progress in neurobiology

Peer Review Overview

Manuscript Title: "IL-10 normalizes aberrant amygdala GABA transmission and reverses anxiety-like behavior and dependence-induced escalation of alcohol intake"

Received	17-Apr-2020
1st Decision	12-Jun-2020
Revision Submitted	13-Sep-2020
Accepted	06-Nov-2020

Decision Letter

Dear Dr Roberto,

Thank you for submitting your manuscript to Progress in Neurobiology.

We have completed our evaluation of your manuscript. The reviewers recommend reconsideration of your manuscript following major revision. We invite you to resubmit your manuscript after addressing the comments below. Please resubmit your revised manuscript by Aug 11, 2020.

When revising your manuscript, please consider all issues mentioned in the reviewers' comments carefully: please outline every change made in response to their comments and provide suitable rebuttals for any comments not addressed. Please note that your revised submission may need to be re-reviewed.

Progress in Neurobiology values your contribution and we look forward to receiving your revised manuscript.

Kind regards,

Kimberley Raab-Graham
Associate Editor

Sabine Kastner
Editor-in-Chief
Progress in Neurobiology

Editor and Reviewer comments:

Reviewer 1

This is an interesting manuscript that sought to investigate the role of the anti-inflammatory neuroimmune mediator IL-10 in behavioral and neural phenotypes engendered by a mouse model of alcohol use disorder (AUD). Extensive prior work by these investigators has shown that increased GABAergic transmission in the central amygdala (CeA) plays a causal role in some of the behaviors associated with this AUD model. Here, the authors discovered that,

although chronic alcohol exposure increases the number of IL-10 expressing microglia across the brain, it leads to a reduction in IL-10 levels in the CeA. Using an elegant, multidisciplinary approach, the authors go on to demonstrate that over-expression of CeA IL-10 reduces anxiety-like behaviors and prevents chronic alcohol-mediated escalations in alcohol drinking. They also demonstrate that acute IL-10 treatment significantly reduces CeA GABAergic transmission and normalizes the enhancement of inhibitory transmission observed following chronic alcohol exposure.

Overall, this is a well-written, multidisciplinary study and the findings provide strong evidence that IL-10 signaling may play a causal role in some of the maladaptive, behavioral phenotypes that manifest in a well-established mouse model of AUD. I do not have any major concerns with this manuscript. There are, however, several minor issues that need further clarification.

Minor Concerns:

1. A major finding in this paper is that, while chronic alcohol increases IL-10 cell numbers throughout the brain, IL-10 protein is lower in the CeA. Did the authors also observe a decrease in IL-10 cell number in this brain region? If not, did they look at IL-10 levels in any other brain regions? It may be that chronic alcohol increases the number of cells that produce IL-10 throughout the brain but compromises their ability to express this anti-inflammatory cytokine.
2. The authors found that acute IL-10 reduces CeA GABAergic transmission in naïve mice, an effect opposite to that observed with acute alcohol exposure. However, they found that overexpression of IL-10 is anxiolytic. Given that acute alcohol exposure is also anxiolytic, this finding warrants some additional discussion. It would have been informative to conduct some electrophysiological studies in the IL-10 overexpressing mice. However, if this was not done, the authors may want to briefly discuss what they would expect the status of CeA GABAergic transmission would be in these mice.
3. The authors found that acute IL-10 could either enhance or inhibit CeA GABAergic synapses and firing frequency, depending on the alcohol history of the animals. The authors should provide some discussion of what might account for these heterogeneous effects.
4. The authors note that the concentration of IL-10 used in the electrophysiological studies was based on prior studies. However, can they indicate if this is a physiologically relevant concentration of this cytokine? In a related concern, did the authors demonstrate that the viral strategy used to over-express IL-10 actually increased its levels in the CeA?
5. Finally, there appears to be a typo in the legend for Fig 4B. It indicates a concentration of IL-10 (50 ng/mL) yet these experiments involved over-expression of this cytokine.

