nature portfolio

Peer Review File

Stress dynamically modulates neuronal autophagy to gate depression onset

Corresponding Author: Dr Yihui Cui

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

Version 1:

Reviewer comments:

Referee #1

(Remarks to the Author)
In this study, Yang et al. focused on how chronic stress changes the autophagic function in the Lateral Habenula (LHb), which may underlie the pathological mechanism of developing depressive phenotypes. They have performed comprehensive experiments and discovered that chronic stress induced robust change in autophagy signaling only in LHb, which was not observed in other areas of the brain. Treatments of well-known antidepressants also changed the autophagic activity specifically in LHb, which hints the possible role of LHb autophagy as a general mechanism for the treatment of depression.

By targeting LHb autophagy using tet-Beclin-1 peptide, the authors showed a dramatic improvement of both depressive behaviors and correcting hyper-activity of both neuronal and synaptic properties in LHb. These are very promising results that may provide a new therapeutic strategy for treating patients suffering from major depressive disorder. With the following loss-of-function studies using both selective knockdown and knockout of autophagy-related-gene (Atg7), they concluded that autophagy dysfunction in LHb is lowering the endocytosis of membrane receptors such as synaptic GluR, which likely leads to an impaired neuronal and synaptic homeostasis in LHb.

Despite the previous literature suggesting the role of LHb in depression and proposed theories of the possible role of autophagy in depression, this is a proof-of-concept study to show that autophagy in specific area of the brain may play a critical role in developing depressive phenotypes. This study also suggests targeting area-specific autophagy as potential treatment that is a novel approach in the field of depression and stress research studies. They conducted extensive work to screen the multiple areas of the brain and thoroughly investigated the changes in behavior, molecular and physiological function followed by targeting LHb autophagy function with chronic stress behavioral paradigm. They have provided solid experimental data that supports the rationale of targeting LHb autophagy as a new therapeutic target.

However, I have serious concerns regarding their conclusions along with their proposed mechanism. Authors confidently conclude that autophagy-dependent habenular homeostasis gates depression onset. From their presented data, it is convincing that autophagy regulates habenular homeostasis. They also showed that the artificial suppression of habenular autophagy results in abnormal behaviors that resemble depression-like behaviors. However, my concern is that this does not necessarily mean whether autophagy causally regulates these behavioral outputs through habenular homeostasis. Their current data are not sufficient and do not provide the proof of causality in which autophagy regulates depressive behavior that requires habenular homeostasis.

In addition, they conclusively propose the mechanism in which autophagy-dependent-GluRs endocytosis/degradation underlies habenular homeostasis and depressive behaviors. The authors previously reported the other mechanisms underlying hyperexcitability of LHb neurons (Yang et al., 2018 and Cui et al., 2018). Considering that the current mechanism is synaptic and previous two are more of intrinsic properties of neuron, there is a discrepancy between the currently proposed mechanism and the other mechanisms (Yang et al., 2018 and Cui et al., 2018) that underlie habenular homeostasis in LHb. How does autophagy- dependent endocytosis of synaptic GluRs interact with other reported

mechanisms of neuronal homeostasis (neuronal hyperexcitability) in LHb? Does autophagy correct/regulate habenular homeostasis (synaptic to neuronal) through one common pathway? Is autophagy-dependent endocytosis of synaptic GluRs the upstream of every other reported mechanism underlying habenular homeostasis (neuronal/synaptic)? My worry is that this study lacks experimental data nor provides strong evidence to explain whether and how autophagy-dependent GluRs endocytosis causally regulate other known intrinsic properties of LHb neurons, basal synaptic transmission, and behavioral phenotypes. Moreover, we cannot rule out the possibility that autophagic enhancer acting on the intrinsic properties of LHb through different mechanisms which some may not require the endocytosis of synaptic GluRs. We can agree that autophagy enhancer rescues various types of physiological properties in LHb which hints the involvement of autophagy in LHb homeostasis in general. But the missing point is that whether autophagy improving electrophysiological/molecular properties of LHb all require synaptic GluRs endocytosis or not has not been addressed. This is the critical weakness that questions the validity of their conclusions that autophagy-dependent LHb homoeostasis gate depression onset.

Another serious concern is the lack of mechanistic link between autophagy in LHb and well-known acting mechanisms of other antidepressants and mTOR inhibitors (Paroxetine, Ketamine, and Rapamycin). Antidepressant experiments in this study did not provide the proof of the causal role of LHb autophagy in these effects of antidepressants. Despite the authors providing the change in autophagic signaling which coincides with the effects of treatment, this is rather an observational data and outcome-based interpretation. Whether the effect of treatment of other antidepressants requires the causal role of LHb autophagy or not and if there is, how LHb autophagy mechanistically interacts with other molecular mechanisms of antidepressants need to be demonstrated. They must logically elaborate these issues to generalize the mechanism of the causal role of LHb autophagy in depression.

Lastly, is LHb autophagy-dependent habenular homeostasis the common mechanism for all types of depression models? Authors showed interesting observational data which display similar tendency of p62 change across different type of stressors (Figure 2b). We recommend authors perform the additional experiments to address the causality of LHb autophagy in different depression models such as social defeat. This is essential to address whether LHb autophagy is a common target in depression and applicable for all types of stress.

Major points:
1. In all of antidepressant i.p. injection experiments, Is LHb autophagy causally required for the acting mechanisms of the drugs? Do behavioral rescue effects of the antidepressants mechanistically require the activity of LHb autophagy? If it is required, how do you explain the causal role of LHb autophagy with already known acting molecular mechanisms of the drugs? Authors need to demonstrate strong additional experiments to validate the causal role of LHb in the behavioral rescue effect and interaction with other acting mechanisms of various antidepressants.

2. In Figure 4, authors show that enhancing LHb autophagy by tBP (TAT-beclin-1 peptide) dramatically rescues hyperexcitability of LHb neurons (Figure 4c-d , Extended Figure 7&9) by lowering bursting neurons, lowering sEPSC (Figure 4g) with rescuing synaptic LTD experiments which are GluRs endocytosis dependent. From this data, it is clear that tBP can rescue various physiological properties of LHb. While it is convincing that the autophagy enhancer may rescue synaptic LTD through endocytosis of AMPARs, authors do not explain how this mechanism is related to lowering hyperexcitability of neurons, yet they emphasize the mechanistic role of autophagy in endocytosis of GluRs which may underlie the habenular homeostasis and the onset of depressive behaviors in this paper.
• The intrinsic excitability of neurons also relies on different mechanisms other than GluRs endocytosis or synaptic inputs. Previous reports already suggested different mechanisms leading to LHb hyperexcitability induced by chronic stress (Yang et al., 2018 and Cui et al., 2018). This leads to a critical question that LHb autophagy enhancer, although globally correcting neuronal, synaptic hyperexcitability and impaired synaptic plasticity, we cannot exclude the possibility that tBP rescues these properties with distinct mechanisms (other channels or receptors important for intrinsic neuronal excitability, basal synaptic transmission) or targeting different pathways other than endocytosis of GluRs considering autophagy is also expressed in non-postsynaptic area. (Kuijpers et al., 2021) (Yang et al., 2022).
• Authors need to carefully demonstrate whether the rescue of hyperexcitability of all properties is caused by the improvement of endocytosis of GluRs. Does failed induction of synaptic NMDAR-dependent LTD (LFS stimulation protocol) play a causal role in the hyperexcitability of LHb neurons and other basal synaptic transmissions that eventually lead to a behavior?

3. It is striking that only the LHb autophagy is affected but no other regions display robust changes in the autophagy pathway. What makes LHb autophagy so distinct from other emotional-related areas of the brain? What would be the unique mechanism that makes LHb autophagy sensitive to chronic stress? If they find a specific mechanism that distinguishes LHb autophagy from other brain areas, this will strengthen the paper.
• Some previous literature has already reported that chronic stress induces hyperexcitability of neurons in the amygdala (Rosenkranz et al., 2010) or POMC neurons in hypothalamic areas (Fang et al., 2023). I recommend authors look at other brain areas that were reported to display hyperexcitability or hyper-synaptic functions induced by chronic stress and validate whether there is a causal relationship between autophagy impairment and neuronal/synaptic homeostasis in these areas.

4. According to the authors' proposed theory, we would theoretically expect that acute stress might show totally different or even opposite physiological properties compared to chronic stress mice. Does acute stress have increased synaptic LTD and reduction of hyperexcitability of LHb neurons? This would be another good control experiment to test their theory regarding the temporal dynamics of LHb autophagy during the stress period.

5. Some experiments have a small number of samples. I recommend adding the number of samples (minimum should be at least over 5-6 animals for both western blots and over 7-8 animals for electrophysiology) for the following experiments.

Figure 1a-b, western blot for LHb, all groups
Figure 3j, Western blot, all groups
Figure 4 c-h, electrophysiology, all groups.
Figure 4t-w, electrophysiology, all groups
Figure 5m-r, electrophysiology, all groups

Minor points:
1. Is autophagy treatment long-lasting? How long do these effects last? Can prophylactic treatments before stress prevent these behaviors?

2. What would be the merit of targeting autophagy compared to other known antidepressants? What is the advantage of targeting autophagy, and can they outperform other treatments? Appealing these points by new experiments or discussions would increase the impact and significance of this study.

3. For loss-of-function studies, the authors targeted Atg7 to impair autophagic functions specifically in LHb. From their extended data Figure 2, transcriptome data show a statistically significant difference for BECN1 and Atg10, which was not observed in Atg7. Is there any specific reason for targeting Atg7 but not BECN1 or Atg10? The authors provided evidence of involvement of Atg7 in their introduction (line 85) with references (30-32) but this was mainly from human patients, while the transcriptomic analysis in this study does not show any change.

4. It is interesting that Atg7 deficiency shows some elevated levels of GluRs expression (Extended Fig 14), along with abnormal synaptic plasticity (Figure 5q & Figure 15), Is there any possibility of deficiency of Atg7 causing other impairments other than autophagy or endocytosis of GluRs which may lead to these defects? It is also noteworthy that there was a report of Atg7 regulating different cell types of spiny neurons in both autophagy-dependent and independent manner (Lieberman et al., 2020). In this study, there is also a change in sIPSC transmissions (Figure 5p) which may raise the possibility of the effects of Atg7 deficiency other than autophagy-dependent GluRs endocytosis. Similar to point No.2, I recommend authors perform experiments to support that these impairments are caused by dysfunctional autophagy-dependent endocytosis.

5. To provide strong evidence of change in autophagic activity, I suggest authors use one additional representative marker for autophagy activity (such as LC3-II and Beclin-1) along with the p62 in all western blot experiments. For the immunostaining data, quantitative data would be much stronger if they performed LC3-II staining with other dendritic markers or spines to show that autophagic activity controls synaptic properties related to Figure 2e.

Referee #2

(Remarks to the Author)
In this study, Yang and colleagues investigated how brain autophagy is engaged in stress response in acute and chronic stress models in mice. They found that acute stress activates autophagy, whereas chronic stress suppresses autophagy selectively in the lateral habenula (LHb). Different antidepressants, including paroxetine and ketamine, both can restore autophagy function specifically in LHb, although they work through distinct mechanisms. They further found that genetic attenuation of LHb neuronal autophagy increases stress susceptibility, whereas enhancing LHb autophagy exerts rapid “antidepressant” effects. Mechanistically, they found that LHb autophagy influences LHb neuronal activity, synaptic transmission and plasticity, and regulates glutamate receptor endocytosis.

Overall, this study is quite comprehensive. It provides some compelling evidence that LHb autophagy plays an important role in LHb function and animal stress coping, which are novel mechanisms. Nevertheless, I have some comments and questions that need to be addressed.

First of all, I have some general comments and questions that the authors should discuss or reconcile.

1. The study relies on forced swim test (FST) and sucrose preference test (SPT) as redouts of “depression” in mice. These are “established” and “traditional” assays, which have been very useful and led to a great deal of knowledge in the field of stress research. However, their limitations are also obvious, and currently there has been some consensus in the field that such tests in animals can hardly be related to depression in humans. FST is not comparable to human behavior. Even for SPT, it does not parallel human anhedonia. For example, a person, even if depressed, will still prefer a delicious meal to a boring one. Therefore, some paradigm shift is needed. To a minimum, the authors should use “stress coping” or its maladaptation other than “antidepressant” or depression throughout the paper.

2. Lateral habenula (LHb) is only one of the brain areas activated by different stressors. There are many other brain areas that are hyperactive in response to stress. Given that autophagy appears to be a general mechanism regulating synaptic function in different neuronal types, why only LHb neuronal function is regulated by autophagy during stress?

3. Why different antidepressants, which act through very different mechanisms, similarly activate autophagy?

4. Ketamine’s antidepressant effects have been attributed to its activation of the mTOR pathway (e.g., Li, …, Duman, 2010, Science), but here the authors show that Ketamine suppresses mTOR. How to reconcile with the literature?

Specific questions:
1. The authors report that activation of LHb autophagy temporally coincides with the onset of antidepressant effects of paroxetine in chronic stressed mice. Are there studies showing that paroxetine decreases LHb activity in “depressed” mice following similar duration of treatment? If so, these studies need to be cited and commented. If not, the authors need to test it experimentally.

2. mRNA sequencing results show significantly decreased expression levels of genes involved in autophagy activation processes (e.g., BECN1 (gene name of beclin-1) and autophagy-related gene Atg10) (Extended Data Fig. 2a,b1,b2), as well as decreased tendencies in Atg4d, Atg12, Atg7, Atg5 and LC3 (light chain 3) (Extended Data Fig. 2b3-b7). There were also increased tendency of expression level of mTOR (Extended Data Fig. 2b9). These results need to be validated, for example via qPCR.

3. Related to #2 above: Fig. 1d shows that LHb and several other brain areas have similar downregulation of genes involved in endocytosis, protein digestion and absorption, and synaptic vesicle cycle. These results seem to be inconsistent with the idea that LHb phagocytosis is selectively affected. The authors need to reconcile this.

4. Related to #2 above: does acute stress induce changes in gene expression opposite to those induced by chronic stress? In other words, does acute stress selectively increase LHb autophagy at the transcriptomic level? This should be the case, if indeed that acute stress activates, whereas chronic stress suppresses autophagy selectively in the LHb.

5. Are the RNAseq data contaminated by glia cells?

6. In chronically stressed mice, LHb neurons show increased bust and overall spontaneous activity. Are these changes due to changes in intrinsic excitability or synaptic inputs? In addition, after incubation with tBP in LHb slices of CRS mice, burst (Fig. 4c,d) and the overall spontaneous (Fig. 4e) firing activity were both largely attenuated. Are these effects solely mediated by normalization of the enhanced synaptic inputs, or may also by normalization of intrinsic excitability?

7. If I understand correctly, phagocytosis and internalization should be two separate processes. In Fig. 4w, the pharmacological LTD was completely blocked by TAT-GluA23Y peptide, suggesting that endocytosis occurs before phagocytosis during LTD. But then why promoting phagocytosis can directly induce LTD? A model needs to be proposed to explain the results.

8. Line 68: “that controlling” should be “that control”.

9. Line 178: “…in naïve mice” should be “…compared with naïve mice”.

Version 2:

Reviewer comments:

Referee #1

(Remarks to the Author)
The authors have added comprehensive experiments in response to all my concerns, and I am satisfied with the revised manuscript. This paper is both conceptually improved and technically sound along with exciting perspective. I would like to appreciate their rigorous efforts. I have a few comments.

1. Fig. R1.1 panels b-e have not been added to the revised manuscript, but these panels should be included in Fig. 6 or Extended Data Fig. These data quantitatively demonstrate the degree of chemogenetic inhibition in neuronal hyperactivity and are essential for evaluating the results of the behavioral experiments in panels f-i. Fig. R1.1 is very important because it shows a causal link between LHB autophagic deficit, LHB hyperexcitability (homeostasis), and the depressive behaviors.

2. If possible, adding Figs. R1.13 and R1.14 to the revised manuscript would strengthen the authors' discussion. However, this is not mandatory.

3. In main Figure 6 c,d, western blot analysis shows p62 is increased while Beclin-1 is decreased in Cre-mice which is mentioned in the text line 392, but in Figure 6 description, it is written as Becline-1 is increased.

Kaoru Inokuchi

Referee #2

(Remarks to the Author)
The authors have done a substantial number of new experiments to substantiate their conclusions and have thoroughly addressed all my questions. Hats off to the authors for such tremendous effort. This is an important study that makes a strong case for LHb autophagy in stress and depression-like behavior.

I only have two (related) suggestions which I think are quite important, as autophagy in regulating brain function is relatively new and more information is helpful for readers to have a more complete and unbiased view:

1. The results on autophagy in the basolateral amygdala (BLA) and arcuate nucleus (Arc) (Figure R2.4 and Figure R2.5) are quite interesting and important, and are an integral part of the paper. Therefore, they should be included in the paper.

2. In many places in the paper, including the abstract (lines 41, 43, 98, 102, 598, and maybe other places throughout the paper), it is stated that autophagy is “selective” to the LHb. The authors should tone down such a claim, as autophagy also occurs at least in the Arc, and potentially in other yet-to-be identified brain regions.

Referee #3

(Remarks to the Author)
This is an interesting paper that suggests autophagy in the lateral habenula (LHb) is uniquely sensitive to acute and chronic stress. Autophagy in the LHb is regulated by AMPK and mTOR, and modulators of these kinases infused into the LHb alters the expression of depression-related behaviors in mice. Similarly, modulating the expression of autophagy-related proteins alters the expression of depression-related behaviors in mice. It is suggest that autophagy regulates depression-related behaviors by a mechanism involving the rapid regulation of local excitatory synaptic transmission. The paper is multidisciplinary in nature and focused on an important topic of research. I have the following suggestions for the authors’ consideration:

1. The manuscript needs to be thoroughly proof-read to correct the many instances of grammatical errors and sometimes strained prose.

2. Figure 1, panel a1: It is unclear why there is a greater number of sample for Western blot analyses from the LHb (7 or more) relative all other brain regions (5 in all other cases).

3. Figure 1: Which cells in the LHb demonstrate stress-induced changes in autophagy? Is this effect restricted to local neurons?

4. Figure 1: How were transcriptional profiles of LHb cells generated? By bulk RNA-seq, scRNA-seq, some other method? Please provide more details in the relevant section of the manuscript.

5. Figure 1: Are altered markers of autophagy in LHb secondary to stress-induced increases neural activity in the LHb? In other works, would chemogenetic or optogenetic stimulation of neural activity in the LHb elicit the same alterations in p62, beclin, etc., observed in LHb lysates?

6. Figure 2: It is unclear why markers of autophagy should be impacted by AMPK and mTOR manipulations only in the LHb. As autophagy is under the control of these kinases in most/all cells, surely autophagy-related markers should have been induced in brain regions other than the LHb? Do AMPK and mTOR show higher basal activity in the LHb relative to other brain regions?

7. Figure 3e-h: The effects of knocking down Atg7 alone (in the absence of rapamycin treatment) should be assessed in the same behavioral tests. Without this group, it is not possible to determine whether Atg7 alone modified behavior or blocked the effect of rapamycin.

8. Figure 3i-l: Once again, the effects of knocking down Atg7 alone (in the absence of paroxetine treatment) should be assessed. Without this group, it is not possible to determine whether Atg7 blocked the effect of paroxetine or alone exerted effects on the same behavioral endpoints.

9. Figure 3: It is unclear why shRNA-Atg7 was used in one case, while Atg7-floxed mice were used in another. Were analyses performed to confirm that Atg7 was selectively and efficiently knocked out of the LHb by AAV-Cre delivery in Atg7-floxed mice?

10. Figure 5: Data showing that LC3 is in close physical proximity to glutamate receptor complexes in the LHb should be moved from supplementary materials to the main figure. Does LC3 interact with glutamate receptors in the same manner in other brain regions, or is this relationship unique to the LHb?

11. Figure 5: Does phasic and tonic activity of LHb neurons recover after tBP has been washed from slices?

12. Figure 5: Does tBP have “direct” pharmacological actions on AMPA receptors that can explain its unexpectedly rapid inhibitory effect on excitatory transmission in the LHb?

13. Figure 5: Does tBP have a similar inhibitory effect on excitatory synaptic transmission in brain regions other than the LHb, or is this effect unique to the LHb?

14. Figure 5: The effects of Dyngo-4a alone on behavior were not assessed. Thus, the interpretation that it blocks the antidepressant-like effect of tBP cannot be make with confidence, as Dyngo-4a may well have effects independent of tBP.

Version 3:

Reviewer comments:

Referee #3

(Remarks to the Author)
The authors have thoroughly addressed the concerns I raised on the previous version of their manuscript by generating substantial amounts of new data. These new data further strengthen what was already a compelling data-set. I have no further comments for the authors' consideration and commend them on a fine contribution.

