Video Article

Trace Fear Conditioning in Mice

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Abstract

In this experiment we present a technique to measure learning and memory. In the trace fear conditioning protocol presented here there are five pairings between a neutral stimulus and an unconditioned stimulus. There is a 20 sec trace period that separates each conditioning trial. On the following day freezing is measured during presentation of the conditioned stimulus (CS) and trace period. On the third day there is an 8 min test to measure contextual memory. The representative results are from mice that were presented with the aversive unconditioned stimulus (shock) compared to mice that received the tone presentations without the unconditioned stimulus. Trace fear conditioning has been successfully used to detect subtle learning and memory deficits and enhancements in mice that are not found with other fear conditioning methods. This type of fear conditioning is believed to be dependent upon connections between the medial prefrontal cortex and the hippocampus. One current controversy is whether this method is believed to be amygdala-independent. Therefore, other fear conditioning testing is needed to examine amygdala-dependent learning and memory effects, such as through the delay fear conditioning.

Video Link

The video component of this article can be found at https://www.jove.com/video/51180/

Introduction

In fear conditioning a neutral stimulus (NS) is paired with an aversive unconditioned stimulus (US). The NS is normally a tone and becomes a conditioned stimulus (CS) through repeated pairings with the US. The CS can then elicit a conditioned response (CR), such as freezing, in the absence of the aversive US. A commonly used fear conditioning protocol is delay conditioning. In this protocol the onset of the NS and the US is contiguous or with some overlap in stimulus presentation. Even though delay fear conditioning is one of the most commonly used types of temporal associative conditioning, there are several other types of associative conditioning temporal arrangements: simultaneous conditioning, backward conditioning, and trace conditioning[1]. In trace fear conditioning there is a stimulus-free interval between the NS and the US of several seconds resulting in a "trace" period.

Several studies have reported deficits in trace fear conditioning when neurotoxic lesions are produced in the structures that input into the hippocampus[2-5] or when pharmacological agents are used to block receptor function in the hippocampus. Lesion to the hippocampus results in deficits in trace conditioning and contextual conditioning, but does not impair delay fear conditioning[8]. There are several benefits to using trace fear conditioning. The fear conditioning protocol can be achieved over a three-day testing period and allows for hippocampal-dependent memory that is not spatially dependent. Trace fear conditioning can be used as a complementary test to the Morris water maze, novel object recognition test, or other maze tests in investigating hippocampal-dependent memory.

Protocol

The mice used in the following experiment were generated and housed at Baylor University at an ambient temperature of 22 °C, with a 14 hr light and 10 hr dark (20:00 to 6:00 hr) diurnal cycle. The mice were given *ad libitum* access to food and water. All procedures to the mice were in compliance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals and the animal protocol was approved by Baylor University Animal Care and Use Committee.

Overview

The trace conditioned fear task is based on procedures described by Wiltgen and colleagues[9].

1. Preparation of Equipment

The fear conditioning apparatus chamber (26 cm x 22 cm x 18 cm) consists of two sides that are acrylic, two sides that are metal, and a grid floor bottom which is used to deliver a mild foot shock. The testing chamber is housed in a sound attenuated chamber. The chamber is also light tight to prevent outside light from influencing the motion detection software.

1. Calibrate the shock levels, light levels, and sound intensity levels for the testing chamber. Measure the background level of the testing chamber. The background noise in this chamber is 65 dB. Use a sound meter to measure this level. The meter should be set to 70 dB, set to C, and to detection setting slow.
    1. Calibrate the shock level to 0.5 mA. Use an external calibration device to correctly calibrate the shock level (see Materials Table). The internal measurement of the shock generator is not accurate. The shock generator administers a scrambled shock which cannot be accurately measured by a standard amp meter.
    2. Place one lead on one of the grid bars and place the other lead 3 or 4 bars over. Use the shock generator to administer the shock. Adjust the shock level until the correct level is achieved. Do this for each fear conditioning chamber.
    3. Close the door to the chamber when measuring the light level of the chamber. Calibrate the light level to approximately 1.0. This is a number specific to the FreezeFrame software used in this experiment. An external light meter will read this as 2 lux. The light level can be adjusted by moving the location of the house light or by adjusting the condenser of the lens. Make sure to tighten the adjusting screws for the lens after adjusting.
    4. Calibrate the sound decibel level to 85 dB. Use an external decibel meter inside the testing cage to calibrate the decibel level (see Materials Table). The sound presented will be a 2,700 Hz tone. Note: If using mice older than 6 months old it may be better to use white noise since older mice can have hearing deficits .
2. After the apparatus is ready take the subjects to a separate holding room. Note: Do not house the mouse in the same room as the testing room.
3. Label the tail of the mice that will be tested in order of testing. It is best to reduce excessive handling before testing. Alternatively, the tails can be labeled the day before the experiment to reduce handling stress. After the mice have been labeled allow them to acclimate to the room for 30 min. Have extra clean cages for housing the mice after testing is completed.