Reviewer 2

Summary: The manuscript "IL-10 normalizes aberrant amygdala GABA transmission and reverses anxiety-like behavior and dependence-induced escalation of alcohol intake" by Patel et al. demonstrated a critical role of the anti-inflammatory cytokine IL-10 in alcohol use disorder pathology. The authors used a well-characterized model of alcohol dependence, chronic intermittent ethanol vapor exposure (CIE) in addition to two-bottle choice alcohol drinking (2BC). Their data revealed a whole brain upregulation and a central amygdala (CeA) specific downregulation in IL-10 expression. Alcohol dependence further disrupted IL-10 modulation of GABAergic neurotransmission in the CeA. Finally, the authors showed that overexpression of IL-10 could rescue the elevation in alcohol drinking following CIE

exposure. Overall, this is a well-designed and well-written manuscript that will further our understanding of the neuroimmune response in the alcohol dependence. I have a few major comments related to the discussion and interpretation of the data. I believe that text revisions are sufficient.

Major Comments:

1. A major concern for this manuscript is the lack of female animals. There are well known sexual dimorphism in alcohol-induced behavioral adaptations, neuroplasticity and neuroimmune responses. With the emergence of the COVID epidemic, I recognize it is unreasonable to request experiments in females at this time. However, this should be mentioned in the discussion.
2. The introduction should be expanded to include information on differences between medial and lateral CeA, as well as the known microcircuitry of the medial CeA. Given the discussion includes a mention of anti-inflammatories and alcohol, this should mentioned in the introduction as well.
3. There needs to be a greater discussion of the biphasic effects seen. Previous work from the Roberto lab showed CRF+ neurons and non-expressing (CRF1-) neurons in the CeA have divergent adaptations in GABAergic transmission following CIE. These two population also have distinct membrane properties (e.g., capacitance, time constant tau). The authors could examine whether CeA neurons in dependent mice that showed increased mIPSC frequency could have different membrane properties from neurons that showed decreased mIPSC frequency. This potentially could indicate that a circuit and cell-type specificity of IL-10 signaling post-CIE. If this data is available, it should be mentioned. If not, a discussion is warranted.
4. The authors mentioned null PFC data. I would encourage the authors to include it in the paper if it does not otherwise have publication plans, but this is certainly not necessary and up to the authors.
5. The authors state that "IL10 does not alter general drinking behavior…" this conclusion needs to be tampered down to be specific to this model.
6. The effect of CIE on IL-10 modulation of miniature GABAergic neurotransmission could be via a IL-10 receptor signaling cascade mechanism or a circuit mechanism, the latter of which is supported by the sIPSC and firing data. Further investigation into the molecular and circuit mechanism of this disruption in IL-10 modulation could help explain the behavior adaptations. This should be elaborated in the discussion.
7. It seems odd to use a one-sample t test to examine the mIPSC response following IL-10 bath application in 'increase' and 'decrease' categories. Given that the results went both directions, it seems improbable that the authors a-priori hypothesized this. These stats should be corrected.
8. Representative images for punches, injections, etc. should be included if available. Hit rate (% missed injections) should be included. For figure 3, the injection looks to be largely in the BLA - is this true for all of the data? If so, the paper may need large revisions to separate BLA and CEA effects.

Minor comments:

9. The language regarding cannulation is confusing. Were cannulas actually used, or just a syringe?
10. Behavior - be explicit - was video tracking software (i.e. ethovision) used or hand scored?

11. Figure 1B: bars should be darkened, not just outlined. They are a little hard to see.
12. Figure 2: Was the marker of activated microglia Iba-1 examined in non-dependent and dependent mice?
13. Figure 3,5,6: The BEC and alcohol intake of non-dependent and dependent mice should be reported if available.
14. mIPSC baseline frequency and amplitude in naïve, non-dependent and dependent mice should be reported.
15. Figure 7: the authors labeled the groups as 'dependent' vs 'non-dependent' in the other graphs, yet "AIR" vs "VAPOR" in this. The labels should be kept consistent.

Author Response Letter

Reviewer 1

This is an interesting manuscript that sought to investigate the role of the anti-inflammatory neuroimmune mediator IL-10 in behavioral and neural phenotypes engendered by a mouse model of alcohol use disorder (AUD). Extensive prior work by these investigators has shown that increased GABAergic transmission in the central amygdala (CeA) plays a causal role in some of the behaviors associated with this AUD model. Here, the authors discovered that, although chronic alcohol exposure increases the number of IL-10 expressing microglia across the brain, it leads to a reduction in IL-10 levels in the CeA. Using an elegant, multidisciplinary approach, the authors go on to demonstrate that over-expression of CeA IL-10 reduces anxiety-like behaviors and prevents chronic alcohol-mediated escalations in alcohol drinking. They also demonstrate that acute IL-10 treatment significantly reduces CeA GABAergic transmission and normalizes the enhancement of inhibitory transmission observed following chronic alcohol exposure.

