Dear Dr. Garofalo,

We are very grateful for your and the reviewers' assessment of the manuscript and we are pleased to submit a revised version of the preprint "A meta-analysis of the effect of protein synthesis inhibitors on rodent fear conditioning".

We were very happy with the positive feedback and we believe the changes suggested by the reviewers greatly improved our work. Below we respond to each comment and point to the revisions in the manuscript.

We hope you will find the revised content suitable for recommendation at PCI Neuroscience.

Best regards,

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REVIEWER 1

This paper by Carneiro and colleagues attempts to use meta-analysis to tackle the heterogeneity of the basic literature on fear conditioning, especially with respect to the role of protein synthesis inhibition in extinction, consolidation and reconsolidation. While taking a meta-analytic approach to the basic literature is an interesting idea, it appears to me that its main benefit at present is to provide validation to the consensus in the literature - e.g., that protein synthesis is required for consolidation and reconsolidation, especially in the hippocampus and amygdala. It was also comforting, as a behavioural neuroscientist, to read their findings that there was little evidence of publication bias on these topics. As discussed by the authors, where the literature was less clear, such as on the role of protein synthesis in extinction, their meta-analysis found fewer significant effects. I suggest some fairly minor revisions with the aim of improving the clarity of the paper and foregrounding its benefit to basic scientists - which in my view is to help identify blindspots or areas of contention where further research is required.

We appreciate the reviewer's dedication to reviewing our manuscript. We agree that identifying blind spots is a worthy contribution of the paper and we revised the introduction and discussion to highlight this point **(p. 1, line 39; p. 21, lines 718-721).**

We provide a response to each point below.

Main Concerns

I have a few main concerns or suggestions which I think might help improve the paper.

My main suggestion here is for the discussion to do a bit more on disagreement between the literature and the meta-analysis. I may be wrong, but it seems to me that this is where the value of the approach is - and it's something that is alluded to in the introduction (line 77). I think that emphasising these points of disagreement between meta-analysis and the general thrust of the literature (which can often be dominated by a small number of thought leaders) can help the field to progress. By pointing out areas where things are definitely unclear, meta-analysis can help scientists to generate hypotheses to test. Without this element, it seems to me that the meta-analysis essentially repeats the consensus points in the literature and is relatively silent on the difficult or contentious topics.

We appreciate the suggestion. We extended the discussion on memory age and memory strength as moderators **(p. 19-20, lines 639-659)** and highlight the key disagreements in the conclusion **(p. 21, lines 718-721)**.

In the introduction (lines 39-43), it is suggested that scientists assume protocol discrepancies are responsible for divergent results (I would further suggest that it is also the polite assumption, rather than suggesting that colleagues have found divergent result because they are wrong, even if they are inadvertent victims of statistical noise). However, the discussion doesn't seem to consider whether this is a valid assumption, although the authors make comments along these lines (lines 660-663). Would the authors care to comment a bit more on this?

We believe that protocol discrepancies are almost always valid as a hypothesis, even though our impression is that they are somewhat overrated in comparison to statistical noise. True heterogeneity unquestionably exists among studies, although the protocol variables we included account for roughly half of it (slightly less for training, slightly more for reactivation). However, the average power of studies also suggests that statistical noise can explain many divergences (as discussed on **p. 20, lines 693-700**).

For the main effects, we have found the most classic protocol elements to be relevant – but these are not really controversial points among studies. For the more controversial topics (e.g. boundary conditions), the absence of interactions in the model does not allow us to detect whether moderators are dependent on other protocol variables.

We now highlight in the conclusion subsection that our analyses cannot eliminate the potential effects of non-linearity in the relationship of protocol variations and effect sizes, or the potential effects of interaction between two or more protocol variables **(p. 21, line 724)**.

Along those lines, I believe some sub-headings within the discussion would be helpful.

Thanks for the suggestion, we have included the subheadings.

Amnestic agents are raised in the introduction (line 69) but their importance is not explained. It would be helpful to mention prior to this point why they are important (e.g., potential clinical utility) in order to make it clearer why we would care about the robustness of their effects.

