

An amygdala to anterior hypothalamic circuit gates stress sensitivity

Corresponding Author: Dr Denise Cai

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

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

Version 0:

Reviewer comments:

Referee #1

(Remarks to the Author)

In this paper by Pennington et al, the authors investigate the role of the anterior hypothalamic nucleus (AHN) and its associated circuitry in stress vulnerability. First, the authors perform a whole-brain cfos immediate early gene screening to identify structures showing correlated activity changes in animals that have or have not experienced a prior stressor. Next, they use in-vivo miniscope calcium imaging to show that AHN neurons respond to aversive stimuli and that these responses scale with aversive intensity. Furthermore, they report that prior stress increased the number of aversive responsive neurons to the second stressor, without affecting the amplitude of responding.

Turning to causal manipulations, the authors next used a chemogenetic approach to show that artificially increasing AHN activity amplifies a freezing to an aversive stimulus without affecting baseline anxiety levels. In contrast, optogenetically silencing AHN activity at various task timepoints reduced the ability of the first stressor to modulate subsequent behavior, the recall of conditioned defensive responses to the context associated with that stress and the ability of the second stressor to produce freezing and produce subsequent defensive responses.

Finally, they examined the role of a pathway from the basolateral amygdala to AHN. They observed that while chemogenetically inhibiting this pathway during stressor 1 recall or during stressor 2 induction does not acutely alter freezing, it does reduce freezing during subsequent recall of Stressor 2.

While the optogenetic/chemogenetic findings suggest that the BLA-AHN pathway participates in stress vulnerability, the design of the experiments and the presented data do not support broad claims about the role of this circuit in behavior. For example, the authors suggest that the AHN encodes negative valence, but there is very little evidence that supports this claim and many alternative explanations exist. Furthermore, the circuit analysis of if or how AHN coordinates downstream pathways as well as studies on the role of the BLA-AHN pathway in different aspects of aversive processing or stress vulnerability are superficial and preliminary. For these reasons, I do not support publication of the manuscript in Nature. Detailed comments are provided below:

Major concerns:

1) The authors use a binomial regression analysis with an FDR (False Discovery Rate? this should be defined in text) correction. They include a great deal of analysis material as Tables and a description in the Methods regarding these approaches. However, it would be useful for the reader if they clearly described how they controlled for multiple comparisons given the large number of comparisons across brain regions and groups (e.g. they should state whether corrections were made only within a brain region analysis or across all possible brain-region/group comparisons, why FDR correction was selected, etc).

2) Related to Fig. 1e, the authors suggest that the AHN represents a central node in a network whose coactivity patterns are upregulated by prior stress and imply that it serves a coordinating function in this network. However, it is unclear whether AHN serves a type of coordinating function in this network or whether AHN simply participates in correlated activity, similar to other brain regions in this network. Furthermore, whether the functional connectivity of AHN with the rest of the network is

remodeled by prior stress is not clear. If the authors want to make these claims they need more in-depth analysis of how AHN could be driving global network changes. One idea would be to examine the weight of AHN relative to other brain regions in the network in driving these distributed correlations or they could use some sort of inactivation approach to determine whether the larger network is affected.

3) Based on data in Fig. 2, the authors argue that AHN neurons encode 'negative valence'. However, because they only test aversive stimuli (shocks and loud noises (100/130 dB) which are naturally aversive) it is not possible to make these claims. For example, it could be that AHN cells respond to a variety of sensory stimuli including those which are non-aversive. The fact that they exhibit declining responses to shocks across trials even though shocks likely do not lose their aversiveness across repeated presentations suggest something more complex than simple aversion being encoded. The authors would need to examine whether AHN cells respond to non-aversive stimuli such as neutral auditory or visual stimuli and compare these responses to aversive shocks and loud noises. In addition, the use of the term valence suggests that these cells specifically encode the unpleasantness of stimuli. However, even if they don't respond to non-aversive sensory stimuli, they may encode general salience and not aversion per-se. To test valence, they would need to examine how these cells encode rewarding and aversive stimuli side-by-side.

4) Also related to Fig. 2, the authors have a rich calcium imaging dataset, but very little data is shown on the dynamics, single neuron response properties of these cells or population activity. The authors should show heat plots of individual cells allowing for the comparison of the different types of stimuli at the single cell level. This should also be done for both the characterization of sensory responses (Fig. 2B-E) as well as how stress changes response properties. It is also important to examine whether AHN neurons are generally encoding aversion or whether they can resolve distinct types of aversive stimuli. This is hinted at in the single neuron response properties in Fig. 2C, but population decoding or low-dimensional analyses would expand this idea. They should also examine how the prior stress paradigm changes this coding.

5) The logic behind the selection of mouse models in Figure 4 is unclear. Optogenetic experiments start in wild-type mice, then shift to GAD2-Cre mice in panel G without justification. Given the hypothesis in Figure 2 implicating GABAergic neurons, it seems important to perform all experiments in GAD2-Cre mice.

6) The BLA-AHN circuit analysis in Fig. 5 is superficial. First, the authors use systemically administered chemogenetic inhibition of BLA cells that project to AHN, but these cells may collateralize elsewhere and the effects of the CNO may not be specific to BLA inputs to AHN. Second, the selection of when the inhibition is performed (e.g. Stressor 1 and Stressor 2) is not well reasoned with respect to specific hypotheses about the function of this pathway. Is it important in conveying general information about stressor 1 to produce stress upregulation? Does it mediate stressor 1 recall? Does it undergo plasticity in some way and participate solely in producing enhanced freezing to Stressor 2 after stressor 1? Is it involved specifically in stressor 2 recall? As it stands, only a few of these ideas are tested leaving open many interpretations of what this pathway does. Third, the response properties of BLA-AHN cells are not examined to stressors or during the stressor sensitization experiments. To understand how this circuit operates some type of recordings in this pathway is important.

7) Related to the above comment, the data presented don't fit with the model in Fig. 6. In this model the authors argue that BLA transmits aversive 'valence' to the AHN and that AHN then provides an output/gating mechanism for the production of defensive responding which can be turned up or down depending on prior stress. If true, then inactivating BLA inputs should reduce acute responses to stressor 1, which doesn't occur. Are the authors suggesting that BLA inputs require strengthening in some way? If not, how does the AHN alter its stressor representation following stressor 1? None of these ideas are considered in the model/paper.

Additional concerns:

1) For the data in Fig. 1B, it is not clear when freezing was measured during Stressor 1 and 2. For example, was freezing in response to Stressor 1 measured during ITI? For Stressor 2, since the loud noise is 3 sec long and animals were removed 10 sec later, it's unclear how freezing could be measured.

2) A related criticism relates to all experiment reporting freezing behavior. The exact time periods where freezing was measured should be reported and terms like 'pre' and 'post' should be defined.

3) For the data in Fig. 1, I could not find a description of the timeline (e.g. when Stressor 2 was given relative to when animals were sacrificed). This information should be included. Generally speaking, the experimental timeline for different experiments appears to vary and the authors don't provide enough information about individual experiments for an assessment of whether this is an issue or not. They should state (and maybe show in the experimental design portion of their graphs) the exact timeline (e.g. number of days between phases) for each experiment.

4) The correlation matrix graphs in Fig. 1E, right are not described in the figure legend.

5) For the data in Fig. 2, the authors should report the number of animals recorded from, the number of male and female animals used and the number of cells recorded in each experiment.

6) The authors use male and female animals for some of their studies. It would be valuable to the reader to know if any sex differences exist.

Referee #2

(Remarks to the Author)

Review of "An amygdala to anterior hypothalamic circuit gates negative valence and stress vulnerability"

Summary: This is an interesting and well written manuscript describing the potential role of the anterior hypothalamic nucleus (AHN) in mediating the stress sensitization model previously defined by this group. Stress sensitization is an interesting and translationally relevant behavior in which a shock stress leads to a later increased threat response to a different aversive modality, e.g. loud noise in a different context. The authors use whole brain clearing and cfos analyses to examine brain regions with correlated activity to the sensitized stress. After identifying the AHN as a potentially important area, they use a combination of calcium imaging and chemogenetic activation and inhibition to address the role of the AHN in the stress sensitization process. While overall I feel that these are interesting and relevant findings to our understanding of stress generalization and sensitization in mammals, I feel that, as it currently stands, the interpretations extend beyond the results for a number of reasons outlined below.

Comments / Concerns:

Throughout the paper, the authors refer to the whole brain cfos studies as indicative of 'functional connectivity' across the brain. I think it is important to use more precise language since what it really shows is brain regions with 'correlated activity' at this timepoint based on cfos, but not necessarily connectivity, functional or otherwise.

In the section on 'Augmenting AHN activity amplifies stressor valence' it is not clear to me that the experiments truly prove that 'valence' is amplified or altered. If anything, I would interpret the results as amplifying salience or strength of the stressor cue or stress response. Altering valence would traditionally be interpreted as altering direction of positive or negative emotion.

Note that valence is defined as 'the positive or negative feeling associated with a stimulus, object, event, or situation', whereas salience is defined as 'the degree to which something (a stimulus, event, or idea) stands out due to its emotional impact...'.

The general term of increased or decreased stressor valence is also used later (and throughout) the manuscript, and I really don't think this is the proper term to use as per above. If so, then I think other experiments are needed where differential valence (e.g. positive vs negative) emotional responding is addressed, vs. salience – how strong the signal is – are directly compared.

On a similar note, the work doesn't really clarify if it is a true effect on emotional valence (or salience) vs. altering the regulatory process of freezing behavior. Both of these issues could be addressed with such tasks as approach / avoidance, operant conditioning, conditioned place preference, or others beyond freezing measures that also get more directly at valence encoding.

Would an alternative interpretation of Figure 5f be that the BLA to AHN projection is required for context threat learning during the stressor 2 experience, such that inhibiting this pathway is interrupting the threat learning – and not necessarily that the BLA to AHN projection is underling the stressor valence process?

The model in Figure 6 isn't fully supported by the data presented. For example, "In mice with a history of prior stress, a larger number of AHN neurons are recruited in response to stress, possibly as a result of stronger amygdala input." The authors have the ability to measure this – does silencing the BLA to AHN inputs decrease the number of calcium imaged activated cells? This could also be addressed electrophysiologically with AHN recordings.

There are also no data presented demonstrating that the BLA-to-AHN projections connect to the AHN cells visualized with Gcamp6f.

Minor:

For the miniscope experiments, neither the figure nor the main text make it clear if this is a viral vector vs. transgenic expt. I'm assuming that this is a virus infused in hypothalamus of the GAD2-Cre line, but it should be made more clear in the figure legend and/or text.

Similarly in the next section, lines 188-189, the method could be more clear – currently states, "we expressed the excitatory chemogenetic receptor HM3Dq in GABAergic AHN neurons, or a control virus expressing mCherry..." but the first half of the sentence doesn't state anything about a virus or the type of virus.

Referee #3

(Remarks to the Author)

The authors use a variety of approaches to identify a region playing a role in behavior. Overall, this was a strong use of complementary methods to explore rigorously.

A few comments:

1) The framing in the very first paragraph is odd because the authors do not look at stress susceptible vs resilient populations: "Notably, there is substantial 36 variation in how individuals cope with stressful life events. Exemplifying this, only a small 37 proportion of individuals who experience a traumatic event at some point develop PTSD or other stress-related illness³⁸. How variability in the brain's stress circuits confers susceptibility to ³⁹ stress-related illnesses is poorly understood." It would seem that given this framing, there would be experiments exploring this.

2) In figure 1, is this the heatmap for the cluster? To me this looks like the heatmap for the entire dataset bc of the number of regions– if so can they denote the cluster within the larger heatmap? If it's just the heatmap for the cluster then label the regions? In extended data they have a no-stress control – where is the heatmap for that group? It is curious because the whole heatmap is red which maybe reflects the stress state but the unstressed comparison might be interesting?

3) Figure 2 says "AHN neurons reflect stressor valence" but I read the data as reflecting intensity rather than valence – they are not testing any positive stimuli for instance. Do we know this isn't a response to any salient stimulus that scales with intensity or is it specific to negatively valenced stimuli? This is a critical question.

4) Response decays with repetition of the stimulus – does the behavioral response (freezing) change over time with this decay of neuronal response? They don't really discuss the time effect here. Is there a functional link?

5) Minor point: Figure 3: Light dark box test is repeated? This is somewhat confusing as I was under the impression that novelty was important here.

6) Figure 4 – experiments with pan neuronal vs gaba neurons, but don't repeat the same experiments to compare the 2 (eg laser on/off during stressor 1), so unclear what the rationale is for studying the 2 diff populations as they are not explored.

7) Where are the outputs that drive freezing behavior? Does AHN project to PAG?

Version 2:

Reviewer comments:

Referee #1

(Remarks to the Author)

The authors have conducted new experiments and analyses that strengthen the manuscript. The extended test of valence coding in AHN neurons is a valuable addition, and the new analyses suggesting that AHN neurons primarily encode aversive intensity rather than distinguishing among distinct aversive stimuli are important. However, I have several remaining concerns about some of the new additions that should be addressed.

1) I thank the authors for clarifying their False Discovery Rate (FDR) procedures (data described in Fig. 1 and Ext. Data Fig. 1). The revised description is substantially improved. However, because the analysis uses a two-stage procedure, first correcting the omnibus group effect across all 454 regions and then correcting the S versus NS post-hoc contrast only across the 90 regions that passed the initial screen, it would be helpful to explicitly justify this hierarchical correction strategy and possibly also show data with corrections across all 454 regions rather than only the screened subset.

2) Although the addition of the sucrose/water valence assay is important, it is not clear that rewarding stimuli actually inhibit AHN neurons as the only comparisons are across stimulus condition. To support this claim, the authors should compare activity during the evoked response period with a comparable pre-stimulus baseline period for each stimulus. More generally, this baseline comparison should be performed whenever the authors make claims about the significance or direction of responses to specific stimuli.

3) For the optogenetic experiments shown in Fig. 3i–l, AHN neurons were inhibited during both Stressor 1 recall and the subsequent Stressor 2 exposure, with reductions in freezing observed at both timepoints and during the later Stressor 2 recall test. Because these manipulations occur serially within the same animals, the results are somewhat difficult to interpret. One possibility is that inhibition during Stressor 1 recall reduced the expression or updating of the Stressor 1 memory, thereby indirectly reducing the effect of Stressor 1 on subsequent Stressor 2 responding. Ideally, these manipulations would be tested separately. At minimum, the authors should acknowledge this interpretive limitation.

Referee #2

(Remarks to the Author)

The authors have satisfactorily addressed my prior concerns.

Referee #3

(Remarks to the Author)

The authors use a whole brain fos approach to identify the AHN as a potential site of action for heightened stress sensitivity.

They then use miniscope imaging to explore the activity of these neurons, focusing on the connection from the BLA. A series of experiments using optogenetic inhibition and activation of Gq signaling using DREADD technology suggest a causal role of these BLA to AHN circuit in this behavior. The authors were very responsive to previous concerns, however I still do not think that the data, while interesting, supports their model.

Specifically, the authors are focused on VALENCE. The neurons that they are exploring are very responsive to aversive stimuli, and appear to scale with intensity, both sound and shock. However, the responses to positive stimuli, are minimal, and while statistically significant, their biological significance is unclear and there is no evidence supporting a role provided. Even when the data is critically assessed, there is little to no differences between sucrose and water, with no response to quinine. One potential reason for this scaling to shock and loud intensity is that this may actually represent a pain signal. For example, loud sounds can drive nociceptive responses (<https://pubmed.ncbi.nlm.nih.gov/25639244/>). It may be that this BLA to AHN is involved in pain aversiveness and that is driving the behavioral changes, but it is not something that the current experiments have been designed to assess.

Also...relatively minor point. The authors used a DREADD vs optogenetics to drive activity, this seems like a missed opportunity to probe behaviors with a more granular time resolution. Did the authors use a Chr2 to drive activity in this pathway or in the AHN, and if so, what were the results?