Overall, this is a well-written, multidisciplinary study and the findings provide strong evidence that IL-10 signaling may play a causal role in some of the maladaptive, behavioral phenotypes that manifest in a well-established mouse model of AUD. I do not have any major concerns with this manuscript. There are, however, several minor issues that need further clarification.

Response: We thank the reviewer for the positive and enthusiastic evaluation of our manuscript and the appreciation of its multidisciplinary approach. We have addressed the minor concerns and significantly strengthened the manuscript.

Minor Concerns:

1. *A major finding in this paper is that, while chronic alcohol increases IL-10 cell numbers throughout the brain, IL-10 protein is lower in the CeA. Did the authors also observe a decrease in IL-10 cell number in this brain region? If not, did they look at IL-10 levels in any other brain regions? It may be that chronic alcohol increases the number of cells that produce IL-10 throughout the brain but compromises their ability to express this anti-inflammatory cytokine.*

Response: The reviewer raised important points. Indeed, based on accumulating evidence from our lab, we hypothesize that though there is an increase in IL-10-producing cells, the overall expression level of IL-10 is compromised by chronic alcohol. We previously found that chronic alcohol exposure trends toward an increase in CeA microglia, which likely include IL-10-producing cells (see Figure 1 below from Warden et al., 2020, Biological

Psychiatry). Indeed, in this study we found that ~55% of microglia in the brain produce IL-10, and this was unchanged by dependence.

In addition, we found that even though there is a significant increase in the number of microglia in the mPFC (see Figure 2 below from Warden et al., 2020, Biological Psychiatry), we found no change in IL-10 levels measured in the mPFC (see below Fig. 3, unpublished data for an ongoing study).

In this study, due to the comparatively low numbers of immune cells in the brain and limitations of multiparameter flow cytometry, we were not able to achieve amygdala-level granularity in our analysis of IL-10-producing cells. Thus, we attempted to use in situ hybridization for quantification of IL-10 expressing neurons, microglia, and astrocytes. Unfortunately, we experienced that IL-10 in situ hybridization probes were not optimal and expression was unclear and limited.

