle responses in the light-enhanced paradigm (Walker & Davis 1997). These findings suggest that two separate pathways mediate the startle response in fear versus anxiety. A study on anxiety using a shock sensitization showed that it is mediated by the BNST, not by the CeA (Gewirtz et al 1998). The CRH-enhanced startle response is also mediated by the BNST, not the CeA (Lee & Davis 1997).

An alternative interpretation of these findings is that CeA and BNST mediate conditioned and unconditioned fear responses, respectively. Indeed, phasic startle potentiation is obtained in aversive conditioning studies, whereas light-enhanced startle, shock sensitization, and CRH-enhanced startle are unconditioned responses. However, recent studies relying on measures of anxiety other than startling have also demonstrated the role of BNST in sustained forms of aversive conditioning such as context conditioning (Sullivan et al 2004, Waddell et al 2006) and long-duration conditioned stimuli (Waddell et al 2006), suggesting that BNST is involved in sustained aversive states rather than in unconditioned responses.

Some studies suggested that the amygdala is important for both anxiety within the normal range and pathological anxiety. Activation of

amygdala in anxious individuals is stronger than in normal individuals during backward masking (Kim et al 2010). Tye suggested that amygdala circuitry mediates unconditioned anxiety in the elevated plus maze and open field tests (Kim et al 2010, Tye et al 2011). These findings suggest that amygdala also mediates fear and anxiety whether conditioned or not. For elucidating the roles of BNST and amygdala in various aspects, fear and anxiety must be extensively studied by precise behavioral paradigms.

In contrast, many studies have shown that structures and downstream of the amygdala and BNST are connected (Dong & Swanson 2005, Krettek & Price 1978, Price & Amaral 1981, Sun & Cassell 1993, Veinante & FREUND-MERCIER 2003). These findings suggest that fear and anxiety are distinct but related psychological states.

Purpose of the study

Previous studies have showed that subjects with anxiety disorders exhibited stronger fear responses during the acquisition of conditioned fear when compared to non-anxious individuals (Grillon 2008, Lissek et al 2005, Mineka & Oehlberg 2008). These findings suggest that pathological anxiety as a diathesis can be detected by fear conditioning. However, it remains unclear whether basal anxiety within the normal range is related to conditioned fear in rodents. Considering

the close relationship between fear and anxiety, basal anxiety is often tested before measuring conditioned fear in experiments to exclude the effects of high basal anxiety in a fear memory experimental group (Crawley 2008). However, individual differences in basal anxiety have not been studied in relation to conditioned fear. A previous study showed that behavioral patterns of individual animals are correlated across time and context (Lewejohann et al 2011), implying a psychological meaning to inter-individual variances in laboratory mice with the same genetic background.

On the basis of these findings, we hypothesized that individual differences in conditioned fear are related to the basal anxiety level of each individual. To test this hypothesis, we sequentially performed the elevated plus maze (EPM), open field test (OFT), and contextual fear conditioning (CFC) tests and then analyzed correlations between individual performances on each test. If basal anxiety was related to conditioned fear, positive correlations would exist between the freezing level in the CFC test and the time spent in the peripheral area of the OFT, and between the freezing level in the CFC test and the time spent in the closed arm of the EPM. Additionally, we correlated baseline freezing during preconditioning with both time spent in the peripheral area during the OFT and time spent in the closed arm of the EPM. Some mice show a high baseline freezing level before they receive shocks during preconditioning phase of CFC, but it is still not known

whether individual baseline freezing is related to the freezing level during retrieval phase of CFC test. The correlation between baseline freezing during preconditioning and the freezing level during retrieval phase of CFC test was investigated to uncover the meaning of baseline freezing during preconditioning in the CFC test.

Methods

Animals

All mice (n = 49) were from a C57BL/6J genetic background. Some of the mice (n = 13) were obtained commercially and the others (n = 36) were the wild type littermates obtained by crossing heterozygotes with C57BL/6J genetic background. All the mice used in the experiments were 8 to 9 weeks or older. All mice were housed in groups and had access to food and water ad libitum. All experiments were conducted according to the guidelines of the Institutional Animal Care and Use committee of Seoul National University.

