nature portfolio

Peer Review File

The basolateral amygdala decodes visceral pain and itch via distinct circuits and molecules

Corresponding Author: Professor Guang-Yin Xu

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

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

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
This manuscript investigates the neural representation of visceral pain and itch within the BLA. Several fundamental concerns regarding its scientific premise, experimental design, and interpretation significantly limit the impact and validity of the findings.
1. The core premise of comparing visceral pain and itch at the BLA is not adequately justified. Itch is predominantly a somatosensory sensation, typically originating from the skin. Since itch doesn't occur from internal organs, it is highly unlikely that the neuronal pathways for visceral pain and itch are significantly overlapped.
The introduction fails to present a compelling argument for this specific comparison. Existing literature, as even cited by the authors, strongly suggests that comparing somatic pain and itch pathways would be a more scientifically sound and straightforward approach, given their known interactions and shared anatomical substrates in the somatosensory system.
2. The assertion that "visceral pain and itch frequently co-occur in various diseases" is problematic and requires stronger evidence. The provided example of Inflammatory Bowel Disease (IBD) and itch is not generally supported by clinical observations. Itch in IBD patients is more commonly associated with drug-induced skin lesions (e.g., anti-TNF therapy) rather than directly with the bowel inflammation itself. This misrepresentation undermines the translational relevance of the study.
3. The use of colorectal distention as a model in the context of comparing visceral pain and itch pathways is unclear and needs substantial justification.
4. Given that histamine-dependent and histamine-independent itch pathways are distinct, it is crucial to investigate whether these different pruritogenic stimuli activate the same or distinct populations of BLA neurons. The current study does not provide this important distinction.
5. The observation that innocuous abdominal pressure activates the BLA raises concerns regarding the specificity of BLA neuron activation. It is possible that the activated BLA neurons are associated with the topological location of the stimulus (i.e., visceral vs. somatic). If so, any observed differences in BLA populations activated by visceral pain versus itch might merely reflect this topographical difference (e.g., back skin vs. internal organ) rather than distinct processing mechanisms for the two sensations.
6. The experimental setup involves anesthetized and firmly immobilized mice during visceral pain application. Considering the known role of the CeA in anesthesia, and the potential for BLA activation by stress associated with immobilization, there is a significant risk that BLA activity captured by the Tet-off system is confounded by anesthesia- and immobilization-induced stress responses, rather than being solely attributable to visceral pain.
7. The 36-hour window for labeling activated neurons via the Tet-off system appears excessively long and could capture neuronal activity unrelated to the acute experimental stimuli. This prolonged window increases the likelihood of capturing baseline activity or activity related to other non-specific stressors.
8. Crucial control groups are missing. The study should include a control group for the intra-dermal (id) injection of PBS (for itch controls) and a control group for the insertion of the pressure machine for CRD without increasing pressure. These controls are essential to differentiate specific neuronal activation related to the stimuli from non-specific responses to injection or mechanical presence.
9. The justification for using only male mice to "avoid hormonal variability" is weak and insufficient. It is imperative to acknowledge the importance of sex as a biological variable and to include female mice or provide a more robust scientific rationale for their exclusion, particularly given the known sex differences in pain and itch processing.

10. Several statements throughout the manuscript are overstatements. Examples include:
"The present study provides compelling evidence that the basolateral amygdala (BLA) serves as a sorting center for the processing of visceral pain and itch." This conclusion is not supported given the limitations discussed above.
"various abdominal organ diseases - including hepatobiliary pancreatic disorders, renal diseases, and inflammatory bowel diseases - frequently present with concurrent visceral pain and itch through distinct yet overlapping pathophysiological mechanisms." This broad generalization is unsubstantiated and misrepresents the clinical reality, particularly regarding the co-occurrence of primary visceral itch.
Minor concerns:
1. The methods section lacks critical details regarding the delivery of CNO via cannula. This includes:
-The timing of cannula implantation relative to the experimental procedures.
- Specific stereotaxic coordinates for cannula placement.
- The size and type of cannula used.
- The precise timing and duration of CNO delivery.
- Confirmation of whether an infusion cannula was indeed implanted.
2. In Figure 5M, the Notch2 signal shown next to the CRD-labeled image appears considerably weaker compared to the Notch signal adjacent to the histamine-labeled image. This visual discrepancy needs to be addressed or explained.

Reviewer #2

(Remarks to the Author)
In the manuscript titled "The basolateral amygdala decodes visceral pain and itch via distinct circuits and molecules," Zhao et al. demonstrated that distinct neuronal groups within the BLA are responsible for the regulation of visceral pain and itch, respectively. The neurons activated by pain projected to the VMH, while those activated by itch projected to the DMS. They also screened for gene specificity and identified that Fgf2 was highly selectively expressed in BLA-VMH projecting neurons, whereas Notch2 was selectively expressed in BLA-DMS projecting neurons. Finally, through functional gene knockdown experiments, the regulatory effects of Fgf2 and Notch2 on visceral pain and itch were identified. Overall, this work is interesting and presents significant novelties. The experiments were well conducted, and the results are reliable. However, there are several concerns that need to be addressed to further improve the quality of the work.

1. The sample size of behavioral animals in each group in Figure 4 is relatively small. In fact, I found that the number of animals in each behavioral test is six, which may increase the uncertainty of the data. It is suggested to conduct repeated experiments to supplement the data.
2. There is a lack of basal expression levels of Fgf2 and Notch2 in the sham groups in Figures 5K-M. Additionally, it is recommended to include high-magnification images to better illustrate the co-localization of Fgf2 and Notch2 with FOS cells in different groups.
3. In Figure 6, the authors demonstrated that knockdown of Fgf2 in CRD-labeled BLA neurons projecting to VMH attenuates visceral pain, while knockdown of Notch2 in His-labeled BLA neurons projecting to DMS inhibits itch. Has the author considered examining the impact of the overexpression of Fgf2 and Notch2 on visceral pain and itch? Furthermore, similar to the suggestion for Figure 4, it is recommended to increase the number of animal experiments.
4. How did the authors select the most efficient sequence of the shRNA for knocking down the expression of Fgf2 and Notch2? The knockdown efficacy for different candidates should be demonstrated.
5. What about the FOS expression induced by CQ and His stimulation in the BLA? Is the same group of neurons responding to CQ and His stimulation?
6. The authors divided BLA neurons into pain-activated neural ensembles and itch-activated neural ensembles. How should we understand the statement, "The results revealed that 47.6% of BLA neural ensembles only expressed itch-induced mCherry, while 46.5% only expressed visceral pain-activated c-Fos, and 5.9% co-expressed both itch-induced mCherry and visceral pain-activated c-Fos" (page 8, line 162)? In this sentence, it seems that 'neural ensembles' may represent the total neurons within the BLA. The authors should specify the definition of 'neural ensembles'.
7. The article's expression is not entirely accurate and contains some grammatical errors in English. It would be beneficial to have a native English speaker meticulously proofread and refine it.

Reviewer #3

(Remarks to the Author)
This manuscript presents an extensive and technically sophisticated investigation into how the basolateral amygdala (BLA) decodes visceral pain and itch via distinct glutamatergic neuronal ensembles. The authors employ a wide range of cutting-edge tools-including activity-dependent viral labeling (Tet-off), projection-specific chemogenetics, fiber photometry, and function-based single-cell transcriptomics (fLCM-seq)-to identify two parallel circuits: a BLA-VMH pathway for visceral pain and a BLA-DMS pathway for itch. They further show that Fgf2 and Notch2 are selectively expressed in these respective ensembles and functionally contribute to modality-specific behaviors. The central claims are conceptually interesting and the data are largely convincing. The demonstration of circuit-level and molecular segregation in the BLA is of potential importance for understanding the central processing of visceral sensory signals. However, several key aspects of the experimental design, data interpretation, and anatomical precision need to be clarified or strengthened. In particular, control data are missing in critical figures, circuit specificity may be overstated without further anatomical validation, and the rationale for molecular selection is unclear.

