

# Neuronal excitation-inhibition imbalance in the basolateral amygdala is involved in propofol-mediated enhancement of fear memory

Corresponding Author: Dr Cheng-Hua Zhou

This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Attachments originally included by the reviewers as part of their assessment can be found at the end of this file.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

In this manuscript, Chen et al. investigate propofol-mediated effects on excitation/inhibition balance in the bilateral amygdala (BLA) as a potential mechanism underlying propofol-mediated enhancement of fear memory. The authors report that multiple injections of propofol shortly after fear conditioning result in enhanced fear behavior upon re-exposure to the conditioned stimulus. Mice that received fear conditioning and propofol administration exhibited significantly higher c-Fos expression in the BLA when compared with mice that received fear conditioning and vehicle injection, indicating that propofol increases neural activity in the BLA shortly after training and suggesting that this propofol-mediated enhancement of neural activity may result in enhanced fear memory. When administered after fear conditioning, propofol significantly increased co-expression of c-Fos and Vglut2; in contrast, propofol significantly decreased c-expression of c-Fos and GAD67 in the BLA, suggesting that propofol increases the activity of glutamatergic cells while simultaneously decreasing the activity of GABAergic cells. These results were substantiated with electrophysiological experiments demonstrating that the effects of propofol in the BLA are dependent on GABAA receptors, which suggests that the drug may increase activity in the region through disinhibition. Finally, using optogenetic and chemogenetic techniques, the authors show that propofol-mediated enhancement of fear memory is attenuated by activating inhibitory interneurons or inhibiting glutamatergic neurons in the BLA. Overall, the authors' findings are timely, thorough, and of interest to readers. However, limitations with study design, which are listed below, significantly dampen enthusiasm for this manuscript.

Major comments

4. In Figure 2, the authors do not show results in Untrained groups. How do trained+vehicle animals potentially differ from untrained+vehicle animals, and how does propofol potentially alter neural dynamics of glutamatergic/GABAergic neurons in the BLA in the absence of training?
5. The authors provide 50 minutes of light stimulation in the BLA following CFC training. How did the authors avoid potential heat-related damage to neurons, particularly in such a ventral region?
6. The rationale/purpose for the chemogenetic experiments is not stated. While the results reinforce the data from the optogenetic experiments, it is unclear what neural/behavioral nuances or other results the authors expected to glean from conducting these experiments. Why would the authors choose to conduct this experiment? What additional information might the chemogenetic experiments provide? Indeed, even the conclusions from the chemogenetic experiments are worded almost exactly the same as the conclusions from the optogenetic experiments (e.g., "...fear memory in mice might be mediated by strengthening the excitability of BLA glutamatergic neurons." "...enhancing fear memory in mice might be accomplished by lowering the excitability of GABAergic interneurons in the BLA.").
7. The behavior within this study is limited. The timing of propofol infusions suggests that propofol alters the consolidation of a fear memory; however, the authors do not conduct further experiments to confirm this hypothesis. Additional experiments administering propofol at different timepoints relative to fear conditioning training or re-exposure would help confirm whether propofol alters fear memory consolidation or recall. Additionally, it is unclear if these effects are limited to fear memory specifically. For instance, if the valence of the experience is changed to a positive memory, would propofol enhance the positive memory? Would propofol also enhance spatial or object memory? Finally, all of the results in the manuscript are limited to short-term fear memory. How long would this enhancement last? Does this affect learning/memory in the long-

term? Would this effect influence the later formation of a novel memory? Can propofol potential affect reconsolidation? In short, it would behoove the authors to determine whether propofol enhances learning/memory or fear, and to determine a time course for the behavioral effects of the drug.

#### Minor comments

1. Did propofol injections lead to anesthesia, or perhaps alter motor function in a manner that may have confounded the results of the optogenetic experiments?
2. The behavioral results in Figure 1 are noisy. What might be contributing to this?
3. The GAD67 staining in Figure 2D do not look specific to neural cells.
3. In the conclusion, the authors state that anesthetic drugs, such as propofol and ketamine, may increase the likelihood of developing stress-related disorders; however, this is at odds with a study indicating that propofol may protect against stress-related disorders (Niu et al., 2022, Life Sci) and several studies showing that ketamine may enhance stress resilience and does not increase the incidence of PTSD and may prevent postpartum depression (Brachman et al., 2016, Biol Psychiatry; McGhee et al., 2008, J Trauma; McGhee et al., 2014, Mil Med; Chen-Li et al., 2022, Ann Clin Psychiatry; Li et al., 2024, J Affect Disord). How would the authors reconcile their results with the conflicting literature?

#### Reviewer #2

##### (Remarks to the Author)

This paper, by Chen et al. is a well written, thoroughly researched study showing that:

- Fear conditioning (FC) increases c-fos expression in the basolateral amygdala (BLA) of propofol-treated mice, but not in non-FC mice.
- Propofol alone does not enhance c-fos expression in the BLA.
- Propofol modulates c-fos expression in the BLA after fear conditioning, with increased freezing behavior observed in the propofol group.
- Propofol increases c-fos expression in VGlut2 (glutamate) neurons and decreases c-fos expression in GAD67 (GABA) neurons in the BLA of trained mice.
- Optogenetic and chemogenetic inhibition of the BLA reduces c-fos expression and freezing behavior.
- Chemogenetic activation of BLA GABAergic interneurons attenuates propofol-enhanced fear memory.
- Electrophysiology data showed that propofol excites glutamate neurons and attenuates GABA neurons.
- Mechanistically, propofol reduces the excitability of GABAergic neurons through GABAA receptors. Propofol exerts its effects through potentiation of the inhibitory neurotransmitter  $\gamma$ -aminobutyric acid (GABA) at GABAA receptors, thus prolonging the inhibitory postsynaptic GABAergic currents.

##### Minor Comments

- Line 52 - In the intro one of the citations is "Morena et al. (2017). It may be helpful to go into detail regarding what this study found - that propofol anesthesia enhanced 48h memory retention, induced enduring traumatic memory enhancement, and anxiogenic effects – to introduce the rationale for this paper.
- Line 55 – consider saying something to the effect of – "we believe these actions may be mediated by the amygdala"
- Line 60 not necessary to say the word cluster each time
- Line 64 there is an extra space
- Line 64 – perhaps the word fear here is better than freezing / shock as an example of an emotional response
- Line 84 – FCT is abbreviated and only defined in the methods which come after the results. This is fine if the methods come before the results. Same with CFC. Check abbreviations.
- Line 89: saying "up to" makes it seem like the results are reversed. Do you mean "greater than"?
- Line 113 remove the word "of"
- Line 144 – what virus was used? State in the text.
- Line 160 – replace the word "obviously" with consequently or something similar
- Line 206-208 - state this in the abstract since it's a major finding of the paper
- "The effect of propofol on enhancing mouse fear memory might be mediated by strengthening glutamatergic neuronal excitability and decreasing the excitability of GABAergic neurons in the BLA."
- Line 241 – retrieval instead of retrieve
- Line 244 – write Hauer et al., (2011) instead of Hauer D.
- Line 246 – Write Morena et al., not Morena M.
- Line 302 – this study did not employ contextual fear conditioning
- In the methods it says the boxes were cleaned with ethanol and in a different time acetic acid, why were both used?