Behavioral test procedures

Elevated-plus maze

A plus-shaped maze made of white acrylic was used for this experiment. The device comprised 2 opposing closed arms (66 cm × 7 cm) and 2 opposing open arms (66 cm × 7 cm) with 16-cm-high walls. The maze was 54 cm from the floor. Mice were placed in the center of in the maze, and the time spent in each arm was recorded for 5 min. The experiment was carried out under fluorescent light. A smaller version of the EPM was made of white acrylic, and the walls of the closed arms were made of black acrylic. The device comprised 2 opposing closed arms (30 cm × 5 cm) and 2 opposing open arms (30

cm × 5 cm) with 15-cm-high walls. The maze was 50 cm from the floor. The experimental procedure was the same as mentioned above except for the light condition (dim light).

Open field test

An open field made of white acrylic was used for this experiment. The device comprised a surface area (40 cm × 40 cm) enclosed by 40-cm-high walls. The activity of each mouse in the central (within a 20 cm × 20-cm area around the center) and peripheral zones was recorded for 10 min. The experiment was performed in dim light.

Contextual fear conditioning

The mice were handled for 4 consecutive days and then placed in the conditioning chamber (Coulbourn, H10-24T). The test lasted for 3 min and consisted of the presentation of a single 2-s shock (0.8 mA) at 148 s after the start of the test. Freezing during preconditioning was measured before the shock was presented. Twenty-four hours later, the mice were placed in the conditioning chamber for 5 min. Freezing during retrieval was measured for 180 s after the start of the test. The freezing behaviors were recorded by a Freeze Frame fear conditioning system.

Statistical analysis

Graphs were created, and statistics were calculated using GraphPad Prism 5.01. Individual performances were normalized to the mean group performance in each experiment. Linear regressions were used to analyze the data. We excluded a trial from the analysis if a mouse fell onto the floor during the EPM test; therefore, the number of animals in each correlation is different.

Results

Performances in the OFT and CFC are significantly correlated

The mice were sequentially subjected to the EPM, OFT, and CFC tests. Correlations between individual performances in each experiment were analyzed. A significant correlation was observed between the time spent in the peripheral area during the OFT and freezing during the retrieval phase of the CFC ($r^2 = 0.1326$, $p = 0.0290$, $n = 36$; **Figure 3**). Because the OFT performance reflects basal anxiety, this result suggests that the possibility that contextually conditioned fear may be related to the basal anxiety level of each individual.

Baseline freezing and contextual freezing are significantly correlated

In addition, baseline freezing during preconditioning was also correlated with freezing during the retrieval phase of the CFC ($r^2 = 0.2449$, $p = 0.0022$, $n = 36$; **Figure 4**). This result suggests that individual baseline freezing may be related to freezing during the retrieval phase of the CFC test. However, there was no positive correlation between the time spent in the peripheral area during the OFT and baseline freezing during preconditioning ($r^2 = 0.0986$, $p = 0.0622$, $n = 36$; **Figure 5**). These results collectively suggest that the correlation between the time spent in the peripheral area during the

OFT and freezing during the retrieval phase of the CFC is not simply due to the correlation between the time spent in the peripheral area during the OFT and baseline freezing.

Performances in the EPM and CFC are not correlated

No correlation was observed between the time spent in the closed arm of the EPM and freezing during the retrieval phase of the CFC (r^2 = 0.008358, p = 0.6638, n = 25; **Figure 6**), and baseline freezing during preconditioning was not correlated with the time spent in the closed arm of the EPM (r^2 = 0.008358, p = 0.9950, n = 25; **Figure 7**).

Performances in the EPM and OFT are not correlated

Although basal anxiety is measured by both the EPM and OFT, there was no positive correlation between the time spent in the closed arm of the EPM and the time spent in the peripheral area of the OFT (r^2 = 0.01884, p = 0.5130, n = 25; **Figure 8**). To rule out a possibility that different versions of EPM may yield the different results, we carried out a smaller version of EPM. We therefore measured the time spent in the closed arm of the small EPM and correlated the time spent in the closed arm of the small EPM with the time spent in the peripheral area in OFT. C57BL/6J male mice (n = 13) were exposed to the small EPM and the time spent in each arm was measured. There was no correlation

between the time spent in the closed arm of the small EPM and the time spent in the peripheral area during the OFT ($r^2 = 0.02694$, $p = 0.5921$, $n = 13$; **Figure 9**). These results suggest that the EPM and OFT cannot be correlated even though they are both basal anxiety tests.