Major Comments:

1. Figure 1 lacks appropriate controls to confirm that c-Fos expression in the BLA is specifically induced by CRD or histamine. Without a baseline group (e.g., unstimulated or saline-injected animals), it is not possible to conclude that the observed activation is stimulus-specific. Given that c-Fos is used to label and define functional ensembles throughout the study, the absence of a control group substantially weakens this foundation. The authors must include data from control animals and provide a discussion of basal Fos expression in BLA under resting conditions.
2. In Figure 3B, viral expression appears to extend well beyond the BLA into the central amygdala (CeA). Given the proximity of these nuclei and the known roles of CeA in pain, itch, and emotional processing, this raises the possibility that some of the observed behavioral and circuit effects may result from unintended manipulation of CeA neurons. The authors must provide the following for each mouse: Injection volumes for each virus; Raw images showing viral spread, optical fiber placements, and cannula placements; Summary maps (per group) showing targeting success and spatial coverage. Particular attention should be paid to delineating BLA from neighboring CeA in all datasets. Without such anatomical precision, the specificity of the conclusions is in question. For experiments involving local infusion of CNO into VMH or DMS, no data are provided on the extent of spread. The authors should report how far CNO diffuses from the injection site (e.g., through co-injection of dye or prior validation) to confirm that the effects are due to local modulation of axon terminals and not off-target actions.
3. The conclusion that pain and itch ensembles in the BLA project exclusively to VMH and DMS, respectively, is a central claim. However, the current tracing methods do not definitively rule out collateral projections. The degree of exclusivity in projection patterns is not entirely clear. Do any BLA neurons collateralize to both VMH and DMS? If not, were intersectional approaches used to test this? Some clarification or quantification here would be valuable. Dual retrograde tracing (e.g., CTB-488/594 injected into VMH and DMS) or single-neuron projectom analysis could help determine whether BLA neurons send axon collaterals to both targets. The manuscript would benefit from a brief discussion of other potential downstream targets involved in processing visceral signals, such as the central amygdala, PAG, or parabrachial nucleus, and how they might interact with the circuits described.
4. The authors characterize the outputs of the BLA ensembles in detail but provide little information about their upstream inputs. Understanding what drives the selective activation of these ensembles is important for interpreting their role in the broader circuit. Are these ensembles recruited by distinct ascending sensory pathways, or are they selected locally within the BLA based on convergent inputs? Some mention of how thalamic, cortical, or brainstem afferents may contribute to the specificity of pain vs. itch responses would strengthen the Discussion.
5. While the use of fLCM-seq is technically impressive, the rationale for focusing on Fgf2 and Notch2 is not clear. Were these the most significantly differentially expressed genes? If not, why were they prioritized over top-ranking candidates? A table or supplementary figure showing the top DEGs would help readers understand the selection process. Moreover, whether other highly enriched genes also contribute functionally remains an open question. Knockdown of Fgf2 and Notch2 produced modest effects. Without gain-of-function experiments or rescue studies, it is difficult to conclude that these genes are functionally sufficient to drive modality-specific behavior. This should be tested.
6. The Introduction and Discussion emphasize that pain and itch often co-occur in clinical conditions such as cholestasis or inflammatory bowel disease. However, the experimental design models the two sensations separately, and the key conclusion is that BLA circuits are segregated. This somewhat contradicts the clinical motivation. To more directly test the hypothesis of convergence in comorbid states, the authors could consider using a disease model in which both symptoms co-occur. Would such a model recruit overlapping BLA ensembles? This remains unexplored.

Minor Comments:
1. Several panels (e.g., Figures 1G, 1N, 3H, and 3M) do not specify the number of mice used for calcium imaging, and “trials plot” style is not ideal for interpreting mean population dynamics. Additionally, in Figures 3J and 3N, seven data points are shown despite the legend stating n = 6 mice. This discrepancy should be corrected. Accurate reporting of sample size, error bars, and data distribution is essential.
2. The manuscript inconsistently states that mice were perfused 2 hours after CRD in some sections and 1.5 hours in others. As c-Fos kinetics are time-sensitive, this inconsistency must be resolved and corrected throughout.

Version 2:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
I appreciate the authors’ efforts in performing additional experiments in response to prior comments. However, my major concerns have not been sufficiently addressed.
Although the neural mechanisms underlying somatic pain and itch have been extensively investigated, it remains unclear whether distinct neuronal subpopulations within the BLA differentially mediate these two modalities. One of the newly added experiments provides important evidence that somatic pain and itch stimuli activate partially overlapping neuronal populations in the BLA (8.3% overlap), as demonstrated using the Tet-off system. These findings are valuable and should be incorporated into the manuscript. Furthermore, the authors should expand the discussion to address whether these results suggest a potential topographical organization versus distinct processing mechanisms for pain and itch within the BLA.
The revised Introduction places strong emphasis on the clinical relevance of visceral pain and non-cutaneous itch. However, the present study primarily examines neuronal mechanisms of acutely induced cutaneous itch rather than non-cutaneous itch. This conceptual discrepancy should be clarified and better aligned throughout the manuscript. In addition, the use of irritable bowel syndrome mouse model is not fully justified, as this condition is not typically associated with pruritus. Finally, references 9 and 10 do not appear to discuss inflammatory bowel disease as implied in the text and should be corrected or

replaced.

Reviewer #2

(Remarks to the Author)
The authors have satisfactorily addressed all my concerns with additional experiments. I have no more questions for the work.

Reviewer #3

(Remarks to the Author)
See the attachment

Version 3:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The manuscript has been significantly improved by addressing the reviewers' concerns.

Reviewer #3

(Remarks to the Author)
I have no additional comments and recommend acceptance.

Responses to reviewers' comments

Reviewer #1: This manuscript investigates the neural representation of visceral pain and itch within the BLA. Several fundamental concerns regarding its scientific premise, experimental design, and interpretation significantly limit the impact and validity of the findings.

>>>Response: Thank you for the insightful and constructive comments. In accordance with the suggestions, we have carefully revised the manuscript. The revised sections are highlighted. Our point-by-point responses follow:

1. The core premise of comparing visceral pain and itch at the BLA is not adequately justified. Itch is predominantly a somatosensory sensation, typically originating from the skin. Since itch doesn't occur from internal organs, it is highly unlikely that the neuronal pathways for visceral pain and itch are significantly overlapped. The introduction fails to present a compelling argument for this specific comparison. Existing literature, as even cited by the authors, strongly suggests that comparing somatic pain and itch pathways would be a more scientifically sound and straightforward approach, given their known interactions and shared anatomical substrates in the somatosensory system.

>>>Response: Thank you for this critical point. Our premise is not based on the itch occur from internal organs, but on the following rationales, which we have now more clearly articulated in the revised introduction (Page 3, Lines 51–63):

1. Although the neural mechanisms of somatic pain and itch have been extensively and deeply explored, research on visceral pain and itch remains relatively preliminary and limited. This pronounced disparity reflects the long-term underemphasis on the study of related neural mechanisms in the field of visceral sensation. Therefore, this study focuses on the brain's processing mechanisms for visceral pain and itch information, aiming to provide an in-depth analysis of the underlying neural circuits and molecular underpinnings, with the goal of offering theoretical support and experimental evidence for the development of specific therapeutic strategies targeting visceral sensory abnormalities.

2. From a clinical perspective, certain visceral diseases often present with complex symptoms co-occurring pain and itch, such as primary biliary cholangitis and chronic kidney disease [1-2]. Furthermore, during the treatment of related visceral diseases (e.g., e.g., anti-TNF therapy in IBD patients [3]), intractable secondary itch can also be induced. Since the mechanisms underlying these comorbid or secondary symptoms remain poorly understood, clinical management often lacks specific and effective interventions, imposing a considerable physiological and psychological burden on patients. Therefore, an in-depth exploration of the neural and molecular mechanisms of pain and itch in

visceral diseases is of crucial clinical significance for the development of novel therapeutic approaches.