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Reviewers' comments are in black. Responses to reviewers are in blue.

Referee #1 (Remarks to the Author):

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While the optogenetic/chemogenetic findings suggest that the BLA-AHN pathway participates in stress vulnerability, the design of the experiments and the presented data do not support broad claims about the role of this circuit in behavior. For example, the authors suggest that the AHN encodes negative valence, but there is very little evidence that supports this claim and many alternative explanations exist. Furthermore, the circuit analysis of if or how AHN coordinates downstream pathways as well as studies on the role of the BLA-AHN pathway in different aspects of aversive processing or stress vulnerability are superficial and preliminary. For these reasons, I do not support publication of the manuscript in Nature. Detailed comments are provided below:

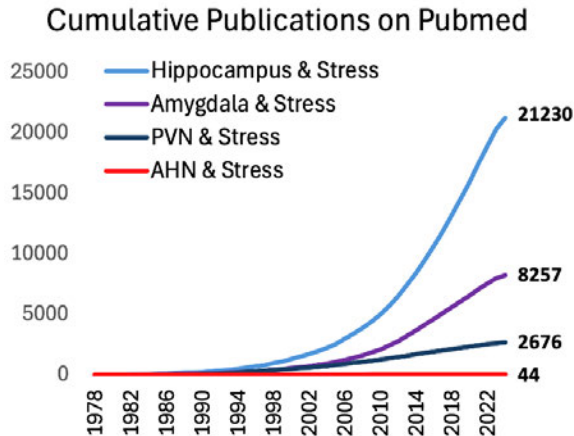
Major concerns:

1) The authors use a binomial regression analysis with an FDR (False Discovery Rate? this should be defined in text) correction. They include a great deal of analysis material as Tables and a description in the Methods regarding these approaches. However, it would be useful for the reader if they clearly described how they controlled for multiple comparisons given the large number of comparisons across brain regions and groups (e.g. they should state whether corrections were made only within a brain region analysis or across all possible brain-region/group comparisons, why FDR correction was selected, etc).

We will clarify this point in the methods and in the main text. FDR was applied across all brain regions to ensure that any detected changes stand up to brain-wide significance.

2) Related to Fig. 1e, the authors suggest that the AHN represents a central node in a network whose coactivity patterns are upregulated by prior stress and imply that it serves a coordinating function in this network. However, it is unclear whether AHN serves a type of coordinating function in this network or whether AHN simply participates in correlated activity, similar to other brain regions in this network. Furthermore, whether the functional connectivity of AHN with the rest of the network is remodeled by prior stress is not clear. If the authors want to make these claims they need more in-depth analysis of how AHN could be driving global network changes. One idea would be to examine the weight of AHN relative to other brain regions in the network in driving these distributed correlations or they could use some sort of inactivation approach to determine whether the larger network is affected.

The reviewer makes an excellent point regarding whether the AHN plays a central, coordinating, function in the identified stress network, or merely participates in this network. It was not our intention to suggest that the AHN is the main node in this network, but rather, to highlight the AHN as a novel player in this network. Given the dearth of papers on the AHN and stress (see comparison of publications on AHN and stress & amygdala, hippocampus, paraventricular nucleus of the hypothalamus), we feel that showing that the AHN is correlated with a known stress network is novel. Additionally, our causal manipulations clearly demonstrate that the AHN is able to influence stress-evoked behavior through interactions with this network. We will amend the text to highlight this novelty, and reduce wording that might suggest that the AHN plays a coordinating, function. In the future, we hope to perform studies in which we manipulate the AHN while simultaneously recording these other regions, but this is a multiyear endeavor.



3) Based on data in Fig. 2, the authors argue that AHN neurons encode 'negative valence'. However, because they only test aversive stimuli (shocks and loud noises (100/130 dB) which are naturally aversive) it is not possible to make these claims. For example, it could be that AHN cells respond to a variety of sensory stimuli including those which are non-aversive. The fact that they exhibit declining responses to shocks across trials even though shocks likely do not lose their aversiveness across repeated presentations suggest something more complex than simple aversion being encoded. The authors would need to examine whether AHN cells respond to non-aversive stimuli such as neutral auditory or visual stimuli and compare these responses to aversive shocks and loud noises. In addition, the use of the term valence suggests that these cells specifically encode the unpleasantness of stimuli. However, even if they don't respond to non-aversive sensory stimuli, they may encode general salience and not aversion per-se. To test valence, they would need to examine how these cells encode rewarding and aversive stimuli side-by-side.

To address this concern, we have since collected data where we simultaneously imaged the AHN's responses to positive and negative stimuli. The AHN was recorded while water-deprived animals forage for liquid and receive stimuli of positive (sucrose), 'neutral' (water), and negative (quinine) valence. In the same session, animals also received footshock, allowing us to compare AHN responses to all of these stimuli. Crucially, shock-responsive AHN neurons (i.e., cells that show a reliable increase in activity in response to footshock), actually show decreased activity in response to water/sucrose. Moreover, these events scale with stimulus intensity (greater inhibition to sucrose than quinine). These results strongly support that AHN is encoding valence and not simply responding to the salience of events.

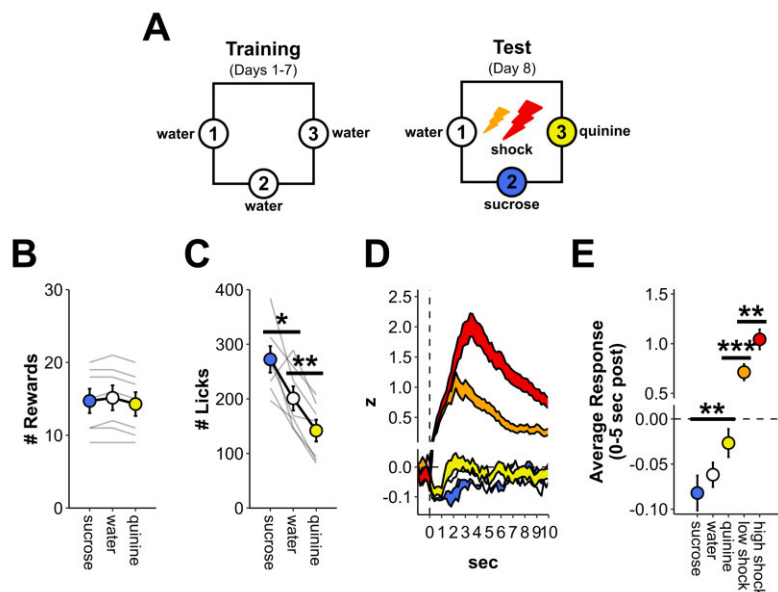


Figure showing AHN neurons encode valence:

- A) Water-deprived animals were initially trained to respond at 3 ports, in order from 1 to 3, to obtain water. Subsequently, during a test session, one water port was swapped for 20% sucrose and another water port was swapped for 0.5 mM quinine, providing a bidirectional manipulation of valence. After 15 minutes of drinking at the ports, animals additionally received low and high amplitude footshocks (0.25 mA and 1 mA).
- B) Animals obtained an approximately equal number of each solution during the test (sucrose, water, and quinine).
- C) Animals licked more at the sucrose port and less at the quinine port, relative to the water port.
- D) Average response of shock-responsive neurons to each stimulus (blue=sucrose, white=water, yellow=quinine, orange=weak shock, red=strong shock).
- E) Shock responsive AHN neurons showed a significant reduction in activity in response to sucrose (relative to quinine), and a significant increase in activity in response to low/high shock.

4) Also related to Fig. 2, the authors have a rich calcium imaging dataset, but very little data is shown on the dynamics, single neuron response properties of these cells or population activity. The authors should show heat plots of individual cells allowing for the comparison of the different types of stimuli at the single cell level. This should also be done for both the characterization of sensory responses (Fig. 2B-E) as well as how stress changes response properties.

It is also important to examine whether AHN neurons are generally encoding aversion or whether they can resolve distinct types of aversive stimuli. This is hinted at in the single neuron response properties in Fig. 2C, but population decoding or low-dimensional analyses would expand this idea. They should also examine how the prior stress paradigm changes this coding.

We will add heatmaps to show the responses of individual neurons. Additionally, although our sample size is small, we have data showing that prior stress enhances the number of stress responsive neurons in the test examining multiple aversive stimuli and will include this as supplementary data.

5) The logic behind the selection of mouse models in Figure 4 is unclear. Optogenetic experiments start in wild-type mice, then shift to GAD2-Cre mice in panel G without justification. Given the hypothesis in Figure 2 implicating GABAergic neurons, it seems important to perform all experiments in GAD2-Cre mice.

We are redoing this experiment in GAD-Cre mice so that everything is consistent. Given the majority of AHN neurons are GABAergic, and our findings from Figure 4 and 5 showing that causally manipulating GABAergic neurons influences stress behavior, we are fairly confident that this experiment will work.

6) The BLA-AHN circuit analysis in Fig. 5 is superficial. First, the authors use systemically administered chemogenetic inhibition of BLA cells that project to AHN, but these cells may collateralize elsewhere and the effects of the CNO may not be specific to BLA inputs to AHN. Second, the selection of when the inhibition is performed (e.g. Stressor 1 and Stressor 2) is not well reasoned with respect to specific hypotheses about the function of this pathway. Is it important in conveying general information about stressor 1 to produce stress upregulation? Does it mediate stressor 1 recall? Does it undergo plasticity in some way and participate solely in producing enhanced freezing to Stressor 2 after stressor 1? Is it involved specifically in stressor 2 recall? As it stands, only a few of these ideas are tested leaving open many interpretations of what this pathway does. Third, the response properties of BLA-AHN cells are not examined to stressors or during the stressor sensitization experiments. To understand how this circuit operates some type of recordings in this pathway is important.

We fully agree with the reviewer. We have new data showing that inhibition of BLA terminals in the AHN is able to blunt the immediate response to stressor 2, showing specificity of this projection (see below). Additionally, we are performing several other experiments to address the reviewer's other concerns:

1. We are performing patch-clamp electrophysiology on BLA→AHN projection neurons to assess if these neurons undergo plasticity in response to stress.
2. We are performing fiber photometry on BLA→AHN neurons to assess if BLA neurons respond to stress.
3. We are assessing the impact of chemogenetically silencing the BLA while simultaneously performing calcium imaging on the AHN.
4. We are testing if chemogenetically exciting BLA → AHN neurons enhances stress responses.

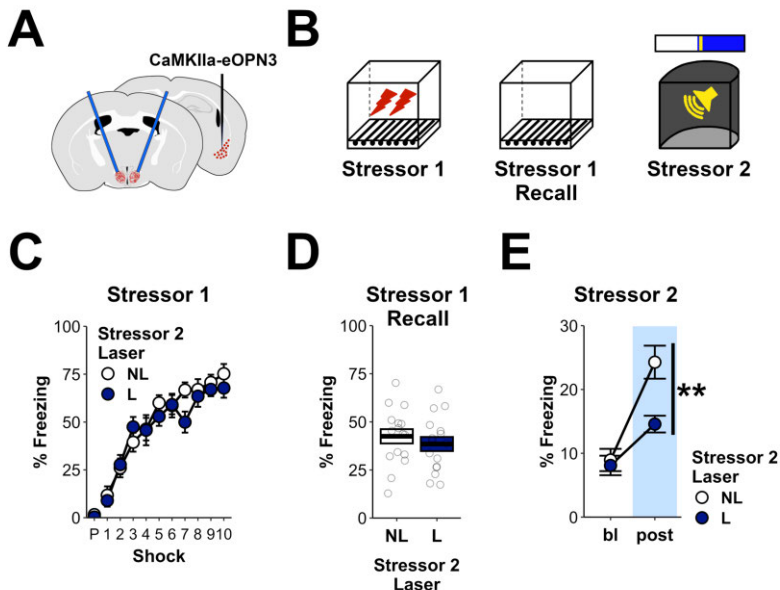


Figure caption: Inhibition of BLA→AHN terminals reduces stress sensitivity:

- A)** Animals received a viral infusion of the axonally trafficked inhibitory opsin eOPN3 into the BLA. Fiber cannula were implanted overlying the AHN to inhibit terminals from the BLA.
- B)** Behavioral schematic.
- C)** Animals that would subsequently have light exposure in the AHN during Stressor 2 did not show differences from no light controls in response to Stressor 1.

- D) Animals that would subsequently have light exposure in the AHN during Stressor 2 did not show differences from no light controls in response to Stressor 1.
- E) Inhibition of BLA→AHN terminals blunts response to Stressor 2.

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Because the period after the aversive stimulus is quite brief (10 sec), it is sometimes difficult to detect reductions in the acute stress freezing response. However, we have an new experiment lengthening the post-stress period to 3 minutes and using terminal inhibition of BLA→AHN projections, we observed a decrease in the acute response. This is consistent with the proposed model.

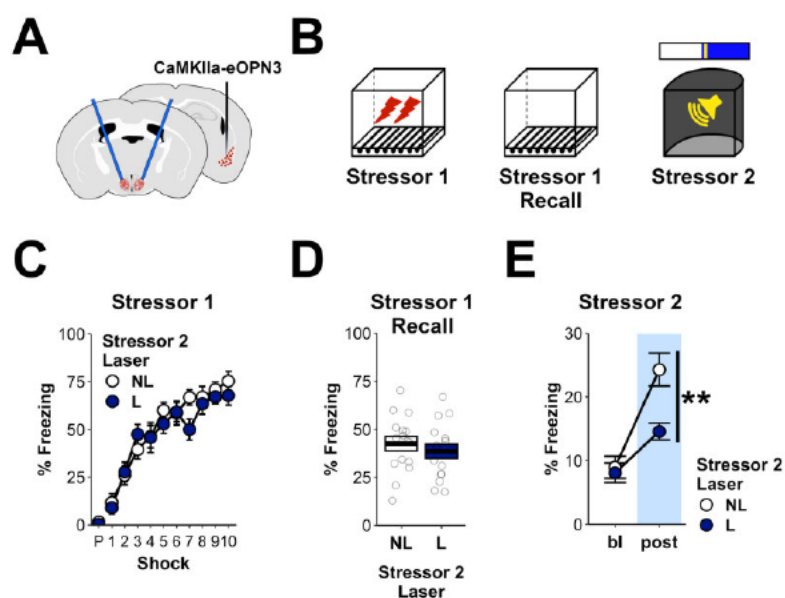


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This will be clarified.

2) A related criticism relates to all experiment reporting freezing behavior. The exact time periods where freezing was measured should be reported and terms like 'pre' and 'post' should be defined.

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3) For the data in Fig. 1, I could not find a description of the timeline (e.g. when Stressor 2 was given relative to when animals were sacrificed). This information should be included. Generally speaking, the experimental timeline for different experiments appears to vary and the authors don't provide enough information about individual experiments for an assessment of whether this is an issue or not. They should state (and maybe show in the experimental design portion of their graphs) the exact timeline (e.g. number of days between phases) for each experiment.

This will be clarified.

4) The correlation matrix graphs in Fig. 1E, right are not described in the figure legend.

We will add a description of the correlation matrices.

5) For the data in Fig. 2, the authors should report the number of animals recorded from, the number of male and female animals used and the number of cells recorded in each experiment.

We will amend the text to ensure that the number of animals, their sex, and the number of cells is included for each experiment.

6) The authors use male and female animals for some of their studies. It would be valuable to the reader to know if any sex differences exist.

We have published data showing that males and females do not behaviorally differ using the proposed behavioral procedures (Pennington, et al, 2024), and we similarly do not see sex differences in any of our behavioral or neural recordings here. We will add this information to the manuscript.