Dear reviewers and editor,

Thank you very much for your comments regarding our manuscript "Stress Dynamically Modulates Neuronal Autophagy-Dependent Habenular Homeostasis to Gate Depression-like State" (2023-07-12605A). The editorial comments stated that "*While the referees find your work of some interest, they raise concerns about the strength of the novel conclusions that can be drawn at this stage.*" Upon careful review and consideration of all reviewers' comments, we recognize their constructive nature. We believe that many of the suggested experiments will greatly enhance the manuscript. Therefore, we are providing a detailed response to all reviewers for your consideration.

Please find our point by point response to comments from each reviewer below (Reviewer's original comments in *italic*; revision data are presented with rebuttal figures below (Figure R1.x or R2.x. corresponding to rebuttal figures for either Reviewer 1 or 2, respectively):

Referees' comments:

Referee #1 (Remarks to the Author):

In this study, Yang et al. focused on how chronic stress changes the autophagic function in the Lateral Habenula (LHb), which may underlie the pathological mechanism of developing depressive phenotypes. They have performed comprehensive experiments and discovered that chronic stress induced robust change in autophagy signaling only in LHb, which was not observed in other areas of the brain. Treatments of well-known antidepressants also changed the autophagic activity specifically in LHb, which hints the possible role of LHb autophagy as a general mechanism for the treatment of depression.
By targeting LHb autophagy using tet-Beclin-1 peptide, the authors showed a dramatic improvement of both depressive behaviors and correcting hyper-activity of both neuronal and synaptic properties in LHb. These are very promising results that may provide a new therapeutic strategy for treating patients suffering from major depressive disorder. With the following loss-of-function studies using both selective knockdown and knockout of autophagy-related-gene (Atg7), they concluded that autophagy dysfunction in LHb is lowering the endocytosis of membrane receptors such as synaptic GluR, which likely leads to an impaired neuronal and synaptic homeostasis in LHb.
Despite the previous literature suggesting the role of LHb in depression and proposed theories of the possible role of autophagy in depression, this is a proof-of-concept study to show that autophagy in specific area of the brain may play a critical role in developing depressive phenotypes. This study also suggests targeting area-specific autophagy as potential treatment that is a novel approach in the field of depression and stress research studies. They conducted extensive work to screen the multiple areas of the brain and thoroughly investigated the changes in behavior, molecular and physiological function followed by targeting LHb autophagy function with chronic stress behavioral paradigm.

They have provided solid experimental data that supports the rationale of targeting LHb autophagy as a new therapeutic target.

We genuinely appreciate Reviewer 1's enthusiasm for the significance and originality of our study. We are also extremely grateful for the insightful comments provided by the reviewer.

However, I have serious concerns regarding their conclusions along with their proposed mechanism. Authors confidently conclude that autophagy-dependent habenular homeostasis gates depression onset. From their presented data, it is convincing that autophagy regulates habenular homeostasis. They also showed that the artificial suppression of habenular autophagy results in abnormal behaviors that resemble depression-like behaviors. However, my concern is that this does not necessarily mean whether autophagy causally regulates these behavioral outputs through habenular homeostasis. Their current data are not sufficient and do not provide the proof of causality in which autophagy regulates depressive behavior that requires habenular homeostasis.

We thank the reviewer for raising such an important question. To investigate whether lateral habenula (LHb) hyperactivity directly links autophagy deficit with depression-like behaviors, we examined whether these depression-like phenotypes in *Atg7*$^{LHb-/-}$ mice were reversed by normalizing LHb hyperactivity. To achieve this, we employed a dual-viral approach to express both AAV-DIO-hM4D and AAV-hSyn-Cre in the LHb of *Atg7*$^{flox/flox}$ mice (Figure R1.1a). This method allows us to precisely silence LHb neurons with *Atg7* ablation. Our chemogenetic strategy effectively reduced neuronal hyperactivity in *Atg7*$^{-/-}$ LHb slices (Figure R1.1b-e) and alleviated depression-like behaviors without affecting locomotion ability in *Atg7*$^{LHb-/-}$ mice (Figure R1.1f-i). This finding indicates that LHb neuronal hyperactivity is the cellular basis for depression-like behaviors in *Atg7*$^{LHb-/-}$ mice.

We hope these experiments will provide evidence of causality, demonstrating that LHb autophagy copes with stress-induced depression-like behaviors by maintaining habenular homeostasis.

Now we integrate these results into Fig. 6r-u and Extended Data Fig. 6g, and add relevant text (line 428-438).

[FIGURE REDACTED]

Figure R1.1. Chemogenetic inhibition of lateral habenula (LHb) neuronal hyperactivity rescued depression-like behaviors in *Atg7*$^{LHb-/-}$ mice. a, Experimental design. **b-d,** Representative traces (**b**) and quantifications (**c,d**) of spontaneous excitatory post-synaptic currents (sEPSCs) before and after Deschloroclozapine (DCZ) perfusion (n = 7 neurons, mice = 3; Paired *t* test). **e,** quantification of spontaneous firing activity before and after DCZ perfusion (n = 7 neurons, mice = 3; Wilcoxon test). **f-i,** Chemogenetic silencing LHb neurons leads to a decreased immobile duration in forced swim test (FST) (**f**), an increased sucrose preference in sucrose preference test (SPT) (**g**), and an increased social interaction ratio in social interaction test (SIT) (**h**), without affecting locomotion and anxiety-like behavior in open-field test (OFT) (**i**) in *Atg7*$^{LHb-/-}$ mice. (n = 5 mice; Unpaired *t* test). *P < 0.05; **P < 0.01; n.s., not significant. Error bars indicate SEM.

In addition, they conclusively propose the mechanism in which autophagy-dependent-GluRs endocytosis/degradation underlies habenular homeostasis and depressive behaviors. The authors previously reported the other mechanisms underlying hyperexcitability of LHb neurons (Yang et al., 2018 and Cui et al., 2018). Considering that the current mechanism is synaptic and previous two are more of intrinsic properties of neuron, there is a discrepancy between the currently proposed mechanism and the other mechanisms (Yang et al., 2018 and Cui et al., 2018) that underlie habenular homeostasis in LHb. How does autophagy-dependent endocytosis of synaptic GluRs interact with other reported mechanisms of neuronal homeostasis (neuronal hyperexcitability) in LHb? Does autophagy correct/regulate habenular homeostasis (synaptic to neuronal) through one common pathway? Is autophagy-dependent endocytosis of synaptic GluRs the upstream of every other reported mechanism underlying habenular homeostasis (neuronal/synaptic)? My worry is that this study lacks experimental data nor provides strong evidence to explain whether and how autophagy-dependent GluRs endocytosis causally regulate other known intrinsic properties of LHb neurons, basal synaptic transmission, and behavioral phenotypes. Moreover, we cannot rule out the possibility that autophagic enhancer acting on the intrinsic properties of LHb through different mechanisms which some may not require the endocytosis of synaptic GluRs. We can agree that autophagy enhancer rescues various types of physiological properties in LHb which hints the involvement of autophagy in LHb homeostasis in general. But the missing point is that whether autophagy improving electrophysiological/molecular properties of LHb all require synaptic GluRs endocytosis or

not has not been addressed. This is the critical weakness that questions the validity of their conclusions that autophagy-dependent LHb homoeostasis gate depression onset.

We thank Reviewer 1 for raising this essential point. Indeed, synaptic components in LHb crucially modulate both neuronal excitability and synaptic transmission (Li et al., 2013; Meye et al., 2015; Yang et al., 2018)**. Under various aversive conditions (e.g., depression, cocaine withdrawal), increased membrane insertion of AMPARs enhances not only synaptic transmission, but also neuronal firing rate and excitability** (Li et al., 2013; Meye et al., 2015)**. Moreover, administering either AMPA or NMDA pharmacologically enhances bursting activity, a pacemaker-like activity that promotes theta wave synchronization in the LHb of depression-like animals** (Yang et al., 2018)**.**

Our current study aligns with these prior discoveries, as we observed an accumulation of glutamate receptors (GluRs), specifically AMPARs and NMDARs, in both chronic stressed and autophagy-deficient mice. In addition, we also found that both excitatory synaptic transmission and bursting activity were enhanced in autophagy-deficient mice. Improving autophagy function restores GluR levels, normalizes both synaptic and intrinsic hyperactivity, and produces antidepressant-like effects in chronic stressed mice. Using super-resolution microscopy imaging, we observed co-localization of LC3 puncta and GluRs. This led us to propose that autophagy normalizes LHb neuronal and synaptic activity by modulating the endocytosis/degradation of GluRs.

However, we agree with Reviewer 1 that, although our findings indicate GluRs are the primary degradation targets of LHb autophagy, we cannot rule out the possibility that autophagy may also target other molecules for regulating LHb homeostasis. To address these critical points, additional experiments have been carried out in this revised manuscript:

1) **We examined the mRNA expression levels of T-type calcium channels (T-VSCC) and inwardly rectifying potassium channel (Kir2.1), potential molecules influencing habenular neuronal intrinsic activity, as recommended by Reviewer 1. Consistent with the very depolarized resting membrane potential (~ -50 mV) of LHb neurons, we found that the mRNA level of *Kir2.1* in the LHb is scarce (Figure R1.9a). We therefore speculated that the contribution of Kir2.1 to neuronal excitability is low. Besides, the mRNA level of T-VSCC in the LHb is unaltered by chronic restraint stress (CRS) (Figure R1.9b), consistent with the unaltered current intensity of T-VSCC** (Yang et al., 2018)**, suggesting that T-VSCC function is not affected by impaired LHb autophagy in CRS mice.**
2) **Confocal imaging analysis revealed that acute stress-induced autophagic flux (indicated by LC3 puncta staining) is predominantly co-localized with PSD95, suggesting that autophagic flux in the LHb is**

primarily located at excitatory post-synaptic sites (Figure R1.8). Additionally, immunoelectron microscopy experiments showed that autophagosomes are co-localized with AMPARs (Figure R1.8). These findings further characterized the subcellular localization of LHb autophagy and its selective targeting onto GluRs.

3) **We found that Dyngo-4a, a general membrane protein endocytosis blocker, and TAT-GluA2$_{3Y}$ peptide, which specifically blocks GluRs endocytosis, both abolished the effects of TAT-beclin-1 peptide (tBP) on behavioral outcome and neuronal activity through cannular infusion in LHb (Figure R1.6 and R1.7), suggesting the involvements of GluR endocytosis for the effects of LHb autophagy on LHb homeostasis and depression-like behaviors.**
4) **We also found that a naturally occurred synaptic depression/de-potentiation in LHb neurons during stress is eliminated by blocking LHb autophagy activation (Figure R1.10).**

Our findings suggest a potential cellular mechanism where autophagy regulates LHb synaptic/intrinsic activity by primarily modulating GluRs endocytosis and degradation.

Another serious concern is the lack of mechanistic link between autophagy in LHb and well-known acting mechanisms of other antidepressants and mTOR inhibitors (Paroxetine, Ketamine, and Rapamycin). Antidepressant experiments in this study did not provide the proof of the causal role of LHb autophagy in these effects of antidepressants. Despite the authors providing the change in autophagic signaling which coincides with the effects of treatment, this is rather an observational data and outcome-based interpretation. Whether the effect of treatment of other antidepressants requires the causal role of LHb autophagy or not and if there is, how LHb autophagy mechanistically interacts with other molecular mechanisms of antidepressants need to be demonstrated. They must logically elaborate these issues to generalize the mechanism of the causal role of LHb autophagy in depression.

We appreciate Reviewer 1's request for additional clarification on the causal role of LHb autophagy in the antidepressant-like effects of paroxetine and ketamine, as well as its potential relevance to their established mechanisms.

1) **To address this crucial point, we conducted the following experiments: We tested the causal role of LHb autophagy in ketamine's and paroxetine's antidepressant-like effects via an *Atg7* knock-out strategy specifically in the LHb. It appears that the therapeutic impacts of both paroxetine and ketamine on depression-like behaviors require the activation of LHb autophagy (Figure R1.2 and R1.4).**
2) **We discovered that both ketamine and paroxetine induce autophagy via inhibiting Akt-mTOR (mechanistic target of rapamycin) signaling in the LHb (Figure R1.5). As Akt-mTOR signaling is crucial for the**

antidepressant actions of various medications, including selective serotonin reuptake inhibitors (SSRIs), ketamine, and psychedelics (Autry et al., 2011; Casarotto et al., 2021; Li et al., 2010; Moliner et al., 2023)**, our characterizations bridge the gap between autophagy-related mechanisms and the well-known antidepressant mechanisms.**

3) **We included Beclin-1 as an additional biomarker in western blot analysis to validate the specificity of autophagy signaling under antidepressants treatments (Figure R1.19).**

Lastly, is LHb autophagy-dependent habenular homeostasis the common mechanism for all types of depression models? Authors showed interesting observational data which display similar tendency of p62 change across different type of stressors (Figure 2b). We recommend authors perform the additional experiments to address the causality of LHb autophagy in different depression models such as social defeat. This is essential to address whether LHb autophagy is a common target in depression and applicable for all types of stress.

Great suggestion! The following experiments have been conducted to further address whether LHb autophagy is a common target in depression and applicable for different stressors as recommended by the reviewer:

1) **We unraveled that specific activation of LHb autophagy directly alleviates depression-like behaviors in mice after experiencing chronic social defeat stress (CSDS) (Figure R1.15).**
2) **We discovered that knocking down any one of other autophagy core genes in LHb also leads to abnormal electrophysiological properties and depression-like behaviors in mice (Figure R1.17 and R1.18).**
3) **Beclin-1 has now been included as an additional biomarker in all Western Blot experiments to confirm the specificity of autophagy signaling (Figure R1.19).**
4) **Corticosterone levels in mice with genetic ablation of LHb autophagy are comparable to chronic stressed mice, suggesting a consistent endophenotype induced by autophagy deficit or chronic stress (Figure R2.2).**

Collectively, these new results suggest that LHb autophagy might be a common target in depression, and applicable to multiple types of stress.

Major points:

1. In all of antidepressant i.p. injection experiments, Is LHb autophagy causally required for the acting mechanisms of the drugs? Do behavioral rescue effects of the antidepressants mechanistically require the activity of LHb autophagy?

To fully address Reviewer 1's critical concern, we measured the behavioral effects of paroxetine or ketamine in mice with impaired LHb autophagy. Firstly, we performed LHb-specific knock-out of *Atg7*,

followed by 14-day CRS and 7-day chronic administration of paroxetine (Figure R1.2a). Notably, LHb-specific autophagy impairment partially abolishes antidepressant-like effects of paroxetine, manifested by increased immobile duration in the forced swim test (FST) and reduced social interaction ratio in the social interaction test (SIT), without affecting the sucrose preference test (SPT) (Figure R1.2b-d). As FST, SIT, and SPT are established to model distinct aspects of depression-like phenotypes, including despair-like (FST), social avoidance-like (SIT), and anhedonia-like behaviors (SPT), these findings suggest that activation of LHb autophagy is indispensable for paroxetine's antidepressant-like effects on despair-like and social avoidance-like phenotypes, but not anhedonia-like phenotype.

[FIGURE REDACTED]

Figure R1.2. Activation of LHb autophagy is partially required for antidepressant-like effects of paroxetine. a, Experimental design. **b-d,** Conditional knock-out of *Atg7* in LHb abolishes antidepressant-like effects of paroxetine in FST (**b**) and SIT (**d**), but not in SPT (**c**) (n = 6 to 8 mice; One-way ANOVA with Uncorrected Fisher's LSD). *P < 0.05; **P < 0.01; ***P<0.001; n.s., not significant. Error bars indicate SEM.

In depressed patients, SSRIs rapidly elevate serotonin levels, whereas exert antidepressant effects typically after weeks to months (Monteggia et al., 2014)**. Consistent with clinical situations, chronic rather than acute administration of SSRIs (e.g., paroxetine or fluoxetine) ameliorates depression-like behaviors in rodents** (Autry et al., 2011; David et al., 2009; Zhu et al., 2021**;** see also our current study)**. These lines of evidence suggest that SSRIs may influence neural mechanisms beyond serotonin elevation. Interestingly, we observed that 7-day but not 1-day treatment of paroxetine enhances LHb autophagy, and further decreased excitatory synaptic transmission and bursting activity in LHb neurons from CRS mice (Figure R1.3, also addressing Specific Question 1 in response to Reviewer 2). These findings provide the mechanistic link between antidepressant-like effects of paroxetine and LHb autophagy: paroxetine enhances LHb autophagy, which facilitates GluRs endocytosis, subsequently normalizes LHb neuronal hyperactivity, and finally improves stress-induced depression-like behaviors. Thus, our data underscores the role of LHb autophagy in key aspects of paroxetine's antidepressant-like effects.**

Now we integrate these results into Fig. 3i-l and Extended Data Fig. 1d-i, and add relevant text (line 137-139, 265-271).

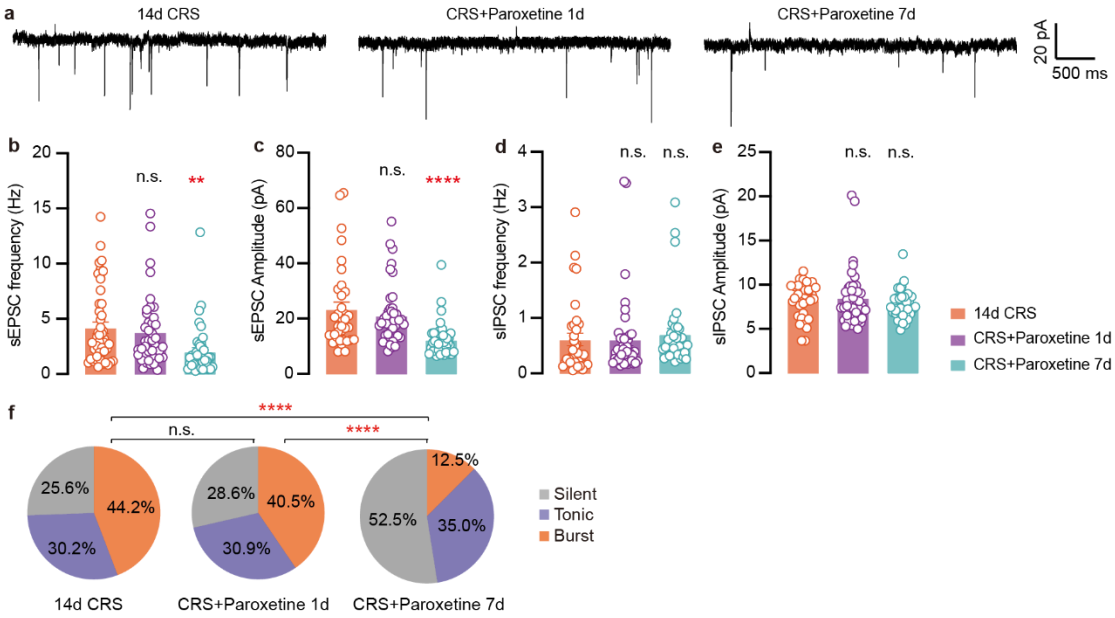

Figure. R1.3. Chronic but not acute administration of paroxetine normalizes LHb neuronal hyperactivity. **a-e**, Representative traces (**a**) and quantifications (**b-e**) of excitatory synaptic activity under 1-day or 7-day intraperitoneal (*i.p.*) injection of paroxetine in chronic restraint stress (CRS) mice (n = 40 to 44, mice = 5; One way-ANOVA with Dunnett's multiple comparisons test). **f**, Pie charts illustrating spontaneous firing activity of LHb neurons under 1-day or 7-day *i.p.* injection of paroxetine in CRS mice (n = 40 to 44, mice = 5; Chi-square test). **P < 0.01; ****P<0.0001; n.s., not significant. Error bars indicate SEM.

To further determine whether activation of LHb autophagy is essential for ketamine's antidepressant-like effects, we performed LHb-specific knock-out of *Atg7*, followed by acute treatment of ketamine. To exclude the immediate and direct effects of ketamine as an NMDAR blocker (Yang et al., 2018)**, we evaluated antidepressant-like effects of ketamine 24 hours after intraperitoneal (*i.p.*) injection, which is well-documented** (Autry et al., 2011; Li et al., 2010)**. Besides, we validated that ketamine increased LHb autophagy (evidenced by decreased p62 and increased Beclin-1) in CRS mice at the time point of 24 hours post-injection (Figure R1.4a). Subsequently, we discovered that *Atg7*$^{LHb-/-}$ mice treated with ketamine showed no antidepressant-like effects compared to vehicle controls (Figure R1.4b-d). These findings indicate the necessity of LHb autophagy for antidepressant-like effects of ketamine.**

[FIGURE REDACTED]

Figure R1.4. Antidepressant-like effects of ketamine are absent in *Atg7*$^{LHb-/-}$ mice. **a,** Antidepressant dosage of ketamine enhances autophagy (i.e., decreased p62 and increased Beclin-1) in LHb 24 h after *i.p.* injection (n = 5 mice; Mann-Whitney test). **b**, Experimental design. **c,d,** Ketamine does not affect FST (**c**) and SPT (**d**) scores in *Atg7*$^{LHb-/-}$ mice (n = 6 to 8 mice; Unpaired *t* test). n.s., not significant. Error bars indicate SEM.