##### Major comments

- Line 83 – Are they getting different doses or the same dose a different number of times, different dosages? Why are mice given multiple doses of propofol – in clinical settings is this the case? It is unclear what you mean by x2, x3, x4, x5. Are the doses in the experiment comparable to a level that achieves anesthesia?
- Line 132 – mention that bicuculline is a competitive GABAA antagonist. Does bicuculline interfere with propofol's enhancing action on freezing by being a competitive antagonist of GABA receptors?
- Lines 140-142 – state this in the abstract since it's a major finding of the paper. It important to be clear from the start of the paper what propofol does to GABA receptors, GABA transmission, and downstream glutamatergic neurons.
- Line 169 – How was the illumination protocol developed? Why was 50 min continuous stimulation chosen?

- To make the paper a bit clearer, in the discussion I would take some time to describe the mechanism of GABA A receptors. This is a good citation: Goetz T, Arslan A, Wisden W, Wulff P. GABA(A) receptors: structure and function in the basal ganglia. *Prog Brain Res.* 2007;160:21-41. doi: 10.1016/S0079-6123(06)60003-4. PMID: 17499107; PMCID: PMC2648504.
- Throughout the paper it says CFC – it's confusing since contextual fear memory was not tested.
- Make a schematic depicting the exact timing of the behavioral experiments (e.g. when shock happened, when cues were presented etc.)

#### Figures:

Figure 1. In the 3 groups (veh, Px2, Px3) there are some mice that show very little freezing (under 20%), were these mice conditioned? In the vehicle group there is one mouse that showed zero freezing. Could this be driving your effect somewhat? Is there a video to ensure they received shock?

Figure 1b. it's a little distracting that some of the points on the graph are colored and some are black.

What do you think changes (does cFos return to baseline?) at 30 min that could be the mechanism for the time course of action of propofol?

Fig 1g. It is unclear what is being depicted here? Is this during the FC session. Is CFC supposed to stand for contextual fear conditioning or cued?

Figure 4a is not detailed enough in terms of the experimental design / word behavior is misspelled / BLA font is very small as are the coordinates. Microscope images are also very small. Yellow is not the best color for bar graph.

Fig 4, what was done in vivo and what was done ex vivo? Make the timeline for this clear.

In figures 5 and 6 how was behavior measured since the experiment seems to take place ex vivo and the way the results are presented, the behavior is presented last.

#### Overall

The paper is very interesting, and the discussion is very good. The intro needs a bit of work to make the rationale clearer and the figures need some clarification as well.

#### Reviewer #3

##### (Remarks to the Author)

The authors showed that intraperitoneal administration of propofol within 30 min after CFC increases freezing during the retention test and the number of c-Fos+ cells in the BLA. Moreover, through ex-vivo electrophysiology they showed that propofol administration leads to increased sEPSC frequency and amplitude of glutamatergic but not GABAergic neurons in the BLA, an effect mediated by GABAA receptors. The effect of propofol in enhancing fear memory were attenuated by both optogenetic and chemogenetic inhibition of glutamatergic neurons and activation of GABAergic neurons in the BLA. Although the paper is generally clear and provides advance in the field, there are some major concerns that should be addressed:

- Figures of immunohistochemistry: it would be useful to see representative pictures also at a lower magnification, showing the localization of the target area.
- It would be useful to have a scheme of virus injection and optic fiber placements for all the animals.
- Line 129: "with the resting potential not significantly changed". However, fig. 3f reports two significance symbols, which are not explained in the caption. Could the authors explain what this significance refers to?
- Line 153-155, authentication of viral expression with electrophysiology during photoinhibition: it is not clear for how long the electrophysiological recordings have been conducted. Considering the evidence that prolonged photoinhibition results in a photoactivation, it is important that the electrophysiological recordings were conducted for the duration as the photoinhibition was performed (50 min). The authors should clarify this methodological point.
- What is the rationale of repeating the inhibition/activation experiments in glutamatergic/GABAergic neurons using chemogenetics, after having already demonstrated an effect with optogenetics and having validated the functionality of the virus used? Those experiments don't seem to add any relevant information to what already shown.
- The methods section refers to "cue memory test" while fig 1 refers to "retention test".
- Line 400: "The average number of positive cells per mouse was counted in three to four brain slices at various levels of the BLA": could the authors give information about those various levels? Possibly referring to AP coordinates of the Mouse Brain Atlas.
- The fact that both photoinhibition and photoactivation decrease the number of c-fos+ cells is intriguing: do the authors have an explanation for this result?
- Line 246: the cited study by Morena et al. is not clinical research.
- Line 295: the authors discuss the inconsistencies between their results and the literature on the effect of propofol in hippocampal-related memories, stating that the present study investigates fear memory which is amygdala-dependent. However, this explanation doesn't seem sufficient, because the paradigm used is a contextual fear conditioning, therefore a hippocampus-dependent task and also in light of BLA-hippocampus projection and its role in fear-related behaviors. In this context, the author should also discuss the literature showing that propofol can accelerate/impair fear memory extinction.
- From the present study it cannot be excluded that the effect on freezing is part of a general effect on locomotion. To strengthen the data, the authors should provide other readouts, such as immobility, darting and fear-related ultrasonic vocalizations (USVs).
- General grammar and punctuation check.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

I thank the authors for their careful effort in addressing the concerns raised during the review process. Overall, I believe that my concerns have been adequately addressed. However, I do believe there are several points that remain that would significantly improve the manuscript for readers.

1. The authors' reasoning for repeating the optogenetic experiments using chemogenetic strategies makes sense; however, I believe that any reader would question this choice. Therefore, I believe the authors should include their rationale for using chemogenetics in the appropriate sections.
2. The discussion of the effects of propofol on long-term memory should be included in the discussion section as an example of a future direction/potential pitfall of this study. Additionally, I am still curious if the authors believe that changing the valence of the encoding experience might affect their experimental findings (this would also be appropriate to include in the discussion section).

Reviewer #2

(Remarks to the Author)

The paper is substantially improved

Lines 236-239 after the author's name the year must go in parentheses

Please include your responses to the first two comments in the "major comments" section in the paper

I am not sure the mice that were not fear conditioned should be included in the analyses

Reviewer #3

(Remarks to the Author)

The authors have addressed my major concerns. However, I still have some minor points that need to be addressed.

- fig 6B: AP, ML, DV coordinates are missing (while present in the other figures);
- "It would be useful to have a scheme of virus injection and optic fiber placements for all the animals.": the schematic diagram in fig 5b mentioned by the author don't provide a full overview of where the fiber was placed in each animal. To make myself clearer on what I meant with "scheme of virus injection for all the animals" please see attached image;
- Line 153-155 (lines 140-143 of revised manuscript): could the authors make it explicit how they chose the laser illumination times of 0.5 and 10 s to validate the virus?
- Line 400: "at the front, center and rear of the BLA" is a bit vague. Could the authors add the exact three AP coordinates?

Version 2:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

I thank the authors for making the suggested changes to the manuscript. The manuscript is much improved, and I have no further concerns or edits at this time.

