nature portfolio

Peer Review File

Feedforward inhibition of stress by brainstem neuropeptide Y neurons

Reviewers' Comments:

Reviewer #1:
Remarks to the Author:
In their manuscript, Yan Zhang and colleagues uncover a previously unknown group of neuropeptide Y (NPY) neurons with significant effects on reducing anorexia and anxiety under stress. First, the authors employ c-Fos whole brain mapping to locate a new cluster of NPY neurons within the dorsal raphe nucleus and ventrolateral periaqueductal gray area (DRN/vlPAG), which are reactive to acute stress from novel environments. Through in vivo fiber photometry using GCaMP6m, they demonstrate that these DRN/vlPAGNPY neurons are more active in novel environments. Using chemogenetic techniques to activate or inhibit these neurons, the study shows that DRN/vlPAGNPY neurons modulate the reduced eating response to acute novelty stress but not regular feeding. Notably, stimulating these neurons reduces stress-related anxiety-like behaviors, potentially influencing the paraventricular nucleus (PVN), as indicated by c-Fos staining. They further reveal that chronic chemogenetic manipulation of these neurons is crucial in managing chronic restraint stress-related under-eating and anxiety. Conditioned knockdown NPY from DRN/vlPAG intensifies stress-induced anxiety in mice. The authors also find that optogenetic modulation of DRN/vlPAGNPY neurons can transmit positive valence, leading to place preference. Through circuit mapping, the study identifies that DRN/vlPAGNPY neurons connect to multiple brain regions, including MPA/LPO, PVT, LH, AH/LA, and DMH. Additionally, systematic optogenetic activation of DRN/vlPAGNPY neuron terminals shows that these neurons influence feeding behaviors and positive valence through different projections to PVT or LH. Overall, this study presents a lot of information, and I have several questions that could enhance the rigor of the study.

Major
1. Regarding the fiber photometry analysis of the DRN/VlPAGNPY neurons, do they respond to other types of stimulation? For example, novel object, tail pinch, or present food to the animal? It will be more informative to annotate other behavior events, e.g., grooming with current data and quantify the calcium dynamic when the animal is in a different environment or state (novel or home cage).
2. In Figure 1m, the in vivo fiber photometry analysis of GCaMP6m in DRN/vlPAGNPY neurons shows increased activity in new cages. Using n= animals rather than trials (average trials per animal) would probably be better.
3. The data presented in Figure 2, which examines acute novelty stress, implies a long-lasting effect on the activity of DRN/vlPAGNPY neurons and their influence on food intake (up to 4 hours). It would be interesting to investigate whether novelty stress alters (for example, at 3 or 4 hours after stress) the firing activity of these DRN/vlPAGNPY neurons.

4. The ongoing debate about desensitization in chronic chemogenetic manipulation (PMID: 35406674) suggests the need for a control experiment to affirm the efficacy of chemogenetic manipulation. Either experiments or discussions should be included to show the effectiveness of long-term chemogenetic manipulation on neuronal activities.
5. For knockdown of NPY in DRN/vlPAG neurons using AAV-Cre. More rigorous histological data or quantification should be done.
6. In Figure 7, concerning neuronal terminal optogenetics, please include a power analysis for panels c and d. The apparent increasing trend suggests that including additional animals might change the conclusions. For example, in LH opto stimulation experiments, limited numbers of animals (4 or 5 according to plots, but 7 stated in the legends) were used. The data show a strong trend of suppression of feeding but with no statistical significance. DMH groups show quite a bit of increase but no significance. The data are quite confusing.

7. Figure 7 e and f, both PVT and LH projecting terminal release GABA, but the kinetics of the postsynaptic response traces are pretty different. Please consider choosing more representative traces if possible.

Minor
1. In Figure 4 b and h, there is inconsistency in the labeling of significance.

Reviewer #2:
Remarks to the Author:
The manuscript by Zhang and colleagues explores the active resistance of the brain to stress and its role in preventing overreactions, a crucial aspect of the well-being of mammals. The authors identify a previously uncharacterized population of neuropeptide Y (NPY) neurons in the dorsal raphe nucleus and ventrolateral periaqueductal gray region (DRN/vlPAG) that exhibits anxiolytic effects in mice. The findings highlight the rapid and strong activation of NPYDRN/vlPAG neurons by various stressful stimuli and their differential yet synergic anxiolytic effects through inhibitory projections to the paraventricular thalamic nucleus (PVT) and the lateral hypothalamic area (LH).

While the significance of the work and the novelty of the conclusions are commendable, several major concerns and minor points need addressing:

Major Comments:

1. The discrepancy in Figure 1c, where the total 24-hour food intake appears to mildly

increase despite novelty stress significantly suppressing feeding in the first 4 hours, requires clarification. The authors should explain this apparent contradiction and assess whether the effect of suppressed feeding persists beyond the initial 4 hours.

2. In Figure 2, the chemogenetic activation of NPYDRN/vlPAG neurons shows conflicting results with Figure 1 regarding suppressed feeding during novelty stress. The authors need to explain this discrepancy and carefully scrutinize raw data to ensure consistency in their conclusions.

3. The intriguing observation of steadily decreasing daily food intake in mice after restraint from day 1 to day 10, followed by a steady increase, warrants further explanation. The authors should explore the implications of this phenomenon and consider whether daily food intake in restraint mice would eventually exceed that of non-restraint mice after prolonged restraint.

4. The authors claim that the employed CNO has greater potency and fewer side effects than other methods, but details about potential side effects in their study are lacking. The authors should provide clarity on any observed side effects or revise the claim accordingly.

5. In Figure 5, the authors conduct extensive efforts to reduce NPY expression in the DRN/vlPAG in vivo. However, it is advisable to include the daily food intake data of restraint mice over the 14-day period to provide a comprehensive view of the impact of NPY reduction.

6. The suggested involvement of NPY receptors in the PVT and LH in mediating separated effects is intriguing but remains speculative. The authors should consider conducting additional experiments or providing a more nuanced discussion to support this hypothesis.

7. The manipulation in Figure 6 should be assessed for its impact on acute or restraint stress-induced hypophagia and anxiety-like behaviors, providing a more comprehensive understanding of its effects.

8. The rationale behind observing total 24-hour food intake is not clearly justified. The authors should provide additional explanations to support this choice.

Minor Comments:

1. More details on imaging, behavior, and quantification, especially regarding the parameters of optogenetics stimulation, should be provided for clarity.

2. In line 221, consider revising "We then asked whether NPYDRN/vlPAG neurons can ameliorate novelty..." to "We then asked whether activation of NPYDRN/vlPAG neurons can ameliorate novelty..."