Figures

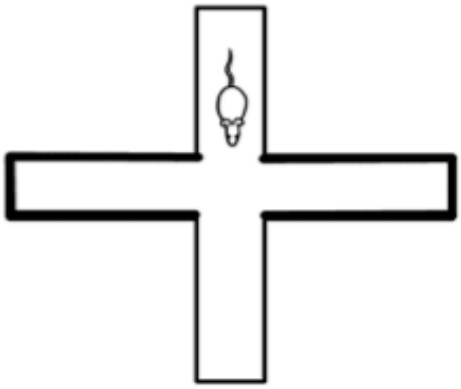
Figure 1.

Behavioral experiments for measuring basal anxiety in mice.

(a) Elevated plus maze (EPM).

(b) Open field test (OFT).

(a)



(b)

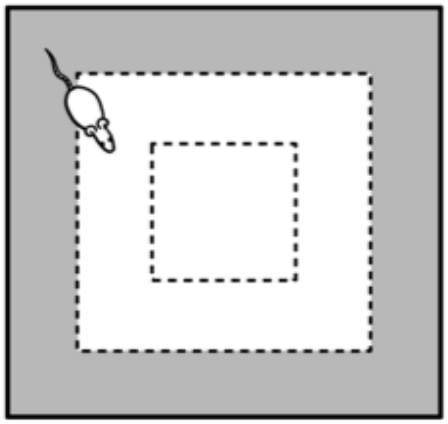


Figure 2.

A behavioral experiment for measuring conditioned fear in mice.

(a) Contextual fear conditioning (CFC).

(a)

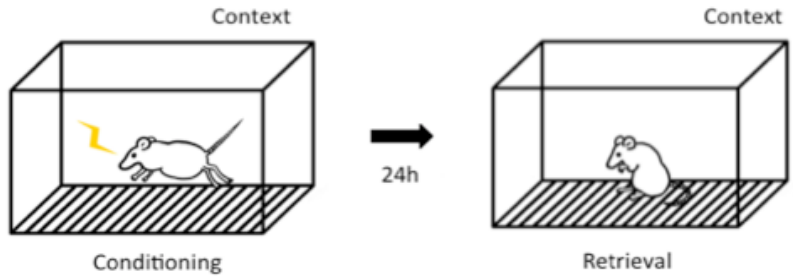


Figure 3.

The correlation between OFT performance and contextual fear conditioning (CFC).

(a) A significant correlation was detected between the time spent in the peripheral area during the OFT and freezing during the retrieval phase of CFC.

(a)

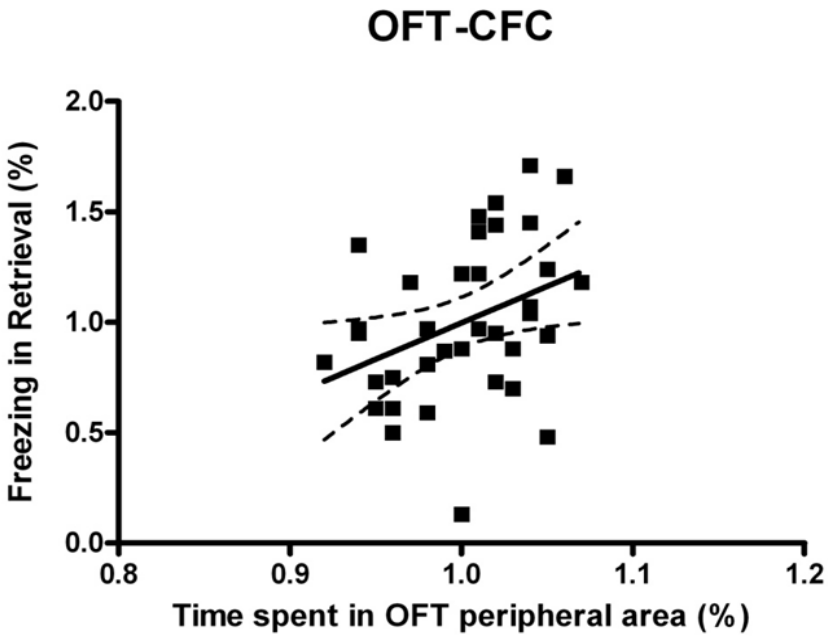

OFT-CFC
Freezing in Retrieval (%)
2.0
1.5
1.0
0.5
0.0
0.8
0.9
1.0
1.1
1.2
Time spent in OFT peripheral area (%)

Figure 4.