Referee #2 (Remarks to the Author):

Summary: This is an interesting and well written manuscript describing the potential role of the anterior hypothalamic nucleus (AHN) in mediating the stress sensitization model previously defined by this group. Stress sensitization is an interesting and translationally relevant behavior in which a shock stress leads to a later increased threat response to a different aversive modality, e.g. loud noise in a different context. The authors use whole brain clearing and cfos analyses to examine brain regions with correlated activity to the sensitized stress. After identifying the AHN as a potentially important area, they use a combination of calcium imaging and chemogenetic activation and inhibition to address the role of the AHN in the stress sensitization process. While overall I feel that these are interesting and relevant findings to our understanding of stress generalization and sensitization in mammals, I feel that, as it currently stands, the interpretations extend beyond the results for a number of reasons outlined below.

Comments / Concerns:

Throughout the paper, the authors refer to the whole brain cfos studies as indicative of ‘functional connectivity’ across the brain. I think it is important to use more precise language since what it really shows is brain regions with ‘correlated activity’ at this timepoint based on cfos, but not necessarily connectivity, functional or otherwise.

We will amend our language in the text accordingly and use correlation and not functional connectivity. Additionally, we are performing slice electrophysiology experiments to test if the excitability of BLA→AHN inputs is increased as the result of prior stress.

In the section on ‘Augmenting AHN activity amplifies stressor valence’ it is not clear to me that the experiments truly prove that ‘valence’ is amplified or altered. If anything, I would interpret the results as amplifying salience or strength of the stressor cue or stress response. Altering valence would traditionally be interpreted as altering direction of positive or negative emotion.

Note that valence is defined as ‘the positive or negative feeling associated with a stimulus, object, event, or situation’, whereas salience is defined as ‘the degree to which something (a stimulus, event, or idea) stands out due to its emotional impact...’. The general term of increased or decreased stressor valence is also used later (and throughout) the manuscript, and I really don’t think this is the proper term to use as per above. If so, then I think other experiments are needed where differential valence (e.g. positive vs negative) emotional responding is addressed, vs. salience – how strong the signal is – are directly compared. On a similar note, the work doesn’t really clarify if it is a true effect on emotional valence (or salience) vs. altering the regulatory process of freezing behavior. Both of these issues could be addressed with such tasks as approach / avoidance, operant conditioning, conditioned place preference, or others beyond freezing measures that also get more directly at valence encoding.

To address this concern, we have since collected data where we simultaneously imaged the AHN’s responses to positive and negative stimuli. The AHN was recorded while water-deprived animals forage for liquid and receive stimuli of positive (sucrose), ‘neutral’ (water), and negative (quinine) valence. In the same session, animals also received footshock, allowing us to compare AHN responses to all of these stimuli. Crucially, shock-responsive AHN neurons (i.e., cells who show a reliable increase in activity in response to footshock), actually show decreased activity in response to water/sucrose. Moreover, these events scale with stimulus intensity. These results argue against the idea of the AHN simply responding to the salience of events and instead support a valence interpretation.

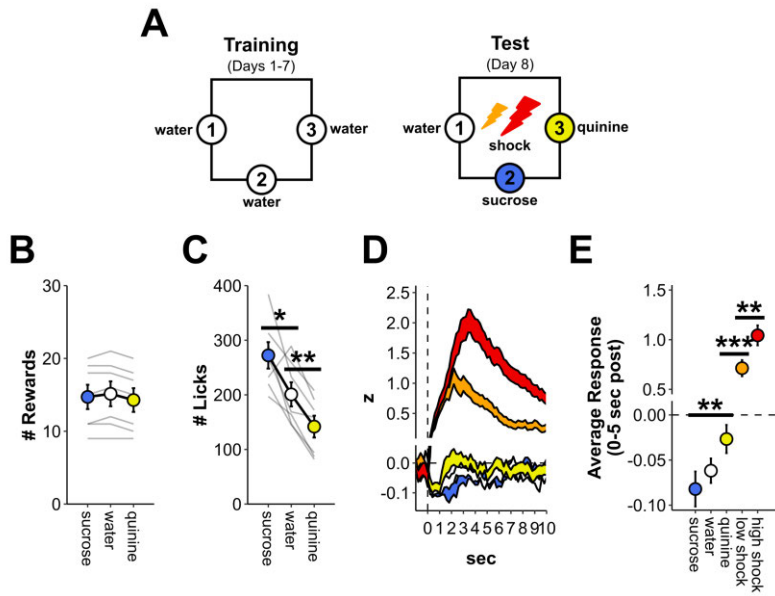


Figure caption: AHN neurons encode valence:

- A)** Water-deprived animals were initially trained to respond at 3 ports, in order from 1 to 3, to obtain water. Subsequently, during a test session, one water port was swapped for 20% sucrose and another water port was swapped for 0.5 mM quinine, providing a bidirectional manipulation of valence. After 15 minutes of drinking at the ports, animals additionally received low and high amplitude footshocks (0.25 mA and 1 mA).
- B)** Animals obtained an approximately equal number of each solution during the test (sucrose, water, and quinine).
- C)** Animals licked more at the sucrose port and less at the quinine port, relative to the water port.
- D)** Average response of shock-responsive neurons to each stimulus (blue=sucrose, white=water, yellow=quinine, orange=weak shock, red=strong shock).
- E)** Shock responsive AHN neurons showed a significant reduction in activity in response to sucrose (relative to quinine), and a significant increase in activity in response to low/high shock.

Would an alternative interpretation of Figure 5f be that the BLA to AHN projection is required for context threat learning during the stressor 2 experience, such that inhibiting this pathway is interrupting the threat learning – and not necessarily that the BLA to AHN projection is underling the stressor valence process?

To address this concern, we are performing fiber photometry recordings on BLA to AHN projection neurons to assess if they encode stressor valence.

The model in Figure 6 isn't fully supported by the data presented. For example, "In mice with a history of prior stress, a larger number of AHN neurons are recruited in response to stress, possibly as a result of stronger amygdala input." The authors have the ability to measure this – does silencing the BLA to AHN inputs decrease the number of calcium imaged activated cells? This could also be addressed electrophysiologically with AHN recordings.

We are currently performing an experiment to assess the impact of chemogenetically inhbiting the BLA while recording from the AHN, in order to test if the BLA directly contributes to AHN responses to stressful stimuli.

There are also no data presented demonstrating that the BLA-to-AHN projections connect to the AHN cells visualized with Gcamp6f.

We will provide images showing innervation of the AHN by BLA neurons. Additionally, we are performing fiber photometry to show if BLA-to-AHN neuron activity reflects stress severity.

Minor:

For the miniscope experiments, neither the figure nor the main text make it clear if this is a viral vector vs. transgenic expt. I'm assuming that this is a virus infused in hypothalamus of the GAD2-Cre line, but it should be made more clear in the figure legend and/or text.

This will be clarified.

Similarly in the next section, lines 188-189, the method could be more clear – currently states, “we expressed the excitatory chemogenetic receptor HM3Dq in GABAergic AHN neurons, or a control virus expressing mCherry...” but the first half of the sentence doesn't state anything about a virus or the type of virus.

This will be clarified.

Referee #3 (Remarks to the Author):

The authors use a variety of approaches to identify a region playing a role in behavior. Overall, this was a strong use of complementary methods to explore rigorously.

A few comments:

1) The framing in the very first paragraph is odd because the authors do not look at stress susceptible vs resilient populations: “Notably, there is substantial 36 variation in how individuals cope with stressful life events. Exemplifying this, only a small 37 proportion of individuals who experience a traumatic event at some point develop PTSD or other stress-related illness³⁸. How variability in the brain’s stress circuits confers susceptibility to 39 stress-related illnesses is poorly understood.” It would seem that given this framing, there would be experiments exploring this.

We will better clarify how our studies directly examine stress vulnerability. Many prior studies of stress vulnerability have examined individual differences in the stress response. However, one of the greatest sources of variation in responding to stressful life events is the experience of prior stress, wherein prior stress increases vulnerability/susceptibility to subsequent stress. Accordingly, in every experiment in the paper, we examined how the prior experience of stress, relative to non-stressed controls, influenced subsequent responding to stress. This approach can be performed at the group level and provided a great deal of power, allowing us to examine neural correlates of these changes using both iDISCO and in vivo calcium imaging. We fully appreciate that this is only one of many sources of variability underlying stress responses (i.e. susceptibility). However, given that it is one of the most replicable sources of variation in humans, and a major correlate of psychiatric illness, understanding this factor is key.

2) In figure 1, is this the heatmap for the cluster? To me this looks like the heatmap for the entire dataset bc of the number of regions– if so can they denote the cluster within the larger heatmap? If it’s just the heatmap for the cluster then label the regions? In extended data they have a no-stress control – where is the heatmap for that group? It is curious because the whole heatmap is red which maybe reflects the stress state but the unstressed comparison might be interesting?

Figure 1 reflects the heatmap for this cluster specifically and we will clarify this point. Additionally, we will denote this cluster within the larger heatmap of all regions in the supplement. We will also provide the heatmap of the non-stressed controls, although, given that there is no activating stimulus, there is very little cfos and it is accordingly questionable whether such correlations are appropriate.

3) Figure 2 says “AHN neurons reflect stressor valence” but I read the data as reflecting intensity rather than valence – they are not testing any positive stimuli for instance. Do we know this isn’t a response to any salient stimulus that scales with intensity or is it specific to negatively valenced stimuli? This is a critical question.

To address this concern, we have since collected data where we simultaneously imaged the AHN’s responses to positive and negative stimuli. The AHN was recorded while water-deprived animals forage for liquid and receive stimuli of positive (sucrose), ‘neutral’ (water), and negative (quinine) valence. In the same session, animals also received footshock, allowing us to compare AHN responses to all of these stimuli. Crucially, shock-responsive AHN neurons (i.e., cells who show a reliable increase in activity in response to footshock), actually show decreased activity in response to water/sucrose. Moreover, these events scale with stimulus intensity. These results argue against the idea of the AHN simply responding to the salience of events and instead support a valence interpretation.

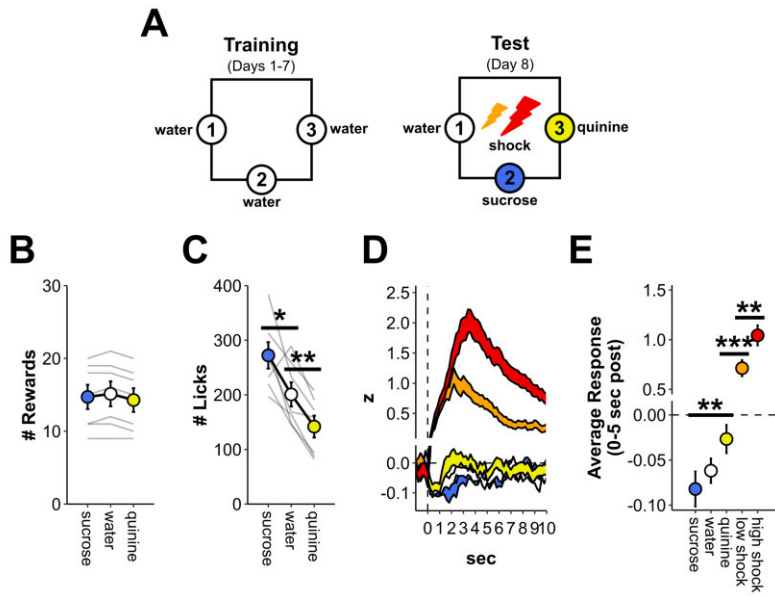


Figure caption: AHN neurons encode valence:

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- B)** Animals obtained an approximately equal number of each solution during the test (sucrose, water, and quinine).
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- E)** Shock responsive AHN neurons showed a significant reduction in activity in response to sucrose (relative to quinine), and a significant increase in activity in response to low/high shock.

4) Response decays with repetition of the stimulus – does the behavioral response (freezing) change over time with this decay of neuronal response? They don't really discuss the time effect here. Is there a functional link?

We will expand on this comment. The answer is that the freezing response to the aversive stimulus does not decrease, but rather, increases across the course of the session, although the rise in freezing after each stimulus is less. It could be that the reduced AHN response corresponds with the smaller change in behavior from one trial to the next. Alternatively, acute stress is known to release endorphins that blunt the response to subsequent stress, and reduced AHN responses may reflect this dampening.

5) Minor point: Figure 3: Light dark box test is repeated? This is somewhat confusing as I was under the impression that novelty was important here.

One of the reasons we have utilized the light-dark test is because we have heard it has better test-retest reliability than similar measures (e.g., elevated plus maze). Indeed, in the test in question, there is no effect of test number on time spent in the dark ($F_{1,19}=1.8$, $p=0.2$).

6) Figure 4 – experiments with pan neuronal vs gaba neurons, but don't repeat the same experiments

to compare the 2 (eg laser on/off during stressor 1), so unclear what the rationale is for studying the 2 diff populations as they are not explored.

The use of C57 instead of GAD-Cre mice for the first experiment in Figure 4 was done purely based upon the availability of GAD-Cre mice in our colony at the time. We are redoing this experiment in GAD-Cre mice so that everything is consistent. Given the majority of AHN neurons are GABAergic, and our findings from Figure 4 and 5 showing that causally manipulating GABAergic neurons influence stress behavior, we are fairly confident that this experiment will work.

7) Where are the outputs that drive freezing behavior? Does AHN project to PAG?

We will expand on upon this in the discussion. The AHN projects to many places that can influence freezing, including the PAG. However, discerning the exact projection is beyond the scope of the

Reviewers' comments are in black. Responses to reviewers are in blue.

We thank the reviewers for their time and thoughtful comments on the initial submission of this manuscript. We found the comments incredibly insightful and fair. In this revision, we have carefully considered the reviewer comments and added several additional experiments. We have also expanded upon the methods and clarified many points in the text. Together, these changes have substantially improved the manuscript and we hope that you will now find it suitable for publication.

Immediately below, we list the major changes made to address reviewer comments. We then respond to each comment, point by point, providing text/figure references.

1. We have added a new calcium imaging experiment recording AHN^{GAD} neurons and found that while these neurons are excited by aversive stimuli, the same neurons are inhibited by positive stimuli (Figure 2k-n). These data strongly suggest that the activity of AHN^{GAD} neurons reflects valence and not salience.
2. We have added a new calcium imaging experiment recording specifically from amygdala neurons that project to the AHN and demonstrated that these neurons' activity reflects the intensity of aversive stimuli, consistent with those of the AHN (Figure 4). These results are consistent with our proposed model, in which valence signals are relayed from the amygdala to the AHN (Figure 5).
3. We have added a new optogenetic experiment showing that terminal inhibition of amygdala to AHN projections reduced sensitized stress responses, suggesting that this specific circuit is necessary for stress sensitization (Figure 4i-n). This addresses the concern that our previous chemogenetic manipulation of amygdala neurons that project to the AHN might act through collaterals to another region.
4. We have also added a new optogenetic experiment showing that terminal inhibition of amygdala to AHN projections does not change locomotor activity when an aversive stimulus is not present (Extended Data Figure 5a-e), consistent with our data showing that activating AHN^{GAD} neurons also does not change freezing levels when an aversive stimulus is not present (Figure 3m-o). Furthermore, additional new data show that neither activating nor inhibiting AHN^{GAD} neurons affect anxiety-related behaviors (e.g., light-dark test; Extended Data Figure 4g-l). These results suggest that increasing or decreasing AHN activity when a stressor is not present does not alter behavior. Rather, the AHN seems to have a modulatory influence on stress responses evoked by environmental stimuli.
5. We have new calcium imaging data showing prior stress increases the proportion of AHN^{GAD} neurons that are responsive to multiple aversive stimuli (Extended Data Figure 3c). Nevertheless, prior stress does not alter the amplitude of stress-responsive neurons (Figure 2e, Extended Data Figure 3f-g).
6. We have replicated the optogenetic inhibition of AHN neurons in GAD-Cre mice (Figure 3a-f) so that everything in the manuscript is internally consistent.
7. We have added a low dimensional analysis (PCA) of AHN activity and shown that the majority of AHN population activity can be explained by a single principal component (PC1=27%, PC2=7%), and PC1 reflects stressor intensity across categorically different stressors (Figure 2j). This suggests AHN activity at the population level broadly reflects stimulus valence.
8. To clarify the text, we have 1) focused the introduction on stress sensitization rather than stress susceptibility. 2) amended that AHN is correlated with, rather than coordinating a known stress network. 3) included additional analysis details in the methods section. 4) expanded the discussion to include downstream targets of AHN.