3. Review sentences in the methods section that may be unclear and ensure they are written more interpretably.

4. Ensure consistency in providing scale bars with notation across all figures for uniformity.

5. Clearly distinguish arrowheads and arrows in figure legends as needed for better clarity and understanding.

Reviewer #3:
Remarks to the Author:
The authors examined the physiological roles of NPYDRN/vlPAG neurons and their downstream projections in counteracting a number of stress responses (e.g. anorexia and anxiety). The experiments were well designed and nicely executed. Multiple genetic models were used to rigorously test the neurons, the circuits and the neuropeptide from the neurons. Results are robust and largely support the conclusions. This reviewer has the following suggestions and comments for the authors to further improve this work and the manuscript.
1. An important question is the timing of NPYDRN/vlPAG neuron activation as relative to stress challenge. For the Fos experiments (Fig. 1j-k and 4d-e), there is no information provided regarding when mice were perfused as relative to the stress, which makes it difficult to interpret the Fos data.
2. As related, the fiber photometry data (Fig. 1m and 4f) only showed a few seconds when mice were stress. What about more long-term responses during the 2-4 hour stress? Are these neurons constantly activated during the stress or show more transient activations during the stress? What about the post-stress (recovery) period? The neural activities during the post-stress period may be even more relevant here, because there is a clear “recovery” phase which allowed mice to catch up the food intake (Fig. 1c). Considering the orexigenic effects of NPYDRN/vlPAG neurons, one would wonder whether these neurons are more activated during the “recovery” phase.
3. The electrophysiological validations for DREADD models need to have quantifications of resting membrane potential and firing frequency. Only showing representative traces from one neuron is not convincing. For example, traces in Fig. 2a may be due to random sampling and would not convince me that there as a CNO-

induced inhibition. It not clear why some experiments used 50 uM CNO but others used 10 uM CNO.
4. Fig. 2g shows that DREADD activation of NPYDRN/vlPAG neurons increased food intake during the 4-hour stress. Is this effect specific for feeding during stress? What are the effects of these neurons on 4-hour food intake in un-stressed mice?
5. Fig. 4j shows body weight changes as %. What about the absolute values?
6. Fig. 5e shows food intake during the 4-hour stress. What about the food intake during 4-24 hours, post the stress? This is related to my point #2, as the signals from NPYDRN/vlPAG neurons may be more relevant during the post-stress period to help animals recover.
7. Fig. 7e-f demonstrated the GABAergic inputs from NPYDRN/vlPAG neurons to their downstream targets in the PVT and LH. Considering the functional relevance of NPY as shown in Fig. 5, the authors should also examine whether NPY receptor inhibitors can affect how NPYDRN/vlPAG neurons regulate the activities of their downstream targets (e.g. those in the PVT and LH).
8. Corticosterone should be measured in multiple models used in the study.

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

In their manuscript, Yan Zhang and colleagues uncover a previously unknown group of neuropeptide Y (NPY) neurons with significant effects on reducing anorexia and anxiety under stress. First, the authors employ c-Fos whole brain mapping to locate a new cluster of NPY neurons within the dorsal raphe nucleus and ventrolateral periaqueductal gray area (DRN/vlPAG), which are reactive to acute stress from novel environments. Through in vivo fiber photometry using GCaMP6m, they demonstrate that these DRN/vlPAGNPY neurons are more active in novel environments. Using chemogenetic techniques to activate or inhibit these neurons, the study shows that DRN/vlPAGNPY neurons modulate the reduced eating response to acute novelty stress but not regular feeding. Notably, stimulating these neurons reduces stress-related anxiety-like behaviors, potentially influencing the paraventricular nucleus (PVN), as indicated by c-Fos staining. They further reveal that chronic chemogenetic manipulation of these neurons is crucial in managing chronic restraint stress-related under-eating and anxiety. Conditioned knockdown NPY from DRN/vlPAG intensifies stress-induced anxiety in mice. The authors also find that optogenetic modulation of DRN/vlPAGNPY neurons can transmit positive valence, leading to place preference. Through circuit mapping, the study identifies that DRN/vlPAGNPY neurons connect to multiple brain regions, including MPA/LPO, PVT, LH, AH/LA, and DMH. Additionally, systematic optogenetic activation of DRN/vlPAGNPY neuron terminals shows that these neurons influence feeding behaviors and positive valence through different projections to PVT or LH. Overall, this study presents a lot of information, and I have several questions that could enhance the rigor of the study.

Response: We are grateful for Reviewer #1's recognition of our study that “presents a lot of information”. We have carefully evaluated and responded to his/her comments as follows.

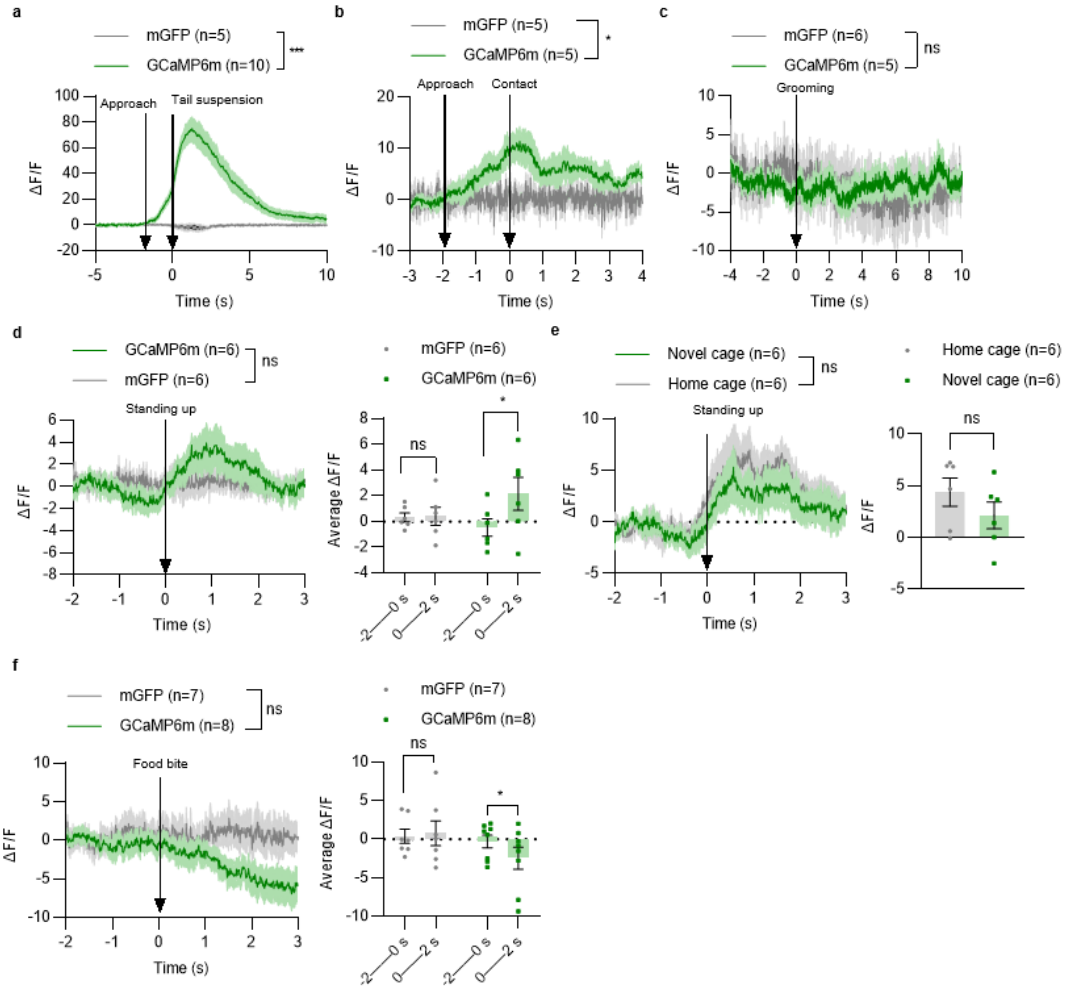
Major

1. Regarding the fiber photometry analysis of the DRN/VlPAGNPY neurons, do they respond to other types of stimulation? For example, novel object, tail pinch, or present food to the animal? It will be more informative to annotate other behavior events, e.g., grooming with current data and quantify the calcium dynamic when the animal is in a different environment or state (novel or home cage).