The correlation between freezing during Pre and CFC retrieval.

(a) A significant correlation was detected between freezing during preconditioning and freezing during the retrieval phase of CFC.

(a)

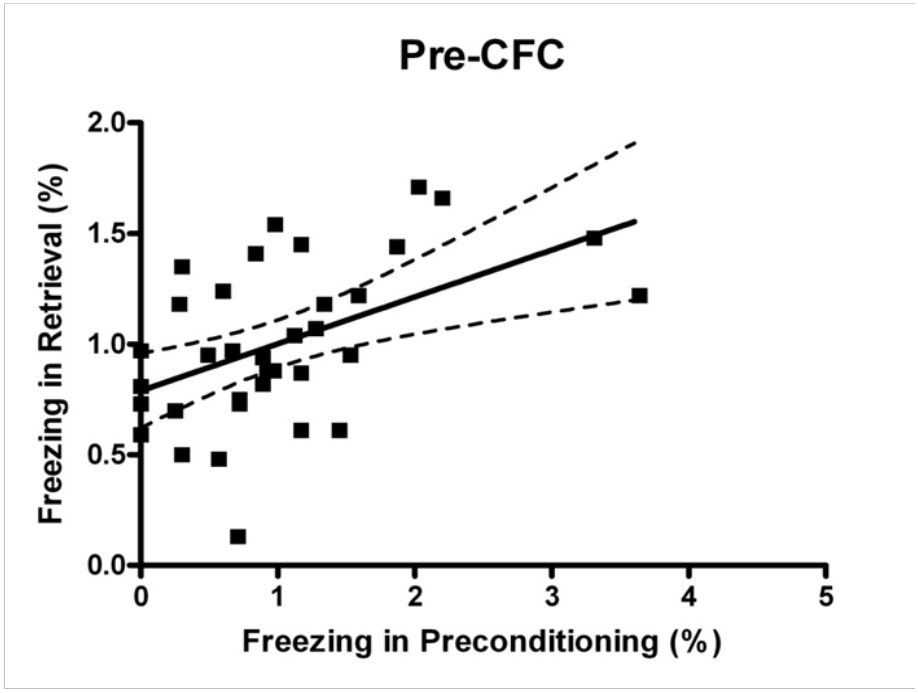

Pre-CFC
Freezing in Retrieval (%)
2.0
1.5
1.0
0.5
0.0
0
1
2
3
4
5
Freezing in Preconditioning (%)

Figure 5.

The correlation between OFT performance and preconditioning (Pre).

(a) No significant correlation was detected between the time spent in the peripheral area during the OFT and freezing during preconditioning.

(a)

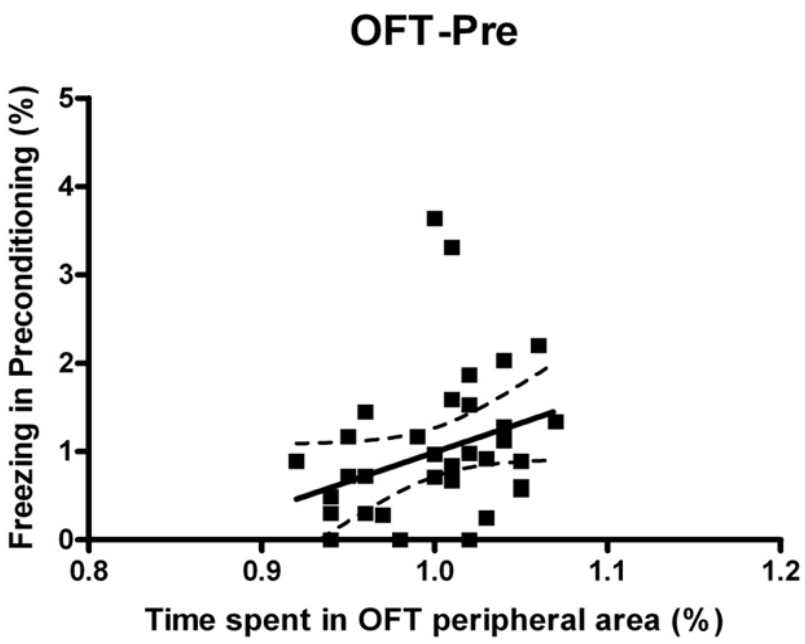

OFT-Pre
Freezing in Preconditioning (%)
5
4
3
2
1
0
0.8
0.9
1.0
1.1
1.2
Time spent in OFT peripheral area (%)

Figure 6.