(1). *Beuers U, Wolters F, Oude Elferink RPJ. Mechanisms of pruritus in cholestasis: understanding and treating the itch. Nat Rev Gastroenterol Hepatol. 2023 Jan;20(1):26-36. doi: 10.1038/s41575-022-00687-7. Epub 2022 Oct 28. PMID: 36307649.*

(2). *Zucker, I., Yosipovitch, G., David, M., Gafter, U. & Boner, G. Prevalence and characterization of uremic pruritus in patients undergoing hemodialysis: uremic pruritus is still a major problem for patients with end-stage renal disease. J Am Acad Dermatol 49, 842-846 (2003).*

(3). *Greuter T, Navarini A, Vavricka SR. Skin Manifestations of Inflammatory Bowel Disease. Clin Rev Allergy Immunol. 2017 Dec;53(3):413-427. doi: 10.1007/s12016-017-8617-4. PMID: 28643285.*

2. The assertion that "visceral pain and itch frequently co-occur in various diseases" is problematic and requires stronger evidence. The provided example of Inflammatory Bowel Disease (IBD) and itch is not generally supported by clinical observations. Itch in IBD patients is more commonly associated with drug-induced skin lesions (e.g., anti-TNF therapy) rather than directly with the bowel inflammation itself. This misrepresentation undermines the translational relevance of the study.

>>>Response: We fully agree with the review. We have been modified the "visceral pain and itch frequently co-occur in various diseases" to "visceral pain and itch frequently co-occur in some diseases" in the revised manuscript (Page 19, Lines 444–446, highlighted in red). Furthermore, we have revised the description of a simple and direct link between IBD and itch (Page 20, Lines 452–454, highlighted in red). Additionally, we have been adopted a more precise clinical context, focusing on conditions like primary biliary cholangitis and chronic kidney disease, where a clear and established link exists between visceral pain and itch[1-2]. This provides a firmer translational foundation for our study.

(1). *Dyson, J. K., Beuers, U., Jones, D. E. J., Lohse, A. W. & Hudson, M. Primary sclerosing cholangitis. Lancet 391, 2547-2559 (2018).*

(2). *Zucker, I., Yosipovitch, G., David, M., Gafter, U. & Boner, G. Prevalence and characterization of uremic pruritus in patients undergoing hemodialysis: uremic pruritus is still a major problem for patients with end-stage renal disease. J Am Acad Dermatol 49, 842-846 (2003).*

3. The use of colorectal distention as a model in the context of comparing visceral pain and itch pathways is unclear and needs substantial justification.

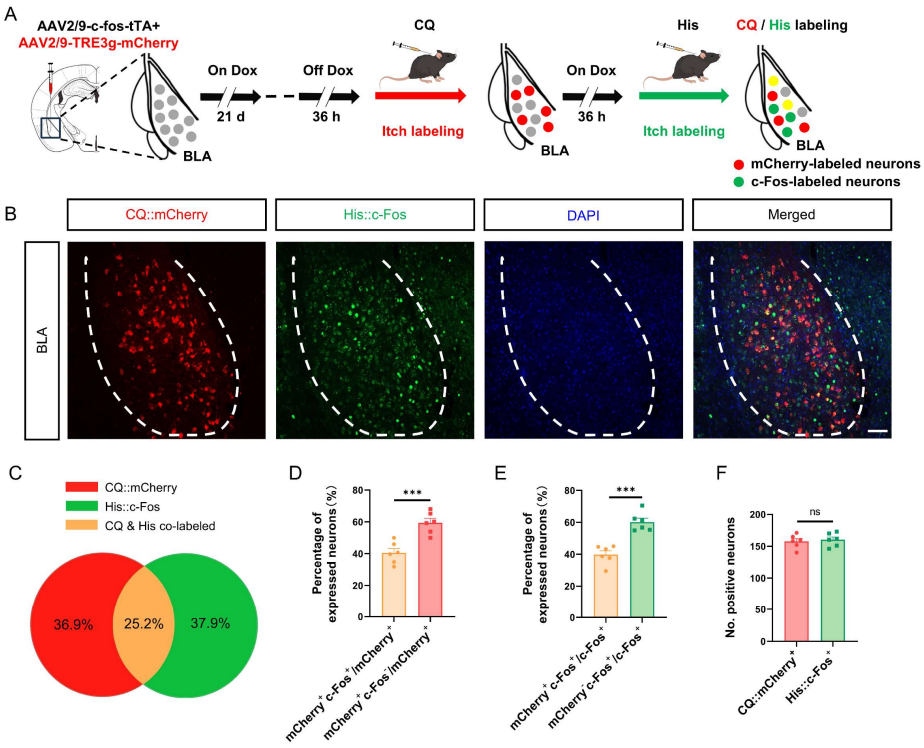
>>>Response: Thank you for this constructive comment and we sincerely apologize for the unclear description. Visceral pain is a broad concept encompassing pain from various internal organs, with irritable bowel syndrome (IBS) representing a key and challenging clinical example. Moreover, Colorectal distention (CRD) is a classic and

reliably induced stimulus, which is commonly used to model visceral pain. Our laboratory has long been dedicated to research on visceral pain. Therefore, we selected CRD to mimic the visceral pain seen in clinical IBS patients. Other types of visceral pain will be explored in our future studies. In addition, we have added this justification in the methods sections, clarifying that our use of "visceral pain" in this study specifically refers to intestinal pain modeled by CRD (Page 24, Line 556, highlighted in red).

4. Given that histamine-dependent and histamine-independent itch pathways are distinct, it is crucial to investigate whether these different pruritogenic stimuli activate the same or distinct populations of BLA neurons. The current study does not provide this important distinction.

>>>Response: Thank you for the comment. To address this issue, we conducted a **new experiment** to evaluate the activation status of BLA neurons in response to histamine-dependent and histamine-independent itch. The results are presented in Supplementary Figure 3.

New experiment: To investigate histamine-independent itch, we utilized chloroquine, a well-established pruritogen commonly employed in models of histamine-independent itch. The results demonstrate that histamine-dependent and histamine-independent itch stimuli activate neuronal populations in the BLA with partial overlap of 25.2%.



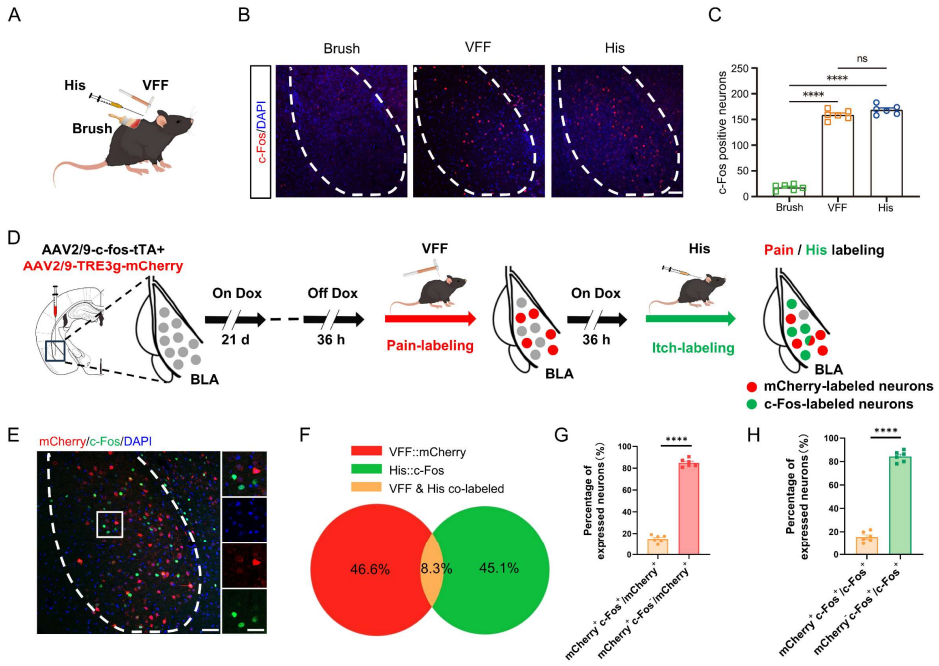
Supplementary Figure 3. Representation of CQ and His-induced itch by distinct neural ensembles in the BLA.

5. The observation that innocuous abdominal pressure activates the BLA raises concerns regarding the specificity of BLA neuron activation. It is possible that the activated BLA neurons are associated with the topological location of the stimulus (i.e., visceral vs. somatic). If so, any observed differences in BLA populations activated by visceral pain versus itch might merely reflect this topographical difference (e.g., back skin vs. internal organ) rather than distinct processing mechanisms for the two sensations.