We did not choose the intervention based on its clinical potential; rather, we chose protein synthesis inhibitors because they have been extensively used as pharmacological tools to investigate mechanistic questions on fear memory (basic research). With that, our goal was mainly to explore the robustness of the findings in terms of consistency or heterogeneity between experiments and papers, including the exploration of potential moderators of the observed heterogeneity, not to estimate a highly precise meta-analytical effect size. Selecting a commonly used, widely accepted treatment allowed us to include a large number of studies, and thus analyze a wide range of variables in meta-regressions with sufficient power. This is now mentioned in **p.2, line 73-82**.

I disagree with the suggestion that there is no easy answer with respect to trusting empirical studies or meta-analysis (line 700). To me, the answer is simple. It's replication.

We fully agree that confirmatory replications trump both individual studies and meta-analysis. The "no easy answer" meant to the situation when an individual study directly testing a moderator does not agree with a meta-analysis, and no further data is available. We reviewed the paragraph to convey this idea more clearly **(p. 21, lines 725-736)**.

Minor Comments

It seems odd to me that the results of drug dose were not described. I would have assumed that drug dosage would be important, if just as an additional example of a sanity check?

The effects of drug doses were included in the univariable and multivariable meta-regressions but was indeed not discussed within the manuscript.

According to the reviewer's suggestion, we returned to the data to try to understand the lack of effect and detected an error in the calculations of the z scores (corrected in **Figure 3, Suppl. Tables 12-21)**. Furthermore, we also revised the calculations to include information regarding species in the Z-scores (**p. 6, lines 260-262**), as effective cycloheximide doses are 100-fold greater in mice than in rats. However, even after the correction, our univariate models still do not yield a significant effect of dose; this is likely an impact of the low variability of the doses used within each route/drug/species.

We have included these results as **Suppl. Figure 12**, and discuss them in the results (**p. 16, lines 527-530**) and discussion (**p. 20, lines 666-671**).

Line 56 - negative unconditioned stimulus - may be better written as "aversive" unconditioned stimulus.

We agree with the comment and have changed this (**p. 2, line 56**).

On the usage of males over females (lines 669-671), the sex as a biological variable (SABV) is relatively recent (the NIH SABV policy only came into effect in 2016), so it seems unfair to criticise a dataset that includes papers up to 2018 for not complying. I would say that this

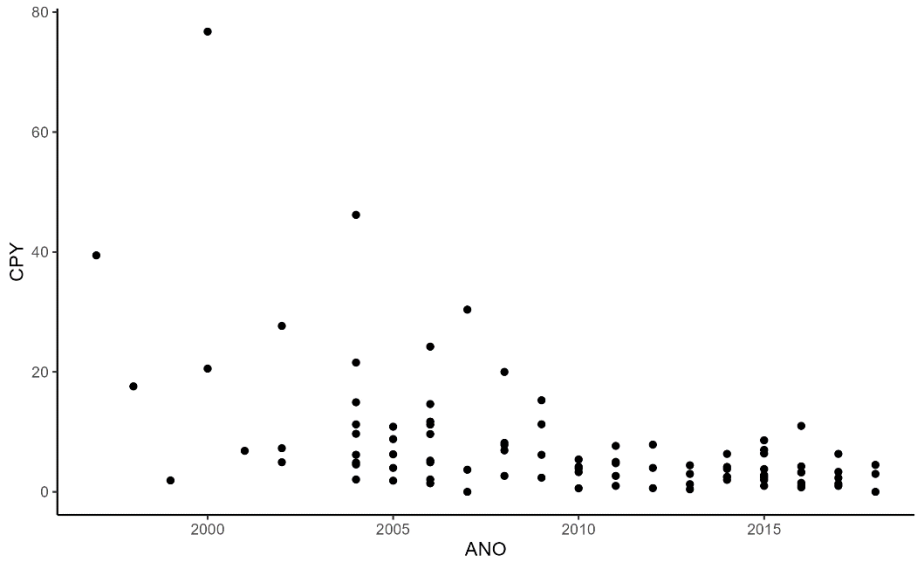
finding isn't interesting, but simply a symptom of the time it takes for policy recommendations to be implemented and for replication studies with female animals to be published.