Response: We thank the Reviewer for this insightful suggestion and have done many of the suggested experiments as shown below. We found that $NPY^{DRN/vlPAG}$ neurons are sensitive to different stimuli and responded to these behaviors differentially (**Attached figure 1**). Specifically, tail elevation and novel object activated $NPY^{DRN/vlPAG}$ neurons. However, when biting chow diet, the activity of $NPY^{DRN/vlPAG}$ neurons decreased. Standing up in the cage is a sign of exploration and was observed in both novel and home cage. We found that $NPY^{DRN/vlPAG}$ neurons were activated during standing up, and the neuronal

activity changes were similar between novel and home cage. There was no changes in calcium signal when grooming. Together these findings indicate that stress related behaviors increase $NPY^{DRN/vlPAG}$ neuronal activity however food consumption suppresses the calcium response, highlighting the key role of $NPY^{DRN/vlPAG}$ neurons in coping stress induced feeding.

We agree that these additional data would provide more insights into understanding how $NPY^{DRN/vlPAG}$ neurons respond to different environment, so we have added these results to the revised manuscript **Supplementary Figure 2d-i** and **lines 158-182, 505-506, 509-511**.



Attached figure 1. $NPY^{DRN/vlPAG}$ neuronal calcium signals in response to tail elevation (a), novel object (b), grooming (c), standing up (d, e) and food bite (f). Specifically, $NPY^{DRN/vlPAG}$ neuronal activity changes were similar between novel and home cage (e).

2. In Figure 1m, the in vivo fiber photometry analysis of GCaMP6m in DRN/vlPAGNPY neurons shows increased activity in new cages. Using n= animals rather than trials (average trials per animal) would probably be better.

Response: Thank you for the review's suggestion. We have changed the analysis in the

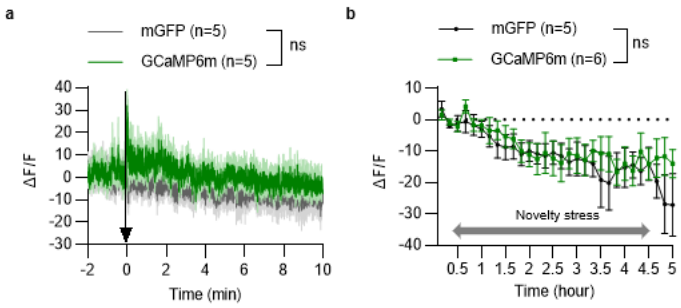
revised manuscript **Fig. 1l, Fig. 4g** and **Supplementary Figure 2**.

3. The data presented in Figure 2, which examines acute novelty stress, implies a long-lasting effect on the activity of DRN/vlPAGNPY neurons and their influence on food intake (up to 4 hours). It would be interesting to investigate whether novelty stress alters (for example, at 3 or 4 hours after stress) the firing activity of these DRN/vlPAGNPY neurons.

Response: We share with the reviewer's interest in seeing the long-term recording. For novelty stress, we found that $NPY^{DRN/vlPAG}$ neurons mildly increased calcium activity for less than 5 minutes and then gradually decreased to the basal level (**Attached figure 2**).

To see a long-term effect, we extended the recording time to 4 hours. To minimize bleaching, 1 minute of the fluorescence signal was collected once every 10 min. The average fluorescence intensity for the last 50 seconds of every 1-minute record was used as the signal value for a given time point. As you see from the Attached Figure 2, there was no significant changes of $NPY^{DRN/vlPAG}$ neurons during long-term recording in response to novelty stress.

Together these results indicates that $NPY^{DRN/vlPAG}$ neurons mainly activated at the beginning of stress for a short time. We have added these results to the revised manuscript **Supplementary Fig. 2b,c** and **lines 150-155, 507-509**.



Attached figure 2. $NPY^{DRN/vlPAG}$ neuronal calcium signals in mice individually transferred to a novel cage without padding for 10 minutes (a) or 4 hours (b).

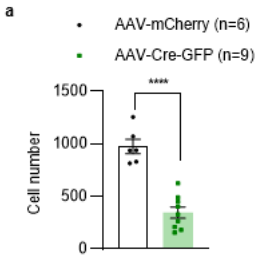
4. The ongoing debate about desensitization in chronic chemogenetic manipulation (PMID: 35406674) suggests the need for a control experiment to affirm the efficacy of chemogenetic manipulation. Either experiments or discussions should be included to show the effectiveness of long-term chemogenetic manipulation on neuronal activities.

Response: We appreciate the reviewer for bringing up this concern. We acknowledge that in some chronic chemogenetic manipulations, receptor desensitization may occur. However, according to the review (PMID: 35406674), functional effects can be observed for several days before desensitization happened. In our study, the effects of chronic manipulations of $NPY^{DRN/vlPAG}$ neurons remained consistent from the beginning of

CNO/clozapine injection (Fig. 4h and Supplementary Figure 3g-h), during which desensitization should have not happened. On the other hand, AAV-mediated delivery can provide persistent and overexpression of receptors, which may compensate such desensitization (PMID: 35406674). Overall, we agree that chemogenetic manipulation may cause reduced efficacy but such effect may unlikely affect our conclusions. We have included this important point to the discussion **lines 645-651**.

5. For knockdown of NPY in DRN/vlPAG neurons using AAV-Cre. More rigorous histological data or quantification should be done.

Response: We appreciate the reviewer for this important suggestion. In addition to the quantification of fluorescent intensity, we looked back to our data and calculated the number of NPY-containing cells in both AAV-Cre-GFP and AAV-mCherry groups. We found that mice in the knockdown group had significantly fewer NPY-containing cells than the control mice (**Attached Figure 3**). This was consistent with our previous statistics based on the fluorescent intensity.
We have added these results to the revised manuscript **Supplementary Figure 4e** and **lines 344-346**.



Attached figure 3. Quantitative comparison of NPY-containing cells in AAV-mCherry and AAV-Cre-GFP *Npy*$^{lox/lox}$ groups.

6. In Figure 7, concerning neuronal terminal optogenetics, please include a power analysis for panels c and d. The apparent increasing trend suggests that including additional animals might change the conclusions. For example, in LH opto stimulation experiments, limited numbers of animals (4 or 5 according to plots, but 7 stated in the legends) were used. The data show a strong trend of suppression of feeding but with no statistical significance. DMH groups show quite a bit of increase but no significance. The data are quite confusing.