Referee #1 (Remarks to the Author):

1) The authors use a binomial regression analysis with an FDR (False Discovery Rate? this should be defined in text) correction. They include a great deal of analysis material as Tables and a description in the Methods regarding these approaches. However, it would be useful for the reader if they clearly described how they controlled for multiple comparisons given the large number of comparisons across brain regions and groups (e.g. they should state whether corrections were made only within a brain region analysis or across all possible brain-region/group comparisons, why FDR correction was selected, etc).

We have now clarified this point in the methods. False Discovery Rate (FDR) correction of p-values was applied to control for multiple comparisons. This correction was applied across all brain regions included in each analysis to ensure that detected changes stood up to brain-wide significance. Moreover, this correction is commonly used in the field when controlling for a large number of statistical comparisons (e.g, PMID: 40175547, 39939746):

*“For each region, count data was modeled as a function of group ($y=B_0+B_1*NS+B_2*S+e$). We first screened whether group membership was a significant predictor of cFos counts by comparing the full model listed above to an intercept only model ($y=B_0+e$). As can be appreciated from Extended Figure 1, this largely served to detect regions activated by Stressor 2. Amongst the 90 regions that displayed group variation in cFos counts following False Discovery Rate (FDR) correction (i.e., across all 454 regions. Benjamini-Hochberg correction), we then assessed whether S and NS groups displayed differential cFos counts using post-hoc contrasts with the multcomp package⁶⁹. For this contrast, we report regions which surpass FDR correction (across the 90 regions that displayed some variation in cFos in our initial screen).”* (Lines 968-977)

2) Related to Fig. 1e, the authors suggest that the AHN represents a central node in a network whose coactivity patterns are upregulated by prior stress and imply that it serves a coordinating function in this network. However, it is unclear whether AHN serves a type of coordinating function in this network or whether AHN simply participates in correlated activity, similar to other brain regions in this network. Furthermore, whether the functional connectivity of AHN with the rest of the network is remodeled by prior stress is not clear. If the authors want to make these claims they need more in-depth analysis of how AHN could be driving global network changes. One idea would be to examine the weight of AHN relative to other brain regions in the network in driving these distributed correlations or they could use some sort of inactivation approach to determine whether the larger network is affected.

The reviewer makes an excellent point regarding whether the AHN plays a central, coordinating, function in the identified stress network, or merely participates in this network. It was not our intention to suggest that the AHN is the main node in this network, but rather, to highlight the AHN as a novel player in this network. Given the dearth of papers on the AHN and stress, we feel that showing that the AHN is correlated with a known stress network is highly novel. Additionally, our causal manipulations clearly demonstrate that the AHN is able to influence stress-evoked behavior through interactions with this network.

We have amended the text to highlight this novelty and reduce wording that might suggest that the AHN plays a coordinating function.

A few key examples:

“Prior stress remodels a distributed stress network that includes the AHN.” (Section Title, Line 70)

“Notably, although these findings do not address the causal contribution of the AHN to this network, or to stress sensitization, they place the AHN within a network that is well-positioned to regulate stress sensitization.” (Lines 91-93)

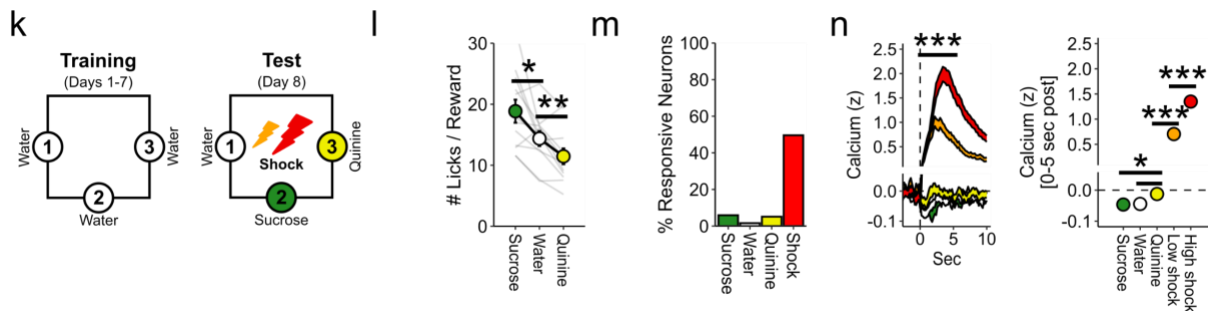
“These findings bolster the idea that prior adversity increases the correlational strength of a threat-related network that includes the AHN, in addition to increasing the AHN’s response to stress.” (Lines 97-99)

3) Based on data in Fig. 2, the authors argue that AHN neurons encode ‘negative valence’. However, because they only test aversive stimuli (shocks and loud noises (100/130 dB) which are naturally aversive) it is not possible to make these claims. For example, it could be that AHN cells respond to a variety of sensory stimuli including those which are non-aversive. The fact that they exhibit declining responses to shocks across trials even though shocks likely do not lose their aversiveness across repeated presentations suggest something more complex than simple aversion being encoded. The authors would need to examine whether AHN cells respond to non-aversive stimuli such as neutral auditory or visual stimuli and compare these responses to aversive shocks and loud noises. In addition, the use of the term valence suggests that these cells specifically encode the unpleasantness of stimuli. However, even if they don’t respond to non-aversive sensory stimuli, they may encode general salience and not aversion per-se. To test valence, they would need to examine how these cells encode rewarding and aversive stimuli side-by-side.

To address this concern, we have now included data where we simultaneously imaged the AHN's responses to both positive and negative stimuli and shown that stress-responsive AHN neurons are inhibited by reward (Fig 2k-n):

“The relationship between AHN^{GAD} activity and stressor intensity may suggest that AHN^{GAD} neurons reflect valence (i.e., how negative/positive a stimulus is), or it could be that they reflect stimulus salience (i.e., the importance of a stimulus, be it positive or negative). To address this possibility, in a separate experiment, we recorded from AHN^{GAD} neurons while water-deprived mice consumed liquids of various valence/palatability (quinine=low, water=moderate, sucrose=high) and also received shocks of different intensities (Fig. 2k). If AHN neurons encode valence, they would be expected to be excited by negative stimuli and inhibited by positive stimuli. Alternatively, if AHN neurons encode salience, they would be expected to be excited by both positive and negative stimuli. Approximately half of all recorded neurons responded to shock, whereas very few neurons responded to any of the liquids, suggesting AHN neurons are biased toward highly aversive stimuli (Fig. 2m). Then, examining the population of shock-responsive neurons, we found that they were inhibited by sucrose and water, showed little response to quinine, and showed an intensity-dependent increase in activity in response to shock (Fig. 2n). These results are in line with prior reports showing AHN neurons are excited by aversive mechanical stimuli⁴¹, as well as their inhibition by rewards⁴². Further, they reveal that the activity of individual AHN^{GAD} neurons reflect valence and are specifically biased towards negative valence. Of interest, although these neurons were not excited by quinine, as one might expect, it is important to note that a very weak concentration of quinine was used to prevent mice from stopping drinking entirely.” (Lines 159-177)

Main Figure 2k-n:

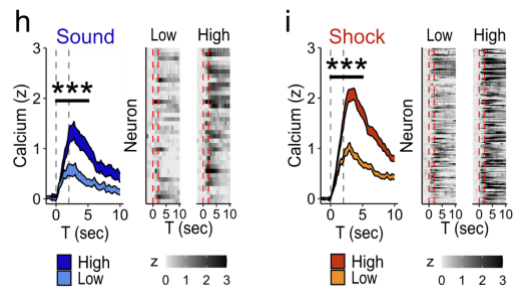


4) Also related to Fig. 2, the authors have a rich calcium imaging dataset, but very little data is shown on the dynamics, single neuron response properties of these cells or population activity. The authors should show heat plots of individual cells allowing for the comparison of the different types of stimuli at the single cell level. This should also be done for both the characterization of sensory responses (Fig. 2B-E) as well as how stress changes response properties. It is also important to examine whether AHN neurons are generally encoding aversion or whether they can resolve distinct types of aversive stimuli. This is hinted at in the single neuron response properties in Fig. 2C, but population decoding or low-dimensional analyses would expand this idea. They should also examine how the prior stress paradigm changes this coding.

We greatly appreciate these comments and have taken several steps to address these concerns:

1. Heat plots are now included in Figure 2h-i to illustrate how individual neurons scale their response with stimulus amplitude:

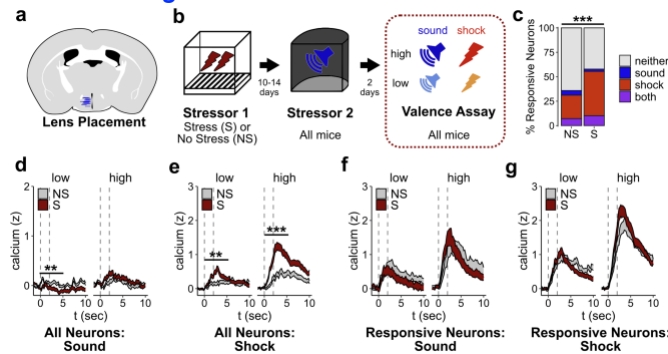
Main Figure 2h-i:



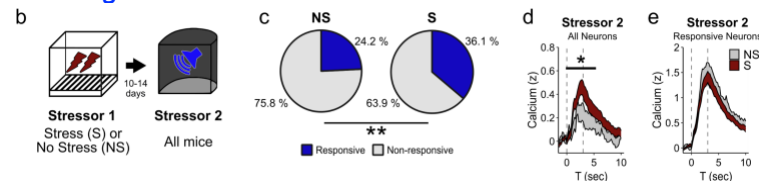
We should also note the statistics for intensity-dependent increases in AHN neuron responses used repeated measures ANOVA, with cells as subjects. Therefore, our results reflect a consistent increase in response from Low to High intensity stimuli across cells.

2. We have now shown prior stress increases the proportion of AHN^{GAD} neurons that are responsive to multiple aversive stimuli. We show that prior stress increases the proportion of neurons responsive to shock and sound (Extended Data Figure 3c), and this results in a larger population-level response of AHN^{GAD} neurons (Extended Data Figure 3e). Nevertheless, as we also show in the main figures (Figure 2e), this does not alter the amplitude of stress-responsive neurons (Extended Data Figure 3f-g).

Extended Figure 3:

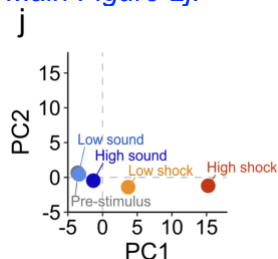


Main Figure 2b-e:



3. We have now performed a low dimensional analysis and shown that the majority of AHN population activity can be explained by a single principle component (PC1=26%, PC2=7%), and PC1 reflects stressor intensity across categorically different stressors (Figure 2j). This argues against the idea of distinct neurons within the AHN encode different stressors. Further suggesting that these neurons are not stimulus-specific, most AHN^{GAD} neurons that responded to sound also responded to shock (70% of these neurons. Noted in manuscript). Although many neurons that responded to shock were not responsive to sound, this is likely the result of shock being a much more aversive stimulus, and therefore recruiting more neurons.

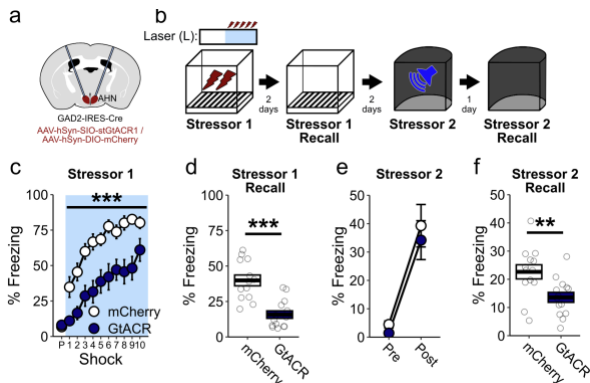
Main Figure 2j:



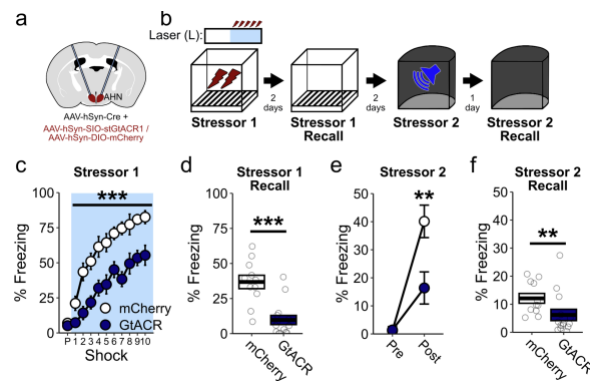
5) The logic behind the selection of mouse models in Figure 4 is unclear. Optogenetic experiments start in wild-type mice, then shift to GAD2-Cre mice in panel G without justification. Given the hypothesis in Figure 2 implicating GABAergic neurons, it seems important to perform all experiments in GAD2-Cre mice.

We have now redone this experiment in GAD-Cre mice and gotten the same pattern of results (Figure 3a-f). We have moved the pan-neuronal data to the supplement to show that – consistent with GABAergic neurons being the dominant cell population in this region – pan-neuronal inhibition has the same impact (Extended Figure 4a-f).

Main Figure 3a-f:



Extended Figure 4a-f:



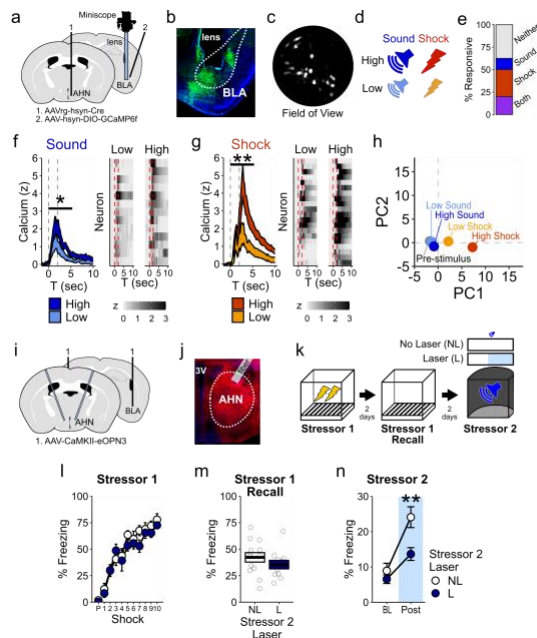
6) The BLA-AHN circuit analysis in Fig. 5 is superficial. First, the authors use systemically administered chemogenetic inhibition of BLA cells that project to AHN, but these cells may collateralize elsewhere and the effects of the CNO may not be specific to BLA inputs to AHN. Second, the selection of when the inhibition is performed (e.g. Stressor 1 and Stressor 2) is not well reasoned with respect to specific hypotheses about the function of this pathway. Is it important in conveying general information about stressor 1 to produce stress upregulation? Does it mediate stressor 1 recall? Does it undergo plasticity in some way and participate solely in producing enhanced freezing to Stressor 2 after stressor 1? Is it involved specifically in stressor 2 recall? As it stands, only a few of these ideas are tested leaving open many interpretations of what this pathway does. Third, the response properties of BLA-AHN cells are not examined to stressors or during the stressor sensitization experiments. To understand how this circuit operates some type of recordings in this pathway is important.