>>>Response: Thank you for the comment. This was a critical point regarding a potential confound. In response, we conducted a **new experiment**. Since itch doesn't occur from internal organs, it is not feasible to deliver visceral pain and itch stimuli to the same visceral site. Instead, we applied somatic pain and itch stimuli to the same location (the neck skin) to investigate whether these two sensory modalities activate shared or distinct neuronal populations.

New experiment: To determine whether different sensory modalities applied to the same site activate shared or distinct neuronal populations, we delivered somatic pain and itch stimuli to the neck skin by Tet-off system. The results demonstrate that somatic pain and itch stimuli activate neuronal populations in the BLA with a partial overlap of 8.3%, indicating that even sensory stimuli applied to the same location do not recruit identical neuronal ensembles. Although we cannot fully rule out the potential influence

of spatial factors, substantial evidence suggests that the observed differences in BLA populations activated by visceral pain versus itch are primarily due to distinct neural processing mechanisms for these two sensations.

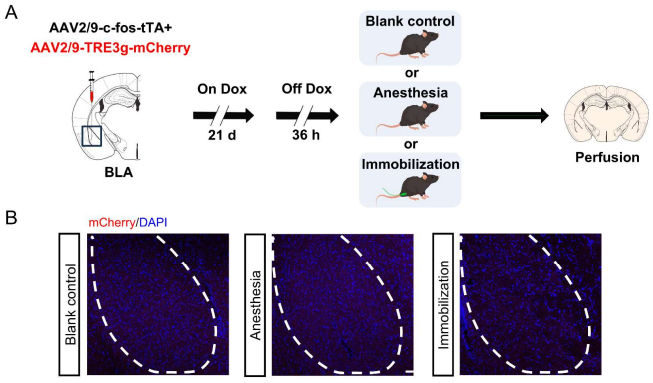


6. The experimental setup involves anesthetized and firmly immobilized mice during visceral pain application. Considering the known role of the CeA in anesthesia, and the potential for BLA activation by stress associated with immobilization, there is a significant risk that BLA activity captured by the Tet-off system is confounded by anesthesia- and immobilization-induced stress responses, rather than being solely attributable to visceral pain.

>>>Response: Thank you for the comment. To address the concern regarding a potential risk that BLA activity could be confounded by anesthesia or immobilization, we have conducted an **additional experiment.** We sincerely apologize for the insufficient methodological detail. To avoid similar misunderstandings among readers, we have explicitly clarified in the revised manuscript's methods section that visceral pain stimuli were applied to awake, partially restrained mice, not completely immobilized or chronically anesthetized animals (Page 24, Lines 560–562, highlighted in red). Isoflurane was used solely during the initial setup phase.

New experiment: To address whether the BLA activation observed in our main experiments could be attributed to procedural stressors rather than the visceral pain, we quantified BLA neuronal activity using Tet-off system in mice exposed only to the transient isoflurane anesthesia (Anesthesia) or partial restraint procedure used

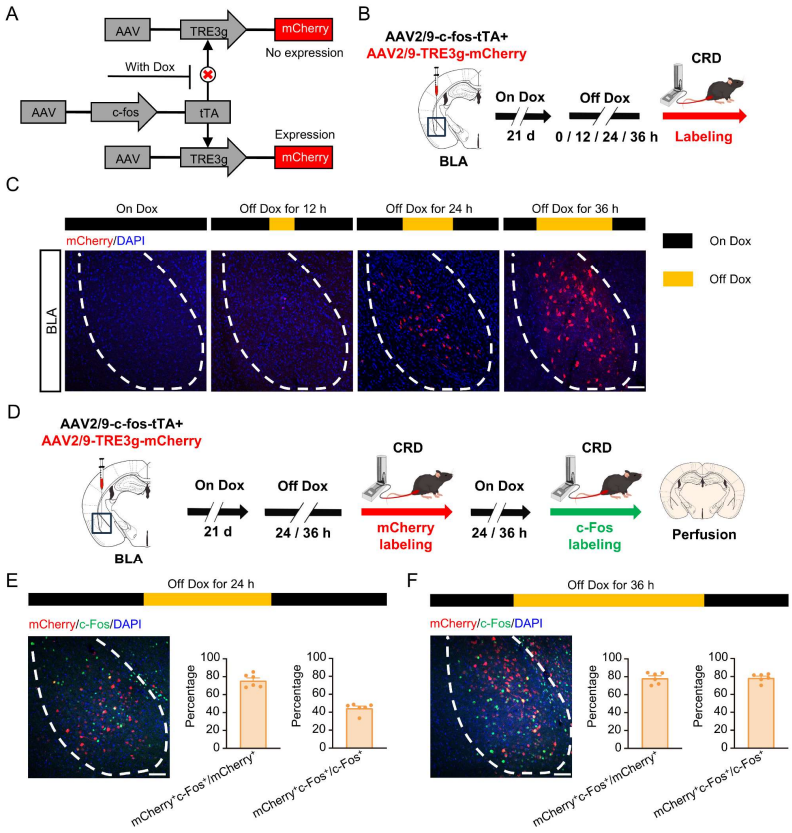
(Immobilization) in our main experimental paradigm. The new results show that neither Immobilization nor Anesthesia meaningfully activates BLA neurons.



7. The 36-hour window for labeling activated neurons via the Tet-off system appears excessively long and could capture neuronal activity unrelated to the acute experimental stimuli. This prolonged window increases the likelihood of capturing baseline activity or activity related to other non-specific stressors.

>>>Response: Thank you for the comment. The 36-hour window was systematically optimized based on our previous work (Neuron, 2024, PMID: 39326407; Neuron, 2025, PMID: 40345185). We performed additional a **new experiment** across multiple time windows (12h, 24h, 36h) and confirmed that the 36-hour window provides the optimal signal-to-noise ratio for activity-dependent labeling in the BLA in our hands. The results are presented in Supplementary Figure 4.

New experiment: To evaluate the optimal drug withdrawal time window for the Tet-off system, we performed new control experiments comparing labeling efficiency and specificity across multiple time windows (12h, 24h, 36h). The new results show that the 36-hour window provides the best balance between the efficiency and specificity.

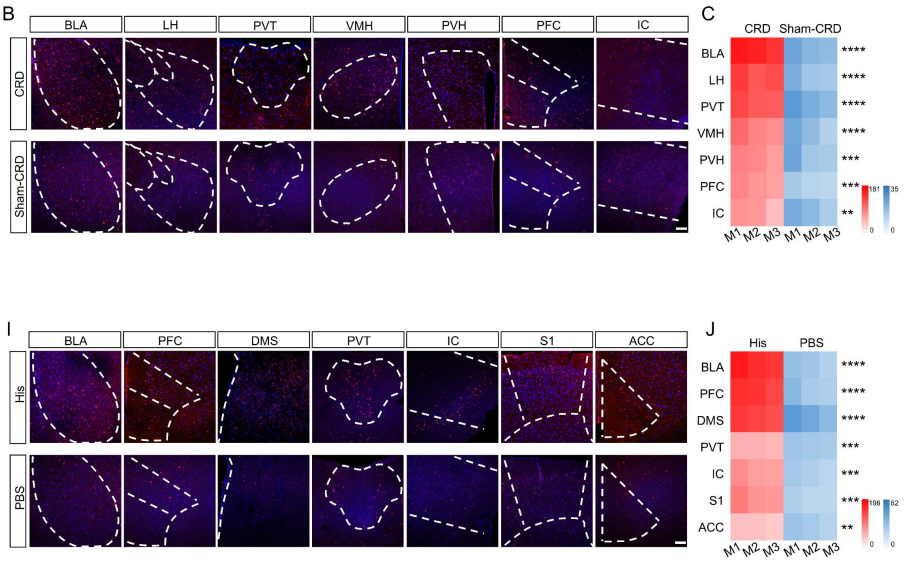


Supplementary Figure 4. Optimization of the time window for signal detection using the Tet-off system.

8. Crucial control groups are missing. The study should include a control group for the intra-dermal (id) injection of PBS (for itch controls) and a control group for the insertion of the pressure machine for CRD without increasing pressure. These controls are essential to differentiate specific neuronal activation related to the stimuli from non-specific responses to injection or mechanical presence.

>>>Response: We fully agree with the comment. We have now included the essential control groups for intradermal PBS injection and CRD device insertion without distension (Sham-CRD). These data have been incorporated into Figures 1B-1C and 1I-1J, confirming that the observed neuronal activation is stimulus-specific.