Reviewer #2

(Remarks to the Author)

For the mice that were not freezing I was referring to figure 1b - vehicle group

Reviewer #3

(Remarks to the Author)

The authors have addressed all the issue and the paper is ready for publication

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Dear reviewers,

Thank you for offering us an opportunity to improve the quality of our submitted manuscript “Neuronal excitation-inhibition imbalance in the basolateral amygdala is involved in propofol-mediated enhancement of fear memory in mice” (Manuscript Number: COMMSBIO-24-3421). Those comments are all valuable and very helpful for revising and improving our paper, as well as providing important guidance for our research. We have studied the comments carefully and have made corrections, which we hope will meet with approval. Revised portions are marked in red on the paper. The main corrections in the paper and the response to the reviewers' comments point-by-point are as follows:

Reviewers' comments:

Reviewer #1 (Remarks to the Author):

In this manuscript, Chen et al. investigate propofol-mediated effects on excitation/inhibition balance in the bilateral amygdala (BLA) as a potential mechanism underlying propofol-mediated enhancement of fear memory. The authors report that multiple injections of propofol shortly after fear conditioning result in enhanced fear behavior upon re-exposure to the conditioned stimulus. Mice that received fear conditioning and propofol administration exhibited significantly higher c-Fos expression in the BLA when compared with mice that received fear

conditioning and vehicle injection, indicating that propofol increases neural activity in the BLA shortly after training and suggesting that this propofol-mediated enhancement of neural activity may result in enhanced fear memory. When administered after fear conditioning, propofol significantly increased co-expression of c-Fos and Vglut2; in contrast, propofol significantly decreased c-expression of c-Fos and GAD67 in the BLA, suggesting that propofol increases the activity of glutamatergic cells while simultaneously decreasing the activity of GABAergic cells. These results were substantiated with electrophysiological experiments demonstrating that the effects of propofol in the BLA are dependent on GABAA receptors, which suggests that the drug may increase activity in the region through disinhibition. Finally, using optogenetic and chemogenetic techniques, the authors show that propofol-mediated enhancement of fear memory is attenuated by activating inhibitory interneurons or inhibiting glutamatergic neurons in the BLA. Overall, the authors' findings are timely, thorough, and of interest to readers. However, limitations with study design, which are listed below, significantly dampen enthusiasm for this manuscript.

We sincerely thank you for the time and effort you spent reviewing our paper. Your significant comments helped us improve the quality of our work greatly. We have studied the comments carefully and tried to fix all the problems you mentioned. The revisions are highlighted in red font in the revised manuscript and the responses to your comments are listed as follows.

## Major comments

4. In Figure 2, the authors do not show results in Untrained groups. How do trained+vehicle animals potentially differ from untrained+vehicle animals, and how does propofol potentially alter neural dynamics of glutamatergic/GABAergic neurons in the BLA in the absence of training?

We sincerely thank the reviewer for careful reading. We also performed statistical studies on untrained+vehicle and untrained+propofol animals previously and found that propofol anesthesia did not change the neural dynamics of glutamatergic/GABAergic neurons substantially in untrained animals. Therefore, we did not present these results. As per your suggestion, We have added experimental results for both Untrained+Vehicle and Untrained+Propofol animals. Please kindly see the revised Fig. 2 and line 102 in the revised manuscript.

5. The authors provide 50 minutes of light stimulation in the BLA following CFC training. How did the authors avoid potential heat-related damage to neurons, particularly in such a ventral region?

Thank you very much for your valuable suggestions. To avoid potential heat-related damage to neurons, we used an illumination (60s)-pause (60s) cycling pattern for the optogenetic manipulation and controlled the irradiation power to be around 4mW prior to the optogenetic manipulation. Referring to our previous experimental protocol and the results of immunofluorescence staining in our experiments, this optical strategy worked without causing heat-related damage to brain regions.



6. The rationale/purpose for the chemogenetic experiments is not stated. While the results reinforce the data from the optogenetic experiments, it is unclear what neural/behavioral nuances or other results the authors expected to glean from conducting these experiments. Why would the authors choose to conduct this experiment? What additional information might the chemogenetic experiments provide? Indeed, even the conclusions from the chemogenetic experiments are worded almost exactly the same as the conclusions from the optogenetic experiments (e.g., "...fear memory in mice might be mediated by strengthening the excitability of BLA glutamatergic neurons." "...enhancing fear memory in mice might be accomplished by lowering the excitability of GABAergic interneurons in the BLA.").

Thank you very much for your valuable comment. We have controlled the laser energy to be around 4mW to minimize the heat-related damage during the optogenetic manipulation. However, this may lead to an attenuation of the laser illumination, which is generally believed to activate neurons 0.35mm-0.75mm away from the light source. The BLA is a long and narrow brain area, and it is possible that part of the brain area may not be able to receive light stimulation efficiently due to the attenuation. Chemogenetics could have avoided this situation so we chose to employ it to further validate the results of the optogenetic manipulation.

7. The behavior within this study is limited. The timing of propofol infusions suggests that propofol alters the consolidation of a fear memory; however, the authors do not

conduct further experiments to confirm this hypothesis. Additional experiments administering propofol at different timepoints relative to fear conditioning training or re-exposure would help confirm whether propofol alters fear memory consolidation or recall. Additionally, it is unclear if these effects are limited to fear memory specifically. For instance, if the valence of the experience is changed to a positive memory, would propofol enhance the positive memory? Would propofol also enhance spatial or object memory? Finally, all of the results in the manuscript are limited to short-term fear memory. How long would this enhancement last? Does this affect learning/memory in the long-term? Would this effect influence the later formation of a novel memory? Can propofol potentially affect reconsolidation? In short, it would behoove the authors to determine whether propofol enhances learning/memory or fear, and to determine a time course for the behavioral effects of the drug.

We sincerely thank the reviewer for careful reading. We have administered propofol to mice immediately or 30, 60 and 90 min after training. The results revealed that fear memory was greatly enhanced in mice that were injected intraperitoneally with propofol 0 and 30 min after training compared with the vehicle group. These findings showed that intraperitoneal injection of propofol within 30 min after training could effectively strengthen fear memory in mice.

Our published paper has shown that propofol anesthesia impairs spatial or object memory in mice so here we did not repeat the experiment again (Yang X. et al., 2022 *Front Aging Neurosci*).

Our experimental methodology for the fear memory test at 48h after fear training refers to previous studies such as Morena et al. (Morena M. et al., 2017, Behav Brain Res, Hauer D. et al., 2011, Anesthesiology). As for the test of long-term memory, it implicates the memory extinction mechanism and is subject to more influencing factors. Our experiments focused on the enhancement impact of fear memory and not memory extinction so we didn't test the long-term memory. Thank you again for your comments which are very helpful and valuable in improving our study. We will definitely follow your comments in our future studies to further investigate in depth in order to elucidate the mechanisms associated with the impact of propofol anesthesia on memory extinction.

#### Minor comments

1. Did propofol injections lead to anesthesia, or perhaps alter motor function in a manner that may have confounded the results of the optogenetic experiments?

Thank you very much for your valuable comment. Propofol is widely used in clinical practice as an anesthetic induction and maintenance drug with rapid onset, short duration of action, and few side effects. Research has shown that propofol anesthesia does not impair animals' mobility upon awakening; neither the distance nor the speed of movement in the Open field test of rats was altered after propofol injection (Li J. et al., 2021, Neurotox Res). Our previous paper also showed that there was no difference in the total time spent exploring new locations or new objects in the novel object recognition test and object location test in mice before and after propofol anesthesia

(Yang X. et al., 2022 Front Aging Neurosci). All these results indicate that propofol anesthesia does not impair the locomotion of the animals upon awakening and that our behavioral tests were performed after the animals had awakened from anesthesia.