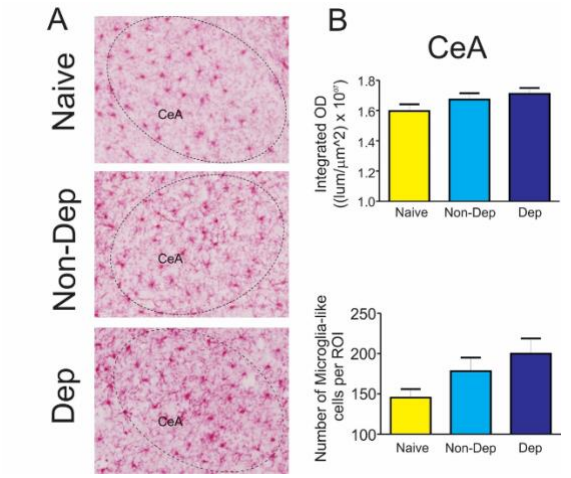

Figure 1. CeA microglia data published in Warden et al., 2020

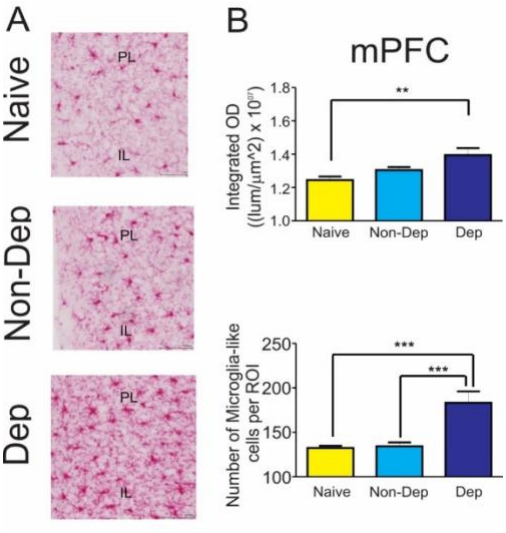

Figure 2. mPFC microglia data published in Warden et al., 2020

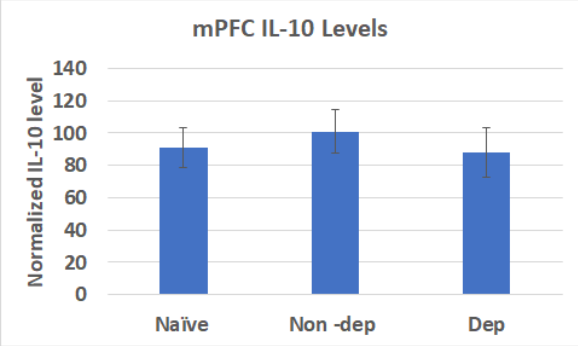

Figure 3. mPFC IL-10 levels in naïve, non-dependent, and dependent mice (n=9-10).

2. *The authors found that acute IL-10 reduces CeA GABAergic transmission in naïve mice, an effect opposite to that observed with acute alcohol exposure. However, they found that overexpression of IL-10 is anxiolytic. Given that acute alcohol exposure is also anxiolytic, this finding warrants some additional discussion. It would have been informative to conduct some electrophysiological studies in the IL-10 overexpressing mice. However, if this was not done, the authors may want to briefly discuss what they would expect the status of CeA GABAergic transmission would be in these mice.*

Response: This is an important point to note, and we have added a brief discussion of this to page 26. We did not test the effect of acute ethanol on CeA GABAergic transmission in IL-10 overexpressing mice. However, we did examine the interaction of acute alcohol and IL-10 on CeA GABAergic transmission. We found that IL-10 decreases GABA transmission in the presence of acute ethanol, and acute ethanol increases GABA transmission in the presence of IL-10 (see below Fig. 4). Therefore, the effects of ethanol and IL-10 do not occlude each other, suggesting their effects are mediated by distinct mechanisms. Indeed, IL-10 canonically signals through PI3K and p38 MAPK mediating its effect on CeA GABA transmission (see Figure 5C of this manuscript; Patel et al.), while we have previously shown that ethanol works through PKA and PKC to increase CeA GABA transmission (1, 2). Together, it is possible that IL-10 and ethanol are working through distinct mechanisms to decrease anxiety-like behavior.

Importantly, under more physiological conditions of intact network activity, we found that IL-10 both increases and decreases sIPSCs and firing in subsets of CeA neurons. It is possible that IL-10 increases sIPSCs, similar to acute ethanol, on the subset of CeA neurons critically regulating anxiety-like behavior. Future studies will clarify this possibility.

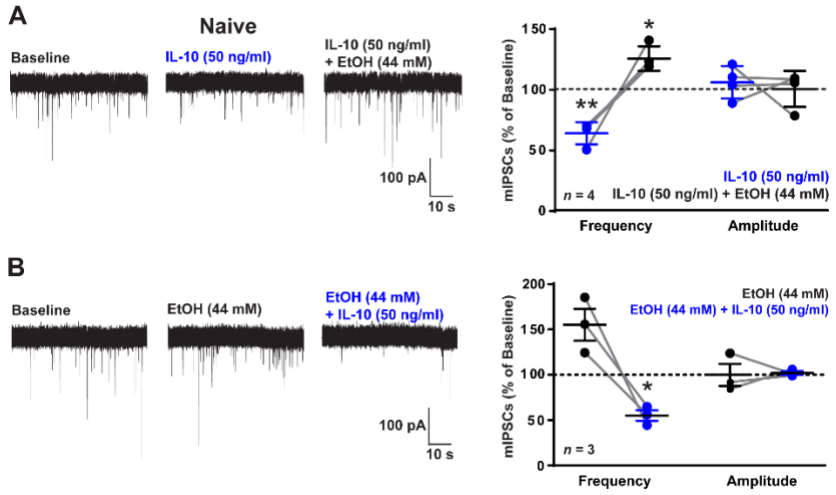

Figure 4. IL-10 and acute ethanol interaction effects on CeA GABA transmission.

3. *The authors found that acute IL-10 could either enhance or inhibit CeA GABAergic synapses and firing frequency, depending on the alcohol history of the animals. The authors should provide some discussion of what might account for these heterogeneous effects.*

Response: We thank the reviewer for this suggestion, and we have added additional commentary about these heterogeneous effects to the discussion on page 25. The CeA contains heterogeneous populations of neurons with intricate microcircuitry, and IL-10 receptors are expressed on a subset of neurons, astrocytes, and microglia, both of which may contribute to the diversity of responses to IL-10.