We fully agree with the reviewer, and have now added several key findings regarding this projection:

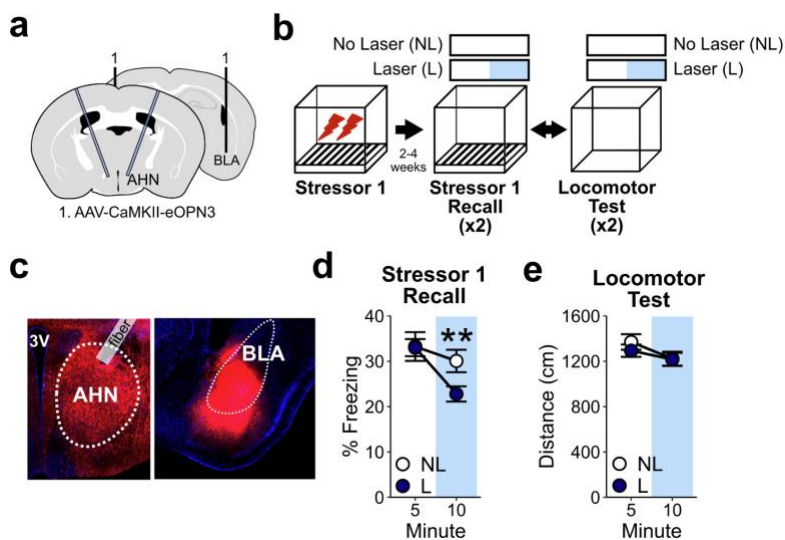
1. First and foremost, we have shown using Miniscopes that a majority of neurons in the BLA that project to the AHN (BLA^{AHN}) respond robustly to stressful stimuli (Figure 4e).
2. We have shown that BLA^{AHN} neurons – like AHN neurons – reflect the intensity of stressful events, both on an individual level and at the population level (Figure 4f-h).
3. We have specifically silenced axon terminals projecting from the amygdala to the AHN. This has revealed that silencing this projection is able to blunt the immediate response to a stressor (Figure 4i-4j).

n), as well as the recall of a stressor (Extended Data Figure 5d). Importantly, this manipulation has no impact on general locomotion (Extended Data Figure 5e).

Main Figure 4:



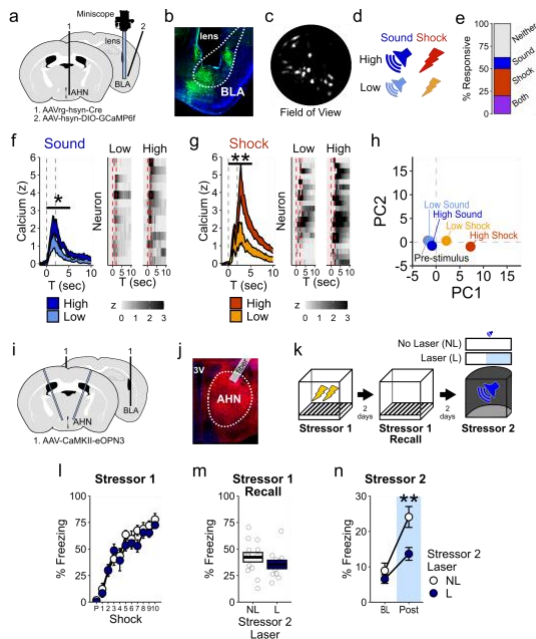
Extended Figure 5a-e:



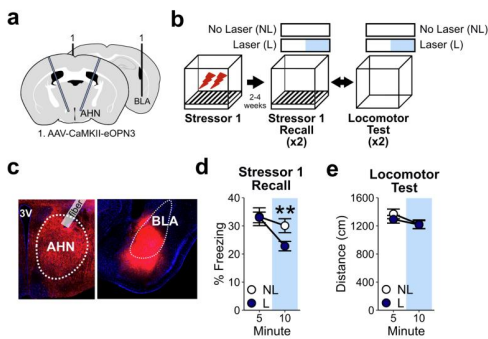
7) Related to the above comment, the data presented don't fit with the model in Fig. 6. In this model the authors argue that BLA transmits aversive 'valence' to the AHN and that AHN then provides an output/gating mechanism for the production of defensive responding which can be turned up or down depending on prior stress. If true, then inactivating BLA inputs should reduce acute responses to stressor 1, which doesn't occur. Are the authors suggesting that BLA inputs require strengthening in some way? If not, how does the AHN alter its stressor representation following stressor 1? None of these ideas are considered in the model/paper.

We have now provided data where direct silencing of amygdala terminals in the AHN using an optogenetic strategy is able to blunt the immediate response to a stressor (Figure 4). Similarly, like our AHN cell body manipulation, inhibition of amygdala to AHN terminals was able to blunt recall (Extended Data Figure 5d). We feel that it is potentially the case that our DREADDs manipulation did not impact the immediate response to the stressor because HM4Di provides relatively weak inhibition. For transparency, we have kept our DREADDs data in the extended data (Extended Data Figure 6). Additionally, we think it provides a useful comparison to silencing AHN inputs from the ventral hippocampus.

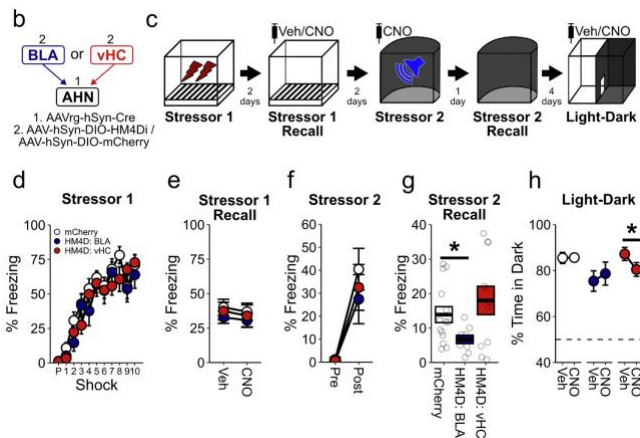
Main Figure 4:



Extended Figure 5a-e:



Extended Figure 6b-h:



Additional concerns:

1) For the data in Fig. 1B, it is not clear when freezing was measured during Stressor 1 and 2. For example, was freezing in response to Stressor 1 measured during ITI? For Stressor 2, since the loud noise is 3 sec long and animals were removed 10 sec later, it's unclear how freezing could be measured. 2) A related criticism relates to all experiment reporting freezing behavior. The exact time periods where freezing was measured should be reported and terms like 'pre' and 'post' should be defined.

When freezing was measured has now been clarified in the methods. Additionally, consistent with many in the field, freezing was defined as the absence of movement lasting at least a half second in length. Importantly, using our automated software for scoring freezing, ezTrack, we previously showed that this definition closely tracks manual scoring of freezing (Pennington et al, 2019), with similar results for the commercial software we used (Anagnostaras et al, 2010). Accordingly, 10 seconds leaves plenty of time to measure freezing.

“For analysis of freezing and motion in conditioning chambers when no fibers/cables were attached to the mice, Med Associates Video Freeze software was used⁶². For measuring freezing and motion in chambers when cables were attached to the animals, as well as motion in the light-dark test, ezTrack was used⁶³. Freezing was defined as the cessation of movement lasting at least a half a second in length. Pre-stress freezing (‘Pre’ in the figures) refers to freezing occurring from the start of the session until the onset of the stressor. Likewise, post-stress freezing (‘Post’ in the figures) refers to freezing occurring from the time of stressor offset until the end of the session. When multiple stressors were given in the same session, freezing measurements reflect the inter-stimulus periods.” (Lines 837-845)

3) For the data in Fig. 1, I could not find a description of the timeline (e.g. when Stressor 2 was given relative to when animals were sacrificed). This information should be included. Generally speaking, the experimental timeline for different experiments appears to vary and the authors don’t provide enough information about individual experiments for an assessment of whether this is an issue or not. They should state (and maybe show in the experimental design portion of their graphs) the exact timeline (e.g. number of days between phases) for each experiment.

This has now been clarified in the experimental schematics for all figures.

4) The correlation matrix graphs in Fig. 1E, right are not described in the figure legend.

This has been clarified:

“Within Cluster 1, previously stressed animals display a larger number of positive correlations. Fisher’s Exact Test – $p < 0.001$. Left shows correlation matrices for all regions in Cluster 1, with significant positive/negative correlations highlighted in red/blue. Right shows the proportion of significant positive correlations.” (Lines 117-119)

5) For the data in Fig. 2, the authors should report the number of animals recorded from, the number of male and female animals used and the number of cells recorded in each experiment.

For all experiments, we have now included this information in the figure caption.

6) The authors use male and female animals for some of their studies. It would be valuable to the reader to know if any sex differences exist.

We have now added this information to the manuscript:

“Male C57BL/6J were obtained from Jackson Laboratories for whole-brain immediate early gene imaging as well as pan-neuronal and axon terminal silencing (Jackson Laboratories, #000664). Studies with homozygous GAD2-IRES-Cre mice (Jackson Laboratories, #028867) utilized an equal mixture of female and male mice that were bred in-house. Critically, we did not observe any sex differences in the behaviors under study, consistent with our prior work¹³, nor did we observe any sex differences in our neuronal recordings.” (Lines 628-634)

Referee #2 (Remarks to the Author):

Summary: This is an interesting and well written manuscript describing the potential role of the anterior hypothalamic nucleus (AHN) in mediating the stress sensitization model previously defined by this group. Stress sensitization is an interesting and translationally relevant behavior in which a shock stress leads to a later increased threat response to a different aversive modality, e.g. loud noise in a different context. The authors use whole brain clearing and cfos analyses to examine brain regions with correlated activity to the sensitized stress. After identifying the AHN as a potentially important area, they use a combination of calcium imaging and chemogenetic activation and inhibition to address the role of the AHN in the stress sensitization process. While overall I feel that these are interesting and relevant findings to our understanding of stress generalization and sensitization in mammals, I feel that, as it currently stands, the interpretations extend beyond the results for a number of reasons outlined below.

Comments / Concerns:

Throughout the paper, the authors refer to the whole brain cfos studies as indicative of ‘functional connectivity’ across the brain. I think it is important to use more precise language since what it really shows is brain regions with ‘correlated activity’ at this timepoint based on cfos, but not necessarily connectivity, functional or otherwise.

We appreciate this criticism and now use the term ‘correlational strength’ throughout the bulk of the paper, although a couple of times we still use the term functional connectivity, in keeping with standard nomenclature in the field. Notably, the term ‘functional connectivity’ is most often used to describe increased correlational strength between regions, without insinuating a structural connection between regions (e.g., in human brain imaging).

As an example:

“A major advantage of whole-brain cFos mapping is the ability to extend beyond group comparisons of individual brain regions and build models of functional connectivity across the brain (here, defined as the correlated activation of brain regions). Given that the AHN is interconnected with established stress circuits²⁵, we investigated whether a prior stressful experience biases the AHN’s correlational strength with other brain regions. Examining the correlations between cFos expression in the AHN and every other mapped structure in response to stress, previously stressed mice demonstrated substantial correlational strength between the AHN and other stress-associated limbic regions, particularly the amygdala, hippocampus, and medial prefrontal cortex (Fig. 1e). Much smaller numbers of correlations between the AHN and other regions were observed in the animals that did not experience Stressor 1 (Fig. 1e). This suggests that prior experience may increase functional connectivity between the AHN and brain regions involved in processing stress” (Lines 71-82).

In the section on ‘Augmenting AHN activity amplifies stressor valence’ it is not clear to me that the experiments truly prove that ‘valence’ is amplified or altered. If anything, I would interpret the results as amplifying salience or strength of the stressor cue or stress response. Altering valence would traditionally be interpreted as altering direction of positive or negative emotion.

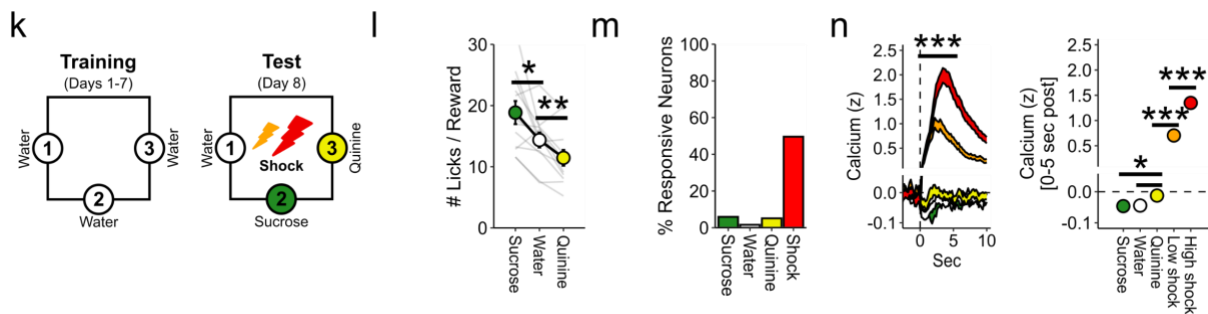
Note that valence is defined as ‘the positive or negative feeling associated with a stimulus, object, event, or situation’, whereas salience is defined as ‘the degree to which something (a stimulus, event, or idea) stands out due to its emotional impact...’. The general term of increased or decreased stressor valence is also used later (and throughout) the manuscript, and I really don’t think this is the proper term to use as per above. If so, then I think other experiments are needed where differential valence (e.g. positive vs negative) emotional responding is addressed, vs. salience – how strong the signal is – are directly compared. On a similar note, the work doesn’t really clarify if it is a true effect on emotional valence (or salience) vs. altering the regulatory process of freezing behavior. Both of these issues could be addressed with such tasks as approach / avoidance, operant conditioning, conditioned place preference, or others beyond freezing measures that also get more directly at valence encoding.

To address this concern, we have now included data where we simultaneously imaged the AHN’s responses to both positive and negative stimuli and shown that stress-responsive AHN neurons are inhibited by reward (Figure 2k-n):

“The relationship between AHN^{GAD} activity and stressor intensity may suggest that AHN^{GAD} neurons reflect valence (i.e., how negative/positive a stimulus is), or it could be that they reflect stimulus salience (i.e., the

importance of a stimulus, be it positive or negative). To address this possibility, in a separate experiment, we recorded from AHN^{GAD} neurons while water-deprived mice consumed liquids of various valence/palatability (quinine=low, water=moderate, sucrose=high) and also received shocks of different intensities (Fig. 2k). If AHN neurons encode valence, they would be expected to be excited by negative stimuli and inhibited by positive stimuli. Alternatively, if AHN neurons encode salience, they would be expected to be excited by both positive and negative stimuli. Approximately half of all recorded neurons responded to shock, whereas very few neurons responded to any of the liquids, suggesting AHN neurons are biased toward highly aversive stimuli (Fig. 2m). Then, examining the population of shock-responsive neurons, we found that they were inhibited by sucrose and water, showed little response to quinine, and showed an intensity-dependent increase in activity in response to shock (Fig. 2n). These results are in line with prior reports showing AHN neurons are excited by aversive mechanical stimuli⁴¹, as well as their inhibition by rewards⁴². Further, they reveal that the activity of individual AHN^{GAD} neurons reflect valence and are specifically biased towards negative valence. Of interest, although these neurons were not excited by quinine, as one might expect, it is important to note that a very weak concentration of quinine was used to prevent mice from stopping drinking entirely.” (Lines 157-177)

Main Figure 2k-n:

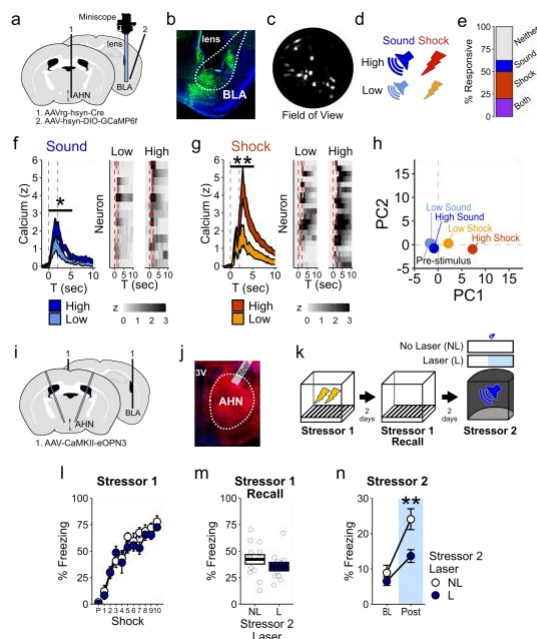


Would an alternative interpretation of Figure 5f be that the BLA to AHN projection is required for context threat learning during the stressor 2 experience, such that inhibiting this pathway is interrupting the threat learning – and not necessarily that the BLA to AHN projection is underlying the stressor valence process?