2. The behavioral results in Figure 1 are noisy. What might be contributing to this?

We sincerely thank the reviewer for careful reading. The more the number of shocks, the stronger the fear memory of the mice. In order to avoid the ceiling effect of fear memory caused by too many shocks, which could not reflect the enhancement impact of propofol, we used the mode of one shock to make the fear memory of the mice mildly enhanced. This may result in a few mice that were not intelligent enough not showing very strong fear memories, and this might be the cause of the noisy in Fig. 1.

3. The GAD67 staining in Figure 2D do not look specific to neural cells.

We sincerely thank the reviewer for careful reading. The BLA contains 80% glutamatergic neurons and 20% GABAergic neurons. Because of the small proportion of GABAergic neurons in the BLA, the background tends to become visible in immunofluorescence due to the influence of other factors. However, as shown in Fig. 2d in the partial magnification we can see a clearer and significantly stronger cellular morphology than in the background.

We utilized GAD67 antibody from Abcam (ab26116) in our experiments and strictly followed the protocol for specific staining of GABAergic cells. To further confirm the immunofluorescence results, we repeated the experimental steps multiple times and

achieved similar experimental images. And our immunofluorescence pictures are similar to the sample pictures provided by Abcam. Not only that, but we reviewed recent published article using the same antibody and found that they stained similarly to what we showed (Kim YJ. et al., 2023, Exp Neurobiol).

3. In the conclusion, the authors state that anesthetic drugs, such as propofol and ketamine, may increase the likelihood of developing stress-related disorders; however, this is at odds with a study indicating that propofol may protect against stress-related disorders (Niu et al., 2022, Life Sci) and several studies showing that ketamine may enhance stress resilience and does not increase the incidence of PTSD and may prevent postpartum depression (Brachman et al., 2016, Biol Psychiatry; McGhee et al., 2008, J Trauma; McGhee et al., 2014, Mil Med; Chen-Li et al., 2022, Ann Clin Psychiatry; Li et al., 2024, J Affect Disord). How would the authors reconcile their results with the conflicting literature?

Thank you very much for your valuable suggestions which have helped us to improve the manuscript. The study indicated that propofol may protect against stress-related disorders. Niu et al. found that propofol can accelerate fear memory extinction and change synaptic plasticity of PTSD mice. These contradictory results may be related to differences in the dose and timing of propofol administration or in the models used. They administered propofol to the mice 30 minutes after fear training was completed and tested contextual memory at weeks 1, 2, 4, and 6 after fear conditioning. Whereas in our experiment the propofol administration was done immediately after the fear

condition training was completed; we tested cue memory rather than contextual memory; and we tested memory 48h after training but not week 1, 2, 3, 4 and 6. Meanwhile Niu et al. also said “One study showed that propofol induced an enduring traumatic memory enhancement and anxiogenic effects in a rat model of PTSD. However, these contradictory results may be related to differences in the dose and timing of propofol administration or in the models used” in the discussion section. Several studies showed that ketamine may enhance stress resilience and does not increase the incidence of PTSD and may prevent postpartum depression. The mechanisms of ketamine and propofol induced anesthesia are different, and our study did not investigate the effects of ketamine on fear memory. These conflicting results may also be related to variations in the dose and timing of ketamine administration or in the models used. As per your suggestion, We have provided additional explanations on these issues in the discussion section. Please kindly see the line 236-242 and line 604-617 in the revised manuscript.

Reviewer #2 (Remarks to the Author):

This paper, by Chen et al. is a well written, thoroughly researched study showing that:

- Fear conditioning (FC) increases c-fos expression in the basolateral amygdala (BLA) of propofol-treated mice, but not in non-FC mice.
- Propofol alone does not enhance c-fos expression in the BLA.

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- Propofol increases c-fos expression in VGlut2 (glutamate) neurons and decreases c-fos expression in GAD67 (GABA) neurons in the BLA of trained mice.
- Optogenetic and chemogenetic inhibition of the BLA reduces c-fos expression and freezing behavior.
- Chemogenetic activation of BLA GABAergic interneurons attenuates propofol-enhanced fear memory.
- Electrophysiology data showed that propofol excites glutamate neurons and attenuates GABA neurons.
- Mechanistically, propofol reduces the excitability of GABAergic neurons through GABAA receptors. Propofol exerts its effects through potentiation of the inhibitory neurotransmitter  $\gamma$ -aminobutyric acid (GABA) at GABAA receptors, thus prolonging the inhibitory postsynaptic GABAergic currents.

We sincerely thank you for the time and effort you spent reviewing our paper. Your significant comments helped us improve the quality of our work greatly. We have studied the comments carefully and tried to fix all the problems you mentioned. The revisions are highlighted in red font in the revised manuscript and the responses to your comments are listed as follows.

Minor Comments:

Line 52 - In the intro one of the citations is “Morena et al. (2017). It may be helpful to go into detail regarding what this study found - that propofol anesthesia enhanced 48h memory retention, induced enduring traumatic memory enhancement, and anxiogenic effects – to introduce the rationale for this paper.

Thank you very much for your valuable suggestions which have helped us to improve the manuscript. We have described in detail the findings of Morena et al. Please kindly see line 35-38 in the revised manuscript.

Line 55 – consider saying something to the effect of – “we believe these actions may be mediated by the amygdala”

We sincerely thank the reviewer for careful reading. We've added a description of your suggestion. Please kindly see line 41-42 in the revised manuscript.

Line 60 not necessary to say the word cluster each time

Thank you very much for your important advice. As per your suggestion, we have removed the redundant “cluster”. Please kindly see line 45-46 in the revised manuscript.

Line 64 there is an extra space

Thank you for your significant comment. We are very sorry for our incorrect writing. We have deleted the extra space. Please kindly see line 49 in the revised manuscript.



Line 64 – perhaps the word fear here is better than freezing / shock as an example of an emotional response

Thank you very much for your significant comment. We have chosen to use “fear” instead of “freezing, shock”. Please kindly see line 49 in the revised manuscript.

Line 84 – FCT is abbreviated and only defined in the methods which come after the results. This is fine if the methods come before the results. Same with CFC. Check abbreviations.

Thank you for your significant comment. As per your suggestion, We have added descriptions of the meanings of FCT and FC (With reference to your subsequent comments, we have changed CFC to FC) when they first appeared in the results section. Please kindly see line 69-70 in the revised manuscript.

Line 89: saying “up to” makes it seem like the results are reversed. Do you mean “greater than”?

Thank you very much for your significant comment. We are very sorry for our incorrect writing. We have chosen to use “greater than” instead of “up to”. Please kindly see line 75 in the revised manuscript.

Line 113 remove the word “of”

Thank you very much for your significant comment. We are very sorry for our incorrect writing. We have removed “of” from the sentence. Please kindly see line 99 in the revised manuscript.

Line 144 – what virus was used? State in the text.

We sincerely thank the reviewer for careful reading. We describe in detail the viruses for mice injection in this section. Please kindly see line 135 in the revised manuscript.

Line 160 – replace the word “obviously” with consequently or something similar

Thank you very much for your significant comment. We have chosen to use “consequently” instead of “obviously”. Please kindly see line 150 in the revised manuscript.

Line 206-208 - state this in the abstract since it's a major finding of the paper “The effect of propofol on enhancing mouse fear memory might be mediated by strengthening glutamatergic neuronal excitability and decreasing the excitability of GABAergic neurons in the BLA.”