2. Trace Conditioning Day 1

1. Remove each mouse from the cage and place them in individual cages for transport to the fear conditioning chamber. Use clean bedding for each cage. Place a sticky note on the transfer cage to keep the order of mice testing correct. Note: If the mice are singly housed then they can be transported in their home cage.
2. Place the mouse in the testing chamber and close the door. Start the software program.
3. On the training day, allow the mice to explore the chamber for 3 min. The software then presents a 20 sec tone (85 dB, 2,700 Hz) to the animal. After a trace period of 20 sec a mild shock (2 sec, 0.5 mA) is administered to the animal.
4. Record the reaction of the subject to confirm that they received the aversive stimuli by viewing the video. A 200 sec inter-trial interval separates the 5 conditioning trials. Each trial consists of a 20 sec tone followed by a 20 sec delay then a shock.
5. After testing is completed allow the animal to remain in the testing chamber for 1 min before removing it from the testing cage.
6. Place the animal back in the transfer cage and return it to its home cage. If there are additional mice in their home cage then individually house the mouse until all mice complete testing. This will reduce the stress to the other mice that have not been tested. An alternative solution would be to singly house all mice one week prior to testing to reduce the influence of continuously removing mice from one cage.
7. Clean the testing chamber with 30% isopropanol after each animal is tested.
8. Repeat steps 2.2-2.7 for all mice in the testing cohort.
9. Return all mice to their colony room after the last mouse in the cohort has been tested.

3. Trace Fear Conditioning Day 2: Trace Memory Testing

1. Trace memory testing will occur on day 2. In this protocol there are 3 tone presentations. Place the mice in a new context for the trace conditioning test.
2. Prepare the software to run a program with a 2 min baseline period followed by three 20 sec tone presentations. There is s a 220 sec ITI between each tone presentation.
3. For the new context condition, place clear acrylic inserts on the floor of the chamber to alter the shape, texture, and color of the conditioning chamber.
4. Change the odor in the chamber by placing vanilla extract in a weigh boat under the floor insert.
5. Clean the chamber with 70% ethanol instead of 30% isopropanol. Note: This will help to create a novel context.
6. Bring the mice to the holding room and relabel their tails for testing if necessary.
7. Prepare novel context transfer cages by replacing the bedding with shredded paper. Note: This will help in creating a novel context.
8. Place the mice in the testing chamber then start the program. Clean the chamber with 70% ethanol after testing is completed.
9. Return the mice to their home cage after the trace conditioning is completed. Return all mice to their colony room when all mice have been tested.

4. Trace Fear Conditioning Day 3: Contextual Memory Testing

1. On the third day contextual conditioning is conducted. Prepare the software to run a program to record freezing behavior for 8 min.
2. Clean the chamber with 30% isopropanol before testing and after testing each mouse. The context should be identical to that of day 1. The transfer cages should be the same as in day 1.

3. Bring mice to the holding room and label their tails for testing.
4. Place each mouse in the testing chamber then start the program. Clean the chamber with 30% isopropanol after testing is completed.
5. Return all mice to their colony room when completed.

Representative Results

For the representative results we present data from control C57BL/6J adult mice that received the neutral stimulus pairings with the unconditioned stimulus (shock condition) compared to mice that received the neutral stimulus but did not receive the unconditioned stimulus (no shock condition). It is important to run this condition when first setting up this behavioral test to determine whether the protocol has been performed correctly.

The data in **Figure 1** represent the training day of C57BL/6J mice in the trace fear conditioning test. It is not generally necessary to compare the groups across all conditions during the training day. However it is useful to examine the baseline period to evaluate whether there are initial differences in baseline. We did not observe any differences in freezing levels in the baseline condition $t(1,10) = 0.6$, $p = 0.56$. This demonstrates that there are no initial differences in freezing levels. In general there are low freezing levels at the early learning trials but there is an increase in freezing behavior over the conditioning pairing trials. When we performed a mixed-model ANOVA that analyzed differences between the Shock and No Shock condition over the 16 periods we found a main effect of group $F(1,10) = 60.3$, $p < 0.001$. There was a main effect of group $F(1,10) = 215.9$, $p < 0.001$, and a group by time interaction $F(1,10) = 133.9$, $p < 0.001$. The analysis demonstrates that there was a significance difference in acquisition between the two groups.