We have now incorporated Figure R1.4 into Extended Data Fig. 7a-d, and added relevant text (line 271-273). Thanks for this excellent suggestion!

If it is required, how do you explain the causal role of LHb autophagy with already known acting molecular mechanisms of the drugs? Authors need to demonstrate strong additional experiments to validate the causal role of LHb in the behavioral rescue effect and interaction with other acting mechanisms of various antidepressants.

Various antidepressants engage Akt signaling pathways (Duman et al., 2019)**, which might affect autophagy process. Previous studies unraveled that reduced phosphorylation of Akt inhibits the mTOR signaling, leading to activation of Beclin-1 and the subsequent autophagic process** (Gassen et al., 2014; Wang et al., 2012)**. In our study, we indeed observed that ketamine at antidepressant dosage decreased the levels of phosphorylated Akt (p-Akt), phosphorylated mTOR (p-mTOR) and p62, whereas increased the level of Beclin-1 in the LHb of CRS mice (Figure R1.5a-c), indicating enhanced autophagy signaling via inhibited Akt signaling. Similar mechanisms in the LHb were observed with chronic paroxetine treatment in CRS mice (Figure R1.5d-f).**

Importantly, the effects of different antidepressants on Akt-mTOR signaling depend on brain regions. Previous studies have revealed that activation of brain derived neurotrophic factor (BDNF)-Akt-mTOR signaling in the medial prefrontal cortex (mPFC) or hippocampus is crucial for the antidepressant actions of various medications, including SSRIs, ketamine, and psychedelics (Autry et al., 2011; Casarotto et al., 2021; Li et al., 2010; Moliner et al., 2023)**. In contrast, activation of BDNF signaling in the nucleus accumbens (NAc) or LHb causally mediates depression-like behaviors** (Koo et al., 2019;Lei et al., 2020)**. As BDNF activation strongly activates Akt-mTOR signaling and thus inhibits autophagy, these findings are consistent with ours in the LHb. To align with previous studies and gain quality control of our experiments, we selected the mPFC as a positive control region for measuring ketamine's effects. In line with previous reports, we demonstrated that ketamine activated BDNF-mTOR signaling in the mPFC (Figure R1.5g-h).**

We have now incorporated Figure R1.5 into Extended Data Fig. 7e-h, and added relevant text (line 274-280).

[FIGURE REDACTED]

Figure R1.5. Antidepressants commonly inhibit Akt-mTOR signaling and activates autophagy in the LHb. **a-c,** Ketamine treatment (*i.p.*) decreased phosphorylated Akt (p-Akt), phosphorylated-mechanistic target of rapamycin (p-mTOR) and p62 levels, and increased Beclin-1 level in LHb (n = 5 to 6 mice; Mann-Whitney test). **d-f**, Paroxetine treatment (*i.p.*, 7d) decreased p-Akt, p-mTOR and p62 levels, and increased Beclin-1 level in LHb (n =5 to 7 mice; Mann-Whitney test). **g-h,** Ketamine treatment (*i.p.*) increased brain derived neurotrophic factor (BDNF) and p-mTOR levels in medial prefrontal cortex (mPFC) (n =5 mice; Mann-Whitney test). *P < 0.05; **P < 0.01. Error bars indicate SEM.

2. In Figure 4, authors show that enhancing LHb autophagy by tBP (TAT-beclin-1 peptide) dramatically rescues hyperexcitability of LHb neurons (Figure 4c-d , Extended Figure 7&9) by lowering bursting neurons, lowering sEPSC (Figure 4g) with rescuing synaptic LTD experiments which are GluRs endocytosis dependent. From this data, it is clear that tBP can rescue various physiological properties of LHb. While it is convincing that the autophagy enhancer may rescue synaptic LTD through endocytosis of AMPARs, authors do not explain how this mechanism is related to lowering hyperexcitability of neurons, yet they emphasize the mechanistic role of autophagy in endocytosis of GluRs which may underlie the habenular homeostasis and the onset of depressive behaviors in this paper.

Thanks for these insightful comments. To further elucidate how autophagy is related to lowering hyperexcitability of LHb neurons, we performed the following experiments:

1) **We investigated whether blocking endocytosis with Dyngo-4a prevents tBP's ability to normalize LHb hyperactivity. Notably, reduced excitatory synaptic transmission and spontaneous neuronal activity under tBP application (Figure R1.6a-f) were completely abolished by co-application with Dyngo-4a, an endocytosis blocker (Figure R1.6g-l), revealing a necessary role of endocytosis for decreasing both synaptic and neuronal activity under tBP treatment. Excitatory**

synaptic transmission primarily relies on AMPARs, while LHb bursting activity is largely dependent on NMDARs (Yang et al., 2018**). In our current study, expression levels of both AMPARs and NMDARs were elevated in the LHb of CRS mice, whereas normalized by LHb local infusion of tBP (please refer to Fig. 5a-h in the manuscript). Collectively, these findings suggest that enhancing LHb autophagy promotes degradation of both AMPARs and NMDARs, thereby rapidly normalizing both spontaneous bursting activity and excitatory neurotransmission.**

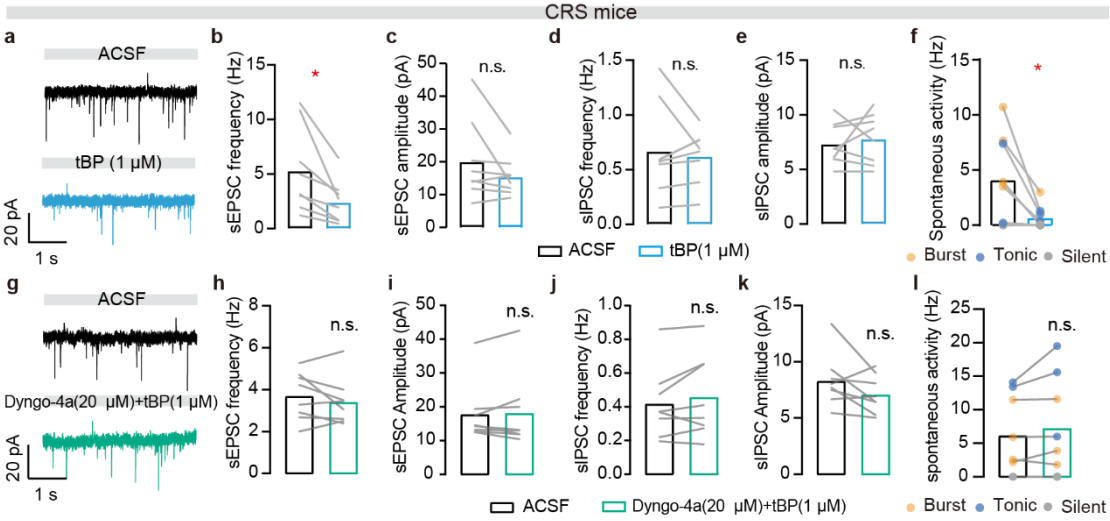

Figure R1.6. An endocytosis blocker, Dyngo-4a, abolishes tBP's normalizing effects on LHb hyperactivity. a-e, Representative traces (**a**) and quantifications (**b-e**) showing spontaneous neural transmission before and right after tBP perfusion in LHb neurons of CRS mice (n = 8, mice = 6; Paired *t* test). **f,** Bar graph showing spontaneous firing frequency before and after tBP perfusion. **g-k,** Representative traces (**g**) and quantifications (**h-k**) showing spontaneous neural transmission before and right after co-perfusion of tBP and Dyngo-4a in LHb neurons of CRS mice (n = 8, mice = 3; Paired *t* test). **l,** Bar graph showing spontaneous firing frequency before and after co-perfusion of tBP and Dyngo-4a. *P < 0.05; n.s., not significant. Error bars indicate SEM.

2) **At the behavioral level, we tested whether antidepressant-like effects of tBP were blocked by Dyngo-4a or TAT-GluA2$_{3Y}$ peptide, a specific blocker of AMPAR endocytosis. We locally infused tBP together with Dyngo-4a or TAT-GluA2$_{3Y}$ peptide into the LHb, in order to induce autophagy while simultaneously inhibiting the endocytosis of membrane proteins. As depicted in Figure R1.7, reduced immobility in FST and increased sucrose preference in SPT under tBP infusion were abolished by co-infusion of tBP and Dyngo-4a or TAT-GluA2$_{3Y}$. These data further suggest that GluRs are the primary targets of autophagy-mediated degradation.**

[FIGURE REDACTED]

Figure R1.7. Endocytosis blockers abolish antidepressant-like effects of tBP on stress-induced depression-like behaviors. a, Experimental design. **b-d,** tBP decreased immobile duration in FST (**b**) and increased sucrose preference in SPT (**c**) while co-application of tBP with Dyngo-4a or TAT-GluA2$_{3Y}$ peptide abolished these effects without affecting locomotion and anxiety-like behavior (**d**). (n = 7 to 12 mice; One-way ANOVA with Uncorrected Fisher's LSD). *P < 0.05; **P < 0.01; ***P < 0.001; n.s., not significant. Error bars indicate SEM.

3) To further substantiate the hypothesis that LHb autophagy primarily targets post-synaptic membrane receptors, we performed immuno-staining to measure the co-localization of LC3 puncta and PSD95, a canonical postsynaptic marker of excitatory synapses (Sampedro et al., 1981). **Notably, 80% of LC3 puncta was co-localized with PSD95, indicating predominant postsynaptic localization of acute stress-induced autophagic flux in LHb (Figure R1.8a,b). Furthermore, immunoelectron microscopy imaging revealed that the number of AMPAR subunit A2 (GluA2)-containing autophagosomes were increased by ARS, whereas decreased by CRS (Figure R1.8c,d), suggesting that LHb autophagy primarily targets post-synaptic GluRs.**

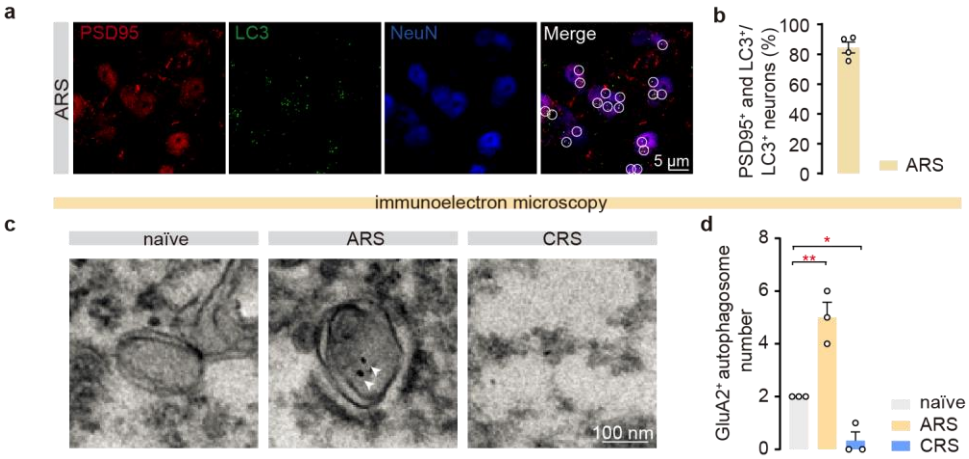

Figure R1.8. Post-synaptic localization of LHb autophagy and its targeting onto GluRs. a,b, Representative images (**a**) and quantification (**b**) showing co-localization of PSD95 and LC3 in LHb neurons of mice treated with acute restraint stress (ARS) (n = 4 mice). **c,d,** Representative immunoelectron microscopy images (**c**) and quantification (**d**) showing GluA2 immunoreactivity co-localized within autophagosomes (quantified from 15 randomly selected neurons) in LHb from

mice treated with ARS or CRS (n = 3 mice; One-way ANOVA with Uncorrected Fisher's LSD). *P < 0.05; **P < 0.01. Error bars indicate SEM.

Now we have integrated Figure R1.6 into Fig. 5l-v, Figure R1.7 into Fig. 5w-y and Extended Data Fig. 6e, and Figure R1.8 into Extended Data Fig. 13. We also add relevant text (line 347-353, 357-360, 362-381).

• The intrinsic excitability of neurons also relies on different mechanisms other than GluRs endocytosis or synaptic inputs. Previous reports already suggested different mechanisms leading to LHb hyperexcitability induced by chronic stress (Yang et al., 2018 and Cui et al., 2018). This leads to a critical question that LHb autophagy enhancer, although globally correcting neuronal, synaptic hyperexcitability and impaired synaptic plasticity, we cannot exclude the possibility that tBP rescues these properties with distinct mechanisms (other channels or receptors important for intrinsic neuronal excitability, basal synaptic transmission) or targeting different pathways other than endocytosis of GluRs considering autophagy is also expressed in non-postsynaptic area. (Kuijpers et al., 2021) (Yang et al., 2022).

To address the reviewer's insightful comment, we investigated how autophagy affects both intrinsic and synaptic properties in LHb neurons. As Kir2.1 and T-VSCC are two potential channels regulating neuronal activity and excitability (Lieberman et al., 2020; Yang et al., 2018)**, we assessed the mRNA expression levels of *Kir2.1* and *T-VSCC*. Utilizing transcriptome screening, we found that the mRNA level of *Kir2.1* in the LHb is scarce (Figure R1.9a), consistent with the very depolarized resting membrane potential (~ -50 mV) of LHb neurons. So we speculate that the contribution of Kir2.1 to neuronal excitability is low. Besides, CRS did not alter the mRNA level of T-VSCC (Figure R1.9b). Consistent with the unaltered T-VSCC current in CRS mice** (Yang et al., 2018)**, these findings suggest that LHb autophagy does not target T-VSCCs to modulate neuronal intrinsic excitability. In contrast, autophagy enhancer decreases synaptic expression levels of both NMDARs and AMPARs, without affecting GABARs (Fig. 5e-h in the manuscript). These findings suggest the selective targeting of LHb autophagy onto GluRs.**

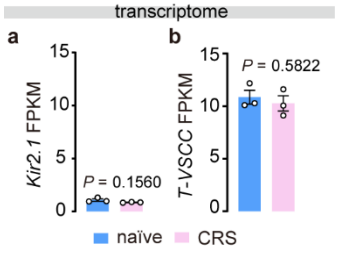

Figure R1.9. Expression levels of *T-VSCC* and *Kir2.1* channels in LHb. a,b, Quantifications of mRNA levels of *Kir2.1* (**a**) and *T-VSCC* (**b**) in mice after CRS (n = 3 mice; Unpaired *t* test). Error bars indicate SEM.

Notably, synaptic components within the LHb crucially modulate both neuronal excitability and synaptic transmission (Li et al., 2013; Meye et al., 2015; Yang et al., 2018)**. Under various aversive conditions (e.g., depression, cocaine withdrawal), increased membrane insertion of AMPARs enhances synaptic transmission, neuronal firings and excitability** (Li et al., 2013; Meye et al., 2015)**. Additionally, pharmacological activation of AMPARs or NMDARs enhances bursting activity, a pacemaker activity that promotes neuronal synchronization in the LHb of depression-like animals** (Yang et al., 2018)**.**

Consistent with these findings, our study revealed a significant increase in the expression levels of both AMPAR and NMDAR in the LHb of CRS mice, which were normalized by tBP infusion (Fig. 5a-h in the manuscript). Combined with our observation that LC3 puncta is predominantly co-localized with the postsynaptic marker PSD95 (Figure R1.8), these findings suggest that LHb autophagy primarily targets AMPAR and NMDAR to regulate both intrinsic and synaptic activity.

Now we have integrated Figure R1.9 into Extended Data Fig. 12, and added relevant text (line 337-346).

• *Authors need to carefully demonstrate whether the rescue of hyperexcitability of all properties is caused by the improvement of endocytosis of GluRs.*

As mentioned above (Figure R1.6), to investigate whether GluRs endocytosis is required for tBP to normalize LHb hyperactivity, we co-perfused tBP with Dyngo-4a in LHb slices from CRS mice. Notably, reduced excitatory synaptic transmission and spontaneous neuronal firings under tBP application (Figure R1.6a-f) were completely abolished by co-application with Dyngo-4a (Figure R1.6g-l), revealing a necessary role of receptor endocytosis for decreasing both synaptic and neuronal activity. Moreover, we co-infused tBP with Dyngo-4a or TAT-GluA2$_{3Y}$ in the LHb of CRS mice and found that antidepressant-like effects of tBP were completely abolished by either Dyngo-4a or TAT-GluA2$_{3Y}$ (Figure R1.7). Considering the fact that tBP rapidly decreases membrane expression of both AMPARs and NMDARs, these findings collectively suggest that rapid antidepressant-like actions of enhancing LHb autophagy requires GluRs endocytosis to rescue LHb hyperactivity.

Does failed induction of synaptic NMDAR-dependent LTD (LFS stimulation protocol) play a causal role in the hyperexcitability of LHb neurons and other basal synaptic transmissions that eventually lead to a behavior?

The reviewer has proposed an intriguing experiment that could potentially elucidate the causal role of NMDAR-dependent long-term

depression (LTD) deficiency in LHb hyperactivity and the depression-like state. However, it is technically challenging to directly block naturally occurring NMDAR-dependent LTD, as its temporal occurrence cannot be precisely predicted under physiological conditions. More importantly, local infusion of NMDAR antagonists (e.g., ketamine, AP5) into the LHb exerts rapid antidepressant-like effects via blocking NMDAR-dependent bursting activity (Yang et al., 2018)**. Therefore, we alternatively utilized SBI, which is a blocker of AMPK signaling and prevents low frequency stimulation (LFS)-induced LTD (Extended data Fig.19e in the current manuscript). Interestingly, we found a naturally occurred LTD-like process during stress exposure. Namely, excitatory synaptic transmission elevated by acute stress returns to basal level in the homecage 1-3 days after stress exposure (Figure R1.10). (Please also refer to the Major Point 4, where Reviewer 1 insightfully suggested to interrogate the temporal correlation between synaptic changes and the dynamics of LHb autophagy following acute stress exposure). We thus investigated the impact of SBI on excitatory neurotransmission during this LTD-like period. Intriguingly, SBI induces increased frequency and amplitude of sEPSC in the LHb, comparable to the levels immediately after acute stress (Figure R1.10), indicating a blockade of LTD-like process by SBI. At the behavioral level, local infusion of SBI into LHb during acute stress facilitated depression-like behavior (Extended Data Fig. 5e-h in the manuscript), suggesting a causal role of LTD deficit in stress susceptibility and depression-like state.**

Now we have integrated Figure R1.10 into Extended Data Fig. 5, and added relevant text (line 229-234).

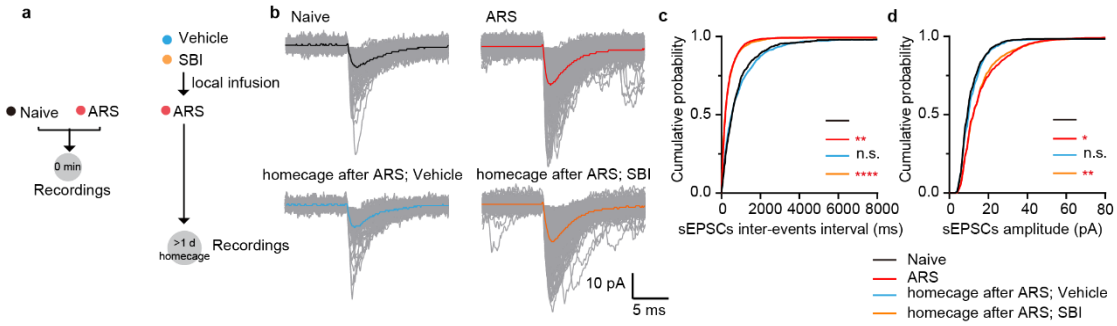

Figure R1.10. SBI prevents the long-term depression (LTD)-like process naturally occurring after acute stress. a, Experimental design**. b-d,** Representative traces (**b**) and quantifications (**c,d**) of excitatory neurotransmission immediately after ARS, or pre-treated with SBI or vehicle and 1-3 days after ARS in the homecage (n = 32 to 43, mice = 5 to 6; one-way ANOVA with Uncorrected Fisher's LSD and Dunn's multiple comparisons test). *P < 0.05; **P < 0.01; ****P<0.0001; n.s., not significant. Error bars indicate SEM.

Additionally, we successfully induced a NMDAR-independent LTD (cannabinoid-dependent LTD) in the LHb of CRS mice via perfusion of

WIN-55, an agonist of cannabinoid receptor type 1 (CB1R) (Figure R1.11a,b), and evaluated its impact on depression-like behaviors (Figure R1.11c-e). Local infusion of WIN-55 into the LHb during 14-day CRS successfully prevented stress-induced depression-like behaviors, including a reduced immobility in FST and an increased sucrose preference in SPT (Figure R1.11d,e). These findings further substantiate the causal role of LTD deficit in mediating depression-like state.