Thank you very much for your valuable suggestions which have helped us to improve the manuscript. We have added descriptions “The effect of propofol on enhancing mouse fear memory might be mediated by strengthening glutamatergic neuronal excitability and decreasing the excitability of GABAergic neurons in the BLA” in the

abstract section and labeled them in red. Please kindly see line 13-18 in the revised manuscript.

Line 241 – retrieval instead of retrieve

Thank you very much for your significant comment. We are very sorry for our incorrect writing. We used the word “retrieval” instead of “retrieve”. Please kindly see line 231 in the revised manuscript.

Line 244 – write Hauer et al., (2011) instead of Hauer D.

Thank you very much for your significant comment. We used the word “Hauer et al., (2011)” instead of “Hauer D”. Please kindly see line 234 in the revised manuscript.

Line 246 – Write Morena et al., not Morena M.

Thank you very much for your significant comment. We used the word “Morena et al.” instead of “Morena M”. Please kindly see line 236-237 in the revised manuscript.

Line 302 – this study did not employ contextual fear conditioning

Thank you very much for your valuable suggestion. As per your suggestion, we have removed contextual to avoid confusion since we did not employ contextual fear conditioning. Please kindly see line 302 in the revised manuscript.

In the methods it says the boxes were cleaned with ethanol and in a different time acetic acid, why were both used?

Thank you very much for your valuable suggestions. Odors are part of the context, and since we were testing cue memory rather than contextual memory, in order to detect sound-induced fear memories we had to change the context including the odor during test. So we wipe the training box with alcohol in the training period and the test box with acetic acid in the testing period.

Major comments:

Line 83 – Are they getting different doses or the same dose a different number of times, different dosages? Why are mice given multiple doses of propofol – in clinical settings is this the case? It is unclear what you mean by x2, x3, x4, x5. Are the doses in the experiment comparable to a level that achieves anesthesia?

Thank you very much for your valuable suggestions which have helped us to improve the manuscript. The experimental protocol for propofol administration in mice was to receive the same dose a different number of times. The clinical use of propofol is continuous pumping, and to simulate the clinical mode of administration we used a multiple administration pattern. x2, x3, x4, x5 means 2, 3, 4 and 5 injections of equal doses (60 mg/kg) of propofol with an interval of 30 min. We use a dosage that maintains a desirable state of anesthesia, which can be maintained for different duration of anesthesia through different number of injections.

Line 132 – mention that bicuculline is a competitive GABAA antagonist. Does bicuculline interfere with propofol's enhancing action on freezing by being a competitive antagonist of GABA receptors?

Thank you very much for your significant comment. The objective of using bicuculline was to utilize its competitive antagonist of GABAA receptors to confirm whether the memory-enhancing effects of propofol were mediated through the enhancement of the inhibitory neurotransmitter  $\gamma$ -aminobutyric acid at GABAA receptors. The experimental evidence that GABAergic neurons originally inhibited by propofol regained activity again after the application of bicuculline verified our conjecture.

Lines 140-142 – state this in the abstract since it's a major finding of the paper. It important to be clear from the start of the paper what propofol does do GABA receptors, GABA transmission, and downstream glutamatergic neurons.

Thank you very much for your valuable suggestions which have helped us to improve the manuscript. We have added descriptions from lines 140-142 in the “Abstract” section and labeled them in red. Please kindly see line 13-18 in the revised manuscript.

Line 169 – How was the illumination protocol developed? Why was 50 min continuous stimulation chosen?

Thank you very much for your valuable suggestions. To avoid potential heat-related damage to neurons, we used an illumination (60s)-pause (60s) cycling pattern for the optogenetic manipulation and controlled the irradiation power to be around 4mW prior to the optogenetic manipulation.

50 min was the effective duration of illumination, since we used an illumination (60s)-pause (60s) cycling pattern, and the duration of fully completed illumination was 100 min, as propofol administration sustained the effective duration for about 100 min. Referring to our previous experimental protocol and the results of immunofluorescence staining in our experiments, this optical strategy worked without causing heat-related damage to brain regions.

To make the paper a bit clearer, in the discussion I would take some time to describe the mechanism of GABA A receptors. This is a good citation: Goetz T, Arslan A, Wisden W, Wulff P. GABA(A) receptors: structure and function in the basal ganglia. *Prog Brain Res.* 2007;160:21-41. doi: 10.1016/S0079-6123(06)60003-4. PMID: 17499107; PMCID: PMC2648504.

Thank you very much for your valuable suggestions which have helped us to improve the manuscript. We have added descriptions of mechanism of GABAA receptors in the discussion section and cited the article you recommend and labeled them in red. Please kindly see line 243-247 and line 618-619 in the revised manuscript.

Throughout the paper it says CFC – it's confusing since contextual fear memory was not tested.

We sincerely thank the reviewer for careful reading. Regardless of whether cue memory or contextual memory is detected, the mice have to be in a fixed context during the training phase, and the context used for detecting contextual memory is exactly the same as that used during training; while the context used for detecting cue memory is completely different from that used during training. As per your suggestion, we have changed the abbreviation “CFC” to “FC (fear conditioning)” to avoid confusion since we did not measure contextual memory. Please kindly see the revised manuscript.

Make a schematic depicting the exact timing of the behavioral experiments (e.g. when shock happened, when cues were presented etc.)

Thank you very much for your valuable suggestions. We depicted in Fig. 1a when an electric shock occurs; and when a cue (sound) occurs. It is possible that some of the descriptions are not very clear, and we have made further refinements in the figure.

Please kindly see the revised Fig. 1a in the revised manuscript.

Figures:

Figure 1. In the 3 groups (veh, Px2, Px3) there are some mice that show very little freezing (under 20%), were these mice conditioned? In the vehicle group there is one

mouse that showed zero freezing. Could this be driving your effect somewhat? Is there a video to ensure they received shock?

We sincerely thank the reviewer for careful reading. The more the number of shocks, the stronger the fear memory of the mice. In order to avoid the ceiling effect of fear memory caused by too many shocks, which could not reflect the enhancement impact of propofol, we used the mode of one shock to make the fear memory of the mice mildly enhanced. This may result in a few mice that were not very intelligent not showing very strong fear memories, and even a case where the freezing ratio was 2.74%. Video recordings of our training sessions were kept to prove that each mouse received shock.

Figure 1b. it's a little distracting that some of the points on the graph are colored and some are black.

Thanks very much for your significant comment. We have changed all the points into black. Please kindly see the revised Fig. 1b in the revised manuscript.

What do you think changes (does cfos return to baseline?) at 30 min that could be the mechanism for the time course of action of propofol?

We sincerely thank you for the time and effort you spent reviewing our paper. As shown in Fig. 1c we respectively injected propofol 0 min (immediate), 30 min, 60 min and 90 min after fear training in mice, and the results showed that propofol injected



immediately or 30 min after fear training induced enhancement of fear memory in mice. So we chose to administrate propofol to the mice immediately after fear training in our experiments.

We chose to examine c-Fos expression in mice sacrificed 1h after fear training. Increased c-Fos expression in propofol-injected compared to vehicle-injected mice after fear training (as shown in Fig. 1d, e and f).

Fig 1g. It is unclear what is being depicted here? Is this during the FC session. Is CFC supposed to stand for contextual fear conditioning or cued?

We sincerely thank the reviewer for careful reading. This is the freezing level of mice during free exploration before shock in FC periods. And we have changed all the abbreviation “CFC” to “FC (fear conditioning)” to avoid confusion since we did not measure contextual memory. Please kindly see the revised manuscript.

