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Trace Fear Conditioning: Procedure for Assessing Complex Hippocampal Function in Mice

Vijendra Sharma[1, *], Noah Cohen[1], Rapita Sood[1], Hadile Ounallah-Saad[2, 3], Shunit Gal-Ben-Ari[2] and Kobi Rosenblum[2, 3]

[1]Department of Biochemistry, McGill University, Montréal, Québec, Canada; [2]Sagol Department of Neurobiology, University of Haifa, Haifa, Israel; [3]Center for Gene Manipulation in the Brain, University of Haifa, Haifa, Israel

*For correspondence: vijendra.sharma@mcgill.ca

[Abstract] The trace fear conditioning protocol is designed to measure hippocampal function in mice. The protocol includes a neutral conditioned stimulus (tone) and an aversive unconditioned stimulus (shock), separated in time by a trace interval. The trace interval between the tone and the shock critically involves the hippocampus and could be used to evaluate hippocampal-dependent learning and memory. In this protocol, we presented mice with five pairings of tone and shock separated by a 20 sec trace interval. Freezing was measured 24 h after conditioning to evaluate contextual memory by placing mice in the conditioned chamber. In addition, 48 h after conditioning, freezing was measured in a dark chamber, which served as a different context. This method enables precise detection of hippocampal-dependent learning and memory following pharmacological and genetic manipulations that impair or enhance hippocampal function.

Keywords: Trace fear conditioning (TFC), Contextual memory, Hippocampus function, Memory enhancement, Learning and memory deficits

[Background] The trace fear conditioning (TFC) paradigm differs from standard fear conditioning paradigms (Heise *et al.*, 2017; Segev *et al.*, 2013 and 2015) by the simple insertion of a trace interval between a conditioned stimulus (CS, *e.g.*, tone) and an unconditioned stimulus (US, *e.g.*, electric foot shock), and repeated application of their combination at fixed intervals. The TFC paradigm involves the formation of temporally non-contiguous associations in both natural and pathological conditions, and is considered a complex, hippocampal-dependent paradigm, in contrast to simple cortical-dependent learning paradigms such as taste learning (Stern *et al.,* 2013; Ounallah-Saad *et al.,* 2014; Rappaport *et al.,* 2015; Levitan *et al.,* 2016; Sharma *et al.,* 2018). A remarkable aspect of trace fear conditioning is that it provides a reliable model of attention-dependent associative learning that reflects the complex processing of the hippocampus and alters the circuitry recruited for learning. Several studies have shown that hippocampal lesions before and after training impair the ability of the animal to associate the CS and US stimuli when they are separated by the trace interval (Bangasser *et al.*, 2006; Esclassan *et al.*, 2009). However, animals with hippocampal lesions could associate the CS and US in a delay situation, where no trace interval separates them, but the CS and US co-terminate (McEchron *et al.,* 1998; McEchron *et al.,* 2000; Quinn *et al.,* 2002). Although other brain regions such as the medial prefrontal cortex (Peters *et al.,* 2009; Beeman *et al.,* 2013), the entorhinal and perirhinal cortices

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(Esclassan *et al.,* 2009; Kent and Brown, 2012), and the amygdala (Pape and Pare, 2010; Gilmartin *et al.,* 2012) are involved in relaying stimulus inputs and response outputs, the hippocampus is selectively involved in trace conditioning rather than general fear learning or expression. Moreover, the acquisition of trace fear conditioning increases intrinsic excitability and facilitates LTP in pyramidal neurons of the hippocampus (Song *et al.,* 2012), which makes trace fear conditioning an ideal paradigm to test hippocampal function in young and aged mice (Sharma *et al.,* 2018).

Materials and Reagents

1. Animals
   Male C57BL/6 mice (Envigo, Jerusalem) weighing 20-25 g and approximately 12 weeks old were used in this study. This protocol can also be used to study the function of the hippocampus in other strains and different age groups of mice (Shoji *et al.,* 2016; Sharma *et al.,* 2018). The mice were housed individually, on a 12/12 h light/dark cycle, and provided with water and standard rodent chow ad libitum.
2. 70% ethanol (Fisher Scientific, catalog number: BP82011)

Equipment

1. TFC chambers (Coulbourn Instruments, model: H10-11M-TC)
   Place TFC chambers measuring 25 x 25 x 25 cm internally inside a larger, insulated plastic cabinet that excludes external light and noise (Panlab, Harvard Apparatus, model: LE116 76-0280).
2. Visual (CCD) and infrared camera (Sensor Technologies America, model: STC-CMB4MPOE) along with an infrared illuminator (Bosch, model: EX12LED-3BD-8W).