[FIGURE REDACTED]

Figure R1.11. [TEXT REDACTED]

3. It is striking that only the LHb autophagy is affected but no other regions display robust changes in the autophagy pathway. What makes LHb autophagy so distinct from other emotional-related areas of the brain? What would be the unique mechanism that makes LHb autophagy sensitive to chronic stress? If they find a specific mechanism that distinguishes LHb autophagy from other brain areas, this will strengthen the paper.

• *Some previous literature has already reported that chronic stress induces hyperexcitability of neurons in the amygdala (Rosenkranz et al., 2010) or POMC neurons in hypothalamic areas (Fang et al., 2023). I recommend authors look at other brain areas that were reported to display hyperexcitability or hyper-synaptic functions induced by chronic stress and validate whether there is a causal relationship between autophagy impairment and neuronal/synaptic homeostasis in these areas.*

We thank Reviewer 1 for these fascinating suggestions! During recent decades, substantial evidence from human studies and animal models have supported the pivotal role of LHb in negative emotion, especially depression (Hu et al., 2020; Nuno-Perez et al., 2018; Proulx et al., 2014)**. Whole-brain screening studies in rodents or zebrafish consistently identified the hyperactivity of LHb in multiple animal models of depression (Figure R1.12, adapted from** Andalman et al., 2019; Caldecott-Hazard et al., 1988**). Besides, systemic treatment of antidepressant ketamine selectively attenuates vHb (LHb homolog in zebrafish) hyperactivity (Figure R1.12b) and rescues behavioral passivity in zebrafish** (Andalman et al., 2019)**.**

[FIGURE REDACTED]

Figure R1.12. LHb or vHb shows consistent hyperactivity cross various brain regions in multiple animal models of depression. a, Glucose metabolic rates for 25 brain regions in three rat models of depression (amphetamine withdrawal-induced depression-like rats (AMP WD), α-methyl-paratyrosine-induced depression-like rats (AMPT) and chronic stress-induced depression-like rats) and controls. The lateral habenula (LHb) is the only brain region that shows consistent hyperactivity under multiple depression models. **b,** Whole-brain calcium imaging showing the ventral habenula (vHb) as the only region with hyperactivity in repeatedly shocked zebrafish. (CC, corpus callosum; vmHippo, ventral medial hippocampus; SN, substantia nigra; VTA, ventral tegmental area; RF, reticular formation; Pyr, pyriform cortex; LS, lateral septum; MFB, medial forebrain bundle; PAG, periaqueductal grey; EC-Sub, entorhinal and subicular cortex; BLA, basolateral amygdala; dHippo pyramid, pyramidal cell layer of the dorsal hippocampus; vHippo pyramid, pyramidal cell layer of the ventral hippocampus; IPN, interpeduncular nucleus; NAc, nucleus accumbens; AVT, anterior ventral nucleus of the thalamus; DTN, dorsal tegmental nucleus; LHb, lateral habenula; Cd, caudate; Cx, cortex; Med Gen, medial geniculate; dmPFC, dorsal medial prefrontal cortex; Inf Col, inferior colliculus; vHb, ventral habenula; dHb, dorsal habenula.) +, significant differences in all three depression models from controls; **, $P < 0.01$; ***, $P < 0.001$. (Modified from Caldecott-hazard et al., 1988; Andalman et al., 2019).

However, as several other brain regions may also exhibit hyperactivity under chronic stress, it becomes highly valuable to interrogate autophagy function in these regions, as suggested by Reviewer 1. Firstly, we measured p62 and Beclin-1 levels after CRS in basolateral amygdala (BLA) and arcuate nucleus (Arc), two brain regions recommended by Reviewer 1. Western blot analysis revealed that CRS impairs autophagy function in the Arc but not the BLA, as indicated by increased p62 and decreased Beclin-1 levels (Figure R1.13).

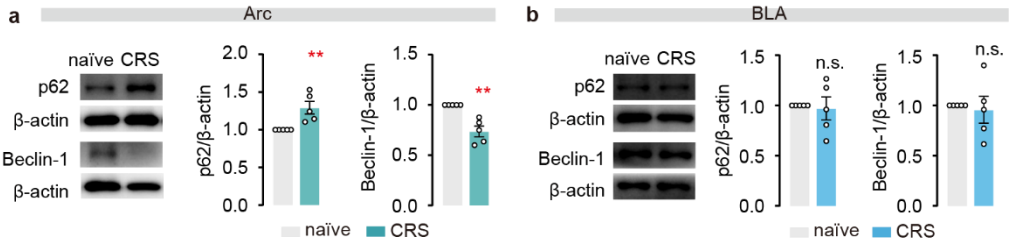

Figure R1.13. Chronic stress impairs autophagy in the arcuate nucleus (Arc) but not basolateral amygdala (BLA). a, Representative images of Western Blot and quantifications of p62 and Beclin-1 levels in the Arc after CRS (n = 5 mice; Mann-Whitney test). **b,** Representative images of Western Blot and quantifications of p62 and Beclin-1 levels in the BLA after CRS (n = 5 mice; Mann-Whitney test). **P < 0.01; n.s., not significant. Error bars indicate SEM.

We then tested whether tBP modulate neuronal activity of pyramidal neurons (PNs) in BLA slices or pro-opiomelanocortin (POMC) neurons in Arc slices. We crossed POMC-Cre mice with Ai14 mice to visualize POMC neurons, and found that tBP perfusion rapidly decreased both neuronal excitability and excitatory neurotransmission in the Arc POMC neurons (Figure R1.14a-e). In contrast, tBP did not alter any of these parameters in the BLA PNs (Figure R1.14f-j).

Notably, the neuronal excitability and excitatory synaptic transmission of Arc POMC neurons are much higher than BLA PNs, whereas comparable to LHb neurons (Figure R1.14k-m). Besides, impaired autophagy signaling was observed in the Arc, similarly to the LHb. These findings suggest that autophagy signaling is more vulnerable in more active neurons. As autophagy acts in an "on-demand" mode, active neurons require more autophagy function and facilitate the exhaustion of autophagy. It would be intriguing to investigate whether this hypothesis extends as a general principle in the brain, potentially enabling autophagy enhancers to specifically target brain regions with higher metabolic demands.

We have expanded our discussion on the "On-Demand" hypothesis in our revision as follows: "*Our findings indicate that very few brain regions may fit the profile of a prime target of autophagy enhancers: they are intrinsically active, become hyperactive in depression, and exhibit severe impairment of autophagy functions after chronic stress.*" (line 599-602)

Thank you for highlighting this potential scenario.

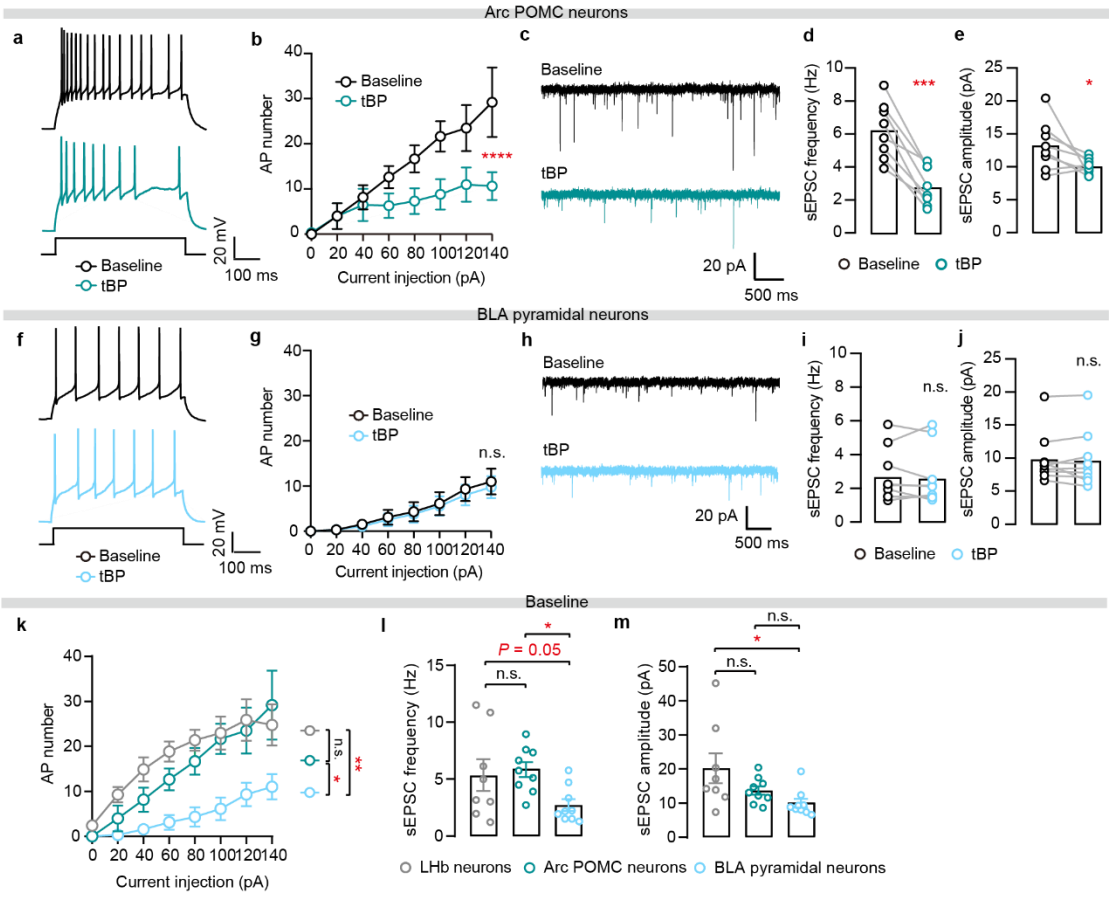

Figure R1.14. tBP attenuates neuronal and synaptic activity in Arc pro-opiomelanocortin (POMC) neurons but not BLA pyramidal neurons (PNs) of CRS mice. a,b, Representative traces (**a**) and quantification of (**b**) neuronal excitability in Arc POMC neurons treated with tBP (n = 9, mice = 4; Paired *t* test). **c-e**, Representative traces (**c**) and quantifications of (**d,e**) excitatory synaptic transmission in Arc POMC neurons treated with tBP (n = 9, mice = 4; Paired *t* test). **f,g,** Representative traces (**f**) and quantification of (**g**) neuronal excitability in BLA PNs treated with tBP (n = 10, mice = 6; Paired *t* test). **h-j,** Representative traces (**h**) and quantifications of (**i,j**) excitatory synaptic transmission in BLA PNs treated with tBP (n = 10, mice = 6; Paired *t* test). **k-m,** Comparison of neuronal excitability (**k**) and spontaneous excitatory neurotransmission (**l,m**) in LHb neurons, Arc POMC neurons and BLA PNs (n = 8 to 9 neurons, mice = 4 to 6; One-way ANOVA with Uncorrected Fisher's LSD). *P < 0.05; **P < 0.01; ***P < 0.001; ****P<0.0001; n.s., not significant. Error bars indicate SEM.

Moreover, to address Reviewer 1's concern that "*whether LHb autophagy is a common target in depression and applicable for all types of stress*", we determined the effects of tBP in mice subjected to chronic social defeat stress (CSDS). Local infusion of tBP in the LHb directly alleviates depression-like behaviors in CSDS mice, evidenced by improvements in the tail suspension test (TST), SPT, and SIT (Figure R1.15).

Now we have integrated Figure R1.15 into Fig. 4f-i and Extended Data Fig. 6d, and added relevant text (line 290-293).

[FIGURE REDACTED]

Figure R1.15. tBP rapidly alleviates chronic social defeat stress (CSDS)-induced depression-like phenotypes in mice. a, Experimental design. **b-e,** Local infusion of tBP into the LHb decreases immobile duration in tail suspension test (TST) (**b**), increased sucrose preference in SPT (**c**) and increased social interaction ratio in SIT (**d**), without affecting locomotion and anxiety-like behavior (**e**). (n = 5 to 7 mice; Unpaired *t* test and Mann-Whitney test). *P < 0.05; **P < 0.01; n.s., not significant. Error bars indicate SEM.

4. According to the authors' proposed theory, we would theoretically expect that acute stress might show totally different or even opposite physiological properties compared to chronic stress mice.

We thank Reviewer 1 for this insightful comment. Indeed, accumulating evidence suggests that acute stress induces opposite physiological profiles compared to chronic stress. For instance, chronic stress induces decreased motivation for reward which is strongly correlated with depression (Lecca et al., 2016; Li et al., 2011)**, whereas acute stress increases motivation for reward** (Zalachoras et al., 2022)**. Besides, stress has also been extensively documented to have a U-shaped effect on cognition. While acute stress enhances cognitive performance, chronic stress leads to cognitive deficits** (Brandner et al., 2022; Quaedflieg and Schwabe, 2018; Yuen et al., 2012)**.**

Now we have expanded our discussion (line 488-492).

Does acute stress have increased synaptic LTD and reduction of hyperexcitability of LHb neurons? This would be another good control experiment to test their theory regarding the temporal dynamics of LHb autophagy during the stress period.

The reviewer's suggestion is insightful and inspires us to test the temporal correlation between synaptic changes and the dynamics of LHb autophagy following exposure to acute stress. Our previous study reported that acute stress instantly increases coincident firings in the LH-LHb pathway, inducing synaptic potentiation which causally mediates depression-like state (Zheng et al., 2022)**. However, acute stress-evoked synaptic potentiation does not persist and acute stress does not elicit depression-like state** (Zheng et al., 2022)**, suggesting the existence of a LTD-like de-potentiation process which copes with acute stress. As our**

current study proposes an "on-demand" action mode of LHb autophagy in stress coping, we tested whether autophagy is required for this LTD-like process to mitigate the temporary synaptic potentiation in the LHb after acute stress. Via measuring excitatory synaptic transmission in LHb slices immediately or 1-3 days after ARS, we discovered that sEPSCs were elevated immediately after ARS, whereas returned to the baseline naïve levels 1-3 days after ARS. Notably, blockade of LHb autophagy by SBI local infusion entirely abolished the mitigation of synaptic potentiation (Figure R1.10), revealing a necessary role of LHb autophagy in the LTD-like process following acute stress.

5. Some experiments have a small number of samples. I recommend adding the number of samples (minimum should be at least over 5-6 animals for both western blots and over 7-8 animals for electrophysiology) for the following experiments.
Figure 1a-b, western blot for LHb, all groups
Figure 3j, Western blot, all groups
Figure 4 c-h, electrophysiology, all groups.
Figure 4t-w, electrophysiology, all groups
Figure 5m-r, electrophysiology, all groups

According to Reviewer 1's suggestion, we have now increased the sample numbers of the mentioned experiments. Here we listed the changes in the following table (Table R1).

Original figure panel	Original sample numbers	Revision figure panel	final sample numbers
Figure 1a	mice=3~5,3~5	**Figure 1a**	mice=5,5 or 7
Figure 1b	mice=3 or 4,4	**Figure 1b**	mice=5,5
Figure 3j	mice=3,3	**Figure 3f left**	mice=5,5
Figure 4c-h	mice=3,2,2,3; neuron=20~30	**Figure 4l-q**	mice=8,6,7,8; neuron=29~45
Figure 4t	mice=2,2; neuron=5,6	**Extended data Figure 19d**	mice=7,7; neuron=9,11
Figure 4u	mice=5; neuron=6	**Extended data Figure 19e**	mice=6; neuron=7
Figure 4v	mice=4,3,3; neuron=7,6,6	**Extended data Figure 19i**	mice=8,7,7; neuron=10,11,10
Figure 4w	mice=3,3; neuron=6,5	**Extended data Figure 19j**	mice=6,7; neuron=9,9
Figure 5m	mice=2,2; neuron=33,36	**Figure 6n**	mice=6,6; neuron=55,64
Figure 5o-p	mice=2,2; neuron=31~36	**Figure 6p-q**	mice=6,6; neuron=51~64

Figure 5q	mice=4,5; neuron=8,8	**Extended data Figure 19g**	mice=6,7; neuron=11,10
Figure 5r	mice=4; neuron=8	**Extended data Figure 19k**	mice=7; neuron=11

Table R1 | Summary of sample numbers in the original and revised manuscripts.

Minor points:

1. Is autophagy treatment long-lasting? How long do these effects last? Can prophylactic treatments before stress prevent these behaviors?

The reviewer raised an excellent point regarding the sustained and prophylactic antidepressant-like effects of autophagy enhancers. To address this critical issue, we have conducted two sets of experiments:

1) **To investigate whether enhancing LHb autophagy exerts long-lasting antidepressant-like effects, we conducted behavioral tests at various time points following tBP infusion into the LHb of CRS mice (namely, at 24 hours, 7 days, and 18-19 days post-infusion, Figure R1.16a). As depicted in Figure R1.16 below, the antidepressant-like actions of tBP on CRS mice maintain at the time points of 24 hours and 7 days post-infusion, whereas disappear 18-19 days post-infusion (Figure R1.16b-g).**
2) **To evaluate the prophylactic effects of enhancing LHb autophagy, we administered local tBP infusions prior to 1st, 5th, and 9th day of restraint during 14-day CRS and subsequently conducted behavioral assessments (Figure R1.16h). Strikingly, prophylactic tBP treatment effectively prevented depression-like behaviors after CRS (Figure R1.16i-m), characterized by reduced immobility in the FST and increased preference in the SPT, while not affecting locomotor activity and anxiety-like behavior in the open field test (OFT). To further validate tBP's prophylactic effects rather than sustained antidepressant-like effects under this dosing regimen, we conducted an additional TST 26 days after CRS (Figure R1.16h), outranging the duration of sustained antidepressant-like effects. Notably, immobile duration in the TST 26 days post-treatment of tBP remains lower than the vehicle control group, thus confirming prophylactic effects of tBP (Figure R1.16l).**

Now we have integrated these results into Extended Data Fig. 9, and added relevant text (line 296-311).

[FIGURE REDACTED]

Figure R1.16. Local infusion of tBP into the LHb exerts sustained and prophylactic antidepressant-like effects. **a,h,** Experimental designs. **b,c,** tBP decreases immobile duration in FST (**b**) and increases social interaction ratio in SIT (**c**) 24h after tBP treatment. **d,e,** tBP decreases immobile duration in TST (**d**) and increases sucrose preference in SPT (**e**) 7d after tBP treatment. **f,g,** tBP did not affect SPT (**f**) and FST (**g**) phenotypes 18-19d after tBP treatment (n = 4 to 9 mice; Unpaired *t* test and Mann Whitney test). **i-m,** Prophylactic treatment of tBP decreases immobile duration in FST and TST (**i, l**), increases sucrose preference in SPT (**j**), without affecting social interaction ratio (**k**), locomotion and anxiety-like behavior (**m**) (n = 6 to 7 mice; Unpaired *t* test). *P < 0.05; ***P < 0.001; n.s., not significant. Error bars indicate SEM.

2. What would be the merit of targeting autophagy compared to other known antidepressants? What is the advantage of targeting autophagy, and can they outperform other treatments? Appealing these points by new experiments or discussions would increase the impact and significance of this study.

Traditional antidepressants targeting monoamine system have the drawbacks of slow actions and limited efficacy. Novel antidepressants targeting NMDAR (e.g., ketamine) take rapid actions and improve efficacy, whereas still have the drawbacks of hallucinogenic side effects. In comparison, antidepressant strategy targeting autophagy system not only exerts rapid (within 30 minutes, Fig. 4a-i in the manuscript) and sustained (> 1 week, Figure R1.16a-g) antidepressant-like actions, but also has the advantage of targeting specific neural circuits and thus minimizing side effects. As illustrated in Fig. 1 in the manuscript, the impairment of autophagy under depression-like state and its enhancement by different antidepressants selectively occurs in the LHb. Indeed, we did not observe hallucination-like behaviors (e.g., head-twitch response) or altered locomotion after enhancing autophagy. Moreover, enhancing autophagy shows promise in preventing depression-like state under chronic stress (Figure R1.16h-m).

Now we have expanded discussion on this point (line 597-609).

3. For loss-of-function studies, the authors targeted Atg7 to impair autophagic functions specifically in LHb. From their extended data Figure 2, transcriptome data show a statistically significant difference for BECN1 and Atg10, which was not observed in Atg7. Is there any specific reason for targeting Atg7 but not BECN1 or Atg10? The authors provided evidence of involvement of Atg7 in their introduction (line 85) with references (30-32) but this was mainly from human patients, while the transcriptomic analysis in this study does not show any change.

