Troy, AL, USA, April 30, 2020

Re: Manuscript
Fear incubation using an extended fear-conditioning protocol for rats

Dr. Vineeta Bajaj
Senior Review Editor
Journal of Visualized Experiments (JoVE)

Dear Dr. Bajaj:

We would like to thank you and the reviewers for the feedback we have received. We are convinced that it has importantly improved the clarity and scope of our paper.

In the attached file we address each of the comments and describe the corresponding changes to the manuscript. We have highlighted the sections that were edited/added in the revised version.

Sincerely,

Camilo Hurtado-Parrado, **PhD** (on behalf of the authors).
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---------- Editor´s comments ----------

1. The editor has formatted the manuscript to match the journal's style. Please retain and use the attached version for revision.

We made all modifications on the format, as requested.

2. Please address all the specific comments marked in the text both for the video and the text manuscript.

We worked on all comments from the reviewers, for both video and text.

3. Please proofread the manuscript before submission.

We edited and proofread the manuscript, as requested.

---------- Editor´s comments on video ----------

1. Please shorten the interview section both in the introduction and conclusion.

We reduced the duration of the introduction and conclusion sections, as requested.

2. 0:41-1:01: Please remove the background noise from the interview section both in the introduction and in the conclusion section.

We made the modification, as requested.

3. 2:53: Please format the degree unit as 20 ˚C. Please include a single space between the number the units as shown here.

Done, as requested.

4. Please use h for hour throughout the video and the figures. Please include a single space between the number and the unit e.g., 2 h.

We made the modification, as requested.

5. Please do not show the commercial term Med associates in the video.

We made the modification, as requested.

6. 11:08: Please reword the discussion subheading to Conclusion instead. Please shorten this section to half. Presently this is more than 4:00 min.

We made the modification, as requested.

7. 10:08-10:51 - The narration audio here is much louder in the right stereo channel, compared to the left stereo channel. The voice should be balanced equally between the left and right channels.

We made the modification, as requested.

Reviewer 1

The authors have responded to reviewer comments, and the manuscript now includes some more information that will be useful for replication.

We appreciate the comments made by the reviewer.

Reviewer 2

The authors significantly improved the manuscript and followed the suggestions of the reviewer. I recommend the paper for publication.

We appreciate the comment made by the reviewer.

typo line 426: Fig. 7A

Typographic error amended.

typo line 507 (spanish question mark)

This fragment was excluded from the text.

Reviewer 3

This is a re-submission by Acevedo-Triana and colleagues described the methodology for expression of fear incubation using an extended fear conditioning protocol. The authors have now mostly described the methods and statistics in adequate detail to allow for replication of this work.

We appreciate the comment made by the reviewer

My main concern that was expressed in the original review, with respect to the lack of rigor in the experimental design, specifically in control conditions still stands. At the very least, please acknowledge in the discussion section that rats with minimal or no exposure to footshock, the time-course of fear incubation etc have not been assessed here.

We added the suggestion of reviewer. Please see lines 636-638.

-Please specify the weights of the Wistar rats used for this experiment.

We specified the range of weights of rats. Please see line 138.

-Please state specifically that the rats were trained during the light cycle.

We added the clarification. Please see line 153.

-Please specify details of how often and how the rats were handled during the incubation period.

We added the clarification. Please see lines 259-261.

Reviewer 4

My concerns with the earlier version have been satisfied.

We appreciate the comments made by the reviewers.

Reviewer 5

The authors described an extended fear conditioning protocol that produces fear incubation. Since fear incubation may be related to delayed post-traumatic stress disorder, this protocol will be useful in neuroscience and psychiatry fields. Overall, this protocol is well written. I have a few minor concerns.

1. The authors should describe brightness level in the experimental chamber.

We added the required information. Please see lines 211-213.

2. The authors should describe size of the experimental chamber.

We added the required information. Please see lines 172-179.

3. Although the authors used extended fear conditioning (25 tone-shock pairings), the freezing level in the context and cue tests looks low or similar compared to the standard protocol. Why?

We agree with the reviewer, and added a possible explanation. Please see lines 553-568 and lines 453-457.

Reviewer 6

The protocol describes a new paradigm using an overtraining method of fear conditioning to assess fear incubation in rats. The authors showed that after 1 session of overtraining rats with 25 tone-shock pairings, rats freeze more to the conditioned context at 6 weeks compared to 48 hours after training.

1) The paradigm described is interesting and useful because there is a growing interest in research investigating fear incubation. However, there is a novelty to this protocol, as a PubMed search for articles using this type of protocol could not be found nor publications from the authors utilizing this protocol. Because of this novelty, the authors need to give more background information for the development of this protocol and describe the pros/cons of this protocol compared to other existing overtraining fear conditioning protocols used to assess fear incubation. In addition, authors should address whether publications are pending or reference any publications using this protocol that may not have found on PubMed to support the validity of the protocol.