4. *The authors note that the concentration of IL-10 used in the electrophysiological studies was based on prior studies. However, can they indicate if this is a physiologically relevant concentration of this cytokine? In a related concern, did the authors demonstrate that the viral strategy used to over-express IL-10 actually increased its levels in the CeA?*

Response: We thank the reviewer for this comment. Serum levels of IL-10 are typically in the 10-200 pg/ml range (3, 4). However, to achieve sufficient perfusion of IL-10 into thick (300µM) slices for ex vivo electrophysiology, it is necessary to increase drug concentrations. Based on recent studies (3), we chose a concentration of 50ng/ml because it achieves a maximal effect on GABAergic transmission. Moreover, it is likely that the effective concentrations of IL-10 at local release sites is much greater than serum levels of IL-10, although the effective local concentrations remain unknown. Importantly, we do not suspect off-target effects of IL-10, as IL-10 receptor inhibition (using IL-10Ra neutralizing antibody) abolished IL-10's effect on mIPSCs (see Figure 5C of the main manuscript). We have added a brief statement about the concentration of IL-10 used in this study to the methods section 'Slice preparation and electrophysiological recordings' on page 8.

Regarding the level of viral-mediated IL-10 overexpression, we have added new data on the levels of CeA IL-10 overexpression achieved in control versus IL-10 overexpressing mice in the results section 'Amygdala IL-10 overexpression decreases anxiety-like behavior.' on page 15.

5. *Finally, there appears to be a typo in the legend for Fig 4B. It indicates a concentration of IL-10 (50 ng/mL) yet these experiments involved over-expression of this cytokine.*

Response: We thank the reviewer for catching this error, and we have fixed it.

Reviewer 2

Summary: The manuscript "IL-10 normalizes aberrant amygdala GABA transmission and reverses anxiety-like behavior and dependence-induced escalation of alcohol intake" by Patel et al. demonstrated a critical role of the anti-inflammatory cytokine IL-10 in alcohol use disorder pathology. The authors used a well-characterized model of alcohol dependence, chronic intermittent ethanol vapor exposure (CIE) in addition to two-bottle choice alcohol drinking (2BC). Their data revealed a whole brain upregulation and a central amygdala (CeA) specific downregulation in IL-10 expression. Alcohol dependence further disrupted IL-10 modulation of GABAergic neurotransmission in the CeA. Finally, the authors showed that overexpression of IL-10 could rescue the elevation in alcohol drinking following CIE exposure. Overall, this is a well-designed and well-written manuscript that will further our understanding of the neuroimmune response in the alcohol dependence. I have a few major

comments related to the discussion and interpretation of the data. I believe that text revisions are sufficient.

Response: We thank the reviewer for the positive and enthusiastic evaluation of our manuscript. We appreciate her/his suggestions to address the comments raised by text revisions, and we believe that the edits significantly strengthened the manuscript.

Major Comments

1. *A major concern for this manuscript is the lack of female animals. There are well known sexual dimorphism in alcohol-induced behavioral adaptations, neuroplasticity and neuroimmune responses. With the emergence of the COVID epidemic, I recognize it is unreasonable to request experiments in females at this time. However, this should be mentioned in the discussion.*

Response: We thank the reviewer for raising this point and for being flexible and reasonable in asking to discuss this limitation. We strongly agree that similar studies in females could be very informative about potential sex differences. While we have not yet completed a full parallel study of the role of CeA IL-10 in anxiety and alcohol dependence in females, we did find that IL-10 similarly reduces CeA GABAergic transmission in female mice. We therefore predict that IL-10 may similarly reduce anxiety-like behavior and dependence-induced alcohol drinking, although this remains to be directly tested. We have added this data as a supplementary figure (see Suppl. Figure 2) and mention this in results and discussion.

2. *The introduction should be expanded to include information on differences between medial and lateral CeA, as well as the known microcircuitry of the medial CeA. Given the discussion includes a mention of anti-inflammatories and alcohol, this should be mentioned in the introduction as well.*

Response: We thank the reviewer for this comment and have now added this information to the introduction.