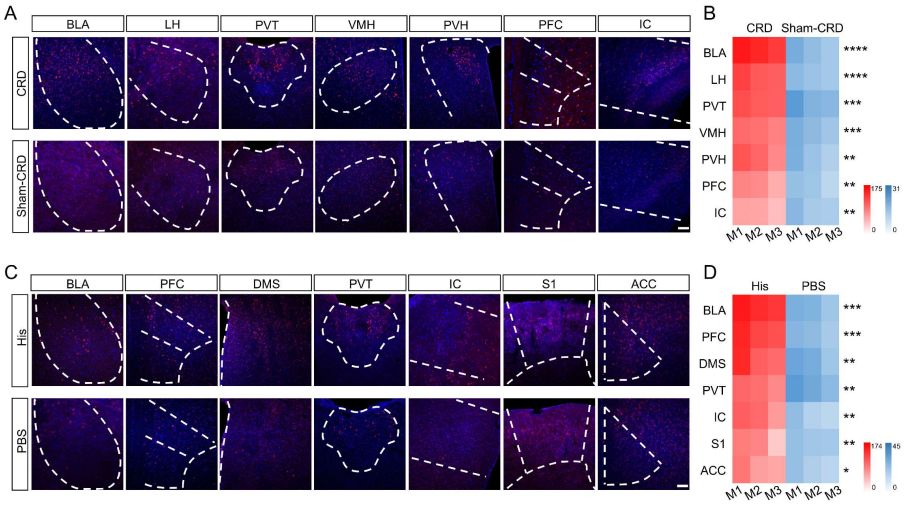
New experiment: To rigorously control for potential nonspecific neuronal activation from procedural artifacts, we have conducted new experiments including intradermal PBS injection and Sham-CRD. These new data, now presented in Figures 1B-1C and 1I-1J, show that the observed neuronal activation is stimulus-specific.



9. The justification for using only male mice to "avoid hormonal variability" is weak and insufficient. It is imperative to acknowledge the importance of sex as a biological variable and to include female mice or provide a more robust scientific rationale for their exclusion, particularly given the known sex differences in pain and itch processing.

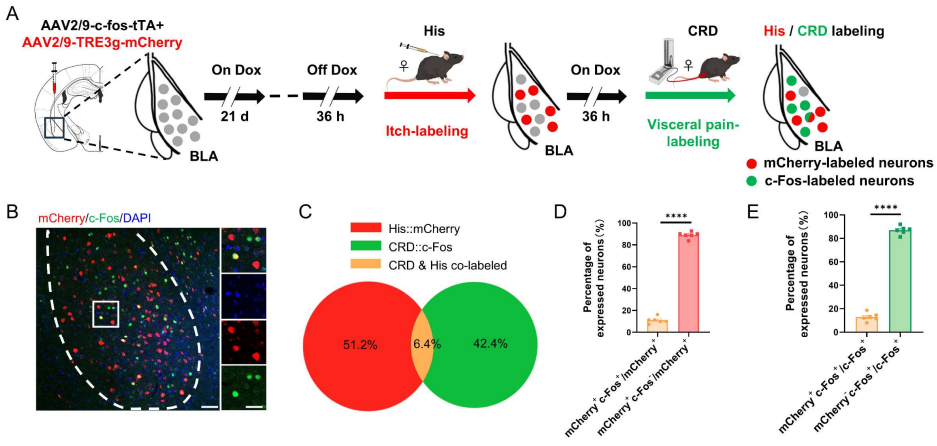
>>>Response: Thanks for your constructive comment. In response, we have conducted **four (4) additional experiments** in female mice to validate our core findings, and we have explicitly clarified in the revised manuscript's methods section (Page 22, Lines 500–502, highlighted in red):

New experiment 1: To further confirm the role of the BLA in female mice, we assessed the expression of CRD- and His-induced c-Fos in the BLA. As shown below, c-Fos induced by CRD or His was primarily expressed in the BLA. These data are consistent with the results from the male mice and have been incorporated into Supplementary Figure 1 of the revised manuscript.



Supplementary Figure 1. Expression of CRD- and His-induced c-Fos in the BLA in female mice.

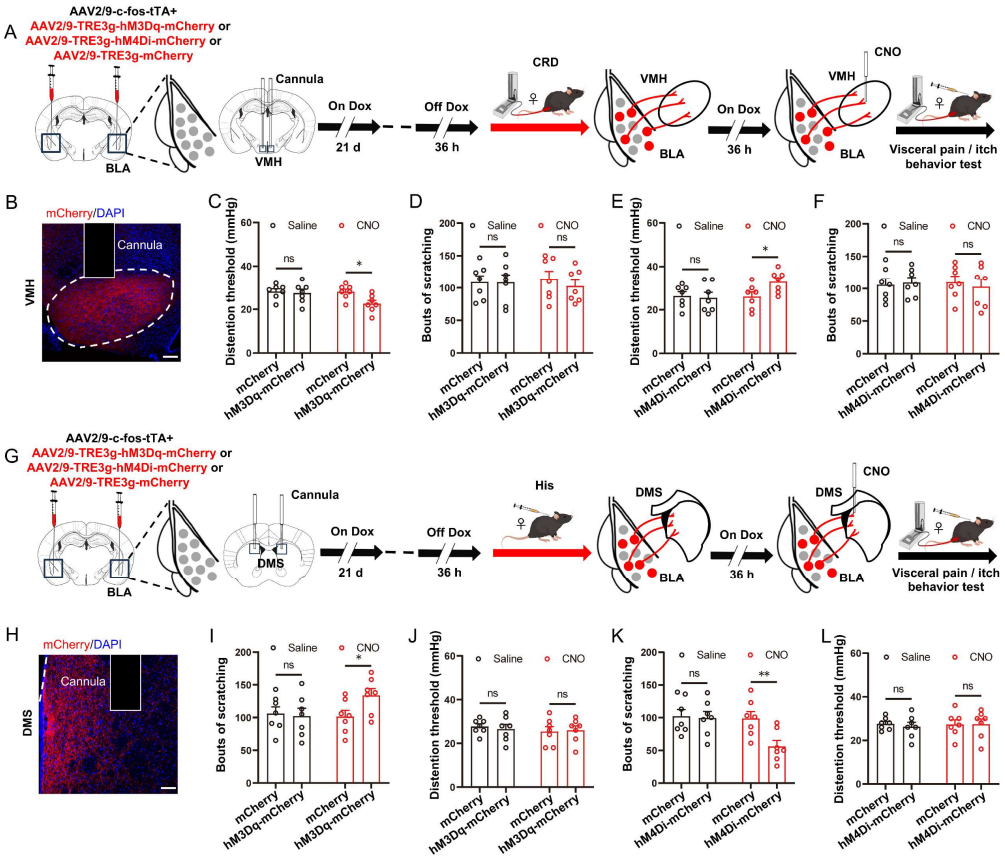
New experiment 2: To investigate the representation of neural ensembles activated by visceral pain and itch in the BLA in female mice, Tet-off system combined with c-Fos staining was utilized. We mapped the distribution of BLA neural ensembles activated by His and CRD using mCherry expression and c-Fos staining. Quantitative analysis showed that the percentage of double-positive neurons for mCherry and c-Fos was significantly lower than single-positive neurons expressing either mCherry or c-Fos alone. As shown below, these data are consistent with the results from the male mice and included in Supplementary Figure 6 of the revised manuscript.



Supplementary Figure 6. His and CRD activated neurons are two distinct ensembles in BLA in female mice.