The correlation between EPM performance and contextual fear conditioning (CFC).

(a) No correlation was detected between the time spent in the closed arm of the EPM and freezing during the retrieval phase of CFC.

(a)

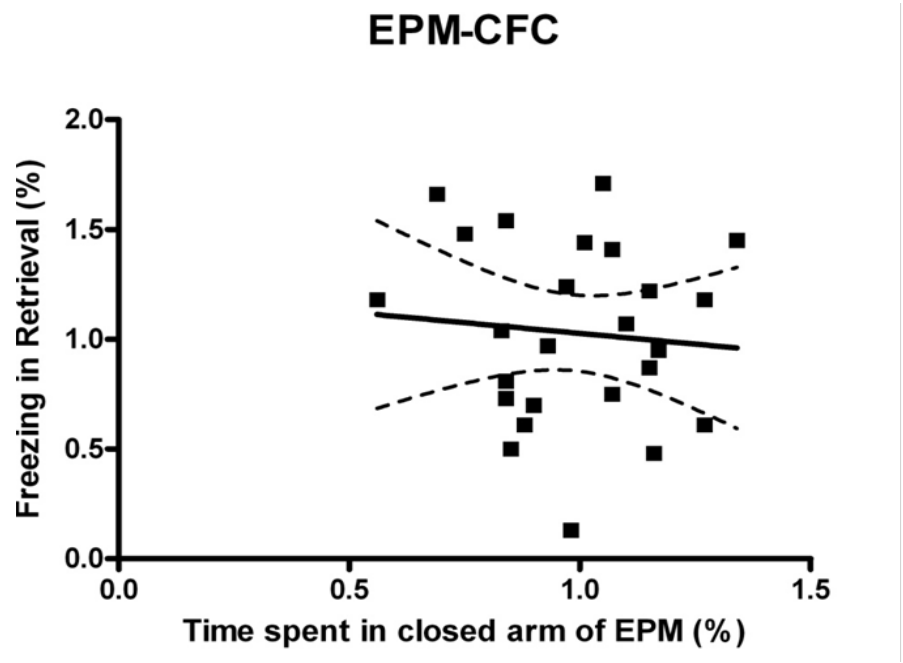

EPM-CFC
Freezing in Retrieval (%)
2.0
1.5
1.0
0.5
0.0
0.0
0.5
1.0
1.5
Time spent in closed arm of EPM (%)

Figure 7.

The correlation between EPM performance and preconditioning (Pre). (a) No correlation was detected between the time spent in the closed arm of the EPM and freezing during preconditioning.

(a)

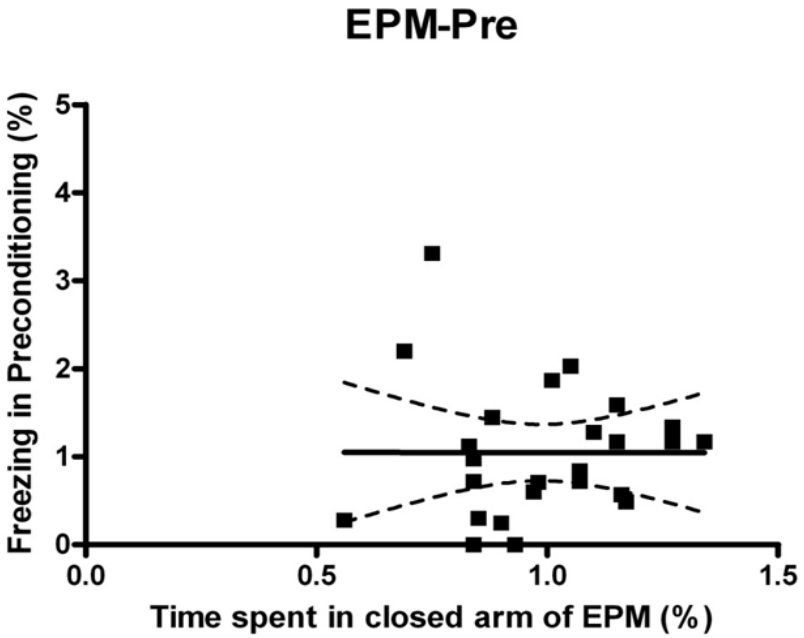

EPM-Pre
Freezing in Preconditioning (%)
5
4
3
2
1
0
0.0
0.5
1.0
1.5
Time spent in closed arm of EPM (%)

Figure 8.