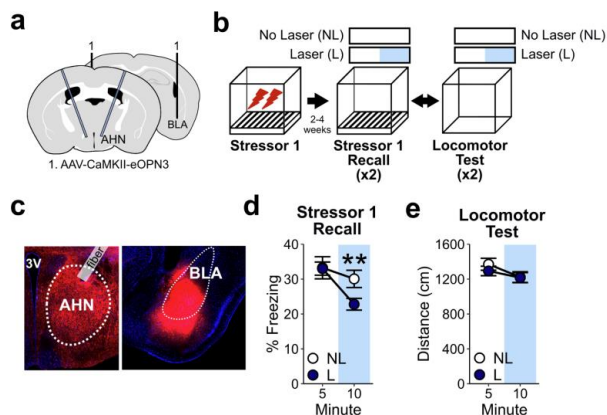
We appreciate this criticism. We have now generated several pieces of evidence that suggest that the projection is indeed conveying valence information, though this certainly influences contextual threat learning as well (in that the aversive stimulus must be associated with the context).

1. We have now shown using Miniscopes that a majority of neurons in the BLA that project to the AHN (BLA^{AHN}) respond robustly and immediately to stressful stimuli (Figure 4e).
2. We have shown that BLA^{AHN} neurons reflect the intensity of stressful events, both on an individual level and at the population level (Figure 4f-h).
3. We have specifically silenced axon terminals projecting from the amygdala to the AHN. This has revealed that silencing this projection is able to blunt the immediate response to a stressor (Figure 4n, as well as the recall of a stressor (Extended Data Figure 5d). However, this manipulation has no impact on general locomotion (Extended Data Figure 5e).

Main Figure 4:



Extended Figure 5a-e:



The model in Figure 6 isn't fully supported by the data presented. For example, "In mice with a history of prior stress, a larger number of AHN neurons are recruited in response to stress, possibly as a result of stronger amygdala input." The authors have the ability to measure this – does silencing the BLA to AHN inputs decrease the number of calcium imaged activated cells? This could also be addressed electrophysiologically with AHN recordings. There are also no data presented demonstrating that the BLA-to-AHN projections connect to the AHN cells visualized with Gcamp6f.

As mentioned above, we have now included data where we specifically recorded from BLA neurons that project to the AHN and found that these neurons respond similarly to stressful stimuli (Figure 4). Moreover, nearly all of the impacts of silencing AHN neurons were mimicked by silencing amygdala to AHN inputs (Figure 4. Extended Data Figure 5). This data supports a model in which valence signals are relayed from the amygdala to the AHN. Additionally, although it could still be that the AHN^{GAD} neurons receive this information through a polysynaptic mechanism within the AHN (e.g., amygdala → glutamatergic AHN neurons → GABAergic AHN neurons), it is nevertheless a direct projection to the AHN.

Minor:

For the miniscope experiments, neither the figure nor the main text make it clear if this is a viral vector vs. transgenic expt. I'm assuming that this is a virus infused in hypothalamus of the GAD2-Cre line, but it should be made more clear in the figure legend and/or text.

[This has now been clarified.](#)

Similarly in the next section, lines 188-189, the method could be more clear – currently states, “we expressed the excitatory chemogenetic receptor HM3Dq in GABAergic AHN neurons, or a control virus expressing mCherry...” but the first half of the sentence doesn’t state anything about a virus or the type of virus.

[This has now been clarified.](#)

Referee #3 (Remarks to the Author):

The authors use a variety of approaches to identify a region playing a role in behavior. Overall, this was a strong use of complementary methods to explore rigorously.

A few comments:

1) The framing in the very first paragraph is odd because the authors do not look at stress susceptible vs resilient populations: “Notably, there is substantial variation in how individuals cope with stressful life events. Exemplifying this, only a small proportion of individuals who experience a traumatic event at some point develop PTSD or other stress-related illness. How variability in the brain’s stress circuits confers susceptibility to stress-related illnesses is poorly understood.” It would seem that given this framing, there would be experiments exploring this.

We appreciate this criticism of the reviewer and have reframed the paper to highlight that we are studying one of the many contributor’s to stress vulnerability: stress sensitization, or prior stress leading to a lasting enhancement in responses to subsequent stress. Notably, nearly every experiment is based upon the observation that following a first stressor, animals display a heightened response to a second stressor.

We now open our manuscript:

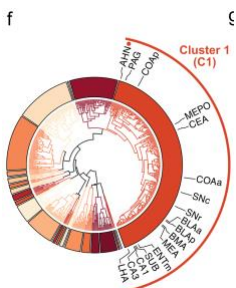
“The brain’s response to stress is fundamentally protective, engaging physiological and behavioral adaptations that promote survival. However, stressful experiences can also precipitate maladaptive brain plasticity, increasing susceptibility to debilitating mental health conditions like post-traumatic stress disorder (PTSD)¹⁻⁵. Notably, there is substantial variation in how individuals cope with stressful life events. Exemplifying this, only a small proportion of individuals who experience a traumatic event go on to develop PTSD or other stress-related illness³. One factor that has been repeatedly shown to contribute to this variation is the experience of prior stress – individuals previously exposed to severe stress are more likely to develop PTSD or depression in response to a subsequent stressor^{1,2,6-10}. Similarly, a core symptom of PTSD is hypervigilance, characterized by heightened reactions to even minor stressors¹¹. These findings point to the sensitizing effects of stress. Importantly, the phenomenon of stress sensitization has been observed across species¹²⁻¹⁴ and could contribute to several of the deleterious consequences of stress. How this sensitization is embedded in the nervous systems remains unclear.” (Lines 2-15)

2) In figure 1, is this the heatmap for the cluster? To me this looks like the heatmap for the entire dataset bc of the number of regions– if so can they denote the cluster within the larger heatmap? If it’s just the heatmap for the cluster then label the regions? In extended data they have a no-stress control – where is the heatmap for that group? It is curious because the whole heatmap is red which maybe reflects the stress state but the unstressed comparison might be interesting?

Figure 1 reflects the correlation for this cluster specifically and we have clarified this point. Figure 1 caption for this panel now reads:

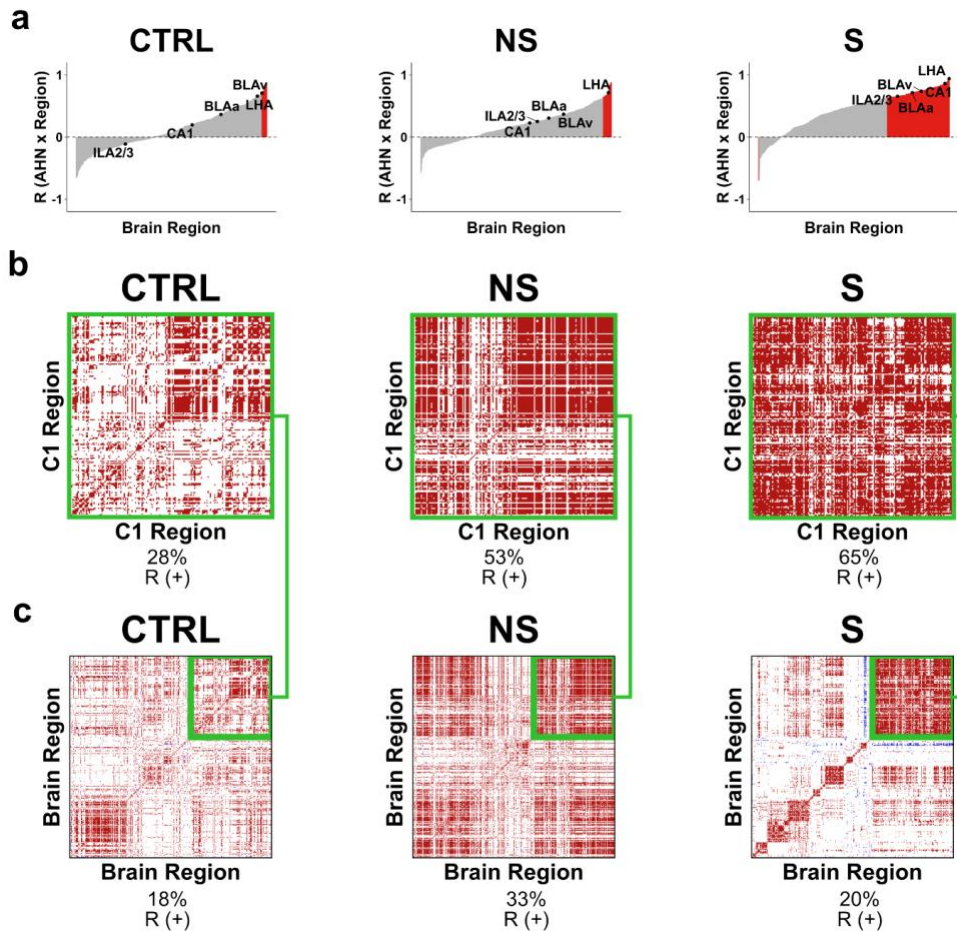
“g) Within Cluster 1, previously stressed animals display a larger number of positive correlations. Fisher’s Exact Test – $p < 0.001$. Left shows correlation matrices for all regions in Cluster 1, with significant positive/negative correlations highlighted in red/blue. Right shows the proportion of significant positive correlations.” (Lines 117-119).

Unfortunately, as can be appreciated from Figure 1f, because Cluster 1 still contains a very large number of regions, we do not have sufficient space to label them all:



We have denoted this cluster within the larger heatmap of all regions in the supplement for each group (Extended Data Figure 2, below. Row 2 [b] shows only the regions in Cluster 1. Row 3 [c] shows all regions included. Green box shows Cluster 1). Additionally, within Extended Data Figure 2, we now include all 3 groups. As can be appreciated, correlational strength is substantially weaker in controls for all correlations examined:

Extended Data Figure 2a-c:



Extended Data Figure 2. Correlational differences across groups and cluster selection for hierarchical clustering. Related to Main Figure 1.

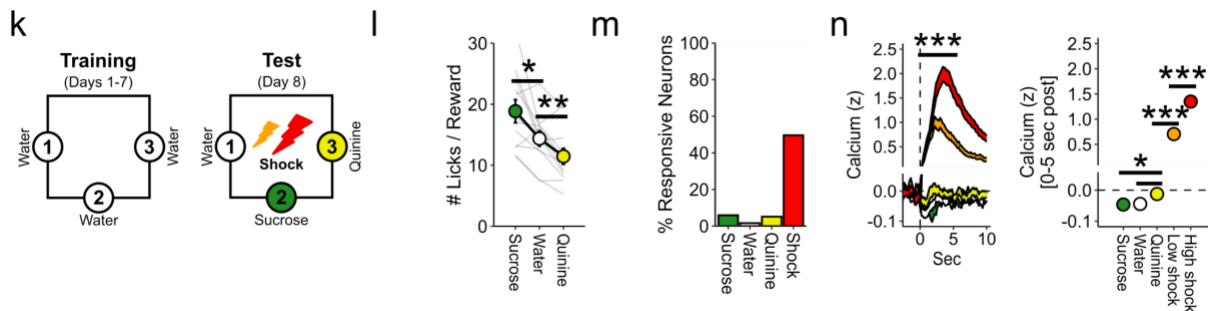
- For each group, the correlation between the AHN and every other structure. Significant correlations are shown in red. There was an increase in the number of positive correlations between S and NS mice (S vs NS: Fisher Exact Test, $p < 0.001$), but no difference between NS and CTRL mice (NS vs CTRL: Fisher Exact Test, $p = 0.16$). $N = 8$ Ctrl, 10 NS, 10 S. All male.
- For each group, the correlation matrix for Cluster 1. Significant positive correlations are shown in red, significant negative correlations are shown in blue, and the percentage of total significant positive correlations is written below each plot. NS mice had a larger number of significant positive correlations than CTRL mice (NS vs CTRL: Fisher's Exact Test, $p < 0.001$), and S mice had a larger number than NS mice (S vs NS: Fisher's Exact Test, $p < 0.001$). Critically, the higher number of correlations in S cannot be explained by a brain-wide increase in correlational strength (see Extended Data Fig. 2c, below).
- For each group, the correlation matrix for all regions. Cluster 1 is outlined in green. Significant positive correlations are shown in red, significant negative correlations are shown in blue, and the percentage of total significant positive correlations is written below each plot. NS mice had a larger number of significant positive correlations than CTRL mice (NS vs CTRL: Fisher's Exact Test, $p < 0.001$), and S mice (NS vs S: Fisher's Exact Test, $p < 0.001$).

3) Figure 2 says "AHN neurons reflect stressor valence" but I read the data as reflecting intensity rather than valence – they are not testing any positive stimuli for instance. Do we know this isn't a response to any salient stimulus that scales with intensity or is it specific to negatively valenced stimuli? This is a critical question.

To address this concern, we have now included data where we simultaneously imaged the AHN's responses to both positive and negative stimuli and shown that stress-responsive AHN neurons are inhibited by reward (Figure 2k-n):

"The relationship between AHN^{GAD} activity and stressor intensity may suggest that AHN^{GAD} neurons reflect valence (i.e., how negative/positive a stimulus is), or it could be that they reflect stimulus salience (i.e., the importance of a stimulus, be it positive or negative). To address this possibility, in a separate experiment, we recorded from AHN^{GAD} neurons while water-deprived mice consumed liquids of various valence/palatability (quinine=low, water=moderate, sucrose=high) and also received shocks of different intensities (Fig. 2k). If AHN neurons encode valence, they would be expected to be excited by negative stimuli and inhibited by positive stimuli. Alternatively, if AHN neurons encode salience, they would be expected to be excited by both positive and negative stimuli. Approximately half of all recorded neurons responded to shock, whereas very few neurons responded to any of the liquids, suggesting AHN neurons are biased toward highly aversive stimuli (Fig. 2m). Then, examining the population of shock-responsive neurons, we found that they were inhibited by sucrose and water, showed little response to quinine, and showed an intensity-dependent increase in activity in response to shock (Fig. 2n). These results are in line with prior reports showing AHN neurons are excited by aversive mechanical stimuli⁴¹, as well as their inhibition by rewards⁴². Further, they reveal that the activity of individual AHN^{GAD} neurons reflect valence and are specifically biased towards negative valence. Of interest, although these neurons were not excited by quinine, as one might expect, it is important to note that a very weak concentration of quinine was used to prevent mice from stopping drinking entirely." (Lines 157-177)

Main Figure 2k-n:



4) Response decays with repetition of the stimulus – does the behavioral response (freezing) change over time with this decay of neuronal response? They don't really discuss the time effect here. Is there a functional link?

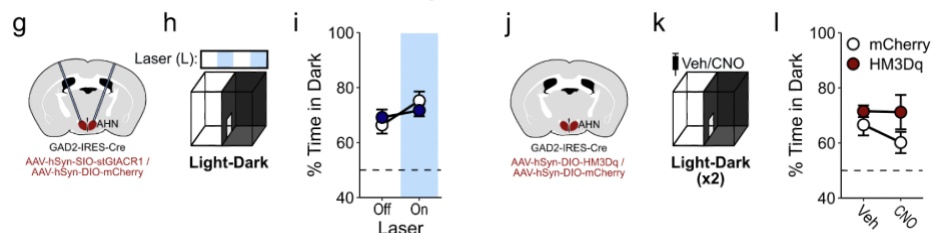
This comment was in response to a decay in AHN^{GAD} amplitude with repeated noise/shock presentations. Unfortunately, because of the large number of additional analyses included in Figure 2 with this revision, we now only have sufficient space to present each neuron's average response to each stimulus.