We agree that policy changes from funders can be expected to have a greater impact, but the discussion around the prevalence of studies in male animals has been taking place in the literature before 2016 (e.g., https://doi.org/10.1371/journal.pone.0007824, https://doi.org/10.1126/science.327.5973.1571, https://doi.org/10.1016/j.neubiorev.2010.07.002). We thus opted for keeping the observation in the discussion, as we do not expect it to be read as a strong criticism.

I also wonder about the use of citations per year (lines 141-143). Since papers tend to accrue most of their citations within a certain window (I would guess it's somewhere between 2-5 years after publication). Did the authors consider using a citation metric that considers paper/citation life-cycle?

We share the reviewer's concerns regarding the limitations of a simple metric such as citations per year. However, we are unaware of an ideal solution and developing a model for the average distribution of citations over time, and trying to obtain a normalized parameter would be beyond the scope of our study.

That said, we examined the distribution of citations by year of publication in our sample (see figure below) and found no indication of bias in favor of recent publications, as would be expected if publications accumulate many citations within the first few years and a low number of citations in subsequent years. On the contrary, the general pattern observed suggest that the earlier publications in the field remain highly cited over time, but such a conclusion should warrant more bibliometric analysis, which we are not confident to make within the current study.



REVIEWER 2

Reviewed by Emiliano Merlo, 09 Jun 2023 13:30

In this manuscript, Carneiro et al. conduct a meta-analysis on the studies reporting the effect of protein synthesis inhibitors (PCI) on fear memory formation and persistence. The manuscript is very well written, clearly explaining the rationale for the analysis, methods, results and data interpretation. The main and supplementary elements are clear and help the reader follow the work and understand the findings.

Overall, I think the study constitutes an important analysis for the field, corroborating some long-standing interpretations (i.e., fear memory formation relies on protein synthesis in some specific brain regions), but also providing additional information for the effect of specific factors in modulating the effect of PSI on memory processing.

I find the study will be of interest to both the learning and memory community and the general reader interested in a synthesis of the role of protein synthesis on memory processing. Besides some suggestions, that I leave for the author's consideration, I can recommend PCI Neuroscience to publish this preprint.

We appreciate the reviewer's dedication to reviewing our manuscript and the positive feedback. We provide a detailed response to each suggestion below.

Suggestions:

- *A definition of memory reconsolidation and extinction should be included. These are central concepts to the manuscript, and the general reader may find it useful to access a definition before delving into the main aspects of the work.*

We definitely agree that this is important, and included a brief definition in the revised preprint (**p. 2, lines 60-63**).

- *In the "Study Selection" section, one of the selection criteria was that protein synthesis inhibitors to be considered should "…directly affect the process of translation…". Antisense oligo deoxynucleotides (ASOs), among other effects, block the mRNA-ribosome interaction and prevent translation. Why did the authors decide to leave these molecules out of the analysis? In the last 20 years, there has been a considerable amount of work using this technology to study memory formation and retrieval dependent processes. Exclusion of ASOs from the analysis should be clarified.*

Our inclusion criteria was focused on broad spectrum protein synthesis inhibitors, rather than inhibition of the synthesis of specific proteins, such as ASOs. We included this clarification in the text **(p. 3, lines 125-127).**

- *Given the cut-off date (31/12/2018), some readers may be wondering why papers from the more recent 4.5 years have not been analysed.*

We share the reviewer's concerns. Unfortunately, however, we lack the resources needed to update the dataset and analyses. The analysis took a long time because it was the first meta-analysis study conducted by our team (the project started in 2016) and since it was completed in 2022 it has gone through other journal submissions.

That said, for the methodological aims of the paper, the lack of inclusion of more recent references might be a relatively minor issue if the included sample is representative of the overall population of existing published studies. In addition, the dataset is already quite large, so that a few new studies are unlikely to change the main conclusions (although they could affect some of the moderator analyses). We now mention this caveat in the conclusions (**p. 21, lines 715-717**).

- *Figure 2: labels on each item indicating the memory phase and manipulation will help the readers understand what each funnel plot is showing at a glance.*

We appreciate the suggestion and have included titles to each plot in **Figure 2**.