The data for **Figure 2** illustrate the freezing behavior between the mice that had tone shock pairings (Shock Condition) and the mice that received the same procedure without the shock pairing (No Shock Condition) across the 5 tone conditioning trials. The mice in the Shock Condition formed an association between the tone shock pairings in trace fear conditioning. A mixed-design ANOVA was used to examine the main effect of learning for group $F(1, 10) = 83.48$, $p < 0.001$. There was also a main effect of conditioning trials (time) $F(3,30) = 24.83$, $p < 0.001$, and group X time interaction $F(3,30) = 4.7$, $p < 0.01$. Since there was a group x time interaction, separate t-tests per time point were conducted to examine the difference between the groups at each time point. Separate t-tests reveal significant difference between the groups at the baseline $t(1,10) = 6.8$, $p < 0.001$; tone $t(1,10) = 8.6$, $p < 0.001$; trace period $t(1,10) = 5.3$, $p < 0.001$, and in the inter-trial interval $t(1,10) = 5.1$, $p < 0.001$. For the tone, trace period, and the inter-trial interval the data was averaged across the three presentations. The data demonstrate that the trace conditioning experiment successfully produced learning differences to the trace conditioning protocol.

The data for **Figure 3** demonstrate the freezing behavior between the mice that had tone shock pairings (Shock Condition) and the mice that received the same procedure without the shock pairing (No Shock Condition) in the context condition. The context condition testing was presented 48 hr after trace conditioning is implemented. The mice in the Shock Condition had significantly more freezing in the original context than the No Shock Condition mice $F(1,10) = 12.5$, $p < 0.01$. There was also a main effect of time $F(7,70) = 5.5$, $p < 0.001$; however, there was no interaction between time and group $F(7,70) = 0.78$, $p = 0.61$. The data demonstrate that the trace conditioning experiment successfully produced contextual learning when the CS was paired with an US in trace fear conditioning.

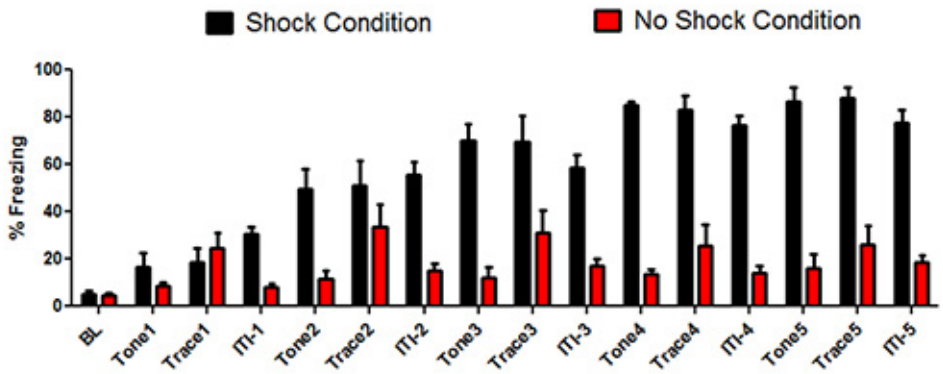

Figure 1. Data from the training day of trace fear conditioning. The black bars represent the data from the mice that received the aversive unconditioned stimulus (shock). The red bars represent mice that did not receive the aversive unconditioned stimulus (no shock) but received the tone stimuli. The bars represent the mean ± Standard error of the mean (SEM) percent freezing for mice during the training day.