Response: We apologize for the confusion. To add up the statistic power, we have repeated the experiments and added mice in LH-, AH/LA- and DMH-optogenetic experiments. These changes have been updated in the revised manuscript **Fig. 7c**. We have also changed the graph of LH optogenetic stimulation result to make every point clear.

7. Figure 7 e and f, both PVT and LH projecting terminal release GABA, but the kinetics of the postsynaptic response traces are pretty different. Please consider choosing more representative traces if possible.

Response: Thank you for pointing out this important issue. We have checked the electrophysiological raw data and replaced representative postsynaptic response traces in the revised manuscript **Fig. 7h**.

Minor

1. In Figure 4 b and h, there is inconsistency in the labeling of significance.

Response: We have corrected this inconsistency in the revised manuscript **Fig. 4b and 4i**.

Reviewer #2 (Remarks to the Author):

The manuscript by Zhang and colleagues explores the active resistance of the brain to stress and its role in preventing overreactions, a crucial aspect of the well-being of mammals. The authors identify a previously uncharacterized population of neuropeptide Y (NPY) neurons in the dorsal raphe nucleus and ventrolateral periaqueductal gray region (DRN/vlPAG) that exhibits anxiolytic effects in mice. The findings highlight the rapid and strong activation of NPYDRN/vlPAG neurons by various stressful stimuli and their differential yet synergic anxiolytic effects through inhibitory projections to the paraventricular thalamic nucleus (PVT) and the lateral hypothalamic area (LH).

While the significance of the work and the novelty of the conclusions are commendable, several major concerns and minor points need addressing:

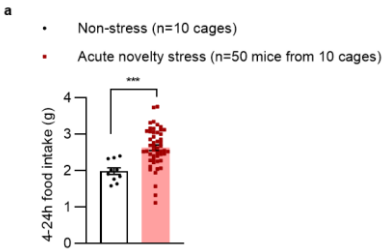
Response: We are grateful for Reviewer #2's comments and the recognition of the significance and the novelty of our work. The questions are carefully answered as below.

Major Comments:

1. The discrepancy in Figure 1c, where the total 24-hour food intake appears to mildly increase despite novelty stress significantly suppressing feeding in the first 4 hours, requires clarification. The authors should explain this apparent contradiction and assess whether the effect of suppressed feeding persists beyond the initial 4 hours.

Response: We apologize for not clearly stating the differences, which have led to the confusion. The unchanged or even mildly elevated 24-h food intake was due to a markable increase of feeding from 4 hours to 24 hours (**Attached figure 4**). We speculate that this subsequent increase may represent a compensatory effect aimed at restoring homeostasis in the body, so that total 24-hour food intake of stressed group was equal or slightly more than non-stress group (without significant difference). Moreover, we believe the increased

food intake observed during the 4-24 hour period could serve as evidence to prove that the initial suppressed feeding no longer persists beyond the initial 4 hours. To avoid further confusion, we have included the data to **Fig.1c** and added this discussion in the revised manuscript **lines 105-108**.



Attached figure 4. 4-24-hour food intake under acute novelty stress and non-stress conditions.

2. In Figure 2, the chemogenetic activation of NPYDRN/vlPAG neurons shows conflicting results with Figure 1 regarding suppressed feeding during novelty stress. The authors need to explain this discrepancy and carefully scrutinize raw data to ensure consistency in their conclusions.

Response: Thank you for raising this intriguing point and we apologize for any confusion. We acknowledge that the increased food intake observed with chemogenetic activation of $NPY^{DRN/vlPAG}$ neurons appears to contradict the suppressed feeding during stress in Figure 1b. However, we hypothesize that the activation of $NPY^{DRN/vlPAG}$ neurons serves as an anti-stress mechanism—aimed at in mitigating stress overactivation and facilitating recovery.

In Fig. 1, the net food intake results from the interplay between stress response-eliciting neurons (e.g. CRH^{PVN} neurons) and stress response-resisting neurons (e.g. $NPY^{DRN/vlPAG}$ neurons). Initially, stress response-eliciting neurons predominate, leading to a temporary inhibition of food intake, as shown in Fig. 1b. Subsequently, the stress-resisting $NPY^{DRN/vlPAG}$ neurons function to mitigate stress overactivation and facilitate recovery, as shown in Figure 1c. Therefore, when $NPY^{DRN/vlPAG}$ neurons are chemogenetically activated, they exhibit an increase in food intake as a stress-resisting effect (Fig. 2g).

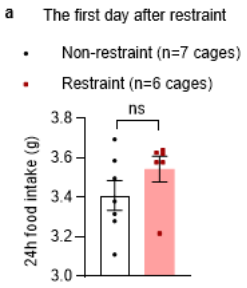
We understand that these results may be confusing, and we have added a detailed interpretation of this to the revised manuscript **lines 220-223, 533-550**. Additionally, we have carefully scrutinized the raw data to ensure consistency in our conclusions.

3. The intriguing observation of steadily decreasing daily food intake in mice after restraint from day 1 to day 10, followed by a steady increase, warrants further explanation. The authors should explore the implications of this phenomenon and consider whether daily food intake in restraint mice would eventually exceed that of non-restraint mice after prolonged restraint.

Response: We appreciate the reviewer's insightful point. The decreasing food intake from

day 1 to day 10 reflects a physiological response to restraint stress, which has been proven to be an effective model for inducing stress-induced food intake decrease (PMID: 24396694; PMID: 31485012). For a longer period, for example from day 11 to day 14, the animals appeared to recover from the stress-induced hypophagia. This recovery is also supported by many other studies with a normalization of food intake (PMID: 24396694; PMID: 8949963) and an adaptation in behavioral and hormonal response (PMID: 18588527; PMID: 25014526) after prolonged restraint stress. In particular, the study (PMID: 24396694) showed that after 10 days of restraint stress, although there was an increased tendency, the food intake of restraint mice did not exceed that of non-restraint mice. These findings suggest the existence of an anti-stress mechanism, potentially involving the $NPY^{DRN/vlPAG}$ neurons, that counteracts the initial stress-induced feeding suppression.

Although we did not observe an overshoot food intake in the restraint group compared to the non-restraint group after 14 days (**Attached figure 5**), we agree that it would be interesting to investigate whether there might be an "overcorrection" of food intake in an extended period in future studies. We have added this discussion to the revised manuscript **Supplementary Figure 4a** and **lines 302-313**.



Attached figure 5. 24-hour food intake between restraint and non-restraint groups on the first day after the end of 14-day restraint.

4. The authors claim that the employed CNO has greater potency and fewer side effects than other methods, but details about potential side effects in their study are lacking. The authors should provide clarity on any observed side effects or revise the claim accordingly.

Response: We apologize for the misunderstanding on this point. Our intention was to show that we employed both chemogenetics and optogenetics based on experiment requirements, with chemogenetic manipulation being more suitable for chronic stimulation. We have corrected the misleading statement in the revised manuscript.

5. In Figure 5, the authors conduct extensive efforts to reduce NPY expression in the DRN/vlPAG in vivo. However, it is advisable to include the daily food intake data of restraint mice over the 14-day period to provide a comprehensive view of the impact of NPY reduction.