Figure 4a is not detailed enough in terms of the experimental design / word behavior is misspelled / BLA font is very small as are the coordinates. Microscope images are also very small. Yellow is not the best color for bar graph.

Thank you very much for your valuable comment. We further refined the details of the experimental design in Fig. 4a and corrected the misspelling of behavior. At the same time, we zoomed in on the microscopic images and described the coordinates in detail in the figure legend. And we changed the yellow part of the bar graph to a dark

brown. Please kindly see the received Fig. 4 and line 738-739 in the revised manuscript.

Fig 4, what was done in vivo and what was done ex vivo? Make the timeline for this clear.

We sincerely thank the reviewer for careful reading. Only electrophysiology was recorded ex vivo, other procedures such as surgery, drug administration and behavioral tests were performed in vivo, and of course immunofluorescence and western blotting were performed after the mice were sacrificed. We further refined the details of the experimental design for the timeline. Please kindly see the received Fig. 4a in the revised manuscript.

In figures 5 and 6 how was behavior measured since the experiment seems to take place ex vivo and the way the results are presented, the behavior is presented last.

Thank you very much for your valuable suggestions. We may not have described the details clearly. Only electrophysiology was recorded ex vivo, other procedures such as surgery, drug administration and behavioral tests were performed in vivo. We further refined the details of the experimental design for the timeline in revised Fig. 5a and 6a. Please kindly see the revised manuscript.

Overall

The paper is very interesting, and the discussion is very good. The intro needs a bit of work to make the rationale clearer and the figures need some clarification as well.

We sincerely appreciate your time and effort in reviewing our paper. We have supplemented the intro section with your feedback and further clarified the images. The revisions in the revised manuscript are highlighted in red font, and your important comments have helped us greatly improve the quality of our work. Thank you again for your hard work!

Reviewer #3:

The authors showed that intraperitoneal administration of propofol within 30 min after CFC increases freezing during the retention test and the number of c-Fos+ cells in the BLA. Moreover, through ex-vivo electrophysiology they showed that propofol administration leads to increased sEPSC frequency and amplitude of glutamatergic but not GABAergic neurons in the BLA, an effect mediated by GABAA receptors. The effect of propofol in enhancing fear memory were attenuated by both optogenetic and chemogenetic inhibition of glutamatergic neurons and activation of GABAergic neurons in the BLA.

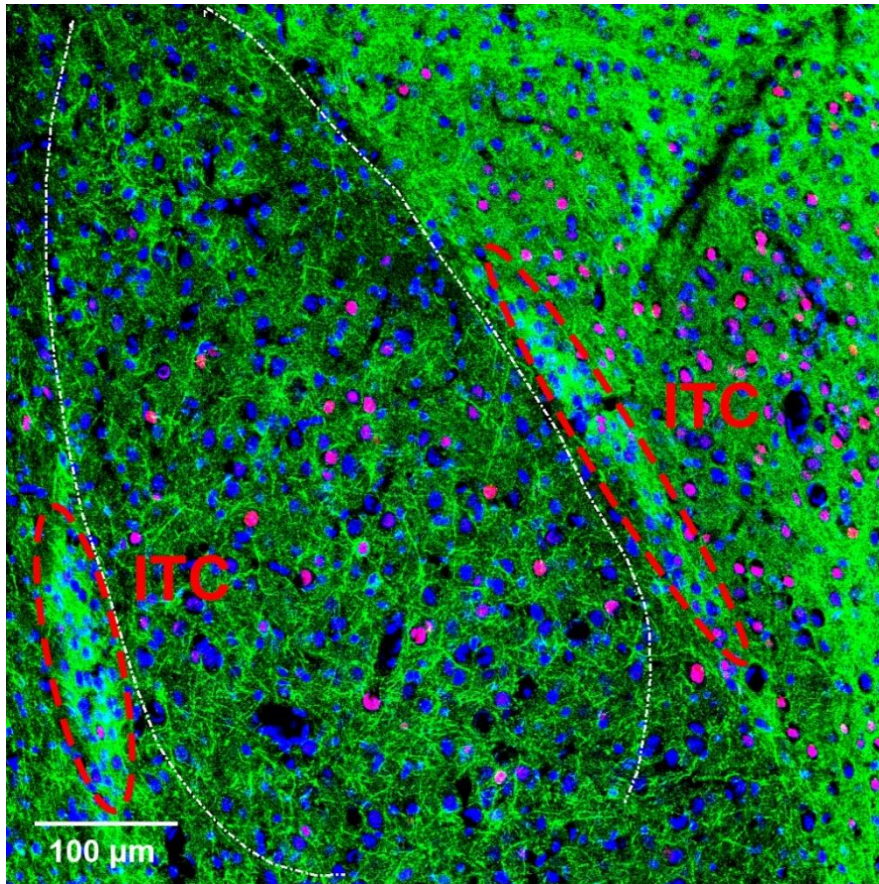
We sincerely thank you for the time and effort you spent reviewing our paper. Your significant comments helped us improve the quality of our work greatly. We have studied the comments carefully and tried to fix all the problems you mentioned. The

revisions are highlighted in red font in the revised manuscript and the responses to your comments are listed as follows.

Major concerns:

Figures of immunohistochemistry: it would be useful to see representative pictures also at a lower magnification, showing the localization of the target area.

Thank you very much for your valuable comment. We have zoomed in on the low magnification figures of immunohistochemistry in the figures to better show the localization of the target area. And in our immunofluorescence diagram, the dense layer of intercalated cells (ITC) can be clearly visualized, which can be considered as a signature structure to localize the BLA (as shown in the figure below). Please kindly see the revised Fig. 4b, 6b and 7b in the revised manuscript.



It would be useful to have a scheme of virus injection and optic fiber placements for all the animals.

Thank you very much for your valuable suggestions. We may not have described the details clearly. The red symbols in Fig. 4e, 6f and 7h are virus expression diagrams, and to avoid confusion we have changed the original word “mCherry” to “virus” for clarity. At the same time we have added the schematic diagram of optic fiber placement in Fig. 5b. Please kindly see the line 755-756, revised Fig. 4e, 5b, 6f and 7h in the revised manuscript.

Line 129: “with the resting potential not significantly changed”. However, fig. 3f reports two significance symbols, which are not explained in the caption. Could the authors explain what this significance refers to?

Thank you for your significant comment. We are very sorry for our incorrect writing. We have changed the sentence “with the resting potential not significantly changed” into “with the resting potential significantly elevated”. Please kindly see the line 115 in the revised manuscript.

Line 153-155, authentication of viral expression with electrophysiology during photoinhibition: it is not clear for how long the electrophysiological recordings have been conducted. Considering the evidence that prolonged photoinhibition results in a photoactivation, it is important that the electrophysiological recordings were conducted for the duration as the photoinhibition was performed (50 min). The authors should clarify this methodological point.

We sincerely thank the reviewer for careful reading. We are very sorry for our misexpression. The experimental strategy we used in the in vivo experiments with animals was light irradiation for 50 min. In the in vitro electrophysiological experiments, both direct photoactivation and photoinhibition as well as indirect photoinhibition due to photoactivation lasted not 50 min, but 0.5 s or 10 s of light exposure to validate the successful expression of the virus. We have added the sentence “Electrophysiological recordings were performed during the laser illumination of the BLA, and we demonstrated the recording of laser illumination for

0.5 s (Fig. 4c and 5d) and 10 s (Fig. 5f) to better visualize the impact of the optogenetic virus before and after light exposure (Fig. 4c)” to the manuscript. Please kindly see the line 140-143, revised Fig. 4c, 5d and 5f in the revised manuscript.