We appreciate the reviewer's insightful comment. All of *Atg7*, *Atg5*, *BECN1* (gene name of beclin1), and *Atg10* are fundamental elements involved in autophagy process. Due to the limitations of viral and transgenic tools, we initially focused on manipulating *Atg7* using a Cre-dependent KO strategy in the *Atg7*$^{flox/flox}$ mouse line or employing an shRNA-*Atg7* virus. Now we have extended the toolkit of autophagy manipulations via developing viral shRNA targeting *BECN1* or *Atg5*.

By locally transfecting LHb neurons with shRNA-*BECN1* (Figure R1.17a), we directly induced depression-like behaviors in naïve mice, including increased immobility in the FST and reduced social interaction ratio in the SIT (Figure R1.17b,c). Consistently, local transfection of LHb neurons with shRNA-*Atg5* (Figure R1.17d) also elicits depression-like behaviors in naïve mice, characterized by increased immobility in the FST, decreased sucrose preference in the SPT, and a statistical trend of reduced social interaction ratio in the SIT (Figure R1.17e-g).

Now we integrate these results into Extended Data Fig. 16d-j, and add relevant text (line 398-402).

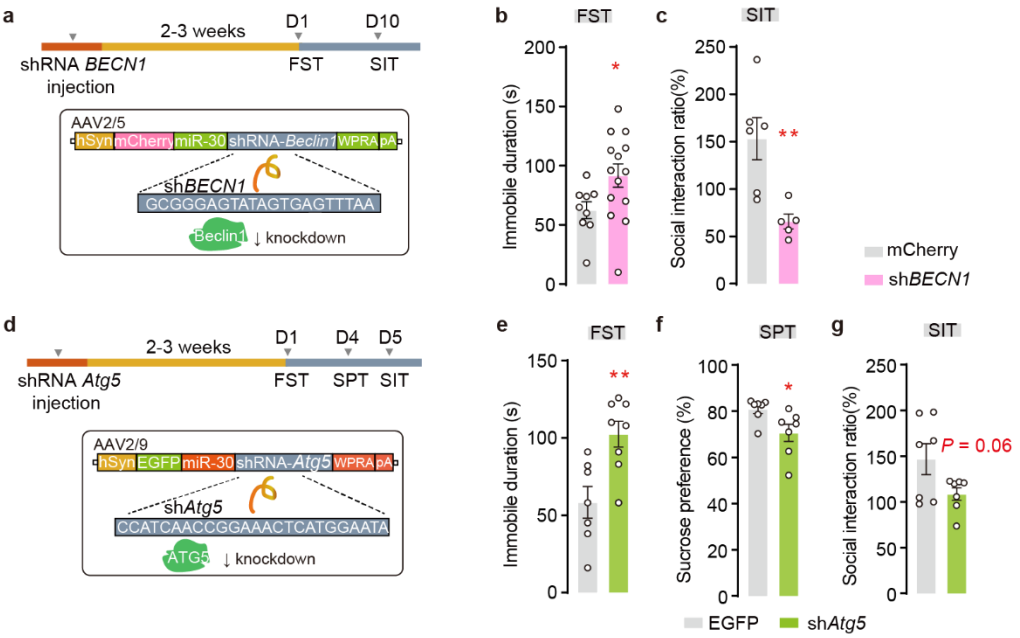

Figure R1.17. Genetic ablation of autophagy-related genes (*Atg5* or *BECN1*) directly leads to depression-like phenotypes. **a,d,** Experimental designs. **b,c,** Conditional knock down of *BECN1*

with shRNA-*BECN1* directly leads to an increased immobile duration in FST (**b**) and a decreased social interaction ratio in SIT (**c**) (n = 5 to 14 mice; Unpaired *t* test and Mann Whitney test). **e-g,** Conditional knock down of *Atg5* with shRNA-*Atg5* directly leads to an increased immobile duration in FST (**e**), a decreased sucrose preference in SPT (**f**), and a decreased trend of social interaction ratio in SIT (**g**) (n = 7 to 8 mice; Unpaired *t* test). *P < 0.05; **P < 0.01; n.s., not significant. Error bars indicate SEM.

4. It is interesting that Atg7 deficiency shows some elevated levels of GluRs expression (Extended Fig 14), along with abnormal synaptic plasticity (Figure 5q & Figure 15), Is there any possibility of deficiency of Atg7 causing other impairments other than autophagy or endocytosis of GluRs which may lead to these defects? It is also noteworthy that there was a report of Atg7 regulating different cell types of spiny neurons in both autophagy-dependent and independent manner (Lieberman et al., 2020). In this study, there is also a change in sIPSC transmissions (Figure 5p) which may raise the possibility of the effects of Atg7 deficiency other than autophagy-dependent GluRs endocytosis. Similar to point No.2, I recommend authors perform experiments to support that these impairments are caused by dysfunctional autophagy-dependent endocytosis.

We appreciate the reviewer's thoughtful comments. Previous studies indicated that *Atg7* deficiency plays varying roles in different types of striatal neurons. *Atg7* deficiency induces synaptic deficits in dopamine D2 receptor-expressing medium spiny neurons (D1-MSNs), while it increases intrinsic neuronal excitability in dopamine D2 receptor expressing medium spiny neurons (D2-MSNs) by reducing the function of Kir2.1 channels (Lieberman et al., 2020)**. However, consistent with the very depolarized resting membrane potential (~-50 mV) of LHb neurons, we found that the expression level of *Kir2.1* in the LHb is scarce (Figure R1.9a). We therefore speculated that the contribution of Kir2.1 to neuronal excitability is low.**

To determine whether *Atg7*$^{LHb-/-}$ induced neuronal dysfunction is specifically caused by autophagy deficit rather than other *Atg7*-related mechanisms, we applied shRNA knock-down strategy onto *Atg5*, another core gene of autophagy, selectively in the LHb. Notably, shRNA-*Atg5* in the LHb induces neuronal phenotypes similar to *Atg7*$^{LHb-/-}$, including enhanced excitatory synaptic transmission and increased bursting activity (Figure R1.18a-c and f). Collectively, these findings suggest autophagy-specific mechanisms in *Atg7*$^{LHb-/-}$ induced neuronal dysfunction.

Moreover, acute treatment of tBP does not alter sIPSCs (Fig. 4q and 5o,p in the manuscript). In contrast, *Atg7* knock-out and *Atg5*-shRNA, whose effects are chronic and contain complex compensatory processes, both decrease sIPSCs (Fig. 6q in the manuscript and Figure R1.18d,e). These results suggest that autophagy-related degradation does not

directly target GABA receptors, whereas autophagy deficit-induced compensatory effects might be involved.

Now we integrate Figure R1.18 into Extended Data Fig. 17 and added relevant text (line 419-424).

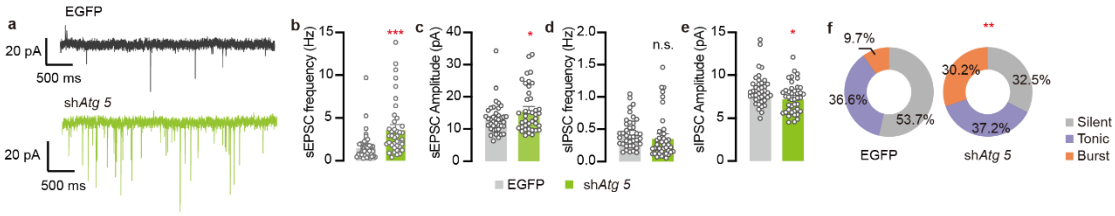

Figure R1.18. Genetic ablation of autophagy-related gene *Atg5* directly leads to LHb hyperactivity. **a-e,** Representative traces (**a**) and quantifications (**b-e**) of spontaneous neurotransmission in LHb neurons with *Atg5* deficiency (n = 41 to 42 neurons, mice = 5; Unpaired *t* test). **f**, Pie charts illustrating the percentage of three types of LHb neurons with *Atg5* knock-down and its EGFP controls (n = 41 to 42 neurons, mice = 5; Chi-square test). *P < 0.05; **P < 0.01; ***P < 0.001; n.s., not significant. Error bars indicate SEM.

5. To provide strong evidence of change in autophagic activity, I suggest authors use one additional representative marker for autophagy activity (such as LC3-II and Beclin-1) along with the p62 in all western blot experiments.

We acknowledge Reviewer 1's thoroughness and have included Beclin-1 as an additional marker in all western blot experiments (Figure R1.19).

For the immunostaining data, quantitative data would be much stronger if they performed LC3-II staining with other dendritic markers or spines to show that autophagic activity controls synaptic properties related to Figure 2e.

In an effort to elucidate the synaptic localization of autophagic flux, we performed immunostaining experiments and observed the co-localization of LC3-II with a postsynaptic marker PSD95 (please refer to Major Point 2, Figure R1.8).

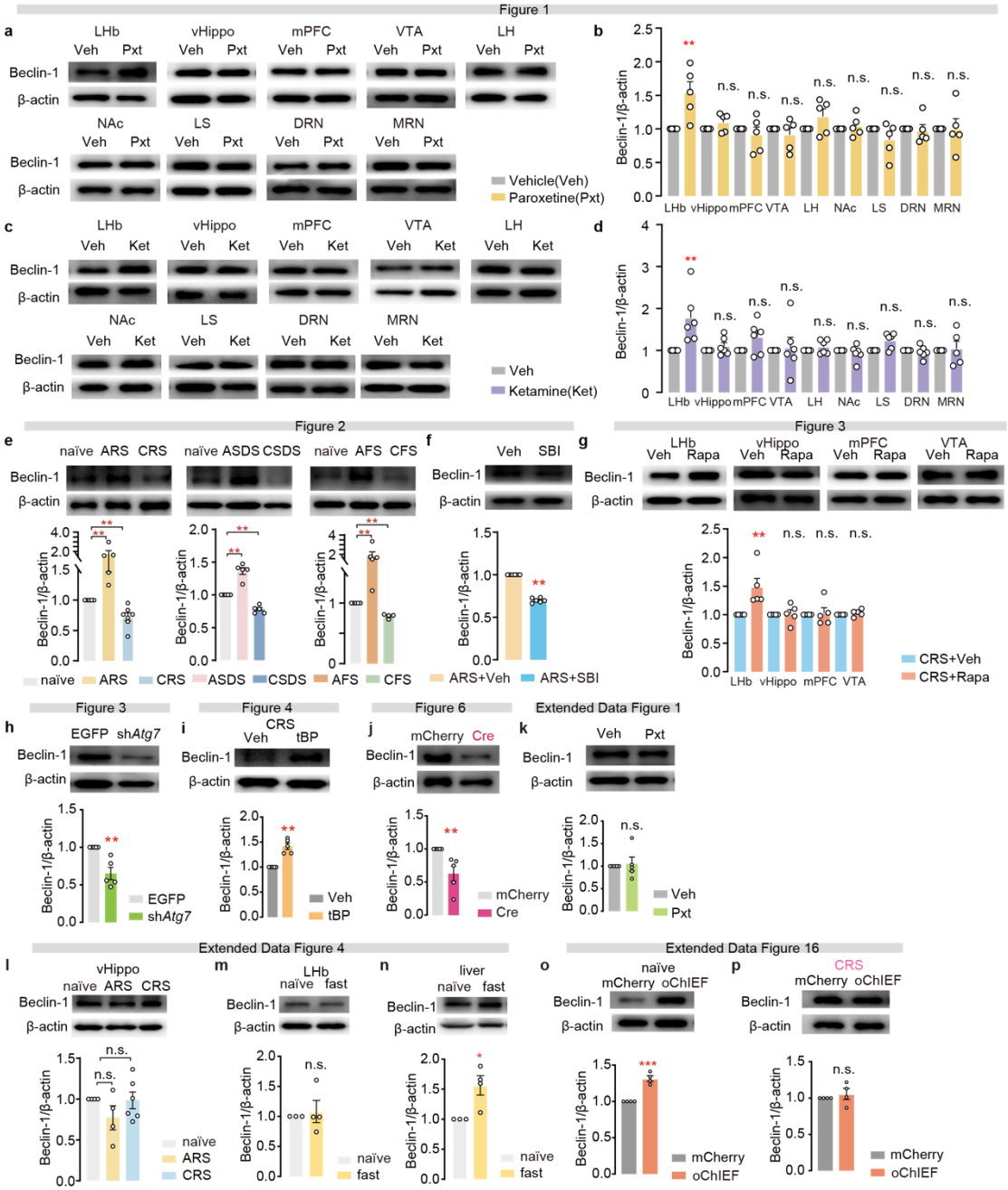

Figure R1.19. Western Blot results of Beclin-1 under various conditions.

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Referee #2 (Remarks to the Author):

In this study, Yang and colleagues investigated how brain autophagy is engaged in stress response in acute and chronic stress models in mice. They found that acute stress activates autophagy, whereas chronic stress suppresses autophagy selectively in the lateral habenula (LHb). Different antidepressants, including paroxetine and ketamine, both can restore autophagy function specifically in LHb, although they work through distinct mechanisms. They further found that genetic attenuation of LHb neuronal autophagy increases stress susceptibility, whereas enhancing LHb autophagy exerts rapid "antidepressant" effects. Mechanistically, they found that LHb autophagy influences LHb neuronal activity, synaptic transmission and plasticity, and regulates glutamate receptor endocytosis.

Overall, this study is quite comprehensive. It provides some compelling evidence that LHb autophagy plays an important role in LHb function and animal stress coping, which are novel mechanisms. Nevertheless, I have some comments and questions that need to be addressed.

We appreciate Reviewer 2's positive evaluation and constructive suggestions. Below, we address each of them accordingly.

First of all, I have some general comments and questions that the authors should discuss or reconcile.

1. The study relies on forced swim test (FST) and sucrose preference test (SPT) as redouts of "depression" in mice. These are "established" and "traditional" assays, which have been very useful and led to a great deal of knowledge in the field of stress research. However, their limitations are also obvious, and currently there has been some consensus in the field that such tests in animals can hardly be related to depression in humans. FST is not comparable to human behavior. Even for SPT, it does not parallel human anhedonia. For example, a person, even if depressed, will still prefer a delicious meal to a boring one. Therefore, some paradigm shift is needed. To a minimum, the authors should use "stress coping" or its maladaptation other than "antidepressant" or depression throughout the paper.

Thank you for raising this essential issue. We have now changed the term "antidepressant" into "stress-coping" or "antidepressant-like", and "depression" into "depression-like", "maladaptation of chronic stress" or "maladaptive behaviors" throughout the manuscript.

In an effort to promote paradigm shift of behavioral assays and identify depression-like behaviors relevant to humans, we explored additional behavioral phenotypes and observed social withdrawal towards the novel conspecifics in C57 mice after experiencing chronic restraint stress (CRS) (Figure R2.1a,b). This phenotype was reversed by the autophagy enhancer TAT-beclin 1 peptide (tBP) in both CRS and chronic social defeat stress (CSDS) models of depression (Figure R2.1c-f, see also Figure R1.15 in response to Reviewer 1). We have extensively tested this

social avoidance phenotype towards C57 conspecifics under various conditions (please refer to Figures R1.1, R1.2, R1.15, R1.16, and R1.17 in response to Reviewer 1 for these data). We hope you appreciate our efforts to enhance the relevance and validity of mouse models in the field of depression.

[FIGURE REDACTED]

Figure R2.1. Chronic stress induces conspecific social avoidance which is reversed by enhancing lateral habenula (LHb) autophagy. a,b, Experimental schematic of social interaction test (SIT) (**a**) and quantification of social interaction ratio (**b**) in chronic restraint stress (CRS) and naïve mice (n = 5 mice; Unpaired *t* test). **c-f,** Local infusion of tBP successfully increased social interaction ratio in CRS (**c,d**) or chronic social defeat stress (CSDS) (**e,f**) mice (n = 5 to 9 mice; Mann-Whitney test). **P < 0.01; ***P < 0.001. Error bars indicate SEM.

In addition, we observed increased corticosterone, a well-established biomarker relevant to clinical depressive disorders and chronic stress-induced maladaptation (McEwen et al., 2015)**, in both CRS and *Atg7*$^{LHb-/-}$ animal models of depression (Figure R2.2). These results suggest a consistent endophenotype of depression in these models.**

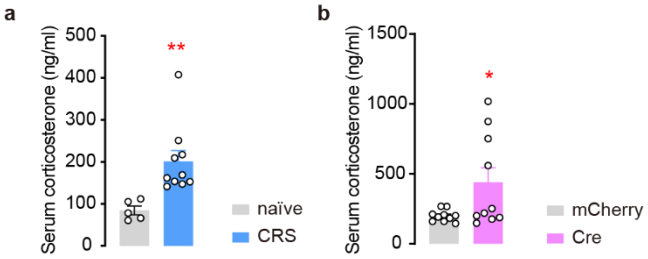

Figure R2.2. Corticosterone levels are increased in CRS or *Atg7*$^{LHb-/-}$ mice. a, Corticosterone levels in naïve and CRS mice (n = 5 to 10 mice; Unpaired *t* test). **b,** Corticosterone levels in *Atg7*$^{LHb+/+}$ and *Atg7*$^{LHb-/-}$ mice (n = 5 to 10 mice; Unpaired *t* test). *P < 0.05; **P < 0.01; n.s., not significant. Error bars indicate SEM.

2. Lateral habenula (LHb) is only one of the brain areas activated by different stressors. There are many other brain areas that are hyperactive in response to stress. Given that autophagy appears to be a general mechanism regulating synaptic function in different neuronal types, why only LHb neuronal function is regulated by autophagy during stress?

We thank Reviewer 2 for these insightful comments. During recent decades, substantial evidence from human studies and animal models have supported the pivotal role of lateral habenula (LHb) in negative emotion, especially depression (Hu et al., 2020; Nuno-Perez et al., 2018; Proulx et al., 2014)**. Whole-brain screening studies in rodents or zebrafish consistently identified the hyperactivity of LHb in multiple animal models of depression (Figure R2.3, adapted from** Andalman et al., 2019; Caldecott-Hazard et al., 1988**). Besides, systemic application of antidepressant ketamine selectively attenuates ventral habenula (vHb, LHb homolog in zebrafish) hyperactivity (Figure R2.3b) and rescues behavioral passivity in zebrafish** (Andalman et al., 2019)**.**

[FIGURE REDACTED]

Figure R2.3. LHb or vHb shows consistent hyperactivity cross various brain regions in multiple animal models of depression. a, Glucose metabolic rates for 25 brain regions in three rat models of depression (amphetamine withdrawal-induced depression-like rats (AMP WD), α-methyl-paratyrosine-induced depression-like rats (AMPT) and chronic stress-induced depression-like rats) and controls. The LHb is the only brain region that shows consistent hyperactivity under multiple depression models. **b,** Whole-brain calcium imaging showing the vHb as the only region with hyperactivity in repeatedly shocked zebrafish. (CC, corpus callosum; vmHippo, ventral medial hippocampus; SN, substantia nigra; VTA, ventral tegmental area; RF, reticular formation; Pyr, pyriform cortex; LS, lateral septum; MFB, medial forebrain bundle; PAG, periaqueductal grey; EC-

Sub, entorhinal and subicular cortex; BLA, basolateral amygdala; dHippo pyramid, pyramidal cell layer of the dorsal hippocampus; vHippo pyramid, pyramidal cell layer of the ventral hippocampus; IPN, interpeduncular nucleus; NAc, nucleus accumbens; AVT, anterior ventral nucleus of the thalamus; DTN, dorsal tegmental nucleus; LHb, lateral habenula; Cd, caudate; Cx, cortex; Med Gen, medial geniculate; dmPFC, dorsal medial prefrontal cortex; Inf Col, inferior colliculus; vHb, ventral habenula; dHb, dorsal habenula.) +, significant differences in all three depression models from controls; **, P < 0.01; ***, P < 0.001. (Modified from Caldecott-hazard et al., 1988; Andalman et al., 2019).

However, we fully acknowledge Reviewer 2's point that while we emphasize the alteration of LHb autophagy function in both chronic stress and antidepressant treatments, we do not discount the possibility that simultaneous changes may occur in other brain regions, either independently or as a consequence of changes in the LHb, during maladaptation and stress-coping conditions. Similar concerns were also raised by Reviewer 1 (Major point 3 in response to Reviewer 1).

In light of Reviewer 1's suggestion, we further examined autophagy functions in two additional brain regions implicated in stress-induced maladaptation and hyperactivity: the basolateral amygdala (BLA) and arcuate nucleus (Arc). Our Western blot analysis revealed that chronic stress did not alter autophagy in the BLA, whereas it impaired autophagy in the Arc, as evidenced by increased p62 and decreased Beclin-1 levels (Figure R2.4).