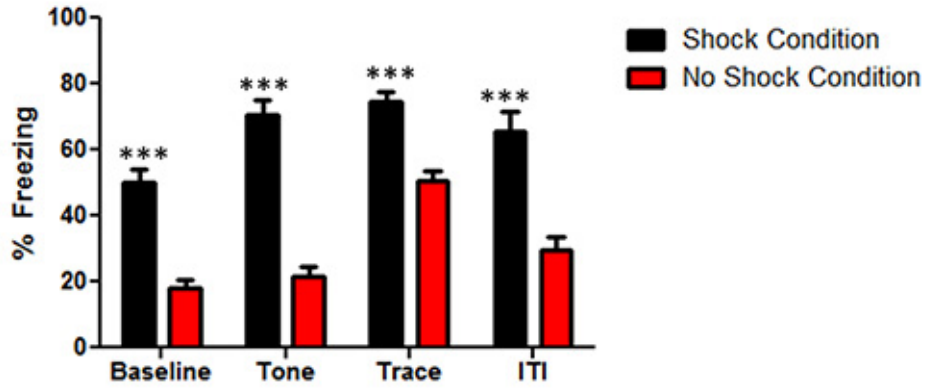

Figure 2. Data from mice in the tone test following trace fear conditioning. The mice that received the unconditioned stimulus (Shock condition) had more freezing compared to the mice that did not receive the shock (No Shock Condition) in the baseline, tone, trace, and intertrial interval (ITI). The bars represent the mean ± Standard error of the mean (SEM) percent freezing for mice during the tone test. Astericks (***) indicate a significant group difference ($p < 0.001$).

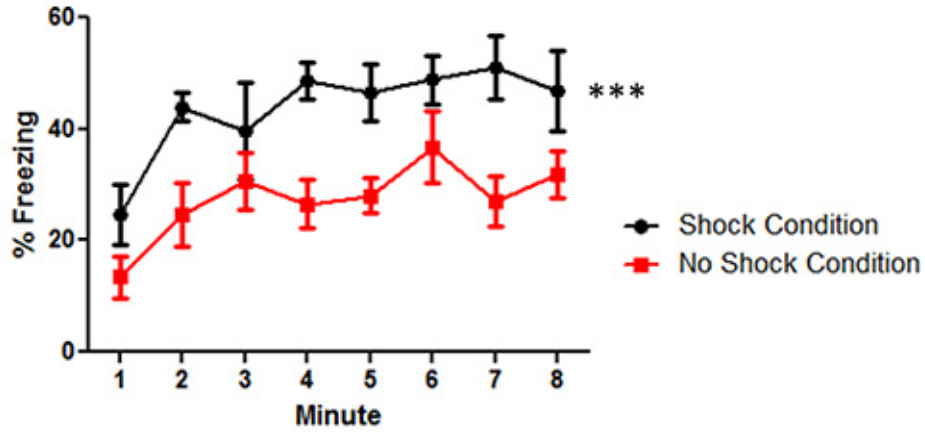

Figure 3. Data from mice tested in the context test following trace fear conditioning. The mice that received the shock had more freezing compared to the mice in the No Shock condition in the context test condition across the 8 min trial. The data points represent the mean ± Standard error of the mean (SEM) percent freezing for mice during the context test. Astericks (***) indicate a significant group difference ($p < 0.001$).

Discussion

There have been several studies that have elucidated the neural circuitry that underlies trace fear conditioning. Trace fear conditioning is believed to involve the CA1 of the hippocampus[12-14]. There is also evidence that the medial prefrontal cortex (mPFC) plays a large role in trace eye-blink conditioning[15], and the mPFC has been found to be involved in trace fear conditioning. One study found that mPFC neurons provide sustained activity during the trace period, thus providing a structure that can sustain the memory during the trace interval[17].

By examining the role of the mPFC it is possible to examine the time line of molecular events in forming an associative memory. Runyan *et al.*[16] found that inhibiting the extracellular signal-regulated kinase (ERK) in the mPFC interfered with memory retention, but did not interfere with memory encoding. Furthermore, an increase in phosphorylated ERK in the prefrontal cortex was reported earlier than in the hippocampus. Since ERK is believed to be involved in long-term memory, the mPFC may be a critical structure involved in the retention of long-term memory. Other studies could examine the role of other signaling pathways in long-term memory and the interaction between the mPFC and hippocampus.

Another use of trace fear conditioning is in examining subtle hippocampal dependent learning and memory alterations. Genetic manipulations that reduce GABAergic inhibition enhance trace conditioning without altering delay fear conditioning. Another study found that trace fear conditioning was enhanced only in female mice that lacked the GABAA δ subunit[9]. A separate study found that trace fear conditioning is enhanced in mice that lack GABAA α4[22]. Therefore, trace fear conditioning may be useful to examine subtle hippocampal-dependent learning and memory enhancements or deficits. Trace conditioning may be sensitive enough to detect the effect of neurosteroids on GABAeric activity similar to what has been found using a contextual fear conditioning protocol[23]. This might be an important consideration when examining learning and memory deficits in a knockout or transgenic mouse where male and female mice are used.