What is the rationale of repeating the inhibition/activation experiments in glutamatergic/GABAergic neurons using chemogenetics, after having already demonstrated an effect with optogenetics and having validated the functionality of the virus used? Those experiments don't seem to add any relevant information to what already shown.

Thank you very much for your valuable comment. We have controlled the laser energy to be around 4mW to minimize the heat-related damage during the optogenetic manipulation. However, this may lead to an attenuation of the laser illumination, which is generally believed to activate neurons 0.35mm-0.75mm away from the light source. The BLA is a long and narrow brain area, and it is possible that part of the brain area may not be able to receive light stimulation efficiently due to the attenuation. Chemogenetics could have avoided this situation so we chose to employ it to further validate the results of the optogenetic manipulation.

The methods section refers to “cue memory test” while fig 1 refers to “retention test”.

We sincerely thank the reviewer for careful reading. We are very sorry for our incorrect writing. We have changed the sentence “retention test” into “cue memory test”. Please kindly see the revised Fig. 1a in the revised manuscript.

Line 400: “The average number of positive cells per mouse was counted in three to four brain slices at various levels of the BLA”: could the authors give information about those various levels? Possibly referring to AP coordinates of the Mouse Brain Atlas.

We sincerely thank the reviewer for careful reading. Our criterion for selecting the slices was to take 3-4 levels at the front, center, and rear of the BLA for counting based on the AP coordinates. We have described this in detail with reference to your comment. Please kindly see the line 401 in the revised manuscript.

The fact that both photoinhibition and photoactivation decrease the number of c-fos<sup>+</sup> cells is intriguing: do the authors have an explanation for this result?

Thank you very much for your valuable comment. The BLA contains 80% glutamatergic neurons and 20% GABAergic neurons (mostly local circuit neurons). When the excitability of GABAergic neurons increases, it may inhibit the excitability of glutamatergic neurons by increasing the release of GABA; When the excitability of GABAergic neurons decreases, glutamatergic neurons may experience an increase in excitability due to the disinhibition of GABAergic neurons. In the experiments we photoactivated and inhibited two different types of neurons; photoactivation of GABAergic neurons and photoinhibition of glutamatergic neurons in the BLA.



As the BLA contains 80% glutaminergic neurons, the excitability of glutaminergic neurons decreased and consequently the number of c-Fos<sup>+</sup> cells decreased when the glutaminergic neurons were directly photoinhibited.

GABAergic neurons released a large amount of GABA when photoactivated, so the excitability of glutamatergic neurons was decreased. Since the BLA contains only 20% GABAergic neurons, the total number c-Fos<sup>+</sup> cells was reduced due to decreased excitability of glutamatergic neurons, which make up 80% of the BLA, despite the activation of GABAergic neurons.

So both photoinhibition of glutamatergic neurons and photoactivation of GABAergic neurons in the BLA lead to a reduction in the number of c-Fos<sup>+</sup> cells.

Line 246: the cited study by Morena et al. is not clinical research.

We sincerely thank the reviewer for careful reading. We are very sorry for our misexpression. We have removed the word “clinical”. Please kindly see the line 237 in the revised manuscript.

Line 295: the authors discuss the inconsistencies between their results and the literature on the effect of propofol in hippocampal-related memories, stating that the present study investigates fear memory which is amygdala-dependent. However, this explanation doesn't seem sufficient, because the paradigm used is a contextual fear conditioning, therefore a hippocampus-dependent task and also in light of BLA-hippocampus projection and its role in fear-related behaviors. In this context,

the author should also discuss the literature showing that propofol can accelerate/impair fear memory extinction.

We sincerely thank the reviewer for careful reading. As you suggested, we have added two literature articles showing that propofol can accelerate/impair fear memory extinction. As per your suggestion, since we were testing cue memory (Fig. 1a) rather than contextual memory, we have changed the abbreviation “CFC” to “FC (fear conditioning)” to avoid confusion since we did not measure contextual memory. Please kindly see the line 35-38 and line 234-242 in the revised manuscript.

From the present study it cannot be excluded that the effect on freezing is part of a general effect on locomotion. To strengthen the data, the authors should provide other readouts, such as immobility, darting and fear-related ultrasonic vocalizations (USVs).

Thank you very much for your valuable comment. Propofol is widely used in clinical practice as an anesthetic induction and maintenance drug with rapid onset, short duration of action, and few side effects. Research has shown that propofol anesthesia does not impair animals' mobility upon awakening; neither the distance nor the speed of movement in the Open field test of rats was altered after propofol injection (Li J, Wu G, Song W, Liu Y, Han Z, Shen Z, Li Y. Prophylactic Melatonin Treatment Ameliorated Propofol-Induced Cognitive Dysfunction in Aged Rats. *Neurotox Res.* 2021 Apr;39(2):227-239. doi: 10.1007/s12640-020-00307-9. Epub 2020 Nov 7. PMID: 33159663). Our previous paper also showed that there was no difference in the total time spent exploring new locations or new objects in the novel object recognition

test and object location test in mice before and after propofol anesthesia (Yang X, Chen C, Qu D, Liu Y, Wang N, Wang H, Fan Y, Zhou Y, Yu B, Xue Q, Wu Y, Lu H. Aberrant expression of FBXO22 is associated with propofol-induced synaptic plasticity and cognitive dysfunction in adult mice. *Front Aging Neurosci.* 2022 Nov 8;14:1028148. doi: 10.3389/fnagi.2022.1028148. PMID: 36425318; PMCID: PMC9680529). All these results indicate that propofol anesthesia does not affect the locomotion of the animals upon awakening.

Thank you again for your comments which are very helpful and valuable in improving our study. We will definitely follow your comments in future studies and further investigate immobility, darting and fear-related ultrasonic vocalizations (USVs) in depth to strengthen the data.

**General grammar and punctuation check.**

We sincerely thank the reviewer for careful reading. We have checked and revised punctuation, grammar and spelling throughout the text as required and are confident that the writing has been greatly improved and refined.

Dear reviewers,

Thank you again for offering us an opportunity to improve the quality of our submitted manuscript “Neuronal excitation-inhibition imbalance in the basolateral amygdala is involved in propofol-mediated enhancement of fear memory” (Manuscript Number: COMMSBIO-24-3421B). Those comments are all valuable and very helpful for revising and improving our paper, as well as providing important guidance for our research. We have studied the comments carefully and have made corrections, which we hope will meet with approval. Revised portions are marked in red on the paper. The main corrections in the paper and the response to the reviewers' comments point-by-point are as follows:

Reviewers' comments:

Reviewer #1 (Remarks to the Author):

I thank the authors for their careful effort in addressing the concerns raised during the review process. Overall, I believe that my concerns have been adequately addressed. However, I do believe there are several points that remain that would significantly improve the manuscript for readers.

Sincerely thank you for all the hard work you put into our manuscript. Your valuable comments have helped us improve the paper considerably. We have studied the comments carefully and tried to fix all the problems you mentioned. The revisions are

highlighted in red font in the revised manuscript and the responses to your comments are listed as follows.

1. The authors' reasoning for repeating the optogenetic experiments using chemogenetic strategies makes sense; however, I believe that any reader would question this choice. Therefore, I believe the authors should include their rationale for using chemogenetics in the appropriate sections.