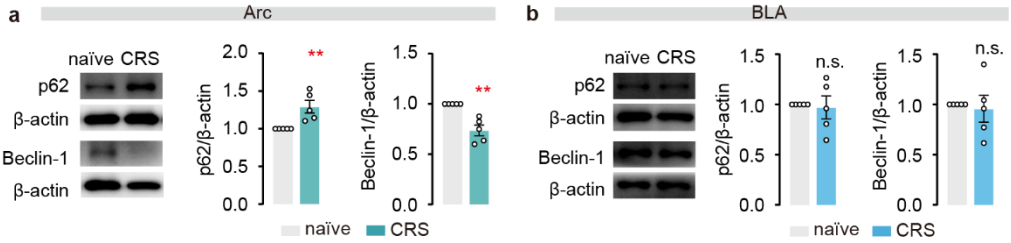

Figure R2.4. Chronic stress impairs autophagy in the arcuate nucleus (Arc) but not basolateral amygdala (BLA). a, Representative images of Western Blot and quantifications of p62 and Beclin levels in the Arc after CRS (n = 5 mice; Mann-Whitney test). **b,** Representative images of Western Blot and quantifications of p62 and Beclin levels in the BLA after CRS (n = 5 mice; Mann-Whitney test). **P < 0.01; n.s., not significant. Error bars indicate SEM.

We then tested whether autophagy enhancer modulates neuronal activity of pyramidal neurons (PNs) in BLA slices or pro-opiomelanocortin (POMC) neurons in Arc slices (Figure R2.5). We crossed POMC-Cre mice with Ai14 mice to visualize POMC neurons, and found that tBP perfusion rapidly decreased both neuronal excitability and excitatory neurotransmission in the Arc POMC neurons (Figure R2.5a-e). In contrast, tBP did not alter any of these parameters in the BLA PNs (Figure R2.5f-j).

Notably, the neuronal excitability and excitatory synaptic transmission of Arc POMC neurons are much higher than BLA PNs, whereas comparable to LHb neurons (Figure R2.5k-m). Besides, impaired autophagy signaling was observed in the Arc, similarly to the LHb. These findings suggest that autophagy signaling is more vulnerable in more active neurons. As autophagy acts in an on-demand mode, active neurons require more autophagy function and facilitate the exhaustion of autophagy. It would be intriguing to investigate whether this hypothesis extends as a general principle in the brain, potentially enabling autophagy enhancers to specifically target brain regions with higher metabolic demands.

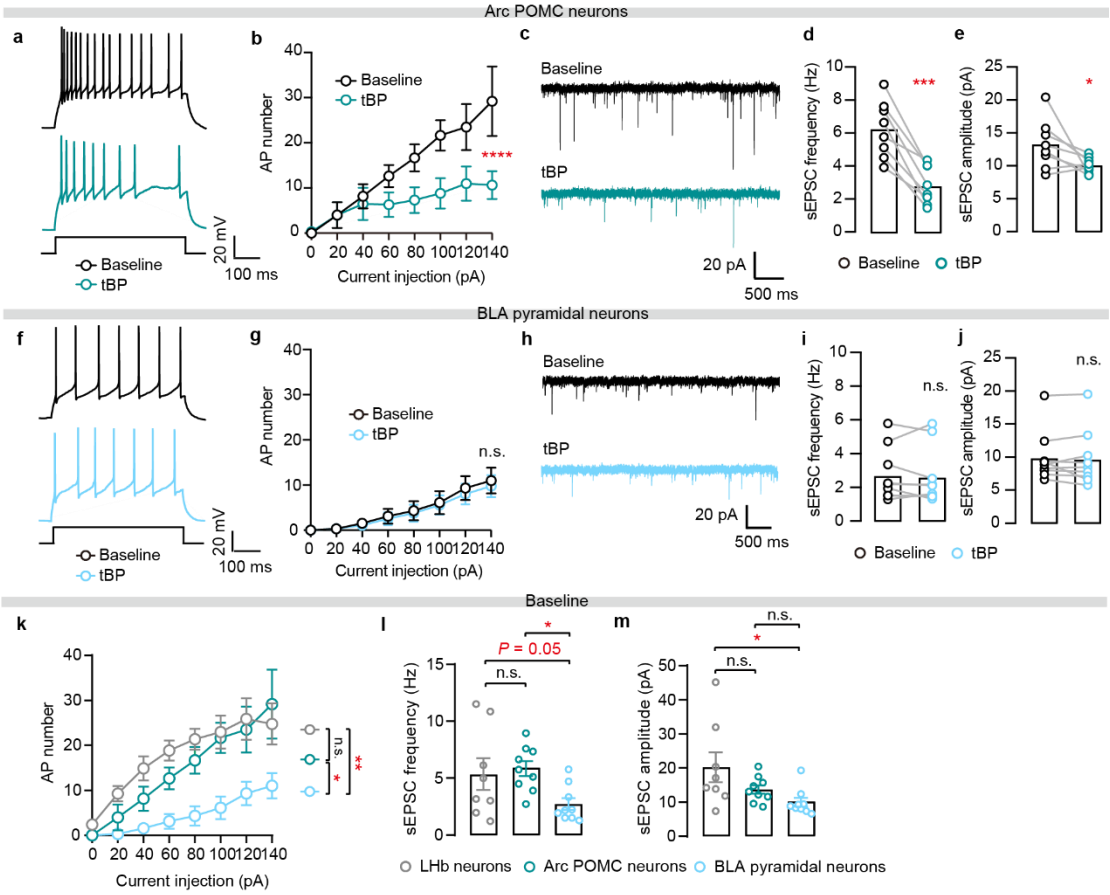

Figure R2.5. tBP attenuates neuronal and synaptic activity in hypothalamic Pro-opiomelanocortin (POMC) neurons but not BLA pyramidal neurons (PNs) of CRS mice. a,b, Representative traces (**a**) and quantification of (**b**) neuronal excitability in Arc POMC neurons treated with tBP (n = 9, mice = 4; Paired *t* test). **c-e,** Representative traces (**c**) and quantifications of (**d,e**) excitatory synaptic transmission in Arc POMC neurons treated with tBP (n = 9, mice = 4; Paired *t* test). **f,g,** Representative traces (**f**) and quantification of (**g**) neuronal excitability in BLA PNs treated with tBP (n = 10, mice = 6; Paired *t* test). **h-j,** Representative traces (**h**) and quantifications of (**i,j**) excitatory synaptic transmission in BLA PNs treated with tBP (n = 10, mice = 6; Paired *t* test). **k-m,** Comparison of neuronal excitability (**k**) and spontaneous excitatory neurotransmission (**l,m**) in LHb neurons, Arc POMC neurons and BLA PNs (n = 8 to 9 neurons,

mice = 4 to 6; One-way ANOVA with Uncorrected Fisher's LSD). *P < 0.05; **P < 0.01; ***P < 0.001; ****P<0.0001; n.s., not significant. Error bars indicate SEM.

We have now discussed our "On-Demand" hypothesis in our revision as follow: "*Our findings indicate that very few brain regions may fit the profile of a prime target of autophagy enhancers: they are intrinsically active, become hyperactive in depression, and exhibit severe impairment of autophagy functions after chronic stress.*" (line 599-602)

Thank you for highlighting this potential scenario.

3. Why different antidepressants, which act through very different mechanisms, similarly activate autophagy?

We appreciate both Reviewers for raising this important question.

Various antidepressants engage Akt signaling pathways (Duman et al., 2019)**, which might affect autophagy process. Previous studies unraveled that reduced phosphorylation of Akt inhibits the mammalian target of rapamycin (mTOR) pathway, leading to activation of Beclin-1 and subsequent autophagic process** (Gassen et al., 2014; Wang et al., 2012)**. In our study, we indeed observed that ketamine at antidepressant dosage decreased the levels of phosphorylated Akt (p-Akt), phosphorylated mTOR (p-mTOR) and p62, whereas increased the level of Beclin-1 in the LHb of CRS mice (Figure R2.6a-c), indicating enhanced autophagy signaling via inhibited Akt signaling. Similar mechanisms in the LHb were observed with chronic paroxetine treatment in CRS mice (Figure R2.6d-f).**

Importantly, the effects of different antidepressants on Akt-mTOR signaling depend on brain regions. Previous studies have revealed that activation of brain derived neurotrophic factor (BDNF)-Akt-mTOR signaling in the medial prefrontal cortex (mPFC) or hippocampus is crucial for the antidepressant actions of various medications, including selective serotonin reuptake inhibitors (SSRIs), ketamine, and psychedelics (Autry et al., 2011; Casarotto et al., 2021; Li et al., 2010; Moliner et al., 2023)**. In contrast, activation of BDNF signaling in the nucleus accumbens (NAc) or LHb causally mediates depression-like behaviors** (Koo et al., 2019; Lei et al., 2020)**. As BDNF activation strongly activates Akt-mTOR signaling and thus inhibits autophagy, these findings are consistent with ours in the LHb. To align with previous studies and gain quality control of our experiments, we selected the mPFC as a positive control region for measuring ketamine's effects. In line with previous reports, we demonstrated that ketamine activated BDNF-mTOR signaling in the mPFC (Figure R2.6g-h).**

We have now incorporated Figure R2.6 into Extended Data Fig. 7e-h, and added relevant text in the Results (line 274-280) and Discussion (line 578-596).

[FIGURE REDACTED]

Figure R2.6. Antidepressants commonly inhibit Akt-mTOR signaling and activates autophagy in the LHb. a-c, Ketamine treatment (*i.p.*) decreased the phosphorylated Akt (p-Akt), phosphorylated mTOR (p-mTOR) and p62 levels, and increased Beclin-1 level in LHb (n = 5 to 6 mice; Mann-Whitney test). **d-f**, Paroxetine treatment (*i.p.*, 7d) decreased p-Akt, p-mTOR and p62 levels, and increased Beclin-1 level in LHb (n =5 to 7 mice; Mann-Whitney test). **g-h,** Ketamine treatment (*i.p.*) increased BDNF and p-mTOR levels in mPFC (n =5 mice; Mann-Whitney test). *P < 0.05; **P < 0.01. Error bars indicate SEM.

4. Ketamine's antidepressant effects have been attributed to its activation of the mTOR pathway (e.g., Li, …, Duman, 2010, Science), but here the authors show that Ketamine suppresses mTOR. How to reconcile with the literature?

Thank you for this intriguing question. Accumulating evidence has indicated that the effects of ketamine on mTOR signaling depend on brain regions. Previous studies revealed that activation of BDNF-Akt-mTOR signaling in the mPFC or hippocampus mediates antidepressant-like actions of ketamine (Autry et al., 2011; Casarotto et al., 2021; Li et al., 2010)**. However, activation of BDNF which presumably activates mTOR in the NAc or LHb causally induces depression-like behaviors** (Koo et al., 2019; Lei et al., 2020)**. Consistent with these findings, we observed that ketamine inhibits Akt-mTOR signaling and activates autophagy in the LHb (Figure R2.6), eliciting antidepressant-like effects.**

Intriguingly, recent studies revealed that systemic blockade of mTOR via rapamycin in depressed patients largely strengthened antidepressant efficacy of ketamine (Abdallah et al., 2020)**, indicating the potential role of autophagy. Besides, previous studies in multiple animal models also discovered the antidepressant-like effects of mTOR inhibition via rapamycin** (Cleary et al., 2008; Zhou et al., 2013)**. Now we have expanded our discussion (line 578-596).**

Specific questions:

1. The authors report that activation of LHb autophagy temporally coincides with the onset of antidepressant effects of paroxetine in chronic stressed mice. Are there studies showing that paroxetine decreases LHb activity in "depressed" mice following similar duration of treatment? If so, these studies need to be cited and commented. If not, the authors need to test it experimentally.

This suggestion is highly insightful. We have investigated the impact of both acute and chronic paroxetine treatment on LHb neuronal activity in CRS mice. Our findings indicate that a single day of acute paroxetine treatment does not alter either the intrinsic or synaptic activity in LHb neurons of CRS mice. However, 7 days of chronic paroxetine treatment significantly reduces excitatory synaptic transmission and bursting activity in LHb neurons of CRS mice (this is also detailed in our response to Reviewer 1, Figure R1.3).

Now we have integrated these results into Extended Data Fig. 1d-i, and added relevant text (line 137-139).

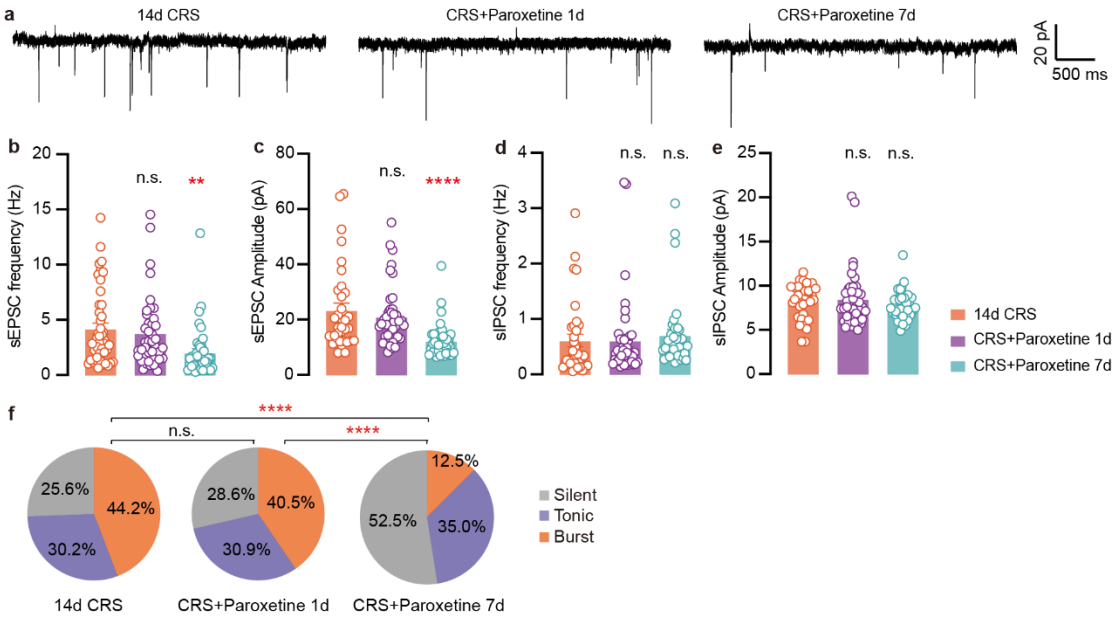

Figure. R2.7. Chronic but not acute administration of paroxetine normalizes LHb neuronal hyperactivity. a-e, Representative traces (**a**) and quantifications (**b-e**) of excitatory synaptic activity under 1-day or 7-day *i.p.* injection of paroxetine in CRS mice (n = 40 to 44, mice = 5; One way-ANOVA with Dunnett's multiple comparisons test). **f**, Pie charts illustrating spontaneous firing activity of LHb neurons under 1-day or 7-day *i.p.* injection of paroxetine in CRS mice (n = 40 to 44, mice = 5; Chi-square test). **P < 0.01; ****P<0.0001; n.s., not significant. Error bars indicate SEM.

2. mRNA sequencing results show significantly decreased expression levels of genes involved in autophagy activation processes (e.g., BECN1 (gene name of beclin-1) and

autophagy-related gene Atg10) (Extended Data Fig. 2a,b1,b2), as well as decreased tendencies in Atg4d, Atg12, Atg7, Atg5 and LC3 (light chain 3) (Extended Data Fig. 2b3-b7). There were also increased tendency of expression level of mTOR (Extended Data Fig. 2b9). These results need to be validated, for example via qPCR.

Thank you for the suggestion. We have now performed qPCR experiments to validate the mRNA sequencing data (Figure R2.8). We observed consistent results with mRNA sequencing data.

Now we have integrated these results into Extended Data Fig. 2d, and added relevant text (line 164-165).

[FIGURE REDACTED]

Figure R2.8. qPCR validations of CRS-altered mRNA levels of autophagy-related genes in the LHb. a, Experimental design. **b1-b9,** Quantifications of qPCR-measured mRNA expression levels of autophagy-related genes after CRS. (n = 6 mice; Mann-Whitney test). *P < 0.05; **P < 0.01. n.s., not significant. Error bars indicate SEM.

3. Related to #2 above: Fig. 1d shows that LHb and several other brain areas have similar downregulation of genes involved in endocytosis, protein digestion and absorption, and synaptic vesicle cycle. These results seem to be inconsistent with the idea that LHb phagocytosis is selectively affected. The authors need to reconcile this.

The KEGG (Kyoto Encyclopedia of Genes and Genomes) pathway is a compilation of genes and proteins found in complete genomes of cellular organisms, derived from publicly available databases. The manually curated pathway maps depict our understanding of molecular interactions and relationships involved in various biological processes. While each KEGG pathway describes distinct mechanistic processes, some processes are interconnected or overlapped, whereas others do not coincide at all. For instance, both autophagy and ubiquitin-proteasome pathways serve as major intracellular protein degradation systems. Although they are initiated and dependent on different molecules, they share common downstream players, including genes involved in endocytosis and protein digestion and absorption (Cohen-Kaplan et al., 2016)**.**

In Fig. 1d, we have demonstrated selective impairment of macroautophagy in the LHb under the condition of chronic stress, accompanied with significant downregulation of other pathways relevant

to autophagy downstream signaling. However, several other brain regions exhibit similar downregulation of genes associated with endocytosis, protein digestion and absorption, and the synaptic vesicle cycle, without concurrent alterations in the autophagy pathway. This suggests that chronic stress impairs these pathways as well, but their upstream signaling mechanisms are independent of autophagy. For example, in the ventral hippocampus (vHippo), we observed impaired ubiquitin signaling (Figure R2.9), which also affects pathways like protein digestion and absorption (Fig. 1d4 in the manuscript), serving as a reference brain region in our study.

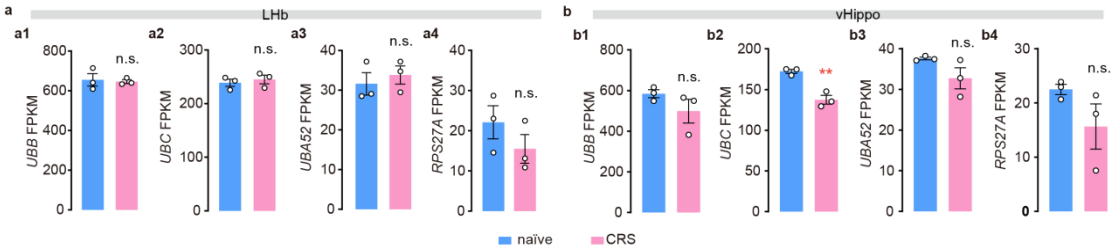

Figure R2.9. Transcriptome data of ubiquitin-related mRNA expression levels. a,b, Bar graphs showing ubiquitin-related mRNA expression levels in the LHb (**a1-a4**) and vHippo (**b1-b4**) from CRS and naïve mice (n = 3 mice; Unpaired *t* test). **P < 0.01; n.s., not significant. Error bars indicate SEM.

4. Related to #2 above: does acute stress induce changes in gene expression opposite to those induced by chronic stress? In other words, does acute stress selectively increase LHb autophagy at the transcriptomic level? This should be the case, if indeed that acute stress activates, whereas chronic stress suppresses autophagy selectively in the LHb.

We agree with Reviewer 2 that it would be a crucial experiment to investigate whether acute stress activates LHb autophagy at the transcriptomic level. To address this, we compared the transcriptomic profiles of naïve mice with those subjected to acute stress (2 hours of restraint). Using KEGG enrichment analysis, we observed that acute stress demonstrated a trend towards activating autophagy signaling in the LHb (Figure R2.10).

[FIGURE REDACTED]

Figure R2.10. [TEXT REDACTED]

5. Are the RNAseq data contaminated by glia cells?

We appreciate the reviewer's insightful comments. To address this concern, we have conducted single-cell sequencing in the LHb, and discovered a statistical trend (p=0.06) of neuron-specific impairment of autophagy signaling by CRS. These results suggest that the bulk RNA sequencing data is not contaminated by glial cells (Figure R2.11).

Now we have integrated these results into Extended Data Fig. 2a, and added relevant text (line 152-154).

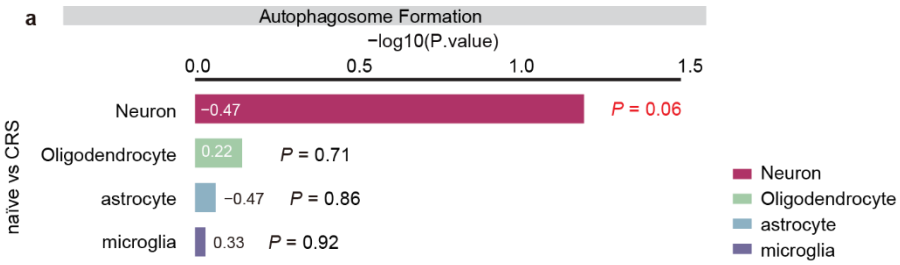

Figure R2.11. Single-cell transcriptomic data reveals neuron-specific autophagy impairment by chronic stress. a, Single-cell sequencing showing neuronal and glial autophagosome formation pathway in naïve vs. CRS mice (n = 3 mice, Gene set variation analysis).