The correlation between EPM (Large) performance and the open field test (OFT).

(a) No correlation was detected between the time spent in the closed arm of the large EPM and the time spent in the peripheral area during the OFT.

(a)

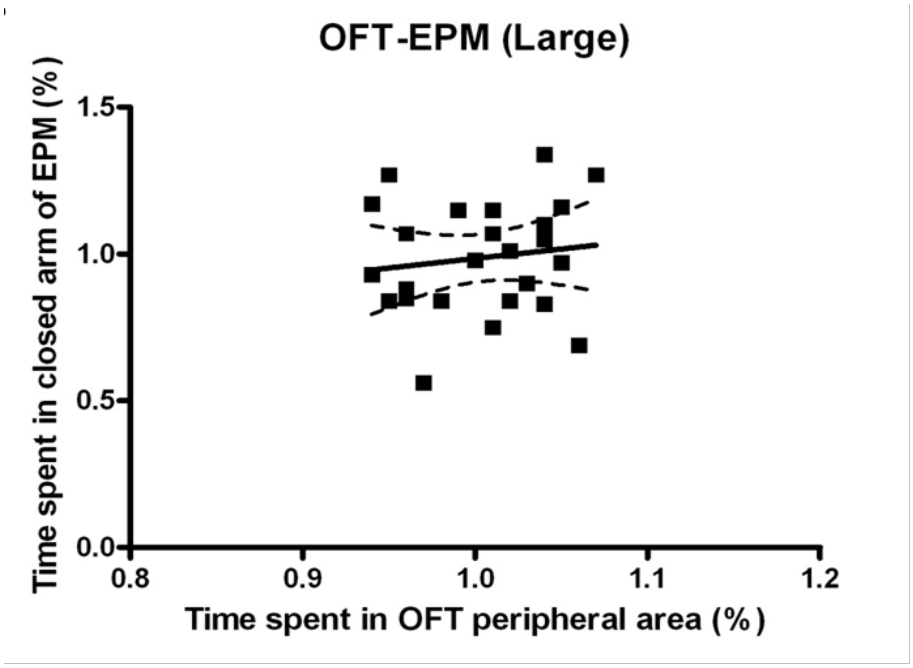
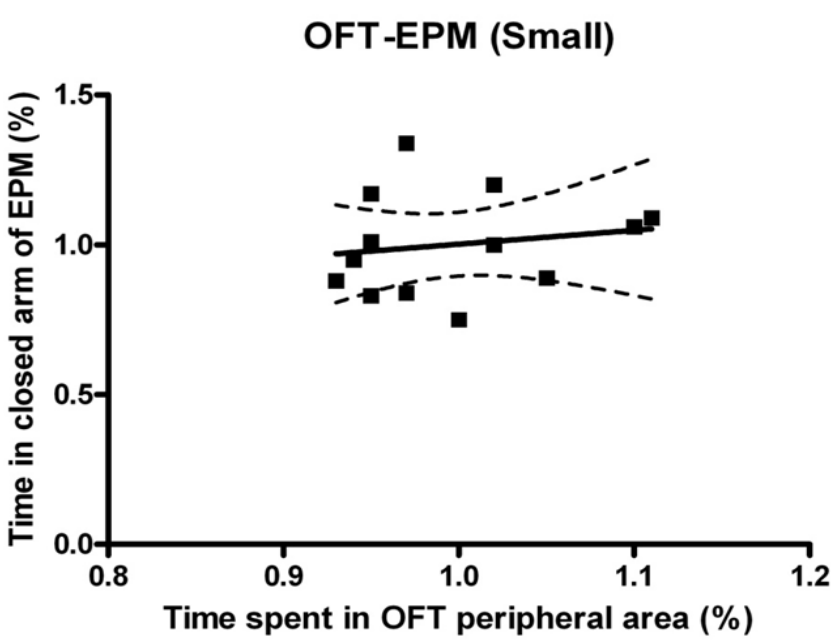

OFT-EPM (Large)
Time spent in closed arm of EPM (%)
1.5
1.0
0.5
0.0
0.8
0.9
1.0
1.1
1.2
Time spent in OFT peripheral area (%)

Figure 9.