Response: We appreciate the reviewer for providing this valuable suggestion. We indeed

recognize the importance of presenting the impact of NPY reduction on daily food intake over the 14-day restraint stress and we attempted to gather this data.

However, to minimize unnecessary stress on the experimental animals, all mice involved in restraint stress-related experiments were group-housed. In this circumstance, average daily food intake is determined by dividing the overall food intake per cage by the number of mice. Regrettably, there were instances where one or two mice within a cage exhibited insufficient $Npy^{DRN/vlPAG}$ knockdown efficiency. Consequently, calculating the average daily food intake became less accurate. We have clarified this limitation in the revised manuscript **lines 624-630**.

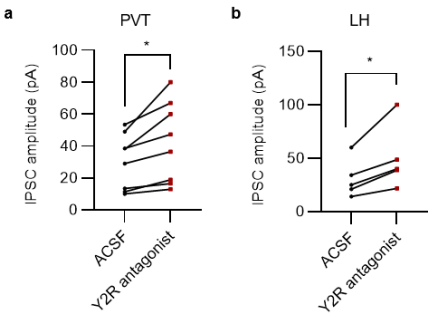
6. The suggested involvement of NPY receptors in the PVT and LH in mediating separated effects is intriguing but remains speculative. The authors should consider conducting additional experiments or providing a more nuanced discussion to support this hypothesis.

Response: We appreciate that the reviewer also finds this hypothesis intriguing. NPY Y1 receptor (Y1R) and NPY Y2 receptor (Y2R) are two well-known NPY receptors. Previously studies (PMID: 16998904, PMID: 12031341) have demonstrated the wide expression of Y2R in the PVT and LH, while Y1R is primarily found in the LH. Notably, Y1R is thought to exhibit anxiolytic-like effect (PMID: 15352219; PMID: 10193650). However, recent studies indicate that Y2R induced either anxiogenic effect when administrated intracerebroventricularly (PMID: 16959374) or anxiolytic effect in the locus coeruleus (LC) (PMID: 9555090), suggesting a complex role for Y2R in stress modulation.

In Fig.7g,h, we illustrated that light stimulation induced inhibitory postsynaptic current (IPSC) in both the PVT and LH. We further found that blocking Y2R with antagonist JNJ-31020028 mainly increased the amplitude of light-evoked IPSC in the PVT (8 in 11 cells) and LH (5 cells) (**Attached figure 6**). In contrast, Y1R receptor antagonist BIBO3304 had a mild effect on the amplitude increase of light-evoked IPSC in the LH (4 in 7 cells). These results suggest that Y2R plays a key role in the inhibitory effect of $NPY^{DRN/vlPAG}$ neurons in the PVT and LH while Y1R in the LH may have little contribution.

We acknowledge that additional experiments would be beneficial to clarify the contradiction that $NPY^{DRN/vlPAG}$ neurons project inhibitory signals to the downstream regions, yet this inhibitory effect could be suppressed by NPY, which exerts an anti-stress effect when released from $NPY^{DRN/vlPAG}$ neurons. However, we believe it is valuable to propose hypothesis based on our current data and the existing literature that Y2R is involved in the stress alleviation mediated by $NPY^{DRN/vlPAG}$ neurons. In the revised manuscript, we have explicitly stated the necessity for further experiments to fully elucidate the details of these differential effects. We will follow your suggestion and encouragement to gather more evidence in future projects.

The current data and nuanced discussion have been added to the revised manuscript **Supplementary Figure 6i.j** and **lines 474-485, 521-530, 631-636**.



Attached figure 6. The effect of Y2R antagonist JNJ-31020028 on light-evoked IPSC in the PVT (a) and LH (b).

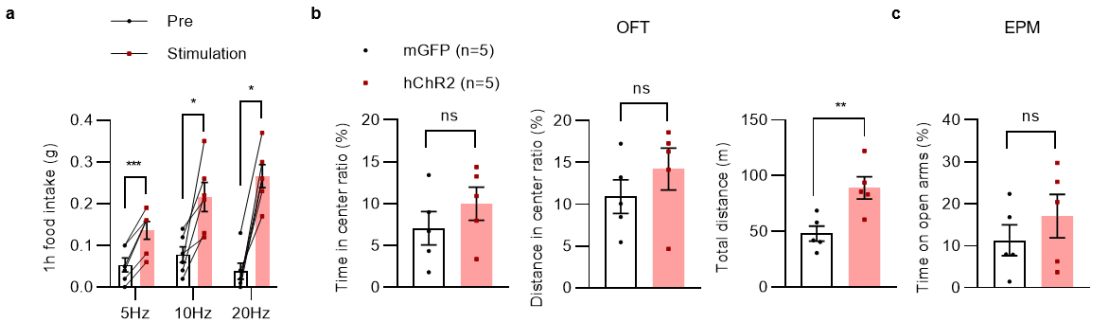
7. The manipulation in Figure 6 should be assessed for its impact on acute or restraint stress-induced hypophagia and anxiety-like behaviors, providing a more comprehensive understanding of its effects.

Response: We appreciate the reviewer for the insightful suggestion. Following this very nice guidance, we have conducted optogenetic activation experiments to access the impact of our manipulation on acute novelty stress induced-hypophagia and anxiety-like behaviors, aiming to provide a more comprehensive understanding of $NPY^{DRN/vlPAG}$ neuronal anti-stress effect.

During novelty stress, optogenetic activation (10 mW, 10 ms pulse, 1-second on and 1-second off) of $NPY^{DRN/vlPAG}$ neurons with various frequencies (5, 10 and 20Hz) significantly increased 1-hour food intake (**Attached figure 7**), demonstrating the alleviation effect of acute stress-induced hypophagia.

Furthermore, we performed optogenetic activation (10 mW, 20Hz, 10 ms pulse, 1-second on and 1-second off) during the entire 2-hour novelty stress paradigm and evaluated anxiety-like behaviors afterward (without light stimulation). No significant increase in the time on open arms of EPM or time/distance in center of OFT was observed, despite an increase in total distance traveled in the OFT (**Attached figure 7**). The discrepancy of outcomes between chemogenetic and optogenetic manipulations may be attributed to potential factors such as light-evoked brain heating and phototoxicity, as highlighted in literatures (PMID: 31209378; PMID: 26192551).

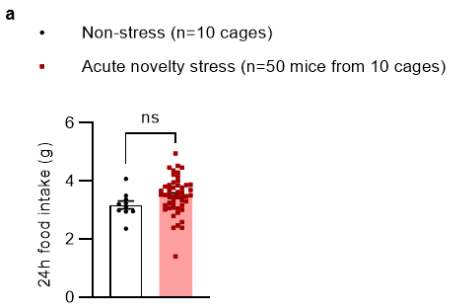
The results above have been added on the manuscript **Supplementary Figure 5b-d** and **lines 377-385**. Overall, our optogenetic results in feeding behaviors and RTPP experiments provide compelling evidence supporting the stress-alleviating effect of $NPY^{DRN/vlPAG}$ neurons.