6. In chronically stressed mice, LHb neurons show increased bust and overall spontaneous activity. Are these changes due to changes in intrinsic excitability or synaptic inputs?

Thank you for raising this intriguing question. Actually, intrinsic excitability and synaptic inputs both contribute to the increased LHb burst firing and overall spontaneous activity. Our previous studies revealed that overexpression of astroglial Kir4.1 induces neuronal hyperpolarization and thus promotes the recruitment of T-type calcium channels (T-VSCCs), eventually enhancing LHb burst firings (Cui et al., 2018). As the activation level of T-VSCCs largely modulates neuronal intrinsic excitability, LHb hyperactivity mediated by the neuron-glia interface is attributed to changes in intrinsic excitability.

CRS increases synaptic expression of both AMPARs and NMDARs in the LHb (Fig. 5a-d in the manuscript). Intriguingly, these synaptic receptors crucially modulate both neuronal excitability and synaptic transmission in the LHb (Li et al., 2013; Meye et al., 2015; Yang et al., 2018)**. Under various aversive conditions (e.g., depression, cocaine withdrawal), increased membrane insertion of AMPARs enhances synaptic transmission, neuronal firing rate and excitability** (Li et al., 2013; Meye et al., 2015)**. Additionally, pharmacological activation of AMPARs or NMDARs both enhances bursting activity, a pacemaker activity that promotes theta wave synchronization in the LHb of depression-like**

animals (Yang et al., 2018)**. Our recent studies discovered that chronic stress selectively enhances excitatory inputs from lateral hypothalamus (LH) to LHb, which presumably contributes to the increased LHb burst firing and overall spontaneous activity** (Zheng et al., 2022)**.**

To investigate whether LH inputs contribute to persistent hyperactivity of LHb, we simulated chronic stress by phasic optogenetic stimulation of LH-LHb pathway (40 Hz, 2 hours per day) for 14 days (Zheng et al., 2022)**. We then examined whether this purely synaptic manipulation led to lasting changes in synaptic transmission and bursting activity in LHb neurons. Our results showed that 14 days of photostimulation significantly enhanced excitatory synaptic transmission and increased the proportion of bursting neurons in the LHb (Figure R2.12), similar to the observations in other animal models of depression** (Cui et al., 2018; Yang et al., 2018)**. These findings strongly indicate that augmenting synaptic inputs alone is sufficient to induce LHb hyperactivity.**

[FIGURE REDACTED]

Figure R2.12. 40 Hz phasic photostimulation of lateral hypothalamus (LH)-LHb projections persistently enhances excitatory neurotransmission and burst firings in the LHb. a,b, Experimental design. **c,** Pie charts showing the percentage of the three types of LHb neurons after 14-day optogenetic manipulations (n = 31 to 55 neurons, mice = 2 to 3; Chi-square test). **d-g**, data adapted from Zheng et al. 2022, showing that 40 Hz opto-stimulation enhances sEPSC, but not sIPSC (n = 31 to 55 neurons, mice = 2 to 3; One-way ANOVA with Tukey's multiple comparisons test). **P < 0.01; ****P<0.0001; n.s., not significant. Error bars indicate SEM.

In addition, after incubation with tBP in LHb slices of CRS mice, burst (Fig. 4c,d) and the overall spontaneous (Fig. 4e) firing activity were both largely attenuated. Are these effects solely mediated by normalization of the enhanced synaptic inputs, or may also by normalization of intrinsic excitability?

This is an intriguing question. LHb hyperactivity is a hallmark of depression-like states, characterized by increased excitatory synaptic transmission, enhanced bursting activity, and heightened excitability (Cui et al., 2018; Yang et al., 2018)**. Notably, bursting activity in LHb neurons depends on the coordinated activation of NMDARs and T-VSCCs, underscoring the importance of both synaptic inputs and intrinsic excitability** (Yang et al., 2018)**. Utilizing transcriptome screening, we found that CRS did not alter the mRNA levels of T-VSCC channels (Figure R2.13). Consistent with the unaltered T-VSCC current under CRS** (Yang et al., 2018)**, these findings suggest that LHb autophagy does not target T-VSCCs to modulate neuronal intrinsic excitability. In contrast, autophagy enhancer decreases synaptic expression levels of both NMDARs and AMPARs, without affecting GABARs (Fig. 5e-h in the manuscript). These findings suggest the selective targeting of LHb autophagy onto synaptic GluRs for normalizing LHb hyperactivity.**

Now we have integrated these results into Extended Data Fig. 12b, and added relevant text (line 337-344).

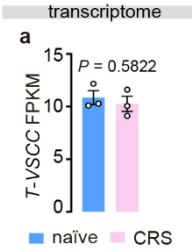

Figure R2.13. Expression of *T-VSCC* channel in LHb is unaltered by CRS. a, Quantification of mRNA levels of *T-VSCC* in mice after CRS (n = 3 mice; Unpaired *t* test). Error bars indicate SEM.

7. If I understand correctly, phagocytosis and internalization should be two separate processes. In Fig. 4w, the pharmacological LTD was completely blocked by TAT-GluA23Y peptide, suggesting that endocytosis occurs before phagocytosis during LTD. But then why promoting phagocytosis can directly induce LTD? A model needs to be proposed to explain the results.

We appreciate Reviewer 2's insightful comment. The process of endocytosis of synaptic receptors, followed by their phagocytosis or recycling back to the synaptic membrane, is suggested to play a constitutive role in maintaining neuronal homeostasis (Overhoff et al., 2021)**. In our study, genetic ablation of autophagy directly leads to the synaptic accumulation of glutamate receptors (GluRs) and induces hyperactivity in LHb neurons of naive mice, highlighting the constitutive role of autophagy-mediated phagocytosis in neuronal homeostasis under basal conditions, likely through an endocytosis-phagocytosis/recycling chain.**

Specifically, autophagy deficiency results in the accumulation of intracellular AMPAR cargo derived from endocytosis, which in turn slows down the endocytosis of synaptic AMPARs. Furthermore, autophagy deficiency reduces competition with the recycling process of AMPARs, leading to a synergistic effect that causes synaptic accumulation of AMPARs.

In contrast, chronic stress, which impairs autophagy in the LHb, can be counteracted by promoting phagocytosis through tBP, thereby alleviating the accumulation of intracellular AMPAR cargo and facilitating the endocytosis of synaptic AMPARs. Additionally, enhanced phagocytosis competes with the recycling of AMPARs, synergistically reducing the synaptic expression of AMPARs and inducing long-term depression (LTD).

Supporting this perspective, we observed that an LTD protocol enhances autophagy (Extended Data Fig. 19a-b). Furthermore, a long-term potentiation (LTP) induction protocol (Zheng et al., 2022) **elicited greater synaptic potentiation in *Atg7*$^{LHb-/-}$ neurons compared to *Atg7*$^{LHb+/+}$ control groups (Extended Data Fig. 19h), indicating that autophagy blockade exacerbates the accumulation of postsynaptic GluRs during LTP induction.**

We have now revised the graphic model in Extended Data Fig. 20 and added this into discussion. In the revised graphic model, we have distinguished receptor degradation with endocytosis and recycling (Figure R2.14). In addition, we modified relevant text to avoid confusion.

[FIGURE REDACTED]

Figure R2.14. Hypothesized model. LHb autophagy is switched on rapidly in an energy-dependent manner (within minutes) via AMPK phosphorylation, acting as the "alarm reaction" phase of stress. As stress exposure persists, excitatory afferents onto LHb are gradually potentiated, and LHb autophagy may be constantly recruited for degrading excessive synaptic GluRs, resulting in the "resistance" phase. During the late phase of chronic stress, LHb autophagy is suppressed by the over-activation of mTOR signaling. Consequently, LHb autophagy is incapable of counterbalancing the excessive synaptic weight through GluRs degradation, therefore slowing down towards GluRs endocytosis and causing the "exhaustion" phase of stress, neuronal hyperactivity and depression-like phenotypes.

8. Line 68: "that controlling" should be "that control".

Corrected. We have now changed "that controlling" into "that control" in the text (line 69-70).

9. Line 178: "…in naïve mice" should be "…compared with naïve mice".

Corrected. We have now changed "…in naïve mice" into "…compared with naïve mice" in the text (line 188).

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Referees' comments:

Referee #1 (Remarks to the Author):

The authors have added comprehensive experiments in response to all my concerns, and I am satisfied with the revised manuscript. This paper is both conceptually improved and technically sound along with exciting perspective. I would like to appreciate their rigorous efforts. I have a few comments.

Great thanks again to Reviewer 1 for the very positive evaluation and continuous strong support of our study.

1. Fig. R1.1 panels b-e have not been added to the revised manuscript, but these panels should be included in Fig. 6 or Extended Data Fig. These data quantitatively demonstrate the degree of chemogenetic inhibition in neuronal hyperactivity and are essential for evaluating the results of the behavioral experiments in panels f-i. Fig. R1.1 is very important because it shows a causal link between LHB autophagic deficit, LHB hyperexcitability (homeostasis), and the depressive behaviors.

We appreciate this great suggestion, and have now integrated these panels into Extended Data Fig. 21.

2. If possible, adding Figs. R1.13 and R1.14 to the revised manuscript would strengthen the authors' discussion. However, this is not mandatory.

We appreciate Reviewer 1's suggestion and have now incorporated these two figures into Extended Data Fig. 13, and added relevant text in results and discussion in the manuscript (Line 336-351, Line 567-575 in the clean version of manuscript).

3. In main Figure 6 c,d, western blot analysis shows p62 is increased while Beclin-1 is decreased in Cre-mice which is mentioned in the text line 392, but in Figure 6 description, it is written as Becline-1 is increased.

We greatly appreciate Reviewer 1's meticulous attention to detail. Thank you for identifying this issue, which has now been corrected.

Kaoru Inokuchi

Referee #2 (Remarks to the Author):

The authors have done a substantial number of new experiments to substantiate their conclusions and have thoroughly addressed all my questions. Hats off to the authors for such tremendous effort. This is an important study that makes a strong case for LHb autophagy in stress and depression-like behavior.

We are immensely grateful to Reviewer 2 for his/her/their continuous strong support and appreciation of our work.

I only have two (related) suggestions which I think are quite important, as autophagy in regulating brain function is relatively new and more information is helpful for readers to have a more complete and unbiased view:

1. The results on autophagy in the basolateral amygdala (BLA) and arcuate nucleus (Arc) (Figure R2.4 and Figure R2.5) are quite interesting and important, and are an integral part of the paper. Therefore, they should be included in the paper.

We thank both Reviewers for highlighting this important point. We have now incorporated these two figures into Extended Data Fig. 13, and included relevant text of results and discussion in the manuscript (Line 336-351, Line 567-575).

2. In many places in the paper, including the abstract (lines 41, 43, 98, 102, 598, and maybe other places throughout the paper), it is stated that autophagy is "selective" to the LHb. The authors should tone down such a claim, as autophagy also occurs at least in the Arc, and potentially in other yet-to-be identified brain regions.

Thank you for your kind suggestion. We have substantially revised the text according to your valuable suggestion.

Referee #3 (Remarks to the Author):

This is an interesting paper that suggests autophagy in the lateral habenula (LHb) is uniquely sensitive to acute and chronic stress. Autophagy in the LHb is regulated by AMPK and mTOR, and modulators of these kinases infused into the LHb alters the expression of depression-related behaviors in mice. Similarly, modulating the expression of autophagy-related proteins alters the expression of depression-related behaviors in mice. It is suggest that autophagy regulates depression-related behaviors by a mechanism involving the rapid regulation of local excitatory synaptic transmission. The paper is multidisciplinary in nature and focused on an important topic of research. I have the following suggestions for the authors' consideration:

We appreciate Reviewer 3's positive evaluation and constructive suggestions. Please find our point-by-point responses to your valuable comments below.

1. The manuscript needs to be thoroughly proof-read to correct the many instances of grammatical errors and sometimes strained prose.

Thank you for the suggestion. We have now substantially revised the manuscript and, for your convenience, have submitted both a clean version and a version with tracked changes.

2. Figure 1, panel a1: It is unclear why there is a greater number of sample for Western blot analyses from the LHb (7 or more) relative all other brain regions (5 in all other cases).

We appreciate Reviewer 3's rigour. Previously, we conducted additional preliminary experiments on LHb samples with paroxetine treatments. In response, we have now increased the sample size across all other brain regions (Figure R1).

We have now incorporated these data into Figure 1 in the manuscript.

[FIGURE REDACTED]

Figure R1. Western Blot results of p62 in multiple brain regions from mice with paroxetine treatment (*i.p.*). **a,** Experimental design. **b,c,** Antidepressant dosing regimen of paroxetine treatment (*i.p.*) enhances autophagy (i.e., decreased p62) in LHb, but does not affect autophagy in vHippo, mPFC, VTA, LH, NAc, LS, DRN and MRN of CRS mice. $^{**}P < 0.01$; n.s., not significant. Error bars indicate SEM (Mann-Whitney test). LHb, lateral habenula; vHippo, ventral hippocampus; VTA, ventral tegmental area; NAc, nucleus accumbens; mPFC, medial prefrontal cortex; LH, lateral hypothalamus; LS, lateral septum; DRN, dorsal raphe; MRN, median raphe; Veh, vehicle; Pxt, paroxetine; CRS, chronic restraint stress.

3. Figure 1: Which cells in the LHb demonstrate stress-induced changes in autophagy? Is this effect restricted to local neurons?

During the first round of revision, Reviewer 2 raised the question, *"Are the RNAseq data contaminated by glia cells?"* To address this, we have conducted single-cell RNA sequencing in the LHb and identified a statistical trend (p = 0.06) of neuron-specific impairment of autophagy signaling under CRS conditions (please refer to Extended Data Fig. 2a in the manuscript).

In response to Reviewer 3's additional concerns, we conducted co-immunostaining of LC3 with markers specific to different neuronal types. Confocal imaging revealed that LC3 puncta is predominantly localized

with Vglut2, a marker for glutamatergic neurons, within the same LHb cells (Figure R2).

[FIGURE REDACTED]

Figure R2. [TEXT REDACTED]

4. Figure 1: How were transcriptional profiles of LHb cells generated? By bulk RNA-seq, scRNA-seq, some other method? Please provide more details in the relevant section of the manuscript.

We analyzed transcriptional profiles by bulk RNA-seq in Figure 1c-f and by scRNA-seq in Extended Data Fig. 2a. Thank you for this valuable suggestion, we have now clarified the details in the legend, methods and results sections.

5. Figure 1: Are altered markers of autophagy in LHb secondary to stress-induced increases neural activity in the LHb? In other works, would chemogenetic or optogenetic stimulation of neural activity in the LHb elicit the same alterations in p62, beclin, etc., observed in LHb lysates?

Thank you for this insightful suggestion. Our previous discovery revealed that various stressors (restraint, tail suspension, footshock, or social defeat) consistently induces 40-Hz clusters of firings (4-5 spikes per cluster) in lateral hypothalamic (LH) neurons, which then leads to burst firings in the LHb neurons (Zheng et al., Neuron 2022). To replicate this stressor-induced neuronal activity, we applied 40-Hz phasic photostimulation (4 pulses per second) at the LH-LHb axonal terminals. We then performed Western blot analysis of p62 and Beclin-1 in LHb samples (Figure R3a). Our results showed that, similar to acute stress, 40-Hz phasic photostimulation at the LH-LHb terminals decreased p62 levels and increased Beclin-1 levels (Figure R3b).

We have now incorporated these data into Extended Data Fig. 3 in the manuscript, and included relevant text in results (Line 191-198).

[FIGURE REDACTED]

Figure R3. 40-Hz phasic photostimulation at LH-LHb axonal terminals rapidly activates LHb

autophagy in naïve mice. **a,** Experimental design. **b,** Representative images (left) and quantification (right) of western blot (n = 4 mice per group; Mann Whitney test). *$P < 0.05$. Error bars indicate SEM.

6. Figure 2: It is unclear why markers of autophagy should be impacted by AMPK and mTOR manipulations only in the LHb. As autophagy is under the control of these kinases in most/all cells, surely autophagy-related markers should have been induced in brain regions other than the LHb? Do AMPK and mTOR show higher basal activity in the LHb relative to other brain regions?

Thank you for raising this thought-provoking question and providing a practical solution. In our manuscript, we have shown that: 1) *i.p.* injection of rapamycin, a specific mTOR inhibitor, enhances autophagy functions in the LHb without affecting autophagy functions in the vHippo, mPFC and VTA; 2) the antidepressant effects of rapamycin require the activation of LHb autophagy; 3) blockade of AMPK specifically in the LHb during acute stress facilitates depression-like behaviors. Please note that since the AMPK blocker is delivered via cannular microinjection into the LHb, we cannot conclude that systemic blockade of AMPK induces brain region-specific effects on autophagy.

Following Reviewer 3's suggestion, we conducted a Western blot analysis to assess the basal expression levels of p-mTOR and p-AMPK in the LHb, vHippo, VTA, NAc, mPFC, and LH. Our results showed that both p-mTOR and p-AMPK levels in the LHb were higher than in the other regions, indicating that the LHb exhibits a greater expression of the active forms of p-mTOR and p-AMPK compared to other brain areas (Figure R4).

Given that rapamycin specifically targets mTOR in the LHb of CRS mice, and antidepressant effects of rapamycin require activation of LHb autophagy, these pieces of evidence further support our "On-Demand" hypothesis: "*Our findings suggest that only a small number of brain regions fit the profile of a primary target for autophagy enhancers: namely that they are naturally active, become overactive in depression, and experience disruption of autophagy functions following chronic stress.*" (Line 634-637)

Thank you again for highlighting this potential scenario.

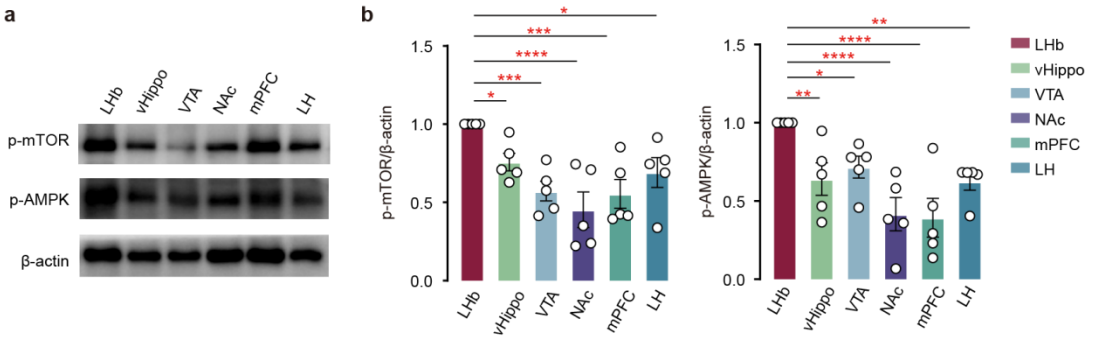

Figure R4. Basal expression levels of p-mTOR and p-AMPK in multiple brain regions from naïve mice. a,b, Representative images (**a**) and quantification (**b**) of western blot in multiple brain regions (n = 5 mice; One-way ANOVA with Uncorrected Fisher's LSD). *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001. Error bars indicate SEM.

7. Figure 3e-h: The effects of knocking down Atg7 alone (in the absence of rapamycin treatment) should be assessed in the same behavioral tests. Without this group, it is not possible to determine whether Atg7 alone modified behavior or blocked the effect of rapamycin.

Thank you for the suggestion. We have now assessed the behavioral effect of knocking down *Atg7* alone in CRS mice. We observed that *Atg7* knock down alone did not modify behavioral outcomes compared to EGFP control group treated with CRS (EGFP+Vehicle vs. sh*Atg7*+Vehicle, Figure R5).

We have now incorporated these data into Figure 3g,h and Extended Data Fig. 7b in the manuscript.

[FIGURE REDACTED]

Figure R5. Activation of LHb autophagy is required for antidepressant-like effects of rapamycin. a, Experimental design and shRNA working model. **b-d,** Knock-down of LHb *Atg7* abolishes antidepressant-like effects of rapamycin in forced swim test (FST) (**b**) and sucrose preference test (SPT) (**c**), without affecting locomotion and anxiety-like behavior (**d**) (n = 8 to 11 mice; One-way ANOVA with Uncorrected Fisher's LSD). *P < 0.05; **P < 0.01; n.s., not significant. Error bars indicate SEM.

8. Figure 3i-l: Once again, the effects of knocking down Atg7 alone (in the absence of paroxetine treatment) should be assessed. Without this group, it is not possible to determine whether Atg7 blocked the effect of paroxetine or alone exerted effects on the same behavioral endpoints.