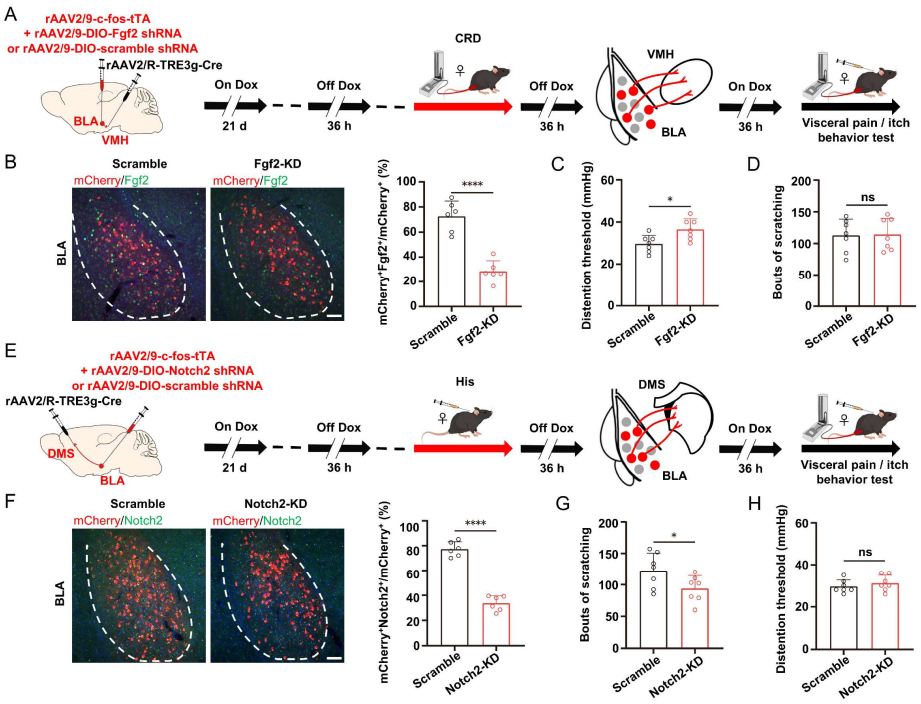
New experiment 3: To further investigate the function of the neural circuits involving CRD-labeled BLA neurons to the VMH and His-labeled BLA neurons to the DMS, chemogenetic manipulations were conducted. The results showed that

manipulation of the projections of CRD-labeled BLA neural ensembles to the VMH specifically modulates visceral pain, whereas manipulation of the projections of His-labeled BLA neural ensembles to the DMS specifically modulates itch. These results are consistent with the results from the male mice and have been incorporated into Supplementary Figure 7 of the revised manuscript.



Supplementary Figure 7. Manipulation of the projections from CRD-labeled BLA neurons to VMH or His-labeled BLA neurons to DMS specifically modulates visceral pain or itch behaviors in female mice.

New experiment 4: To further explore the role of Fgf2 in regulating visceral pain through the BLA-VMH circuit and Notch2 in regulating itch through the BLA-DMS circuit, we selectively knocked down Fgf2 expression in CRD-labeled BLA neurons projecting to VMH and Notch2 expression in His-labeled BLA neurons projecting to DMS. The results showed that knockdown of Fgf2 in CRD-labeled BLA neurons projecting to VMH attenuates visceral pain whereas knockdown of Notch2 in His-labeled BLA neurons projecting to DMS inhibits itch. These results are consistent with the results from the male mice and have been incorporated into Supplementary Figure 8 of the revised manuscript.



Supplementary Figure 8. Knockdown of Fgf2 in CRD-labeled BLA neurons projecting to VMH attenuates visceral pain whereas knockdown of Notch2 in His-labeled BLA neurons projecting to DMS inhibits itch in female mice.

10. Several statements throughout the manuscript are overstatements. Examples include: "The present study provides compelling evidence that the basolateral amygdala (BLA) serves as a sorting center for the processing of visceral pain and itch." This conclusion is not supported given the limitations discussed above. "Various abdominal organ diseases - including hepatobiliary pancreatic disorders, renal diseases, and inflammatory bowel diseases - frequently present with concurrent visceral pain and itch through distinct yet overlapping pathophysiological mechanisms." This broad generalization is unsubstantiated and misrepresents the clinical reality, particularly regarding the co-occurrence of primary visceral itch.

>>>Response: Thank you for the comments. We have thoroughly checked and toned down all instances of overinterpretation or overly broad claims throughout the manuscript. In revised manuscript, we revised " The present study provides compelling evidence that the basolateral amygdala (BLA) serves as a sorting center for the processing of visceral pain and itch." to a more measured and accurate description: " The present study provides evidence that the BLA plays a role in distinguishing and integrating the sensory components of visceral pain and itch." (Page 16, Lines 357–358, highlighted in red). In addition, we revised " Various abdominal organ diseases - including hepatobiliary pancreatic disorders, renal diseases, and inflammatory bowel diseases - frequently present with concurrent visceral pain and itch through distinct yet

overlapping pathophysiological mechanisms." to " In clinical practice, visceral pain and itch frequently co-occur in some diseases-including primary biliary cholangitis, chronic kidney disease, and inflammatory bowel diseases." (Page 19, Lines 444–446, highlighted in red).

Minor concerns:

1. The methods section lacks critical details regarding the delivery of CNO via cannula. This includes:

-The timing of cannula implantation relative to the experimental procedures.

- Specific stereotaxic coordinates for cannula placement.

- The size and type of cannula used.

- The precise timing and duration of CNO delivery.

- Confirmation of whether an infusion cannula was indeed implanted.

>>>Response: Thank you for the comments. These critical details have been added to the method section (Pages 27-28, Lines 639–646, Lines 649–651, highlighted in red).

2. In Figure 5M, the Notch2 signal shown next to the CRD-labeled image appears considerably weaker compared to the Notch signal adjacent to the histamine-labeled image. This visual discrepancy needs to be addressed or explained.

>>>Response: Thank you for the comments. We apologize for the prior selection of an inappropriate representative image and have replaced it with a more suitable one to prevent any potential misinterpretation.

Reviewer #2: In the manuscript titled "The basolateral amygdala decodes visceral pain and itch via distinct circuits and molecules," Zhao et al. demonstrated that distinct neuronal groups within the BLA are responsible for the regulation of visceral pain and itch, respectively. The neurons activated by pain projected to the VMH, while those activated by itch projected to the DMS. They also screened for gene specificity and identified that Fgf2 was highly selectively expressed in BLA-VMH projecting neurons, whereas Notch2 was selectively expressed in BLA-DMS projecting neurons. Finally, through functional gene knockdown experiments, the regulatory effects of Fgf2 and Notch2 on visceral pain and itch were identified. Overall, this work is interesting and presents significant novelties. The experiments were well conducted, and the results are reliable. However, there are several concerns that need to be addressed to further improve the quality of the work.

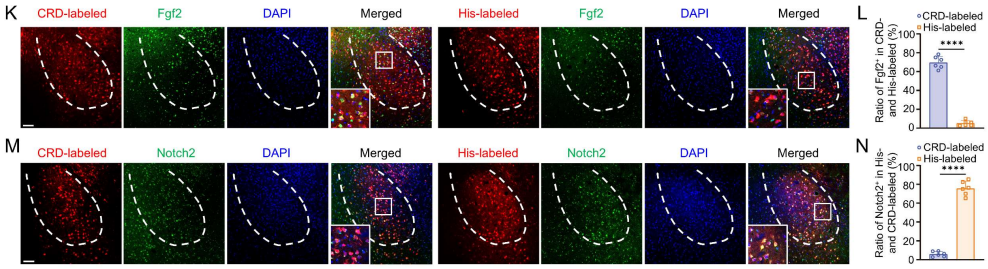
>>>Response: Thank you very much for the positive comments and constructive suggestions.

1. The sample size of behavioral animals in each group in Figure 4 is relatively small. In fact, I found that the number of animals in each behavioral test is six, which may increase the uncertainty of the data. It is suggested to conduct repeated experiments to supplement the data.

>>>Response: Thanks for the constructive comments. We have performed additional experiments to increase the sample size for the key behavioral tests in Figures 4C-4F and 4I-4L, enhancing the statistical power and reliability of our conclusions.

2. There is a lack of basal expression levels of Fgf2 and Notch2 in the sham groups in Figures 5K-M. Additionally, it is recommended to include high-magnification images to better illustrate the co-localization of Fgf2 and Notch2 with FOS cells in different groups.