Thank you very much for your valuable suggestions. We have added description "The BLA is a long and narrow brain area, it is difficult to uniformly regulate all the targeting neurons in BLA by the laser illumination during the optogenetic manipulation, whereas chemogenetics can compensate for this deficiency well." in the result section and labeled them in red. Please kindly see line 181-183 in the revised manuscript.

2. The discussion of the effects of propofol on long-term memory should be included in the discussion section as an example of a future direction/potential pitfall of this study. Additionally, I am still curious if the authors believe that changing the valence of the encoding experience might affect their experimental findings (this would also be appropriate to include in the discussion section).

Thank you very much for your significant comments. We have added description "Finally, the memory of mice was only evaluated 48 h after training by the fear conditioning test. The long-term memory of mice after training remains to be

investigated in the future studies to further elucidate the mechanisms by which propofol affects fear memory.” in the discussion section. In addition, the description “In the present study, the fear memory model of mice was established by one-footshock paradigm and it was found that propofol significantly enhanced fear memory. However, the effect of propofol on fear memory induced by more or stronger footshocks remains to be further explored.” was also added in the discussion section. Please kindly see line 329-334 in the revised manuscript.

Reviewer #2 (Remarks to the Author):

The paper is substantially improved

Sincerely thank you for all the hard work you put into our manuscript. Your valuable comments have helped us improve the paper considerably. We have studied the comments carefully and tried to fix all the problems you mentioned. The revisions are highlighted in red font in the revised manuscript and the responses to your comments are listed as follows.

Lines 236-239 after the author's name the year must go in parentheses

Thank you very much for your valuable suggestions. We have put the year after the author's name in parentheses. Please kindly see line 242 and line 244 in the revised manuscript.

Please include your responses to the first two comments in the "major comments" section in the paper

Thank you very much for your significant comments. As per the major comment 1, we have added description "To explore the effect of different doses of propofol on fear memory, mice received different number of injections of the same single dose of propofol. Multiple intraperitoneal injections of vehicle (vehicle×5) and different number of injections of 60 mg/kg propofol (propofol×2+vehicle×3, propofol×3+vehicle×2, propofol×4+vehicle×1, propofol×5, Libang Pharmaceutical Co., China) with 30 min intervals were administered at the end of FC training to maintain a desirable state of anesthesia." in the paper and labeled them in red. Please kindly see line 464-469 in the revised manuscript.

As per the major comment 2, we have added description "To determine whether the altered excitability of glutamatergic neurons induced by propofol is mediated through activation of GABAA receptors, we preperfused brain slices ex vivo with bicuculline (Bic, 30 μM) to competitively antagonize GABAA receptors prior to propofol perfusion," in the paper and labeled them in red. Please kindly see line 116-119 in the revised manuscript.

I am not sure the mice that were not fear conditioned should be included in the analyses

We sincerely thank the reviewer for careful reading. Actually, this revision of adding data from untrained mice was the response to another reviewer's comment, we have added immunofluorescence data from untrained mice to explore if propofol potentially alter neural dynamics of glutamatergic/GABAergic neurons in the BLA in the absence of training. We included mice that were not fear conditioned in our analyses to explore whether the fear memory-enhancing effect by propofol were only effective in mice that underwent fear conditioning training.

Reviewer #3 (Remarks to the Author):

The authors have addressed my major concerns. However, I still have some minor points that need to be addressed.

Sincerely thank you for all the hard work you put into our manuscript. Your valuable comments have helped us improve the paper considerably. We have studied the comments carefully and tried to fix all the problems you mentioned. The revisions are highlighted in red font in the revised manuscript and the responses to your comments are listed as follows.

- fig 6B: AP, ML, DV coordinates are missing (while present in the other figures);



We sincerely thank you for the time and effort you spent reviewing our paper. We have added AP, ML, and DV coordinates in Figure 6b. Please kindly see the revised Fig. 6b in the revised manuscript.

- " It would be useful to have a scheme of virus injection and optic fiber placements for all the animals.": the schematic diagram in fig 5b mentioned by the author don't provide a full overview of where the fiber was placed in each animal. To make myself clearer on what I meant with "scheme of virus injection for all the animals" please see attached image;

Thank you very much for your valuable suggestions. Referring to the image you provided, we have added a scheme of virus injection and optic fiber placement in Figure 4b. Please kindly see the revised Fig. 4b and line 751 in the revised manuscript.

- Line 153-155 (lines 140-143 of revised manuscript): could the authors make it explicit how they chose the laser illumination times of 0.5 and 10 s to validate the virus?

Thank you very much for your significant comments. In the present study, the duration of the light stimulus was selected based on whether the cell receiving the light stimulus was the same as the cell receiving the recording. If both stimulation and recording were performed in the same cell, light stimulation of 0.5 seconds was selected. If GABAergic neurons were stimulated while the excitability was recorded

in glutamatergic neurons, light stimulation of 10 seconds was selected to ensure that the released GABA neurotransmitter would have enough time to modulate the activity of the glutamatergic neurons. Actually, this stimulation time was supported by the previous study of Wang D et al. (Wang D, Wu J, Liu P, Li X, Li J, He M, Li A. VIP interneurons regulate olfactory bulb output and contribute to odor detection and discrimination. *Cell Rep.* 2022 Feb 15; 38(7): 110383. doi: 10.1016/j.celrep.2022.110383. PMID: 35172159.). We have added this in the results section and labeled them in red. Please kindly see line 141-146 in the revised manuscript.

- Line 400: "at the front, center and rear of the BLA" is a bit vague. Could the authors add the exact three AP coordinates?

We sincerely thank you for the time and effort you spent reviewing our paper. We've added three exact AP coordinates (AP: -0.9 mm, -1.25 mm and -1.6 mm) in the paper. Please kindly see line 412 in the revised manuscript.

Dear reviewers,

Thank you again for offering us an opportunity to improve the quality of our submitted manuscript “Neuronal excitation-inhibition imbalance in the basolateral amygdala is involved in propofol-mediated enhancement of fear memory” (Manuscript Number: COMMSBIO-24-3421B). Those comments are all valuable and very helpful for revising and improving our paper, as well as providing important guidance for our research. We have studied the comments carefully and have made corrections, which we hope will meet with approval. The main corrections in the paper and the response to the reviewers' comments point-by-point are as follows:

Reviewers' comments:

Reviewer #1 (Remarks to the Author):

I thank the authors for making the suggested changes to the manuscript. The manuscript is much improved, and I have no further concerns or edits at this time.

We sincerely thank you for the time and effort you spent reviewing our paper. Your valuable comments have helped us improve the paper considerably.

Reviewer #2 (Remarks to the Author):

For the mice that were not freezing I was referring to figure 1b - vehicle group

Thank you very much for your valuable suggestions. I apologize for not fully understanding the question you asked earlier. There was indeed one mouse with a low level of freezing percentage (2.74%) in the vehicle group of figure 1b. The reason we did not delete this data is that although the freezing percentage of the mouse was low but not zero, the occurrence of this low level could be due to individual differences. In addition, keeping this data did not affect the final results of the experiment, so we decided to retain this data.

Reviewer #3 (Remarks to the Author):

The authors have addressed all the issues and the paper is ready for publication

Sincerely thank you for all the hard work you put into our manuscript. Your valuable comments have helped us improve the paper considerably.

Bregma  
(in mm)

Fibre placement

Viral spread

-5.6

-5.8

-6.0