Even though there is a lot of evidence that trace fear conditioning may be useful to examine the role of the mPFC and the hippocampus, there is some controversy whether trace fear conditioning is amygdala-independent. In one report they performed a double dissociation study to

examine the contribution of the amygdala and the hippocampus to trace and delay fear conditioning[24]. When they inactivated the amygdala by the GABAA agonist muscimol there was impairment in contextual and delay conditioning without disrupting the acquisition or consolidation of trace fear conditioning. Inactivation of the dorsal hippocampus impaired trace conditioning and contextual memory without impairing delay fear conditioning. Even though these results strongly support that trace fear conditioning does not require the amygdala, other studies have found conflicting results[25-27]. Kwapis *et al.*[25] found that consolidation of delay and trace fear conditioning is disrupted by the infusion of the protein synthesis inhibitor anisomysin into the basolateral amygdala. Even though these papers appear to contradict one another there are several procedural differences that need to be taken into account when performing trace fear conditioning.

When examining trace fear conditioning it is important to consider several experimental parameters. One is the type of animal to use in this type of associative conditioning. The study by Kwapis *et al.*[25] used rats and the Raybuck and Lattal[24] study used mice so there may be species differences in the neurocircuitry that underlies trace fear conditioning. The protocol described in this paper is designed for mice. This protocol can be used for rats or other species but validation studies that use a double dissociation experimental design to inactivate the amygdala and the hippocampus will need to be performed to determine the validity in other animals.

Another consideration is the number of trace conditioning trials in the training day. It may be that more or less conditioning trials could influence which neural structures are recruited in trace fear conditioning. In this protocol five conditioning trials were used. If additional conditioning trials are added then there could be additional activation of other neural structures that become recruited with the additional pairings. In addition, the trace period could be lengthened or shortened if the number of pairings is to be altered. However, there is some evidence that short trace intervals do not engage the hippocampus and may produce results similar to delay fear conditioning, which has no interval between the presentation of the CS and the US. Therefore a trace interval ranging from 15-20 sec has been reported in several studies to engage the hippocampus. Another alteration could be the strength of the unconditioned stimulus. In the protocol presented here the shock was set to 0.5 mA. If additional pairings are needed the shock level could be lowered to 0.3 mA. If fewer shocks are needed, then shock level could be set to 0.7 mA. A higher shock level than 0.7 mA should be avoided.

When performing validation studies for hippocampal-dependent trace conditioning a consideration is when the neural structure is inactivated. It has been found that alternative circuits can be used in contextual fear conditioning that do not utilize the hippocampus when it is inactivated prior to training. Therefore, the timing of inactivation of different neural structures during trace conditioning is an important consideration.

Another important consideration for fear conditioning experiments is the baseline level. The baseline level is important to report since many studies subtract the baseline levels from the levels of freezing during the tone presentation[32] or subtract the baseline levels from the inter-trial interval[33]. These manipulations have the underlying assumption that the baseline levels are the same across groups. However, a positive correlation has been reported between baseline fear levels and tone fear levels[34]. The paper by Jacobs *et al.*[34] describes several strategies to reduce baseline levels and how to account for baseline levels during fear conditioning. These are important considerations when using the ratio method of freezing in tone conditioning over baseline levels, using the subtraction method, or when using the baseline covariate method to account for differences in baseline level.

When first establishing fear conditioning, another important control is the use of an unpaired control group. The unpaired control group can consist of an experimental setup where there are random intervals between the presentation of the CS and the US. Another approach can be where the CS and US are presented on different days[29]. The unpaired control group is useful to determine nonassociative effects such as sensitization, novelty stress, pseudoconditioning, and other factors that can contribute to freezing behavior. Smith *et al.*[29], describes the use of different types of trace fear conditioning protocols and how they can be optimized to reduce nonassociative effects in three different strains of mice. These are important considerations when optimizing fear conditioning in mice.

Trace fear conditioning offers several advantages that can complement the findings in delay fear conditioning and spatial learning. However, one must be aware that the choice of rodent, number of trace conditioning trials, and timing of circuit manipulation can significantly alter the outcome and interpretation of the results. Another limitation is that it is controversial whether trace fear conditioning can be used to examine amygdala-dependent learning. This may be a limitation when first examining the effect of a treatment or a knockout animal. In terms of strategy it would be best to first use a delay-feared conditioning protocol that can examine amygdala-dependent learning and memory and contextual-conditioning, which is hippocampus-dependent. One can then use another cohort of subjects to further examine learning and memory differences in trace fear conditioning.

Disclosures

The authors have nothing to disclose.

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