Thank you for the suggestion. We have now evaluated the behavioral

effect of knocking out *Atg7* alone via viral delivery of hSyn-Cre in *Atg7*$^{flox/flox}$ mice treated with CRS. Our results showed that the knockout of Atg7 alone did not alter the behavioral outcomes compared to the mCherry control group treated with CRS (mCherry+Vehicle vs. Cre+Vehicle, Figure. R6).

We have now incorporated these data into Figure 3i-l in the manuscript.

[FIGURE REDACTED]

Figure R6. Activation of LHb autophagy is partially required for antidepressant-like effects of paroxetine. **a**, Experimental design. **b-d** Conditional knock-out of *Atg7* in LHb abolishes antidepressant-like effects of paroxetine in FST (**b**) and social interaction test (SIT) (**d**), but not SPT (**c**) (n = 6 to 8 mice; One-way ANOVA with Uncorrected Fisher's LSD). **P* < 0.05; ***P* < 0.01; ****P* < 0.001; n.s., not significant. Error bars indicate SEM.

9. Figure 3: It is unclear why shRNA-Atg7 was used in one case, while Atg7-floxed mice were used in another. Were analyses performed to confirm that Atg7 was selectively and efficiently knocked out of the LHb by AAV-Cre delivery in Atg7-floxed mice?

The shRNA strategy may encounter off-target effects, so we also explored an alternative loss-of-function strategy by injecting AAV-Cre virus in LHb of *Atg7*$^{flox/flox}$ mice. Studies have shown an efficient knock-out of *Atg7* by injecting AAV-Cre intravenously in *Atg7*$^{flox/flox}$ mice (Fig. 2A in Toledo et al., Cell Metabolism, 2018). Here, to confirm that *Atg7* was also efficiently knocked out in the LHb by AAV-Cre local injection in *Atg7*$^{flox/flox}$ mice. Western blot analysis by micro-dissection of the LHb was performed, and we found that Atg7 expression is significantly decreased in the LHb of AAV-Cre group compared to AAV-mCherry control group (Figure R7).

We have now incorporated these data into Figure 6c,d in the manuscript.

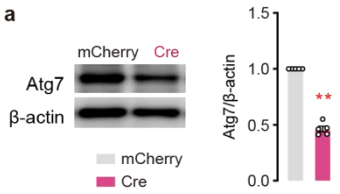

Figure R7. The specific knock-out efficiency of *Atg7* in LHb by AAV-Cre delivery in *Atg7*$^{flox/flox}$ mice. a, Western blot showing decreased Atg7 expression in LHb of AAV-Cre injected mice compare to their mCherry controls (n = 5 mice per group; Mann-Whitney test). $^{**}P < 0.01$. Error bars indicate SEM.

10. Figure 5: Data showing that LC3 is in close physical proximity to glutamate receptor complexes in the LHb should be moved from supplementary materials to the main figure. Does LC3 interact with glutamate receptors in the same manner in other brain regions, or is this relationship unique to the LHb?

Thank you for highlighting this intriguing point. During the first round of revision, Reviewer 2 mentioned that "*Lateral habenula (LHb) is only one of the brain areas activated by different stressors. There are many other brain areas that are hyperactive in response to stress. Given that autophagy appears to be a general mechanism regulating synaptic function in different neuronal types, why only LHb neuronal function is regulated by autophagy during stress?***". Reviewer 2 also made similar comments and mentioned the hyperactivity of amygdala neurons and hypothalamic pro-opiomelanocortin (POMC) neurons under chronic stress. To address both Reviewers' concerns, we performed pharmacological experiments on neurons from the basolateral amygdala (BLA) and hypothalamic arcuate nucleus (Arc), we found region-specific activation of autophagy in the LHb and Arc neurons but not BLA neurons under the treatment of TAT-beclin1 peptide (tBP) (please also refer to Point 13).**

To further investigate whether the LC3-GluR interaction also occurs in the BLA and/or Arc, we performed immunostaining for both LC3 and the GluA2 subunit in these regions. The co-labeling was then visualized using stochastic optical reconstruction microscopy (STORM). We observed substantial co-labeling of GluA2 with LC3 puncta in both the LHb and Arc, while much less co-labeling was seen in the BLA (Figure R8). These findings, consistent with our results in Point 13, support the idea that autophagy specifically targets GluRs in more active brain regions.

We have now incorporated these data into Extended Data Fig. 15c in the manuscript, and included the relevant text in results (Line 383-385).

[FIGURE REDACTED]

Figure R8. The co-labeling of autophagic markers with GluRs in LHb, Arc and BLA. a-c, Representative STORM images showing substantial co-labeling of GluA2 with LC3 puncta in both the LHb (**a**) and Arc (**b**), while much less co-labeling was seen in the BLA (**c**) of ARS-treated mice.

11. Figure 5: Does phasic and tonic activity of LHb neurons recover after tBP has been washed from slices?

Thank you for proposing this great experiment. We have now tested two different dosages (0.5 μM and 1 μM) of tBP, and measured the wash-off effects of tBP on spontaneous synaptic transmission, spontaneous neuronal activity and excitability in LHb neurons. We found that tBP wash-off did not abolish the silencing effects of tBP on LHb neurons in CRS mice (Figure R9). Note that, neither 0.5 μM nor 1 μM tBP altered input resistance of the neurons, illustrating the stability of the recordings throughout the whole process (Figure R9f,m). These findings indicate that tBP promotes the degradation of GluRs, potentially reducing the reservoir of GluRs required to maintain the silencing effect of LHb neurons. Correspondingly, local infusion of tBP into the LHb produces both rapid and long-lasting antidepressant effects (please refer to Fig. 4a-i and Extended Data Fig. 10a-e in the manuscript).

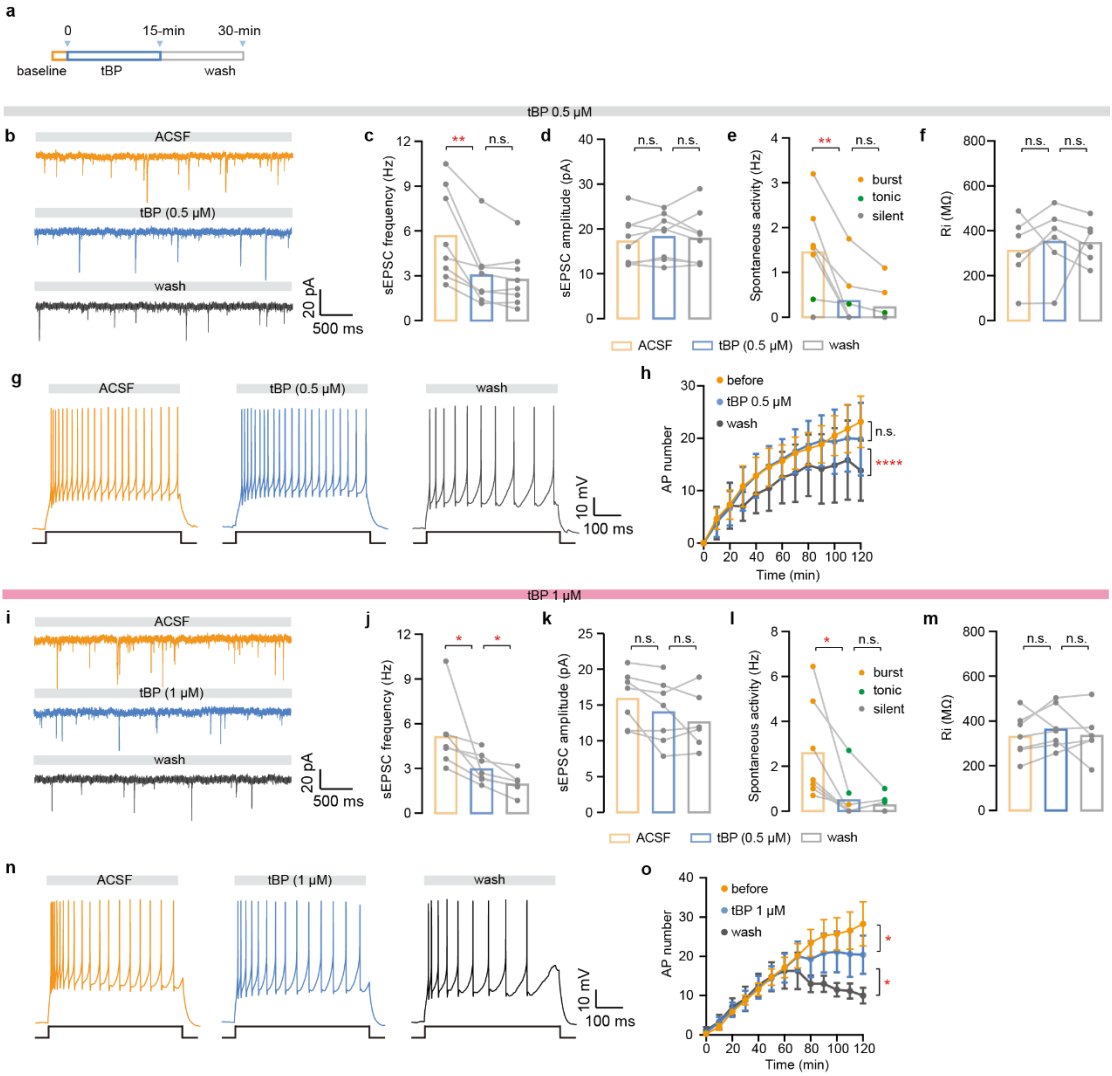

Figure R9. Wash-off does not abolish the silencing effects of tBP on LHb neurons in CRS mice. **a,** Experimental design. **b-d**,**i-k**, Representative traces (**b,i**) and quantifications (**c-d, j-k**) showing spontaneous neural transmission before, during and after tBP application on LHb slices from CRS mice (For 0.5 μM tBP, n = 8 neurons, mice = 5; for 1 μM tBP, n = 7 neurons, mice = 5; Paired *t* test). **e,l,** Bar graph showing spontaneous firing patterns and firing frequency of LHb neurons before, during and after tBP application (For 0.5 μM tBP, n = 8 neurons, mice = 5, for 1 μM tBP, n = 7 neurons, mice = 5; Paired *t* test). **f,m,** Input resistance (Ri) were not altered before, during and after tBP application (For 0.5 μM tBP, n = 8 neurons, mice = 5, for 1 μM tBP, n = 7 neurons, mice = 5; Paired *t* test). **g,n,h,o,** Representative traces **(g,n)** and Quantification **(h,o)** showing neuronal excitability before, during and after tBP application (For 0.5 μM tBP, n = 8 neurons, mice = 5, for 1 μM tBP, n = 7 neurons, mice = 5; Unpaired *t* test). **P* < 0.05; ***P* < 0.01; *****P* < 0.0001; n.s., not significant. Error bars indicate SEM.

12. Figure 5: Does tBP have "direct" pharmacological actions on AMPA receptors that can explain its unexpectedly rapid inhibitory effect on excitatory transmission in the LHb?

Thank you for these thoughtful comments. To experimentally assess the interaction between a drug molecule and its potential target, the ideal

approaches are, in theory, Surface Plasmon Resonance (SPR) and co-immunoprecipitation (co-IP). SPR is an optical technique that detects small changes in the refractive index at the sensor surface. However, this method is not suitable for membrane proteins such as AMPAR, as maintaining their native structure and function requires a lipid or detergent environment, which is challenging to achieve during SPR experiments (Patching et al., 2014). Co-IP is another reliable method for testing protein-protein interactions; however, it is not technically feasible in this case due to the absence of commercially available antibodies against tBP.

Therefore, to preliminarily explore the possibility of direct pharmacological actions of tBP on AMPAR, we simulated the protein-protein interaction of the AMPAR subunit GluA2 (PDB:7LDE) and tBP (blue sticks) in MOE (Molecular Operating Environment). A conformation with the highest affinity was identified by random docking (Figure R10). In this conformation, the binding site of tBP is different from the binding site of antagonists (red area). Therefore, it is unlikely that tBP directly binds to AMPARs.

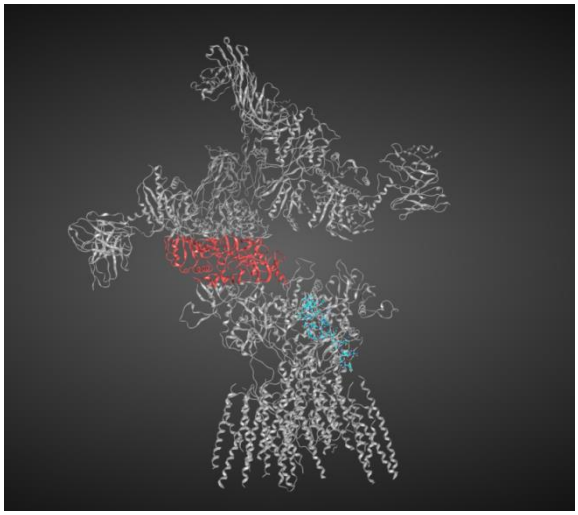

Figure R10. Molecular docking of tBP onto AMPA receptor. Red area indicates the binding site of antagonists, blue sticks indicate tBP.

In addition, we have discovered that blocking endocytosis abolishes tBP's effects on both cellular hyperactivity and behavioral outcomes (Figure 5l-y), suggesting that the effects of tBP on AMPARs are directly linked with endocytosis-degradation.

13. Figure 5: Does tBP have a similar inhibitory effect on excitatory synaptic transmission in brain regions other than the LHb, or is this effect unique to the LHb?

This is a great suggestion. During the first round of revision, Reviewer 1 mentioned that *"Some previous literature has already reported that chronic stress*

induces hyperexcitability of neurons in the amygdala (Rosenkranz et al., 2010) or POMC neurons in hypothalamic areas (Fang et al., 2023). I recommend authors look at other brain areas that were reported to display hyperexcitability or hyper-synaptic functions induced by chronic stress and validate whether there is a causal relationship between autophagy impairment and neuronal/synaptic homeostasis in these areas."
Reviewer 2 also made similar comments. We therefore tested whether tBP modulates neuronal activity of pyramidal neurons (PNs) in the BLA slices, or POMC neurons in the Arc slices (Figure R11). We crossed POMC-Cre mice with Ai14 mice to visualize POMC neurons, and found that tBP perfusion rapidly decreased both neuronal excitability and excitatory neurotransmission in the Arc POMC neurons (Figure R11a-e). In contrast, tBP did not alter any of these parameters in the BLA PNs (Figure R11f-j).

Notably, Arc POMC neurons demonstrate significantly greater neuronal excitability and excitatory synaptic transmission compared to BLA pyramidal neurons, while exhibiting levels comparable to those of LHb neurons (Figure R11k-m). These findings suggest that tBP activates autophagy in more active neurons. Besides, impaired autophagy signaling was observed in the Arc but not BLA (please refer to Extended Data Fig. 13a,b in the manuscript), suggesting that autophagy in more active neurons is more vulnerable to chronic stress. As autophagy acts in an on-demand mode, active neurons require more autophagy function and facilitate the exhaustion of autophagy. It would be intriguing to investigate whether this hypothesis extends as a general principle in the brain, potentially enabling autophagy enhancers to specifically target brain regions with higher metabolic demands.

Thank you for highlighting this potential scenario. Moreover, according to both Reviewer 1 and 2's comments in the second round of revision, we have now incorporated these data into Extended Data Fig. 13, and added relevant text in results and discussion (Line 336-351 and Line 567-575).

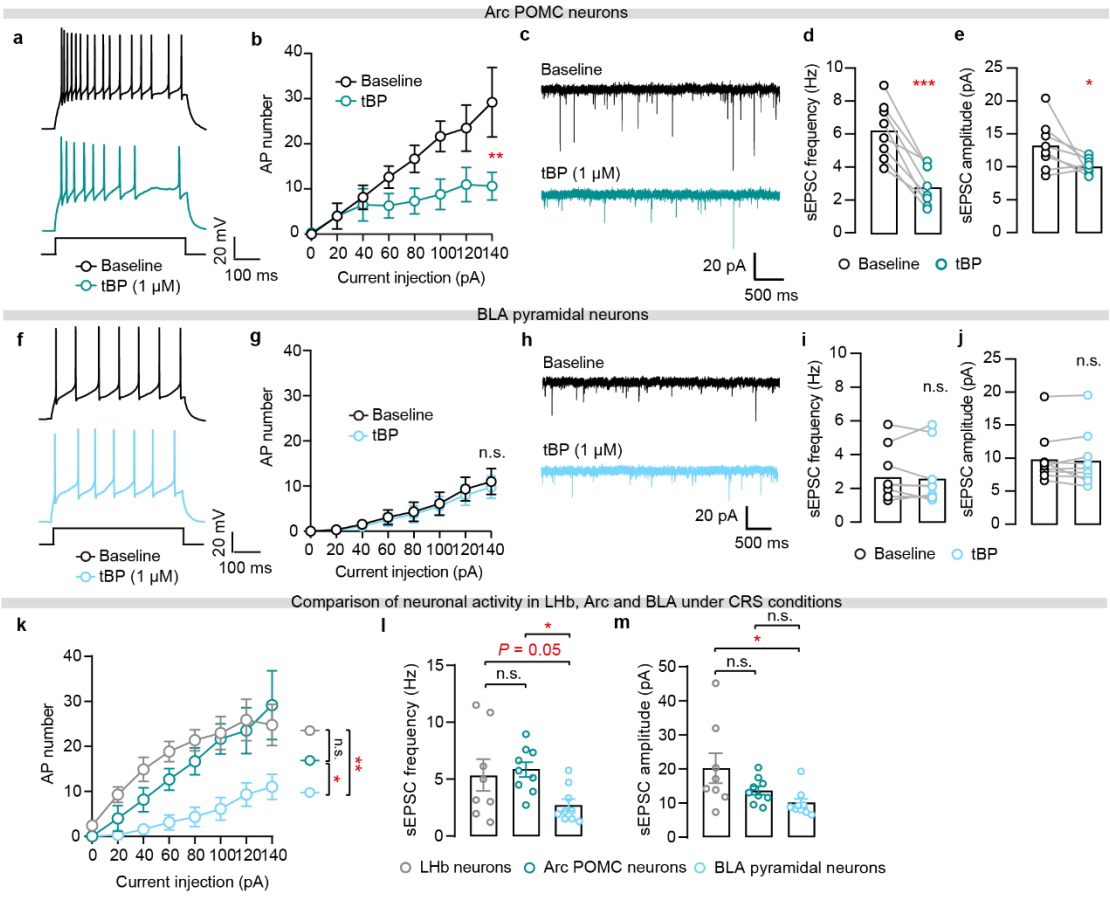

Figure R11. tBP attenuates neuronal and synaptic activity in Arc pro-opiomelanocortin (POMC) neurons but not BLA pyramidal neurons (PNs) of CRS mice. a,b, Representative traces (**a**) and quantification (**b**) of neuronal excitability in Arc POMC neurons treated with tBP (n = 6, mice = 4; Unpaired *t* test). **c-e**, Representative traces (**c**) and quantification (**d,e**) of excitatory synaptic transmission in Arc POMC neurons treated with tBP (n = 8, mice = 4; Paired *t* test). **f,g,** Representative traces (**f**) and quantification (**g**) of neuronal excitability in BLA PNs treated with tBP (n = 9, mice = 4; Unpaired *t* test). **h-j,** Representative traces (**h**) and quantification (**i,j**) of excitatory synaptic transmission in BLA PNs treated with tBP (n = 9, mice = 4; Paired *t* test). **k-m,** Comparison of neuronal excitability (**k**) and spontaneous excitatory neurotransmission (**l,m**) in LHb neurons, Arc POMC neurons and BLA PNs (n = 6 to 9 neurons, mice = 4 to 6; One-way ANOVA with Uncorrected Fisher's LSD). **P* < 0.05; ***P* < 0.01; ****P* < 0.001; n.s., not significant. Error bars indicate SEM.

14. Figure 5: The effects of Dyngo-4a alone on behavior were not assessed. Thus, the interpretation that it blocks the antidepressant-like effect of tBP cannot be make with confidence, as Dyngo-4a may well have effects independent of tBP.

Thank you for the suggestion. We have now evaluated the behavioral effects of Dyngo-4a alone in CRS mice. Local infusion of Dyngo-4a did not alter behavioral outcomes compared to vehicle control group treated with CRS (Fig. R12).

[FIGURE REDACTED]

Figure R.12. Endocytosis blockers abolish antidepressant-like effects of tBP on stress-induced depression-like behaviors. **a,** Experimental design. **b-d,** tBP decreased immobile duration in FST (**b**) and increased sucrose preference in SPT (**c**) while co-application of tBP with Dyngo-4a or TAT-GluA2$_{3Y}$ peptide abolished these effects without affecting locomotion and anxiety-like behavior (**d**) (n = 7 to 12 mice; One-way ANOVA with Uncorrected Fisher's LSD). **P* < 0.05; ***P* < 0.01; ****P* < 0.001; *****P* < 0.001; n.s., not significant. Error bars indicate SEM.

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