The correlation between EPM (Small) performance and the open field test (OFT).

(a) No correlation was detected between the time spent in the closed arm of the small EPM and time spent in the peripheral area of the OFT.

(a)


OFT-EPM (Small)
Time in closed arm of EPM (%)
1.5
1.0
0.5
0.0
0.8
0.9
1.0
1.1
1.2
Time spent in OFT peripheral area (%)

Discussion

Previous studies have frequently reported the existence of weak correlations among anxiety-like behaviors measured in different basal anxiety tests (Hitzemann 2000, Ramos et al 1997, Ramos et al 1998). Factor analysis studies have suggested that the EPM and OFT measure different anxiogenic properties (Carola et al 2002, Ramos et al 2008). Psychological properties in fear conditioning may be more closely related to basal anxiety in the OFT than in the EPM. This may explain why there was no correlation between the time spent in the closed arm of the EPM and the time spent in the peripheral area during the OFT.

The novel finding of the present study is that individual differences within the normal range of basal anxiety levels are related to conditioned fear. We did not use any pharmacological treatments or induce brain lesions before the experiment. Therefore, individual differences in basal anxiety and conditioned fear were subtle, yet still reflecting the different basal anxiety levels of each individual. A previous study showed that behavioral patterns of individual animals with the same genetic background are correlated across time and context (Lewejohann et al 2011), suggesting a psychological meaning to inter-individual variances in laboratory mice.

Recent pathological models of psychiatric disorders highlight the importance of individual differences that confer vulnerability

(Duvarci et al 2009, Grillon 2008, Indovina et al 2011, Lissek et al 2005, Mineka & Oehlberg 2008, Takao et al 2008, Uchida et al 2011, Vialou et al 2010, Yehuda & LeDoux 2007). The development of anxiety disorders is influenced by a variety of factors that include genetic predisposition, environmental risks, early life, and traumatic experiences (Mahan & Ressler 2011).

Several studies have focused on individual differences within the same strain of animals to demonstrate that epigenetic mechanisms can influence vulnerability to psychiatric disorders (Uchida et al 2011, Vialou et al 2010). The correlation between inter-individual differences in different behavioral models suggests that differences among individuals can be measured experimentally. The individual differences within the normal psychological range detected in the present study may therefore be further studied in relation to pathological anxiety and could be elucidated through epigenetic approaches.

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국문 초록

C57B/L6J 생쥐에서의 불안과 조건화된 공포에 대한 행동 분석

안서희

공포 조건화는 불안 장애의 기저에 있는 병인적 기제를 연구하는데 사용되어왔다. 몇가지 연구들은 불안 장애를 가지고 있는 개인이 조건화된 공포를 습득할 때 강한 공포 반응을 보인다는 것을 보여주었다. 하지만 정상 범주의 불안이 조건화된 공포와 관련이 있는지는 알려진바가 없다. 이러한 결과들에 기반하여 우리는 조건화된 공포가 기본 불안 정도의 개인차와 관련되어 있을것이라는 가설을 세웠다. 우리는 elevated plus maze 와 open field test 를 사용하여 생쥐의 기본 불안을 측정하였으며, 이와 공포 조건화에서의 freezing 의 상관관계를 구하였다. 우리는 open field test 에서 나타난 기본 불안이 공포

조건화 실험에서의 공포 반응과 유의미한 상관관계가 있다는 것을 밝혔다. 또한 기본 freezing 은 공포 조건화에서 나타나는 freezing 과 강한 상관관계가 있었다. 그러나 elevated plus maze 실험에서 나타난 기본 불안은 기본 freezing 이나 공포 조건화에서 나타나는 freezing 과 상관관계가 없었다. 이러한 결과들은 OFT 에서 나타나는 기본 불안과 기본 freezing 이 모두 조건화된 공포와 관련이 있다는 것을 보여준다.