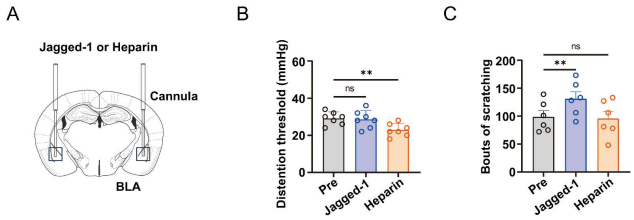
>>>Response: Thank you for the comment. The primary objective of this study is to determine whether Fgf2 and Notch2 expression is predominantly localized to neurons activated by specific stimuli (visceral pain or itch), not to compare whether Fgf2 and Notch2 expression is altered. We sincerely apologize for the visual confusion caused by the color scheme choice in Figures 5L and 5N, and we have modified the color scheme in the revised manuscript. Additionally, as suggested, we have provided high-magnification images (Figures 5K and 5M) to more clearly demonstrate the colocalization of Fgf2 and Notch2 with mCherry-positive cells across experimental groups.



3. In Figure 6, the authors demonstrated that knockdown of Fgf2 in CRD-labeled BLA neurons projecting to VMH attenuates visceral pain, while knockdown of Notch2 in His-labeled BLA neurons projecting to DMS inhibits itch. Has the author considered examining the impact of the overexpression of Fgf2 and Notch2 on visceral pain and itch? Furthermore, similar to the suggestion for Figure 4, it is recommended to increase the number of animal experiments.

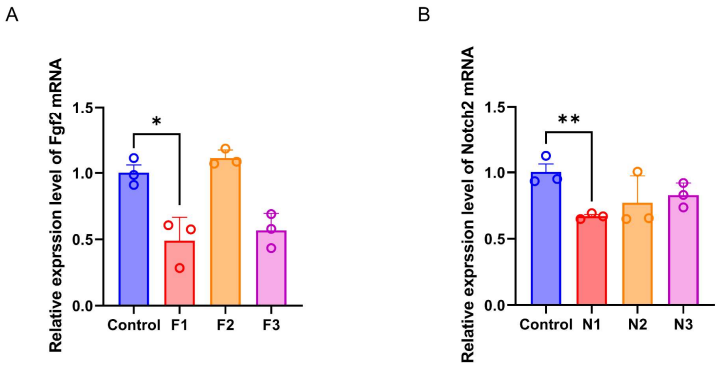
>>>Response: Thank you for the comments. Given that the Notch2 is too large to be packaged into an adenoviral vector, we used agonists to achieve gain-of-function for both targets for consistency. Also, we have performed additional experiments to increase the sample size to enhance the statistical power and reliability of our findings.

New experiment: To further validate the role of Fgf2 and Notch2 in BLA, we performed agonist experiments. The data show that microinjection of the Heparin (agonist of Fgf2) into the BLA promoted visceral pain, while the Jagged-1 (agonist of Notch2) promoted itch.



4. How did the authors select the most efficient sequence of the shRNA for knocking down the expression of Fgf2 and Notch2? The knockdown efficacy for different candidates should be demonstrated.

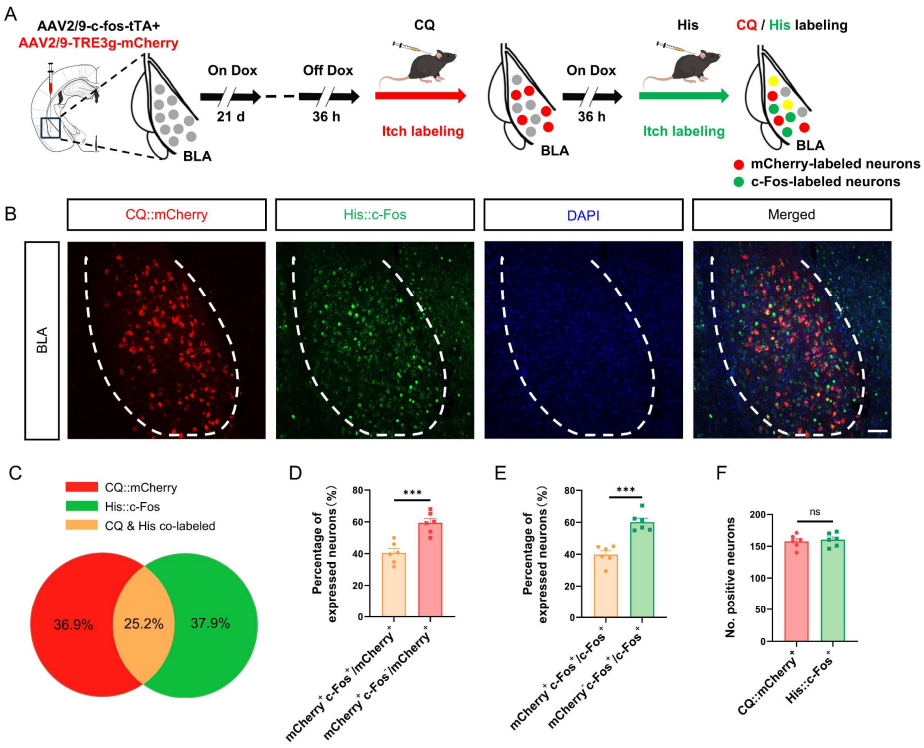
>>>Response: Thank you for the comment. We have provided **additional data** demonstrating the knockdown efficacy and specificity of the selected Fgf2 and Notch2 shRNA sequences used in this study. Representative qPCR results are shown below. The candidate knockdown sequences have been added in the revised manuscript (Page 23, Lines 526–530, highlighted in red). Based on the qPCR results, we selected the one with the highest knockdown efficiency.



5. What about the FOS expression induced by CQ and His stimulation in the BLA? Is the same group of neurons responding to CQ and His stimulation?

>>>Response: Thank you for the comment. To elucidate this issue, **a new experiment** was conducted to evaluate the activation of BLA neurons in response to both histamine-dependent and histamine-independent itch. The corresponding data are presented in Supplementary Figure 3.

New experiment: To investigate histamine-independent itch, we utilized chloroquine, a well-established pruritogen commonly employed in models of histamine-independent itch. The results demonstrate that histamine-dependent and histamine-independent itch stimuli activate neuronal populations in the BLA with partial overlap of 25.2%.



Supplementary Figure 3. Representation of CQ and His-induced itch by distinct neural ensembles in the BLA.

6. The authors divided BLA neurons into pain-activated neural ensembles and itch-activated neural ensembles. How should we understand the statement, "The results revealed that 47.6% of BLA neural ensembles only expressed itch-induced mCherry, while 46.5% only expressed visceral pain-activated c-Fos, and 5.9% co-expressed both itch-induced mCherry and visceral pain-activated c-Fos" (page 8, line 162)? In this sentence, it seems that 'neural ensembles' may represent the total neurons within the BLA. The authors should specify the definition of 'neural ensembles'.

>>>Response: We apologize for the lack of clarity. In our manuscript, "neural ensembles" here refer to functionally defined populations of BLA neurons activated by either visceral pain (c-Fos^{+}) or itch (mCherry^{+}), including non-overlapping and overlapping subsets, and not to the total neurons within the BLA.

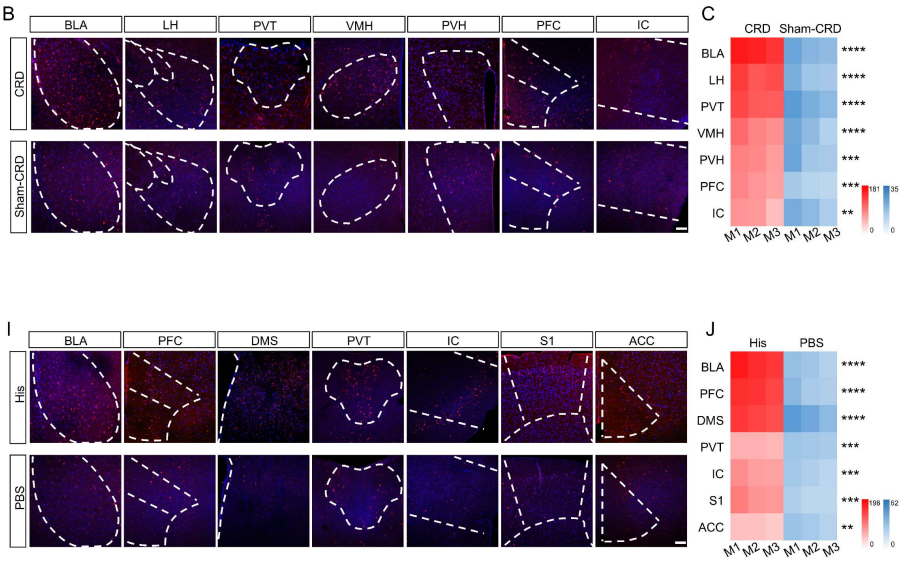
Reviewer #3: This manuscript presents an extensive and technically sophisticated investigation into how the basolateral amygdala (BLA) decodes visceral pain and itch via distinct glutamatergic neuronal ensembles. The authors employ a wide range of cutting-edge tools-including activity-dependent viral labeling (Tet-off), projection-specific chemogenetics, fiber photometry, and function-based single-cell transcriptomics (fLCM-seq)-to identify two parallel circuits: a BLA-VMH pathway for visceral pain and a BLA-DMS pathway for itch. They further show that Fgf2 and Notch2 are selectively expressed in these respective ensembles and functionally contribute to modality-specific behaviors. The central claims are conceptually interesting and the data are largely convincing. The demonstration of circuit-level and molecular segregation in the BLA is of potential importance for understanding the central processing of visceral sensory signals. However, several key aspects of the experimental design, data interpretation, and anatomical precision need to be clarified or strengthened. In particular, control data are missing in critical figures, circuit specificity may be overstated without further anatomical validation, and the rationale for molecular selection is unclear.