Attached figure 7. The effect of optogenetic activation of NPY$^{DRN/vlPAG}$ neurons during acute novelty stress on stress induced-hypophagia (a) and anxiety-like behaviors (b, c).

8. The rationale behind observing total 24-hour food intake is not clearly justified. The authors should provide additional explanations to support this choice.

Response: We deeply appreciate the reviewer's attention to this important point. In our study, novelty stress is a rapid and relatively mild stress type, making it an excellent model for studying the acute stress response and its recovery phase. We measured changes of food intake at various time points during novelty stress and found that the hypophagia induced by novelty stress was transient, with food intake returning to baseline levels within 24 hours (**Attached figure 8**). Based on these observations, we decided to measure food intake over a 24-hour period during a novelty stress. We believed that 24 hours is sufficiently to capture the entire dynamic process of feeding inhibition and subsequent recovery, likely mediated by NPY$^{DRN/vlPAG}$ neurons. The clarification has been induced in the revised manuscript **lines 108-112**.



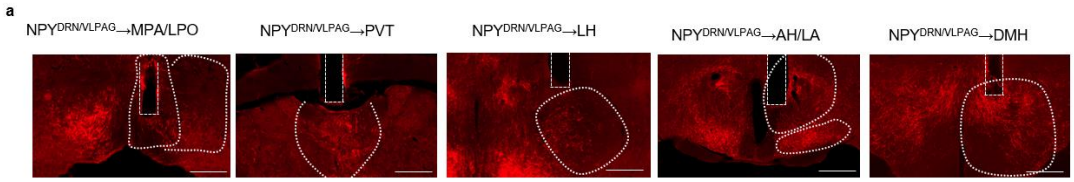
Attached figure 8. 24-hour food intake during novelty stress. After the initial food intake decrease (Figure 1b), the food intake recovered to non-stress state.

Minor Comments:

1. More details on imaging, behavior, and quantification, especially regarding the parameters of optogenetics stimulation, should be provided for clarity.

Response: We appreciate the reviewer's attention to this aspect of our study. In response to his/her nice feedback, we have made revisions to include relevant details in both main text and the method section (For example, **lines 788-791, 890-896, 914-916, 922-923,**

933-935, 989-991, 1032-1034). We have included representative brain slices for optogenetic activation of $NPY^{DRN/vlPAG}$ neuronal terminals in down brain regions (**Attached figure 9**).



Attached figure 9. (a) Expression of $hChR2^{+}$ $NPY^{DRN/vlPAG}$ neuronal terminals in downstream brain regions and optical fiber placement.

2. In line 221, consider revising "We then asked whether NPYDRN/vlPAG neurons can ameliorate novelty…" to "We then asked whether activation of NPYDRN/vlPAG neurons can ameliorate novelty…"

Response: Thank you. We have corrected it in the revised manuscript.

3. Review sentences in the methods section that may be unclear and ensure they are written more interpretably.

Responses: We appreciate the reviewer highlighting this crucial aspect of our manuscript. In response to this valuable suggestion, we have thoroughly revised the methods section to ensure that all sentences are clear and easily interpretable.

4. Ensure consistency in providing scale bars with notation across all figures for uniformity.

Response: We appreciate the reviewer's attention to this matter. After thorough examination, we have ensured that the scale bars in all figures are consistent and uniformly notated as requested. These corrections are now reflected in the revised manuscript.

5. Clearly distinguish arrowheads and arrows in figure legends as needed for better clarity and understanding.

Response: Thank you for your valuable feedback. To enhance clarity and understanding, we have modified all arrows in the figures to make it distinguishable and ensure they can match the descriptions in the figure legends in the revised manuscript.

Reviewer #3 (Remarks to the Author):

The authors examined the physiological roles of NPYDRN/vlPAG neurons and their

downstream projections in counteracting a number of stress responses (e.g. anorexia and anxiety). The experiments were well designed and nicely executed. Multiple genetic models were used to rigorously test the neurons, the circuits and the neuropeptide from the neurons. Results are robust and largely support the conclusions. This reviewer has the following suggestions and comments for the authors to further improve this work and the manuscript.

Response: We are grateful for Reviewer #3's comments that our experiments were well designed and nicely executed. We have carefully responded to each of his/her comments as follows.

1. An important question is the timing of NPYDRN/vlPAG neuron activation as relative to stress challenge. For the Fos experiments (Fig. 1j-k and 4d-e), there is no information provided regarding when mice were perfused as relative to the stress, which makes it difficult to interpret the Fos data.

Response: We appreciate the reviewer for highlighting this important issue. In our Fos immunostaining studies, mice were subjected to either 2-hour novelty stress (Fig. 1i, j) or 2-hour restraint stress (Fig.4 e, f) before sacrifice. We have included this information in both the main text and methods section of the revised manuscript for clarity (**lines 127-129, 316-317, 914-915, 922-923**).

2. As related, the fiber photometry data (Fig. 1m and 4f) only showed a few seconds when mice were stress. What about more long-term responses during the 2-4 hour stress? Are these neurons constantly activated during the stress or show more transient activations during the stress? What about the post-stress (recovery) period? The neural activities during the post-stress period may be even more relevant here, because there is a clear “recovery” phase which allowed mice to catch up the food intake (Fig. 1c). Considering the orexigenic effects of NPYDRN/vlPAG neurons, one would wonder whether these neurons are more activated during the “recovery” phase.

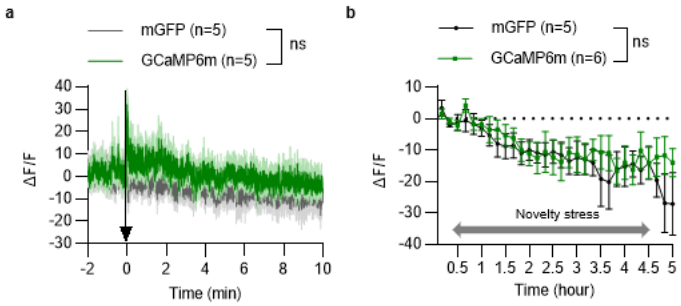
Response: We appreciate the reviewer's insightful inquiry regarding the dynamics of $NPY^{DRN/vlPAG}$ neuronal activity. In response to these questions, we conducted fiber photometry recordings over a 10-minute period during novelty stress. Our findings revealed that $NPY^{DRN/vlPAG}$ neurons maintained a low level of activation for less than 5 minutes before gradually returning to the basal level (**Attached figure 2**). This indicates that $NPY^{DRN/vlPAG}$ neurons are primarily activated at the onset of stress.

To further investigate the long-term responses of these neurons, we extended the recording time to 5 hours (including 4-hour novelty stress). To minimize bleaching, 1 minute of the fluorescence signal was collected once every 10 min. The average fluorescence intensity for the last 50 seconds of every 1-minute record was used as the signal value for a given time point. As shown in the Attached Figure 2, there was no significant changes of $NPY^{DRN/vlPAG}$ neurons during or after long-term recording in response to novelty stress.

We understand the importance of exploring neural activities during both the stress and post-stress period, particularly considering the orexigenic effects of $NPY^{DRN/vlPAG}$ neurons.