3. *There needs to be a greater discussion of the biphasic effects seen. Previous work from the Roberto lab showed CRF+ neurons and non-expressing (CRF1-) neurons in the CeA have divergent adaptations in GABAergic transmission following CIE. These two population also have distinct membrane properties (e.g., capacitance, time constant tau). The authors could examine whether CeA neurons in dependent mice that showed increased mIPSC frequency could have different membrane properties from neurons that showed decreased mIPSC frequency. This potentially could indicate a circuit and cell-type specificity of IL-10 signaling post-CIE. If this data is available, it should be mentioned. If not, a discussion is warranted.*

Response: We thank the reviewer for this excellent suggestion. As suggested we have performed this analysis. We did not find any correlation between membrane properties or baseline GABA transmission and the directional effects of IL-10 on GABAergic transmission or firing (see Fig 5 below). We now explicitly state this in the discussion on page 25.

Given the diversity of neuronal subpopulations in the CeA, discrete microcircuitry, dense expression of neuromodulator systems, and expression of IL-10 receptors across a subset of CeA neurons, astrocytes, and microglia, it is not surprising that IL-10 has diverse effects on GABA transmission and spontaneous firing. We have not added more discussion of this point on page 25 of the discussion.

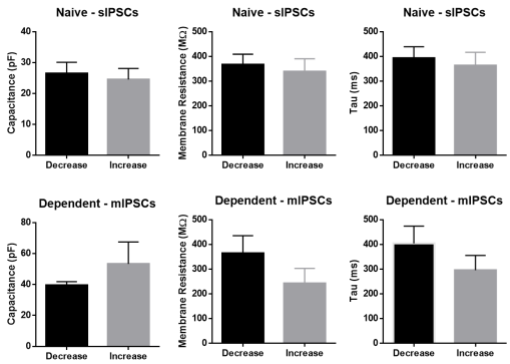

Figure 5. Membrane properties of recorded cells split by directionality of IL-10 effects.

4. *The authors mentioned null PFC data. I would encourage the authors to include it in the paper if it does not otherwise have publication plans, but this is certainly not necessary and up to the authors.*

Response: We thank the reviewer for this suggestion. We have now included the null mPFC data as well as in situ hybridization data showing IL-10R expression in the mPFC into Supplementary Figure 2.

5. *The authors state that "IL10 does not alter general drinking behavior…" this conclusion needs to be tampered down to be specific to this model.*

Response: We apologize for this overstatement and have now tampered our conclusion.

6. *The effect of CIE on IL-10 modulation of miniature GABAergic neurotransmission could be via a IL-10 receptor signaling cascade mechanism or a circuit mechanism, the latter of which is supported by the sIPSC and firing data. Further investigation into the molecular and circuit mechanism of this disruption in IL-10 modulation could help explain the behavior adaptations. This should be elaborated in the discussion.*

Response: We agree with the reviewer that both adaptations in signaling and circuit level effects of IL-10 simultaneously shape activity in the CeA that underlie behavior adaptations, and as suggested we have expanded the discussion of this point.

Please note that for measurements of miniature GABAergic transmission, the sodium channel blocker, tetrodotoxin, is included in the ACSF to block network activity (i.e. action potential firing). Therefore, circuit level effects do not influence IL-10 modulation of miniature GABAergic transmission.

7. *It seems odd to use a one-sample t test to examine the mIPSC response following IL-10 bath application in 'increase' and 'decrease' categories. Given that the results went both directions, it seems improbable that the authors a-priori hypothesized this. These stats should be corrected.*

Response: We did not make any a-priori assumptions about any of the data in this study, all statistical tests were two-tailed, which we have now explicitly stated in the methods section on page 8. In the case of a bimodal distribution in the electrophysiological data, where both increases and decreases are observed, we describe the data separately based on a criteria of greater or less than 15% change compared to baseline control. We then use a one sample t-test to test whether the change observed is significantly different from the null hypothesis set to 100 (i.e. no different than baseline). We use chi-squared to test whether

there is a difference in the overall distribution of responses across groups, and one-way Anova to test whether the magnitude of the changes is different across groups.

8. *Representative images for punches, injections, etc. should be included if available. Hit rate (% missed injections) should be included. For figure 3, the injection looks to be largely in the BLA - is this true for all of the data? If so, the paper may need large revisions to separate BLA and CEA effects.*

Response: We thank the reviewer for this suggestion, we have now added a Supplementary Figure 1 depicting injection sites as well as description of mice number to the following methods sections on 'Chronic-intermittent ethanol two-bottle choice paradigm', 'Viral injections in the brain', and 'Behavioral testing'.