Software

1. FreezeFrame 3.0 and FreezeView software (Coulbourn Instruments)
   Note: Both software components can be downloaded from the Actimetrics website.
2. FreezeView manual
   Note: The manual can be downloaded from the Coulbourn webpage.

Procedure

See Video 1 for the procedure to perform the experiment described in this protocol.

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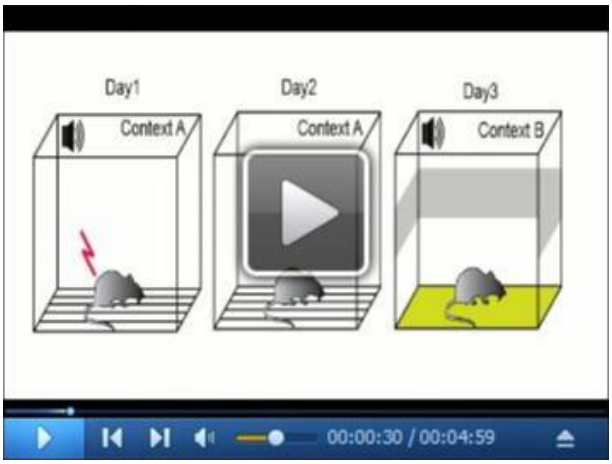

Video 1. Trace Fear Conditioning Protocol. This video describes the trace fear conditioning protocol to assess hippocampal function in mice (Animals were handled according to approved protocols and animal welfare regulations of the University of Haifa Institutional Ethics Committee).

A. Acquisition of TFC

1. Start the FreezeFrame 3.0 software (Coulbourn Instruments) and select the protocol for TFC See Figure 1 for protocol settings in FreezeFrame 3.0.

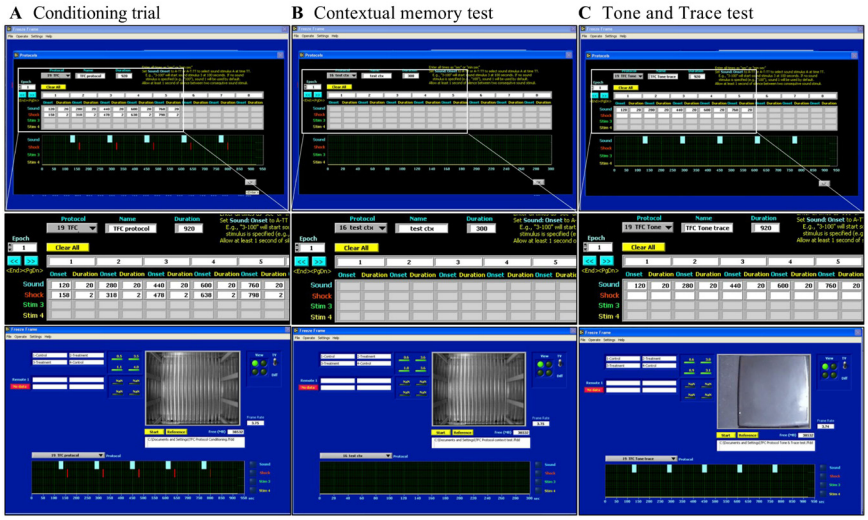

Figure 1. The screenshots from FreezeFrame 3.0 software showing settings used in the TFC protocol. A. Conditioning trial; B. Contextual test; C. Tone and trace test.

2. Turn on the light and fan of the conditioning box and calibrate the shock levels, light levels, and sound intensity levels for the testing chamber.
3. For the TFC protocol, place mice in a chamber (with a 20 W bulb and a 16-bar metal grid floor) for 120 sec. Apply a 2.9 kHz tone for 20 sec at 80 dB (conditioned stimulus, CS) and a 0.5 mA foot shock for 2 sec (unconditioned stimulus, US) at the end of the 20 sec trace interval.

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4. Repeat the previous step four times and separate each trial by a 120 sec inter trial interval (ITI).
5. After administration of the last shock, keep the animals in the chamber for 120 sec before taking them back to the home cage in order to maintain a constant ITI during the conditioning procedure. Do not house the mice in the same room as the testing room.
6. Clean the chambers with 10% ethanol between successive sets of mice.