However, our data suggest that NPY$^{DRN/vlPAG}$ neurons exhibited transient activation in response to stress and does not show pronounced changes during or after the stress phase.

We have added these results to the revised manuscript **Supplementary Fig. 2b,c** and **lines 150-155, 507-509**.



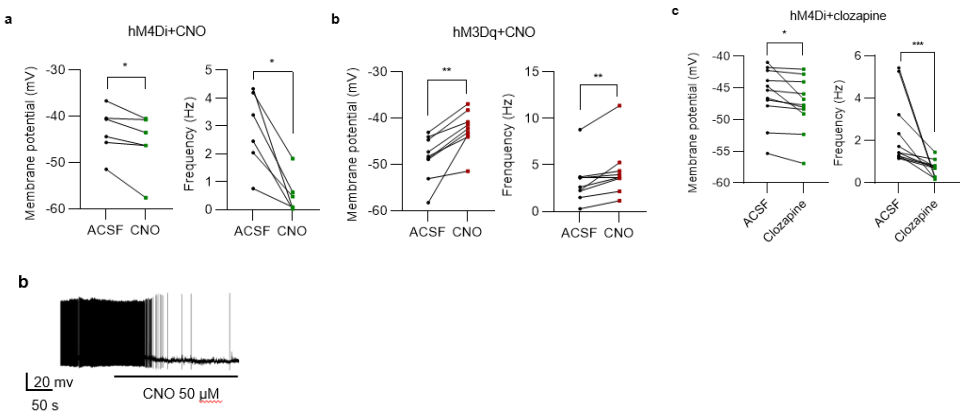
Attached figure 2. NPY$^{DRN/vlPAG}$ neuronal calcium signals in mice individually transferred to a novel cage without padding for 10 minutes (a) or 4 hours (b).

3. The electrophysiological validations for DREADD models need to have quantifications of resting membrane potential and firing frequency. Only showing representative traces from one neuron is not convincing. For example, traces in Fig. 2a may be due to random sampling and would not convince me that there as a CNO-induced inhibition. It not clear why some experiments used 50 uM CNO but others used 10 uM CNO.

Response: We appreciate the reviewer for bringing up this concern. We conducted additional electrophysiological validations for DREADD models, including quantifications of membrane potential and firing frequency before and after CNO/clozapine application. Basal frequency of action potential and membrane potential were defined as the average frequency and membrane potential obtained 3 minutes before CNO/clozapine administration, while post-application frequency of action potential and membrane potential were the average obtained 5 minutes after CNO/clozapine perfusion. We found that 10 μM CNO activated NPY$^{DRN/vlPAG}$ neurons as evidenced by increased neuronal membrane potential and firing frequency, whereas 50 μM CNO or 5 μM clozapine inhibited NPY$^{DRN/vlPAG}$ neurons by decreasing neuronal membrane potential and firing frequency (**Attached figure 10**). To make it convincible, we also replaced the representative trace of CNO-induced inhibition in Fig.2a (**Attached figure 10**).

Regarding the choice of CNO concentration, we explored several concentrations and finally selected 10 μM CNO for activation (PMID: 33171117) and 50 μM CNO for inhibition (reference: PMID: 36070748) to achieve optimal effects.

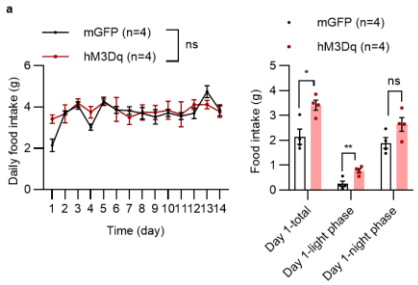
We have added these results to the revised manuscript **Supplementary Figure 3a,b, Supplementary Figure 4c, Fig. 2a** and **lines 192-193, 207-209, 325-327, 864-871**.



Attached figure 10. (a-c) Average membrane potential and firing frequency before and after CNO/clozapine application to activate or inhibit NPY$^{DRN/vlPAG}$ neurons. (d) Representative trace of CNO-induced inhibition of NPY$^{DRN/vlPAG}$ neurons.

4. Fig. 2g shows that DREADD activation of NPYDRN/vlPAG neurons increased food intake during the 4-hour stress. Is this effect specific for feeding during stress? What are the effects of these neurons on 4-hour food intake in un-stressed mice?

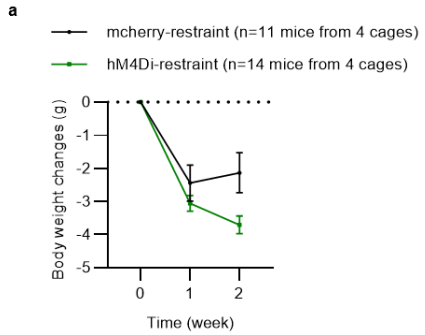
Response: We appreciate the reviewer for highlighting this important issue. To address this, we conducted additional experiments as shown in **Attached figure 11**. In this experiment, mice received 14-day i.p. CNO injection without any artificial stress. On day 1, probably due to an indirect injection stress, there was a slight increase in food intake compared to the control group. However, from day 2 onward, as the mice acclimated to the injection stress, there was no significant difference between two groups. Moreover, the food intake of NPY^{hM3Dq} mice on day 1 did not differ from that on subsequent days, indicating that activating NPY$^{DRN/vlPAG}$ neurons did not increase absolute food intake. In summary, our findings suggest that the effect of NPY$^{DRN/vlPAG}$ neuron activation on increased food intake is specific to feeding during stress and does not manifest in un-stress mice. We have incorporated this discussion to the revised manuscript **Supplementary Figure 3g** and **lines 239-249**.



Attached figure 11. Daily food intake of NPY$^{DRN/vlPAG}$-hM3Dq and NPY$^{DRN/vlPAG}$-mGFP mice injected with CNO once daily for 14 days (a left) and the first day of 14 days (right).

5. Fig. 4j shows body weight changes as %. What about the absolute values?

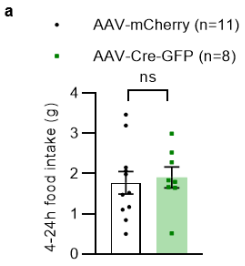
Response: The absolute value of body weight changes from mCherry-restraint and hM4Di-restraint mice over the 14 days of restraint stress was presented below (**Attached figure 12**). Despite observing a tendency for body weight decrease in the hM4Di-restraint mice during week 2, individual variation resulted in no significant difference between mCherry-restraint and hM4Di-restraint mice (Two-way ANOVA with post-hoc Šídák's multiple comparisons test, Group: $F(1, 23) = 4.097$, $p = 0.0547$, $p^{(\text{week 2})}=0.0946$).



Attached figure 12: Absolute body weight changes of mCherry-restraint and hM4Di-restraint mice during 14 days of restraint stress.

6. Fig. 5e shows food intake during the 4-hour stress. What about the food intake during 4-24 hours, post the stress? This is related to my point #2, as the signals from NPYDRN/vlPAG neurons may be more relevant during the post-stress period to help animals recover.