However, the answer is that the freezing response to the aversive stimulus does not decrease, but rather, increases across the course of the session, although the rise in freezing after each stimulus is less. It could be that the reduced AHN response corresponds with the smaller change in behavior from one trial to the next. Alternatively, acute stress is known to release endorphins that blunt the response to subsequent stress (Young and Fanselow, 1992; Zelikowsky and Fanselow, 2010; Ozawa... and Johansen, 2017), and reduced AHN responses may reflect this dampening. These are possibilities we are very interested in pursuing in the future.

5) Minor point: Figure 3: Light dark box test is repeated? This is somewhat confusing as I was under the impression that novelty was important here.

One of the reasons we have utilized the light-dark test is because it has better test-retest reliability than similar measures (e.g., elevated plus maze). Indeed, in the test in question, there is no effect of test number on time spent in the dark ($F_{1,19}=1.8$, $p=0.2$). We have also now included an optogenetic experiment that was conducted between groups, rather than within subjects (Extended Data Figure 4).

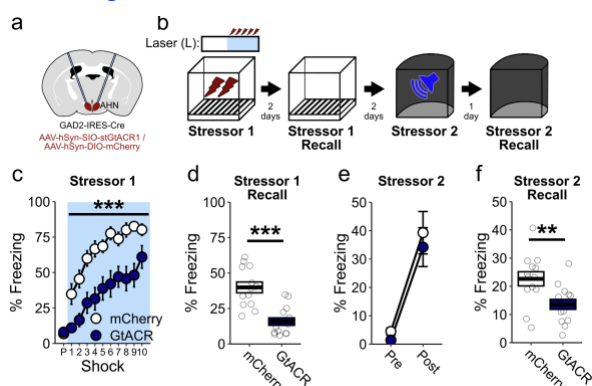
Extended Data Figure 4g-l:



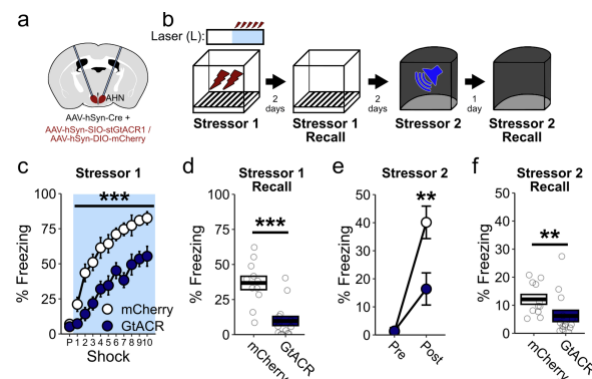
6) Figure 4 – experiments with pan neuronal vs gaba neurons, but don't repeat the same experiments to compare the 2 (eg laser on/off during stressor 1), so unclear what the rationale is for studying the 2 diff populations as they are not explored.

The initial use of C57 instead of GAD-Cre mice for the first experiment in Figure 4 (now Figure 3) was done purely based upon the availability of GAD-Cre mice in our colony at the time. We have now redone this experiment in GAD-Cre mice and gotten the same pattern of results (Figure 3a-f). We have moved the pan-neuronal data to the supplement to show that – consistent with GABAergic neurons being the dominant cell population in this region – that pan-neuronal inhibition has the same impact (Extended Data Figure 4a-f).

Main Figure 3a-f:



Extended Figure 4a-f:



7) Where are the outputs that drive freezing behavior? Does AHN project to PAG

We have now expanded upon this in the discussion. The AHN projects to many places that can influence freezing, including the PAG. However, dissecting the exact projection is beyond the scope of the current manuscript:

Beyond its intrinsic molecular properties, understanding how the AHN fits within the broader circuitry of stress regulation will also be essential for identifying where and how therapeutic intervention might be most effective. Prior work on the hypothalamus' role in stress has most heavily focused on the paraventricular nucleus (PVN), a region known to control release of the stress hormone cortisol via the hypothalamic-pituitary-adrenal axis⁵⁷. Notably, although the PVN receives diverse inputs⁵⁸, the AHN projects directly to the PVN, and in this way can directly modulate stress hormone release³². That said, beyond its neuroendocrine actions, the AHN is likely to regulate stress-

evoked behavior through complex extrahypothalamic interactions. These include monosynaptic projections to the lateral septum, the PAG, and the amygdala^{59,60}, regions known to regulate behavioral responses to stress^{32,61}. Indeed, a recent report found that AHN projections to the PAG can control attack behavior in response to painful stimuli⁴¹. Combined with our observation that different inputs to the AHN can regulate distinct components of defensive behavior (Extended Data Fig. 6), the AHN could be a target for regulating multiple defensive behaviors. Future work is needed to fully disentangle the complex input/output functions of the AHN, as well as how these interactions are modified by prior experience.

“Beyond its intrinsic molecular properties, understanding how the AHN fits within the broader circuitry of stress regulation will also be essential for identifying where and how therapeutic intervention might be most effective. Prior work on the hypothalamus’ role in stress has most heavily focused on the paraventricular nucleus (PVN), a region known to control release of the stress hormone cortisol via the hypothalamic-pituitary-adrenal axis⁵⁷. Notably, although the PVN receives diverse inputs⁵⁸, the AHN projects directly to the PVN, and in this way can directly modulate stress hormone release³². That said, beyond its neuroendocrine actions, the AHN is likely to regulate stress-evoked behavior through complex extrahypothalamic interactions. These include monosynaptic projections to the lateral septum, the PAG, and the amygdala^{59,60}, regions known to regulate behavioral responses to stress^{32,61}. Indeed, a recent report found that AHN projections to the PAG can control attack behavior in response to painful stimuli⁴¹. Combined with our observation that different inputs to the AHN can regulate distinct components of defensive behavior (Extended Data Fig. 6), the AHN could be a target for regulating multiple defensive behaviors. Future work is needed to fully disentangle the complex input/output functions of the AHN, as well as how these interactions are modified by prior experience.” (Lines 432-447).

Referee #1 (Remarks to the Author):

The authors have conducted new experiments and analyses that strengthen the manuscript. The extended test of valence coding in AHN neurons is a valuable addition, and the new analyses suggesting that AHN neurons primarily encode aversive intensity rather than distinguishing among distinct aversive stimuli are important. However, I have several remaining concerns about some of the new additions that should be addressed.

1) I thank the authors for clarifying their False Discovery Rate (FDR) procedures (data described in Fig. 1 and Ext. Data Fig. 1). The revised description is substantially improved. However, because the analysis uses a two-stage procedure, first correcting the omnibus group effect across all 454 regions and then correcting the S versus NS post-hoc contrast only across the 90 regions that passed the initial screen, it would be helpful to explicitly justify this hierarchical correction strategy and possibly also show data with corrections across all 454 regions rather than only the screened subset.

We have now clarified the justification for this two-stage approach, which was established before starting the experiment and was the main reason for including our control group that did not receive Stressor 2. Our central aim was to identify regions whose stress-evoked activation is altered by prior stress history (S versus NS). The great majority of the 454 brain regions in our dataset are not recruited by an acute auditory stressor (Stressor 2). Including them in the S vs NS correction therefore dilutes statistical power by correcting across a large number of biologically uninformative regions. Because of this, we used a two-stage approach: first identifying, across all 454 regions, those showing any evidence of stress-evoked activation; and then restricting the S versus NS contrast to this engaged subset.

Staged approaches of this kind are routine. In human neural imaging analyses, candidate brain regions or features are initially identified and then downstage analyses are selectively performed on these regions (e.g., PMID: 26670285; PMID: 28182017). Likewise, in genetics, an initial screen is often used to restrict which genes are subsequently examined (e.g., PMID: 36646877; PMID: 41382205). More directly, our initial model is essentially an omnibus ANOVA with 3 groups, and when this was significant for a region, we proceeded to the S versus NS contrast as a post-hoc test. It is standard to not conduct post-hoc tests when the initial omnibus test is not significant (Howell, Statistical Methods for Psychology), and our two-stage procedure follows precisely this logic.

The text has been amended accordingly (red is added text):

“As can be appreciated from Extended Figure 1, this largely served to detect regions activated by Stressor 2 relative to controls given no stress at this time point, and to restrict our subsequent comparison of groups NS and S to regions that showed some evidence of activation by Stressor 2, increasing statistical power for this comparison. Critically, the control group was included from the outset of this experiment to limit the number regions examined between our main groups of interest.”

2) Although the addition of the sucrose/water valence assay is important, it is not clear that rewarding stimuli actually inhibit AHN neurons as the only comparisons are across stimulus condition. To support this claim, the authors should compare activity during the evoked response period with a comparable pre-stimulus baseline period for each stimulus. More

generally, this baseline comparison should be performed whenever the authors make claims about the significance or direction of responses to specific stimuli.

We agree and have added these comparisons. Activity in the 5 seconds following the onset of water and sucrose consumption is significantly decreased relative to the pre-stimulus baseline, whereas activity following quinine is not. These tests have been added to the figure caption:

“Shock-responsive neurons were valence sensitive. Left) Average temporal response of shock-responsive neurons, aligned to stimulus onset. Right) Average response during the 5 seconds following stimulus onset. RM-ANOVA – Stimulus: $F_{4,500}=166$, $p<0.001$. High Shock vs Low Shock: $F_{1,125}=43.6$, $p<0.001$. Low Shock vs Quinine: $F_{1,125}=127.7$, $p<0.001$. Quinine vs Water: $F_{1,125}=8$, $p<0.01$. Quinine vs Sucrose: $F_{1,125}=5.5$, $p=0.02$. Water vs Sucrose: $F_{1,125}=0$, $p=0.89$. One sample t-test comparing average response for liquids to the pre-stimulus baseline (i.e., 0): Sucrose: $t_{125}=-3.8$, $p<0.001$. Water: $t_{125}=-5.1$, $p<0.001$; Quinine: $t_{125}=-1.3$, $p=0.21$. 126 neurons.”

This was not necessary for shock because the classification of shock-responsive neurons (and likewise for sound responsive neurons) was already predicated on a consistent change in activity from the pre- to post-stimulus period, as described in the Methods:

“To classify whether a cell was responsive to a particular stimulus (e.g., shock), the average change in activity in the 5 seconds following stimulus onset relative to the 5 seconds preceding stimulus onset was first assessed. This interval was chosen based upon visual inspection of calcium transients. A null distribution was then obtained by circularly shuffling neural activity across the session and recalculating this average difference 1000 times. Stimuli that had a response above the 95th percentile of this null distribution were considered responsive.”

Additionally, we have amended the methods to clarify that a decrease in activity below zero reflects a decrease from the pre-stimulus baseline:

“For each cell and session, activity was normalized by first computing a z-score. For comparing responses of cells to various amplitude stimuli, activity was binned into 200 ms intervals and cells were treated as individual datapoints. Moreover, to more readily examine cellular responses to each stimulus, peri-stimulus activity for each cell was normalized relative to the average activity in the 5 seconds preceding each stimulus by subtracting this average. Accordingly, for all activity traces, data is in standard deviation units (z), with zero reflecting average pre-stimulus activity.”

3) For the optogenetic experiments shown in Fig. 3i–l, AHN neurons were inhibited during both Stressor 1 recall and the subsequent Stressor 2 exposure, with reductions in freezing observed at both timepoints and during the later Stressor 2 recall test. Because these manipulations occur serially within the same animals, the results are somewhat difficult to interpret. One possibility is that inhibition during Stressor 1 recall reduced the expression or updating of the Stressor 1 memory, thereby indirectly reducing the effect of Stressor 1 on subsequent Stressor 2 responding. Ideally, these manipulations would be tested separately. At minimum, the authors should acknowledge this interpretive limitation.

We appreciate this point and will acknowledge the limitation in the text. We will also add the supplementary panel described below. While we agree that, ideally, these manipulations would be separated, three observations (below) indicate that a carry-over effect from Stressor 1 Recall is unlikely to account for the Stressor 2 results. If the Referee considers it essential, we would also perform an experiment in which GABAergic AHN neurons are inhibited during Stressor 2 alone.

1. In our amygdala-to-AHN experiment (Figure 4n), the laser was off during Stressor 1 and Stressor 1 Recall and only on during Stressor 2, yet inhibiting the inputs to AHN substantially reduced post-stress freezing. This is, in effect, the separate test that the Referee describes at the level of the circuit: manipulating the pathway only at the time of Stressor 2 was sufficient to reduce the Stressor 2 response.
2. Inhibition of the GABAergic AHN neurons during Stressor 1 Recall was delivered using an off-on-off-on schedule. The two cycles were collapsed for simplicity in the main text (Figure 3j), but the uncollapsed data (shown below, to be added as a supplementary panel) show that freezing rebounds as soon as the laser is turned off. If inhibition during Stressor 1 Recall had weakened or updated the Stressor 1 memory, freezing would be expected to remain low after the laser was turned off; the immediate rebound argues against such an effect and against carry-over to Stressor 2.

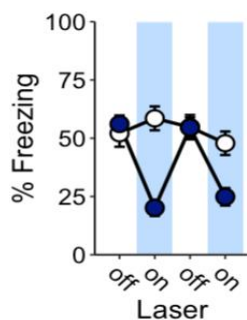


Figure X. Freezing during Stressor 1 recall. Optogenetic inhibition of GABAergic AHN neurons reduced freezing selectively when blue light was administered.

We will both acknowledge the potential for a carry-over effect and highlight data suggesting this is unlikely in text:

“After experiencing an initial footshock stressor (Stressor 1, Fig. 3i), silencing AHN^{GAD} neurons when mice were returned to the stressor context reduced freezing (Stressor 1 Recall, Fig. 3j). This indicates that these neurons are necessary for responding to stress-associated cues, in addition to responding to stressors themselves. Moreover, similar to the effect of silencing AHN neurons during Stressor 1, silencing AHN^{GAD} neurons during a subsequent auditory stressor blunted freezing, both during the stressor and during a laser-free recall test (Stressor 2/Stressor 2 Recall, Fig. 3k-l). Notably, these results are unlikely to result from prior inhibition during Stressor 1 Recall, given the near immediate return of freezing when the laser was turned off during Stressor 1 Recall (Figure X).”

3. Inhibiting the AHN during Stressor 1 (shock) blunts both the immediate and persistent responses to this stressor (Figure 3a-f). Given that AHN neurons respond to both shock and noise, and given the clear effect of silencing BLA-AHN terminals on the Stressor 2 response, there is little reason to expect that inhibiting AHN during Stressor 2 would not influence the response to this stimulus.

Referee #2 (Remarks to the Author):

The authors have satisfactorily addressed my prior concerns.

Referee #3 (Remarks to the Author):

The authors use a whole brain fos approach to identify the AHN as a potential site of action for heightened stress sensitivity. They then use miniscope imaging to explore the activity of these neurons, focusing on the connection from the BLA. A series of experiments using optogenetic inhibition and activation of Gq signaling using DREADD technology suggest a causal role of these BLA to AHN circuit in this behavior. The authors were very responsive to previous concerns, however I still do not think that the data, while interesting, supports their model.

Specifically, the authors are focused on VALENCE. The neurons that they are exploring are very responsive to aversive stimuli, and appear to scale with intensity, both sound and shock. However, the responses to positive stimuli, are minimal, and while statistically significant, their biological significance is unclear and there is no evidence supporting a role provided. Even when the data is critically assessed, there is little to no differences between sucrose and water, with no response to quinine. One potential reason for this scaling to shock and loud intensity is that this may actually represent a pain signal. For example, loud sounds can drive nociceptive responses (<https://pubmed.ncbi.nlm.nih.gov/25639244/>). It may be that this BLA to AHN is involved in pain aversiveness and that is driving the behavioral changes, but it is not something that the current experiments have been designed to assess.