We used several references to build our protocol. Effectively, we reviewed some references and found lengthy protocols, such as Pickens (2013) with 100 pairings and Maren (1998) with considerably shorter versions. We decided to use the protocol used by Maren (1998) with 25 pairing. Unfortunately, in the previous version of our manuscript, we missed including the reference, and appreciate the reviewer's comment that pointed it out. Please see lines 106-109.

2) The overall protocol is difficult to follow. It is more written for an investigator that has the same exact system in their lab and if not, following the protocol becomes difficult. Each step is too detailed and does not give enough information to researchers that may have an alternate setup. This should be kept in mind when writing this protocol.

We have added some NOTES in each step to help generalize the protocol to different users and equipment, we have added information about measurements and information to that same aim.

3) A representative schematic of the protocol (conditioning, context test, and cued test) and photos would be beneficial.

We agree with the reviewer, and added a graphical representation of the study's design. Please see Figure 1.

4) The incubation effects in the cued test were not as robust as the context test. Could it be possible that the context of the cued test may have been too similar to the conditioning and it blunted some of the effects that could have been seen between 48 hours and 6 week groups? In addition, results in figure 3 shows baseline freezing levels in the cued test looks higher than baseline freezing during training. Could the animals be responding to the context before the cues are even tested? Is it possible to change the floor in addition to smell and walls for the cued test?

We consider this lack of effect in the cue test a limitation of the protocol, which we acknowledge and explain as resulting from variability in the 48 h group.

5) The data analysis section is too dependent on the authors' exact methods. This section should include alternate methods for people without Med Associates. For example, describe what parameters should be analyzed if using an alternate setup, what should be scored for freezing, what are the behavioral endpoints people should examine in their results.

We added descriptions aimed at improving generalization to other settings and equipment, including clarifying the measurements and the contents/functions of the processing files. Please see lines 408-415.

6) The authors state on lines 349-350 that there was declining trend of freezing % in the 48 hour group in the context and cued test compared to training freezing. This statement is slightly deceiving, as it sounds more as if the training did not work. If the baseline freezing levels measured during the first 3min of training is being used as the control, then context and cued test freezing should be compared to this control. Therefore, both 48 h and 6 wk groups showed high levels of freezing compared to control baselines, demonstrating learning and memory of the tone-shock pairings. However, the 48 h group did not show statistically significant increases in freezing in the context and cued tests compared to control freezing levels. This should be addressed by the authors. Did the paradigm not work for the 48 hour group, was there too much variability, what was the issue that rats in the 48 h group did not show elevated freezing after conditioning to the context or cue, but the 6 wk group did?

We addressed this absence of statistical significance in the discussion section. We consider it a limitation of our protocol, which is associated with a high behavioral variability in the 48-h group. We made some suggestions to improve the procedure regarding this issue. Please see lines 605-611.

7) In line with comment 6, for context and cued test results, 48 hr and 6 wk freezing levels should be compared to the control, which is described as the first 3 min of the training session. Therefore, figure 2 and 3 should include those levels and statistics compared to those controls as well.

As shown in Figure 4a, baseline measures can be included in the analyses when percentages are used for the comparison. With Figure 3a,b,c and 4,b,c it is not appropriate to include baseline measures because the variables are not proportional (seconds and motion index).

8) Statistics on the figures are confusing to follow. Generally, asterisks and letters are placed above the treated groups to compare to control, not above the control.

We agree with the reviewer, and we have amended the significance information in the figures to avoid confusion. Please see Figures 2-4.

9) Table 1 and 2 are very confusing and difficult to follow. More descriptions of the factors are needed.

We added additional information to improve clarity. Please see lines 553-574.

10) Discussion should address why fear incubation was seen in the context test but not necessarily in the cued test.

We discuss this topic in the discussion. Please see lines 605-611.

11) Are there any publications available that may not have been found on PubMed using this protocol to support the validity of this paradigm?

As was mentioned before, we used information from other protocols and we conducted a study with exercise to stimulate neurogenesis and evaluated its relationship with fear incubation. This study is under review.

12) Have the authors looked at a time point between 48h and 6 weeks? If so, does the incubation effect increase as time increases from conditioning day as seen in the Pickens model?

Unfortunately, we didn't conduct measurements at any point between 48 h and 6 weeks, mainly to avoid effects of reconsolidation.

Introduction - The introduction adequately addresses fear conditioning and fear incubation. However, background of overtraining is unsatisfactory. For fear incubation, they discuss the Pickens method of 100 tone-shock pairing to assess fear incubation, but the methods they use in one session of 25 tone-shock pairing. It is not well understood why and how their method was developed.

We added information to clarify the numbers of sessions. Please see lines 106-108 and lines 115-116.