Response: Thank you again for your insightful feedback. In Fig 5e-g, acute novelty stress lasted 24 hours, indicating that mice remained exposed to novelty stress during 4-24 hours. The 4-24 hour food intake showed no difference between AAV-Cre-GFP and AAV-mCherry groups (**Attached figure 13**). Combining food intake results in the initial 4 hours of acute novelty stress (Fig.5e), we suggest that $NPY^{DRN/vlPAG}$ plays an important role in both onset and recovery period of stress. We have added these results to the revised manuscript **Supplementary Figure 4f** and **lines 350-352**.



Attached figure 13: 4-24 hours of food intake of AAV-mCherry and AAV-Cre-GFP *Npy*$^{lox/lox}$ groups under acute novelty stress.

7. Fig. 7e-f demonstrated the GABAergic inputs from NPYDRN/vlPAG neurons to their downstream targets in the PVT and LH. Considering the functional relevance of NPY as

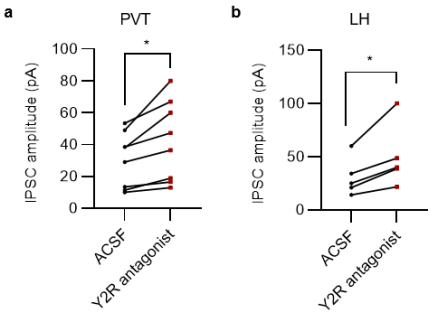
shown in Fig. 5, the authors should also examine whether NPY receptor inhibitors can affect how NPYDRN/vlPAG neurons regulate the activities of their downstream targets (e.g. those in the PVT and LH).

Response: We appreciate that the reviewer also finds this hypothesis intriguing. NPY Y1 receptor (Y1R) and NPY Y2 receptor (Y2R) are two well-known NPY receptors. Previously studies (PMID: 16998904, PMID: 12031341) have demonstrated the wide expression of Y2R in the PVT and LH, while Y1R is primarily found in the LH. Notably, Y1R is thought to exhibit anxiolytic-like effect (PMID: 15352219; PMID: 10193650). However, recent studies indicate that Y2R induced either anxiogenic effect when administrated intracerebroventricularly (PMID: 16959374) or anxiolytic effect in the locus coeruleus (LC) (PMID: 9555090), suggesting a complex role for Y2R in stress modulation.

In Fig.7g,h, we illustrated that light stimulation induced inhibitory postsynaptic current (IPSC) in both the PVT and LH. We further found that blocking Y2R with antagonist JNJ-31020028 mainly increased the amplitude of light-evoked IPSC in the PVT (8 in 11 cells) and LH (5 cells) (**Attached figure 6**). In contrast, Y1R receptor antagonist BIBO3304 had a mild effect on the amplitude increase of light-evoked IPSC in the LH (4 in 7 cells). These results suggest that Y2R plays a key role in the inhibitory effect of $NPY^{DRN/vlPAG}$ neurons in the PVT and LH while Y1R in the LH may have little contribution.

We acknowledge that additional experiments would be beneficial to clarify the contradiction that $NPY^{DRN/vlPAG}$ neurons project inhibitory signals to the downstream regions, yet this inhibitory effect could be suppressed by NPY, which exerts an anti-stress effect when released from $NPY^{DRN/vlPAG}$ neurons. However, we believe it is valuable to propose hypothesis based on our current data and the existing literature that Y2R is involved in the stress alleviation mediated by $NPY^{DRN/vlPAG}$ neurons. In the revised manuscript, we have explicitly stated the necessity for further experiments to fully elucidate the details of these differential effects. We will follow your suggestion and encouragement to gather more evidence in future projects.

The current data and nuanced discussion have been added to the revised manuscript **Supplementary Figure 6i.j** and **lines 474-485, 521-530, 631-636**.



Attached figure 6. The effect of Y2R antagonist JNJ-31020028 on light-evoked IPSC in the PVT (a) and LH (b).

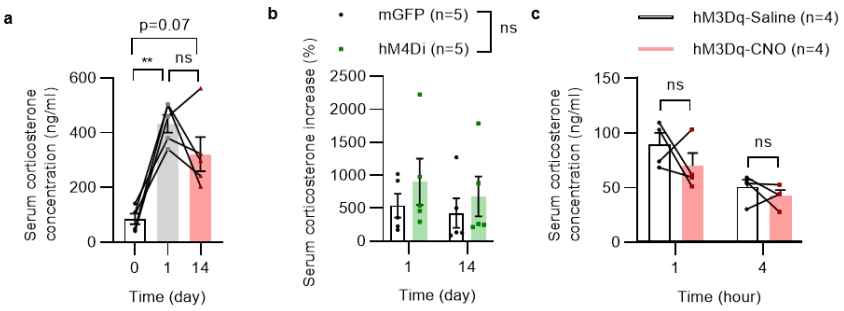
8. Corticosterone should be measured in multiple models used in the study.

Responses: We appreciate the reviewer for providing this important suggestion. Following his/her valuable guidance, we measured corticosterone levels at day 0, day 1 and day 14 of the 14-day restraint stress model. We found a significant increase in corticosterone levels after the first day of restraint compared to the baseline (day 0). After 14 days of restraint stress, the corticosterone still maintained a relatively high level (**Attached figure 14**), which was consistent with previous study (PMID: 25699675; PMID: 27647655).

Interestingly, despite the exacerbation of anxious states, inhibiting $NPY^{DRN/vlPAG}$ neurons during 14-day restraint stress did not influence corticosterone levels (**Attached figure 14**). This result suggested that stress-resistance effect of $NPY^{DRN/vlPAG}$ neurons may not directly modulate the HPA axis.

We also investigated corticosterone levels by directly activating $NPY^{DRN/vlPAG}$ neurons without artificial stress. No difference in corticosterone levels was observed between groups at 1 and 4 hours post saline or CNO injection (**Attached figure 14**). This finding corroborates our anterograde tracing experiment and further indicates that $NPY^{DRN/vlPAG}$ neurons did not directly regulate HPA axis under unstressed conditions.

We have added these results to the revised manuscript **Fig. 4d, Supplementary Figure 3m, Supplementary Figure 4d** and **lines 272-275, 300-302, 334-336, 553-556**.



Attached figure 14: (a) The levels of serum corticosterone in mice at day 0, day 1, and day 14 of the 14-day restraint stress. (b) The increase in serum corticosterone levels at day 1 and day 14 of the 14-dayrestraint stress in CNO-treated $NPY^{DRN/vlPAG}$-mGFP and $NPY^{DRN/vlPAG}$-hM4Di mice. (c) The levels of serum corticosterone in $NPY^{DRN/vlPAG}$-hM3Dq mice 1- and 4- hour after saline or CNO injection.

Reviewers' Comments:

Reviewer #1:
Remarks to the Author:
The authors carefully addressed my concerns about the previous version of the manuscript, and I have no further concerns.

Reviewer #2:
Remarks to the Author:
The authors have thoroughly and effectively addressed all of my previous concerns, significantly enhancing the manuscript's overall quality. Given these substantial improvements, I believe this work is now suitable for publication in Nature Communications.

Reviewer #3:
Remarks to the Author:
The authors adequately addressed my comments, and I support the publication of this manuscript.