We appreciate this point and agree that aversive intensity is the dominant signal these neurons carry. It is clear that AHN^{GAD} neurons respond much more robustly to stimuli with negative valence, as we acknowledge in text: “the activity of individual AHN^{GAD} neurons reflect valence and are specifically biased towards negative valence”. Two features of the data, taken together, indicate that AHN^{GAD} activity reflects negative valence rather than pain or nociception per se.

First, a purely nociceptive account predicts no change in activity to non-painful stimuli. Instead, AHN^{GAD} neurons are significantly suppressed below their pre-stimulus baseline by appetitive and neutral liquids (sucrose and water; one-sample t-tests now reported in response to Referee 1, point 2). Active suppression by reward is a hallmark of a bidirectional valence axis, not of a nociceptor.

Second, the rank ordering of responses tracks valence across modalities that share no common nociceptive channel. Quinine, a bitter tastant that is aversive but not painful, evokes activity significantly greater than water or sucrose (Quinine vs Water: $F_{1,125}=8$, $p<0.01$; Quinine vs Sucrose: $F_{1,125}=5.5$, $p=0.02$), while remaining well below footshock (Low Shock vs Quinine: $F_{1,125}=127.7$, $p<0.001$). An organization of appetitive/neutral taste < mildly aversive taste < strongly aversive shock is a valence signal; nociception does not predict elevated AHN activity to a non-painful bitter tastant relative to water.

Regarding the lack of difference between sucrose and water, it is important to note that AHN^{GAD} calcium transients are relatively sparse, leaving limited dynamic range to detect further decreases in activity, so this comparison may be subject to a floor effect. The mean response to sucrose is lower than that of water, although this difference is not significant. We will clarify this in the text: “although responses to sucrose were not significantly below that of water, this is

potentially attributable to the sparse baseline rate of calcium transients in AHN^{GAD} neurons, limiting the range of a decrease that can be observed.”

We will incorporate these points into the Discussion, and we will frame the AHN as encoding graded negative valence, biased toward aversive stimuli, which is what our data support, rather than a symmetric positive and negative representation.

Should the Referee or editor consider a decisive experimental test necessary, we would perform the following experiment. We would record AHN^{GAD} neurons in response to an innocuous tone (70-75 db), both before and after it is paired with shock, thereby converting a stimulus of neutral valence into one of negative valence, without altering its physical properties. If AHN neurons encode valence independent of pain, they are expected to show an increased response to the tone after it has been paired with shock.

Also...relatively minor point. The authors used a DREADD vs optogenetics to drive activity, this seems like a missed opportunity to probe behaviors with a more granular time resolution. Did the authors use a Chr2 to drive activity in this pathway or in the AHN, and if so, what were the results?

A chemogenetic strategy was used deliberately for theoretical reasons. Because only a subset of GABAergic AHN neurons are responsive to multiple stressors, and because HM3Dq activation is thought to exert a more modulatory impact on neuronal excitability, we reasoned that this manipulation would act more specifically on stress-responsive neurons, amplifying their intrinsic activity. By contrast, optogenetic activation would almost certainly drive all GABAergic neurons – a diverse population – and could produce a range of off-target effects on behavior.

Referee #1 (Remarks to the Author):

The authors have conducted new experiments and analyses that strengthen the manuscript. The extended test of valence coding in AHN neurons is a valuable addition, and the new analyses suggesting that AHN neurons primarily encode aversive intensity rather than distinguishing among distinct aversive stimuli are important. However, I have several remaining concerns about some of the new additions that should be addressed.

1) I thank the authors for clarifying their False Discovery Rate (FDR) procedures (data described in Fig. 1 and Ext. Data Fig. 1). The revised description is substantially improved. However, because the analysis uses a two-stage procedure, first correcting the omnibus group effect across all 454 regions and then correcting the S versus NS post-hoc contrast only across the 90 regions that passed the initial screen, it would be helpful to explicitly justify this hierarchical correction strategy and possibly also show data with corrections across all 454 regions rather than only the screened subset.

We have now clarified the justification for this two-stage approach, which was established before starting the experiment and was the main reason for including our control group that did not receive Stressor 2. Our central aim was to identify regions whose stress-evoked activation is altered by prior stress history (S versus NS). The great majority of the 452 brain regions in our dataset are not recruited by an acute auditory stressor (Stressor 2). Including them in the S vs NS correction therefore dilutes statistical power by correcting across a large number of biologically uninformative regions. Because of this, we used a two-stage approach: first identifying, across all 452 regions, those showing any evidence of stress-evoked activation; and then restricting the S versus NS contrast to this engaged subset.

Staged approaches of this kind are routine. In human neural imaging analyses, candidate brain regions or features are initially identified and then downstage analyses are selectively performed on these regions (e.g., PMID: 26670285; PMID: 28182017). Likewise, in genetics, an initial screen is often used to restrict which genes are subsequently examined (e.g., PMID: 36646877; PMID: 41382205). More directly, our initial model is essentially an omnibus ANOVA with 3 groups, and when this was significant for a region, we proceeded to the S versus NS contrast as a post-hoc test. It is standard to not conduct post-hoc tests when the initial omnibus test is not significant (Howell, Statistical Methods for Psychology), and our two-stage procedure follows precisely this logic.

The text has been amended accordingly (red is added text):

“As can be appreciated from Extended Figure 1, this largely served to detect regions activated by Stressor 2 relative to controls given no stress at this time point, and to restrict our subsequent comparison of groups NS and S to regions that showed some evidence of activation by Stressor 2, increasing statistical power for this comparison. Critically, the control group was included from the outset of this experiment to limit the number of regions examined between our main groups of interest.”

Lines 938-943

2) Although the addition of the sucrose/water valence assay is important, it is not clear that rewarding stimuli actually inhibit AHN neurons as the only comparisons are across stimulus condition. To support this claim, the authors should compare activity during the evoked response period with a comparable pre-stimulus baseline period for each stimulus. More

generally, this baseline comparison should be performed whenever the authors make claims about the significance or direction of responses to specific stimuli.

We agree and have added these comparisons. Activity in the 5 seconds following the onset of water and sucrose consumption is significantly decreased relative to the pre-stimulus baseline, whereas activity following quinine is not. These tests have been added to the figure caption:

“Shock-responsive neurons were valence sensitive. Left) Average temporal response of shock-responsive neurons, aligned to stimulus onset. Right) Average response during the 5 seconds following stimulus onset. RM-ANOVA – Stimulus: $F_{4,500}=166$, $p<0.001$. High Shock vs Low Shock: $F_{1,125}=43.6$, $p<0.001$. Low Shock vs Quinine: $F_{1,125}=127.7$, $p<0.001$. Quinine vs Water: $F_{1,125}=8$, $p<0.01$. Quinine vs Sucrose: $F_{1,125}=5.5$, $p=0.02$. Water vs Sucrose: $F_{1,125}=0$, $p=0.89$. One sample t-test comparing response for liquids to their pre-stimulus baseline: Sucrose: $t_{125}=-3.8$, $p<0.001$. Water: $t_{125}=-5.1$, $p<0.001$; Quinine: $t_{125}=-1.3$, $p=0.21$. N=126 neurons”
Lines 526-532

This was not necessary for shock because the classification of shock-responsive neurons (and likewise for sound responsive neurons) was already predicated on a consistent change in activity from the pre- to post-stimulus period, as described in the Methods:

“To classify whether a cell was responsive to a particular stimulus (e.g., shock), the average change in activity in the 5 seconds following stimulus onset relative to the 5 seconds preceding stimulus onset was first assessed. This interval was chosen based upon visual inspection of calcium transients. A null distribution was then obtained by circularly shuffling neural activity across the session and recalculating this average difference 1000 times. Stimuli that had a response above the 95th percentile of this null distribution were considered responsive.”
Lines 985-991

Additionally, we have amended the methods to clarify that a decrease in activity below zero reflects a decrease from the pre-stimulus baseline:

“For each cell and session, activity was normalized by first computing a z-score. For comparing responses of cells to various amplitude stimuli, activity was binned into 200 ms intervals and cells were treated as individual datapoints. Furthermore, to more readily examine cellular responses to each stimulus, peri-stimulus activity for each cell was normalized relative to the average activity in the 5 seconds preceding each stimulus (by subtracting this average). Accordingly, for all activity traces, data is in standard deviation units (z), with zero reflecting average pre-stimulus activity.”
Lines 977-983

3) For the optogenetic experiments shown in Fig. 3i–l, AHN neurons were inhibited during both Stressor 1 recall and the subsequent Stressor 2 exposure, with reductions in freezing observed at both timepoints and during the later Stressor 2 recall test. Because these manipulations occur serially within the same animals, the results are somewhat difficult to interpret. One possibility is that inhibition during Stressor 1 recall reduced the expression or updating of the Stressor 1 memory, thereby indirectly reducing the effect of Stressor 1 on subsequent Stressor 2 responding. Ideally, these manipulations would be tested separately. At minimum, the authors

should acknowledge this interpretive limitation.

We appreciate this point and have acknowledged the limitation in the text. We have also added a figure panel described below. While we agree that, ideally, these manipulations would be separated, three observations (below) indicate that a carry-over effect from Stressor 1 Recall is unlikely to account for the Stressor 2 results.

1. In our amygdala-to-AHN experiment (Figure 4n), the laser was off during Stressor 1 and Stressor 1 Recall and only on during Stressor 2, yet inhibiting the inputs to AHN substantially reduced post-stress freezing. This is, in effect, the separate test that the Referee describes at the level of the circuit: manipulating the pathway only at the time of Stressor 2 was sufficient to reduce the Stressor 2 response.
2. Inhibition of the GABAergic AHN neurons during Stressor 1 Recall was delivered using an off-on-off-on schedule. The two cycles were originally collapsed for simplicity in the main text (Figure 3j), but the un-collapsed data show that freezing rebounds as soon as the laser is turned off. This uncollapsed data has now replaced Figure 3j. If inhibition during Stressor 1 Recall had weakened or updated the Stressor 1 memory, freezing would be expected to remain low after the laser was turned off; the immediate rebound argues against such an effect and against carry-over to Stressor 2.

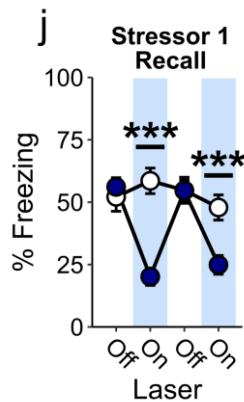


Figure 3j. Inhibiting AHN^{GAD} neurons reduced freezing during Stressor 1 Recall. Omnibus RM-ANOVA – Virus x Time: $F_{1,23}=54$, $p<0.001$. ANOVA for Off time-period – Virus: $F_{1,23}=0.1$, $p=0.72$. ANOVA for On time-period – Virus: $F_{1,23}=30.8$, $p<0.001$.

We have acknowledged the potential for a carry-over effect and highlight data suggesting this is unlikely in text:

“After experiencing an initial footshock stressor (Stressor 1, Fig. 3i), silencing AHN^{GAD} neurons when mice were returned to the stressor context reduced freezing (Stressor 1 Recall, Fig. 3j). This indicates that these neurons are necessary for responding to stress-associated cues, in addition to responding to stressors themselves. Moreover, similar to the effect of silencing AHN neurons during Stressor 1, silencing AHN^{GAD} neurons during a subsequent auditory stressor blunted freezing, both during the stressor and during a laser-free recall test (Stressor 2/Stressor 2 Recall, Fig. 3k-l). **Notably, these results are unlikely to result from prior inhibition during Stressor 1 Recall, given the near immediate return of freezing when the laser was turned off during Stressor 1 Recall (Figure 3j).**”

3. Inhibiting the AHN during Stressor 1 (shock) blunts both the immediate and persistent responses to this stressor (Figure 3a-f). Given that AHN neurons respond to both shock and noise, and given the clear effect of silencing BLA-AHN terminals on the Stressor 2 response, there is little reason to expect that inhibiting AHN during Stressor 2 would not influence the response to this stimulus.

Referee #2 (Remarks to the Author):

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Referee #3 (Remarks to the Author):

The authors use a whole brain fos approach to identify the AHN as a potential site of action for heightened stress sensitivity. They then use miniscope imaging to explore the activity of these neurons, focusing on the connection from the BLA. A series of experiments using optogenetic inhibition and activation of Gq signaling using DREADD technology suggest a causal role of these BLA to AHN circuit in this behavior. The authors were very responsive to previous concerns, however I still do not think that the data, while interesting, supports their model.

Specifically, the authors are focused on VALENCE. The neurons that they are exploring are very responsive to aversive stimuli, and appear to scale with intensity, both sound and shock. However, the responses to positive stimuli, are minimal, and while statistically significant, their biological significance is unclear and there is no evidence supporting a role provided. Even when the data is critically assessed, there is little to no differences between sucrose and water, with no response to quinine. One potential reason for this scaling to shock and loud intensity is that this may actually represent a pain signal. For example, loud sounds can drive nociceptive responses (<https://pubmed.ncbi.nlm.nih.gov/25639244/>). It may be that this BLA to AHN is involved in pain aversiveness and that is driving the behavioral changes, but it is not something that the current experiments have been designed to assess.

We appreciate this point and agree that aversive intensity is the dominant signal these neurons carry. It is clear that AHN^{GAD} neurons respond much more robustly to stimuli with negative valence, as we acknowledge in text: “the activity of individual AHN^{GAD} neurons reflect valence and are specifically biased towards negative valence”. Two features of the data, taken together, indicate that AHN^{GAD} activity reflects negative valence rather than pain or nociception per se.

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Second, the rank ordering of responses tracks valence across modalities that share no common nociceptive channel. Quinine, a bitter tastant that is aversive but not painful, evokes activity significantly greater than water or sucrose (Quinine vs Water: $F_{1,125}=8$, $p<0.01$; Quinine vs Sucrose: $F_{1,125}=5.5$, $p=0.02$), while remaining well below footshock (Low Shock vs Quinine: $F_{1,125}=127.7$, $p<0.001$). An organization of appetitive/neutral taste < mildly aversive taste < strongly aversive shock is a valence signal; nociception does not predict elevated AHN activity to a non-painful bitter tastant relative to water.

Regarding the lack of difference between sucrose and water, it is important to note that AHN^{GAD} calcium transients are relatively sparse, leaving limited dynamic range to detect further decreases in activity, so this comparison may be subject to a floor effect. The mean response to sucrose is lower than that of water, although this difference is not significant. We have clarified this in the text: “although responses to sucrose were not significantly below that of water, this is potentially attributable to the sparse baseline rate of calcium transients in AHN^{GAD} neurons, limiting the range of a decrease that can be observed” (Lines 157-160).

We have incorporated these points into the Discussion, and have framed the AHN as encoding graded negative valence, biased toward aversive stimuli, which is what our data support, rather than a symmetric positive and negative representation.

- “Further, they reveal that the activity of individual AHN^{GAD} neurons reflect valence and are specifically biased towards negative valence” (Lines 154-155)
- “Above, we have identified the AHN as a regulator of negative valence and persistent stress-induced increases in stress sensitivity (i.e., stress sensitization)” (Lines 259-260)
- “By understanding how individual peptide-expressing cells within the AHN regulate negative valence and stress sensitivity, new targets for stress-related mental health conditions might be discovered” (Lines 290-292)
- “Furthermore, direct projections from the amygdala to the AHN were found to reflect negative valence and have robust contributions to stress-associated behaviors.” (Lines 314-315)

Also...relatively minor point. The authors used a DREADD vs optogenetics to drive activity, this seems like a missed opportunity to probe behaviors with a more granular time resolution. Did the authors use a Chr2 to drive activity in this pathway or in the AHN, and if so, what were the results?

A chemogenetic strategy was used deliberately for theoretical reasons. Because only a subset of GABAergic AHN neurons are responsive to multiple stressors, and because HM3Dq activation is thought to exert a more modulatory impact on neuronal excitability, we reasoned that this manipulation would act more specifically on stress-responsive neurons, amplifying their intrinsic activity. By contrast, optogenetic activation would almost certainly drive all GABAergic neurons – a diverse population – and could produce a range of off-target effects on behavior.