Introduction - It would be useful to include reference to the Stephen Maren's 1998 paper (J Neurosci, Overtraining Does Not Mitigate Contextual Fear Conditioning Deficits Produced by Neurotoxic Lesions of the Basolateral Amygdala), which describes asymptotic freezing behavior during overtraining and what is the minimum tone-shock pairings needed for overtraining.

We appreciate the reviewer's feedback and suggestion. We actually used this reference to build our protocol, and as such, we have added to the present manuscript. Please see lines 106-108 and lines 115-116.

-Possibility of usage of other strains of rats should be addressed. Is there a benefit to using Wistar rats over other common strains?

We agree with the reviewer, and have added the clarification in a NOTE. Please see lines 133-136.

-Include a note to why the rats need to be food restricted in a fear conditioning protocol. It should be mentioned that users using the protocol need necessary IACUC approval for food restriction. Will not food restricting rats affect the protocol or results in any way?

We agree and added the clarification in a NOTE. Please see lines 143-148.

-Counterbalancing of animals during testing, using the same exact box for conditioning and context for each rat, and testing rats at the same time of day should be explained.

We agree and added the clarification in a NOTE. Please see lines 157-160.

-Include procedures for animal handling before testing begins, time of day testing (light/dark phase).

We included the clarification, as suggested. Please see line 153 and lines 252-257.

-How long do the rats need to be acclimated to the facility after delivery?

We included the clarification, as suggested. Please see lines 267-268.

-It would be beneficial to describe the components of all the important parts to operate this protocol. Med Associates equipment components should be better explained in these sections. Also, further describe what is needed to execute the protocol.

We edited all protocol and included different NOTEs to enable generalization to different users. By other hand following indications of JoVE papers, we previously received comments to avoid specific indications with a particular equipment.

-Context of conditioning and context test versus cue test should be adequately described.

We added a better description of the context of the training and tests. Please see lines 250-368.

-Animal transportation should be described for each test. Transportation route for cued test should differ from conditioning to further differentiate context for the cued test.

We included the clarification, as requested. Please see lines 252 -257, lines 296 – 301 and lines 333-336.

-Calibration should have their own section. In that section, shock calibration and freezing detection calibration should be described.

We separated the sections, as suggested. We added to section 2 the clarification about shock calibrations. Line 162

-Notes for any possibly issues that may occur with calibration and how to troubleshoot should be included.

We edited some NOTEs to avoid confusion, and edited and improved the section of Troubleshooting. Please see lines 690-712.

-Describe which animals to bring in during testing. Most protocols only bring in the rats to be tested and leave the remaining rats in a holding room to be not affected.

We clarify the information on the transportation of animals. We only moved the animals that were immediately tested and maintained the other rats in a separate holding room to avoid possible affectations. Please see lines 252-257.

-Rats should not be handled by the tail. If this statement is necessary, be specific and state "base of the tail" and never pick up by the end of the tail.

We added the clarification, as requested. We appreciate the reviewer’s comment. Please see lines 263, 318 and 355.

-Include the step, return animals to cage and vivarium

We clarified the transportation system. Please see lines 252-257.

-step 5.1 and 6.1 should be rewritten

We edited the corresponding sections. Please see lines 296-301 and lines 327-329.

-On line 307, should be Figure 1A and not 7A.

We agree with the reviewer, and figures were relabeled. Please see lines 426.

-Where is the data described in lines 304-306?

We added the necessary information. Please see lines 399-403.

-For lines 474-476, reference should be included.

We included the related reference. Please see lines 616.

Veterinary comments

. Please clarify in the text if housing 4 rats per cage is meeting ILAR Guide requirement? If not, what kind of guidelines do you use for housing multiple rats?

We did not follow directly the ILAR guide to housing, instead we follow the requirements of the IACUC of our institution related with housing recommendations. However, it is important to clarify that animals were housed in groups of 4 only for 5 days previous to the start of the training protocol, which required individual housing, since they were maintained at 85% of their weight. Clarification of individual housing was made. Please see lines 133-145.

Under 1.2. Please clarify why you need to maintain rats at 85% of their free-feeding weight for 6 weeks study by giving restricted food access? - - If the animals start losing weight, will you assume feeding animals to ad libitum again? --- How much percentage of body weight loss when you resume ad libitum feeding again?

We added the clarification related to caloric restriction. The procedure to reach the 85% was calculated on the bases of the free-food-consumption weight during five days before fear training. Please see lines 143-146.

Under 6.4. Please add into the text on how you change of the olfactory complex by using 1% of acetic acid to the metal tray below the grid floor?

We added in the manuscript that acetic acid is the standard procedure to change the olfactory context in fear conditioning of rodents, but we did not conduct further measurements below the grid floor. Please see lines 344-345.