While the majority of injection sites centered in the CeA, some mice injections also extended to the BLA and spread into the CeA. Of note, we have added additional data to the results section 'Amygdala IL-10 overexpression decreases anxiety-like behavior.' on page 15, showing measurements of viral-mediated CeA (from 1mm CeA tissue punches) IL-10 overexpression compared to control mice. 6 out of 71 mice were excluded from behavioral analysis from injections that spread outside of the amygdala.

Our ex vivo electrophysiological evidence (specifically IL-10-induced decreased spontaneous CeA GABA transmission and spontaneous firing in dependent mice) motivated the behavioral experiments, as previous studies suggest dampening CeA overactivation regulates dependence-induced drinking (5-7). Coronal CeA brain slices also contain the BLA. Consequently, when measuring IL-10's effects on CeA spontaneous GABA transmission and firing using ex vivo electrophysiology, we cannot rule out the potential contribution of bath application of IL-10's effect in the BLA and its contribution to the observed effects in the CeA. Indeed, the BLA and medial subdivision of the CeA (CeM), where recordings were obtained, are interconnected through several circuits including: lateral amygdala (LA)→lateral subdivision of CeA (CeL)→CeM, LA→intercaleted cells (ITCs)→CeL→CeM, BLA→ITCs→CeL→CeM, BLA→CeM (8, 9). For this reason, we believe it is appropriate to include all mice that demonstrated overexpression of IL-10 that spread across the BLA and CeA. For full transparency, we chose a 'representative image' to include spread across both the BLA and CeA regions. We now have included a statement about our rationale to the methods section 'Viral injections in the brain' on page 9. In addition, we were intentional in stating overexpression of IL-10 in the 'amygdala' rather than 'CeA' throughout the text. We hope the reviewer find our rationale and response satisfactory.

We agree with the reviewer that teasing apart the specific contribution of distinct amygdala subregions and circuits to the effects of IL-10 on regulating anxiety-like behavior and dependence-induced alcohol drinking will be important. Based on the methodological approach used in this study, the logical interpretation of our data is that the effects of IL-10 arise from action of this cytokine on amygdala circuitry that lead to an overall decrease in amygdala output through the CeM, which is the major output nucleus of the amygdala complex (10, 11). Nonetheless, these findings highlight a previously unknown role of IL-10 in regulating neuronal activity and behavior.

Minor comments:

9. *The language regarding cannulation is confusing. Were cannulas actually used, or just a syringe?*

Response: We apologize for the lack of clarity in our methods. Cannulation was not used in these studies. We virally overexpressed IL-10 in the amygdala, and viral injection surgeries were done prior to the mice undergoing 2BC-CIE. We have clarified this point in the methods.

10. *Behavior - be explicit - was video tracking software (i.e. ethovision) used or hand scored?*

Response: We thank the reviewer for this suggestion and have added the exact methods for analysis of behavior.

11. *Figure 1B: bars should be darkened, not just outlined. They are a little hard to see.*

Response: We thank the reviewer for pointing this out. For better visibility, we filled and separated the bars from the scattered individual data points, which now have transparency for better visibility of overlapping points.

12. *Figure 2: Was the marker of activated microglia Iba-1 examined in non-dependent and dependent mice?*

Response: Iba-1 was not used in multiparameter flow cytometry experiments. We used CD45dim and CD11b+ expression to gate for microglia (12). We have added this to the results section 'Chronic alcohol exposure alters the brain immune cell landscape' on page 9.

13. *Figure 3,5,6: The BEC and alcohol intake of non-dependent and dependent mice should be reported if available.*

Response: Figure 1 displays the BEC and alcohol intake of all non-dependent and dependent mice used in the experiments in this study, except for Figs 2 and 7.

14. *mIPSC baseline frequency and amplitude in naïve, non-dependent and dependent mice should be reported.*

Response: We thank the reviewer for this suggestion. We did not find any significant differences in baseline mIPSC frequency, amplitude, rise time, or decay time between naïve, non-dependent and dependent mice. We have now included this data to Supplementary Table 4.

15. *Figure 7: the authors labeled the groups as 'dependent' vs 'non-dependent' in the other graphs, yet "AIR" vs "VAPOR" in this. The labels should be kept consistent.*

Response: We thank the reviewer for catching this, and we have corrected it.

References

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