B. Contextual memory testing
1. Place mice in the conditioning chamber 24 h after conditioning and record freezing for 300 sec (without tone or foot shock) using a visual camera. Analyze the data with FreezeView software (Coulbourn Instruments).
2. Clean chambers with 10% ethanol between successive sets of mice.

C. Tone and Trace memory testing
1. For the tone-trace test, place animals in the dark chambers 48 h after conditioning, but hide the grid floor with black plastic to create another context.
2. Present the animals with the TFC protocol as on the conditioning day, but without shock.
3. Record animal behavior using an infrared camera.
4. Clean the chambers with water between successive sets of mice.

D. Extinction of fear memory

Extinction of TFC refers to a reduction in the freezing response after the repeated presentation of a CS in the absence of the previously paired US.
1. Measure extinction of tone-trace fear response 24 h and 48 h after Tone and Trace testing.
2. Place mice in the dark chambers and hide the grid floor with black plastic to create another context.
3. Present the animals with the TFC protocol as on the conditioning day, but without shock.
4. Record animal behavior using an infrared camera.
5. Clean the chambers with water between successive mice. Do not use 10% ethanol, since the mice may associate the smell with the conditioned context. In this case, water constitutes a different context, allowing to test auditory fear response.

Data analysis

1. The indication for fear memory is the percentage of time spent freezing during the context, tone, and trace event. See Figure 2 for an overview of the experiment and representative results.

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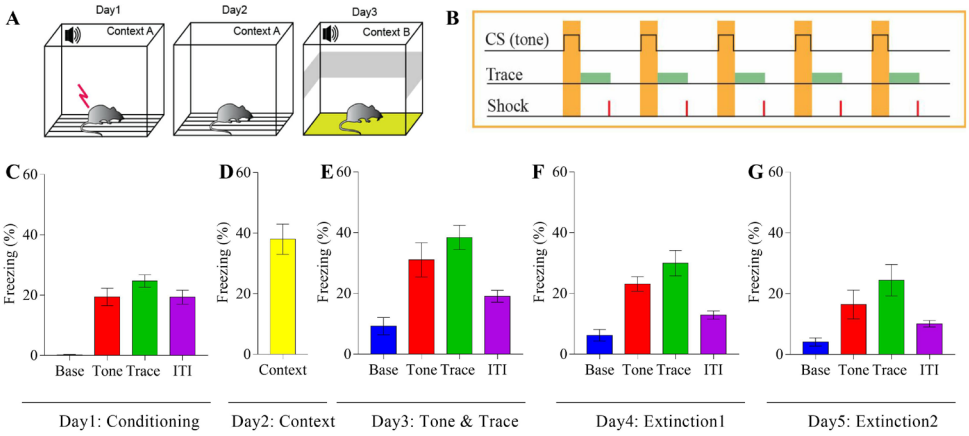

Figure 2. Mice tested in the TFC protocol show evidence of contextual fear and freezing during tone and trace interval. A. Experimental design; B. TFC protocol used for the conditioning; C. Freezing response of mice during the conditioning, Base (% freezing during first 120 sec), Tone (average % freezing during the tones), Trace (average % freezing during the traces) and ITI (average % freezing during the ITI); D. Contextual memory test (% freezing during 300 sec in context); E. Tone and trace fear response, Base (% freezing during first 120 sec), Tone (average % freezing during the tones), Trace (average % freezing during the traces) and ITI (average % freezing during the ITI); F-G. The mice show normal extinction of fear response during tone and trace interval.

2. The software reports freezing behavior to the tone and trace interval as percent freezing across all five trials.
3. Shapiro–Wilk test was used as a numerical means of assessing normality. Independent-samples *t*-test was used as a parametric test and the Mann-Whitney U test was used for the nonparametric equivalent.

Notes

To establish a contextual memory, it is important that the mice be kept in the chamber for 120 sec prior to the stimuli. In addition, a 20 sec trace interval ensures a proper association between the conditional and unconditional stimuli. Five repetitions of this pairing induces a strong response to the trace interval.

Acknowledgments

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Outstanding Post-Doctoral Fellows from China and India (V.S.) and the Ministry of Science and Technology (Eshkol post-doctoral fellowship) and the Tauber foundation fellowship to HO-S. We have modified the protocol in an attempt to make it more efficient to evaluate hippocampus function (Lugo *et al.,* 2014).

Competing interests

The authors declare that there are no conflicting and/or competing interests.

Ethics

Animals were handled according to approved protocols and animal welfare regulations of the University of Haifa Institutional Ethics Committee.

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