>>>Response: Thank you for the insightful comments and positive assessment of our work's technical sophistication.

1. Figure 1 lacks appropriate controls to confirm that c-Fos expression in the BLA is specifically induced by CRD or histamine. Without a baseline group (e.g., unstimulated or saline-injected animals), it is not possible to conclude that the observed activation is stimulus-specific. Given that c-Fos is used to label and define functional ensembles throughout the study, the absence of a control group substantially weakens this foundation. The authors must include data from control animals and provide a discussion of basal Fos expression in BLA under resting conditions.

>>>Response: We fully agree with the comment. We have now included the essential control groups for intradermal PBS injection and CRD device insertion without distension (Sham-CRD). These data have been incorporated into Figures 1B-1C and 1I-1J, confirming that the observed neuronal activation is stimulus-specific.

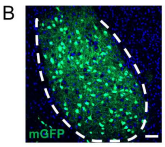
New experiment: To rigorously control for potential nonspecific neuronal activation from procedural artifacts, we have conducted new experiments including intradermal PBS injection and Sham-CRD. These results have been incorporated into Figures 1B-1C and 1I-1J of the revised manuscript.



2. In Figure 3B, viral expression appears to extend well beyond the BLA into the central amygdala (CeA). Given the proximity of these nuclei and the known roles of CeA in pain, itch, and emotional processing, this raises the possibility that some of the observed behavioral and circuit effects may result from unintended manipulation of CeA neurons. The authors must provide the following for each mouse: Injection volumes for each virus; Raw images showing viral spread, optical fiber placements, and cannula placements; Summary maps (per group) showing targeting success and spatial coverage. Particular attention should be paid to delineating BLA from neighboring CeA in all datasets. Without such anatomical precision, the specificity of the conclusions is in question. For experiments involving local infusion of CNO into VMH or DMS, no data are provided on the extent of spread. The authors should report how far CNO diffuses from the injection site (e.g., through co-injection of dye or prior validation) to confirm that the effects are due to local modulation of axon terminals and not off-target actions.

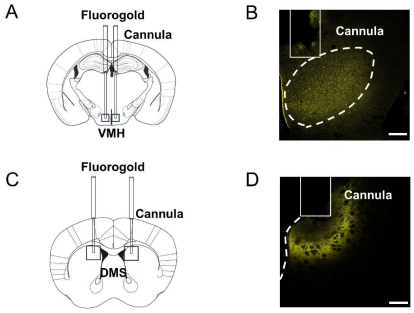
>>>Response: Thank you for emphasizing anatomical precision.

1, In our study, only samples with accurate injection sites were included in the analysis. We regret the prior selection of an inappropriate representative image and have replaced it with a more suitable one to prevent any potential misinterpretation (Figure 3B). In addition, the injection volumes for each virus have been provided in the methods section (Page 23, Line 535, highlighted in red). Raw images will be made available as supplementary material if the manuscript advances to the next stage.



2, For CNO infusion experiments, we performed a **new experiment** co-injecting Fluorogold to visualize spread. The data confirm that the diffusion was localized to the target region (VMH/DMS), supporting circuit-specific effects.

New experiment: To evaluate the extent of CNO diffusion following local infusion, we performed experiments as suggested by the reviewer. Accordingly, we injected an equal volume (300 nl) of Fluorogold into the VMH or DMS via the implanted cannulas and quantified its spread.

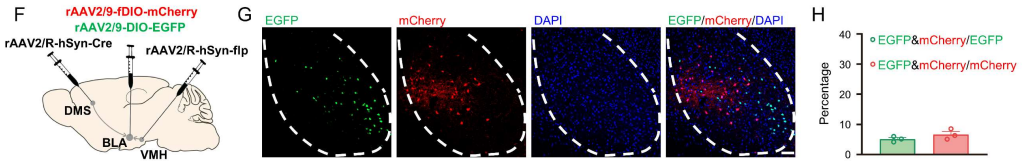


3. The conclusion that pain and itch ensembles in the BLA project exclusively to VMH and DMS, respectively, is a central claim. However, the current tracing methods do not definitively rule out collateral projections. The degree of exclusivity in projection patterns is not entirely clear. Do any BLA neurons collateralize to both VMH and DMS? If not, were intersectional approaches used to test this? Some clarification or quantification here would be valuable. Dual retrograde tracing (e.g., CTB-488/594 injected into VMH and DMS) or single-neuron projection analysis could help determine whether BLA neurons send axon collaterals to both targets. The manuscript would benefit from a brief discussion of other potential downstream targets involved in processing visceral signals, such as the central amygdala, PAG, or parabrachial nucleus, and how they might interact with the circuits described.

>>>Response: Thank you for the constructive comments. To further verify whether the neural ensembles projecting to VMH and DMS are distinct populations, a dual retrograde tracing strategy was employed. In addition, we have expanded the discussion section to include an analysis of downstream targets (Page 19, Lines 426–431, highlighted in red).

New experiment: To directly address the question of projection specificity, we have performed the retrograde tracing experiment, injecting mixed AAV2/9-DIO-EGFP

and AAV2/9-fDIO-mCherry viruses into the BLA and AAV2/R-hSyn-Cre and AAV2/R-hSyn-flp into DMS and VMH, respectively, revealed a low co-localization rate of retrogradely labeled BLA neurons from VMH and DMS. These results have been incorporated into Figures 3F-3H of the revised manuscript.



4. The authors characterize the outputs of the BLA ensembles in detail but provide little information about their upstream inputs. Understanding what drives the selective activation of these ensembles is important for interpreting their role in the broader circuit. Are these ensembles recruited by distinct ascending sensory pathways, or are they selected locally within the BLA based on convergent inputs? Some mention of how thalamic, cortical, or brainstem afferents may contribute to the specificity of pain vs. itch responses would strengthen the Discussion.

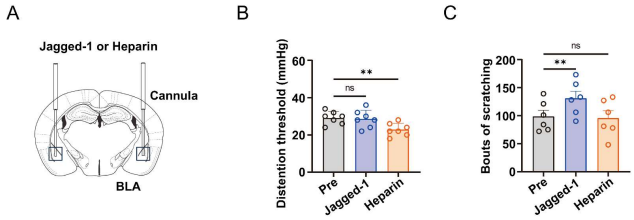
>>>Response: Thank you for the constructive comments. While the current study focused on outputs, we have now expanded the discussion to include a paragraph speculating on potential distinct upstream pathways (e.g., thalamic, cortical) that might drive the selective activation of these BLA ensembles in revised manuscript (Page 17, Lines 385–389, highlighted in red).

5. While the use of fLCM-seq is technically impressive, the rationale for focusing on Fgf2 and Notch2 is not clear. Were these the most significantly differentially expressed genes? If not, why were they prioritized over top-ranking candidates? A table or supplementary figure showing the top DEGs would help readers understand the selection process. Moreover, whether other highly enriched genes also contribute functionally remains an open question. Knockdown of Fgf2 and Notch2 produced modest effects. Without gain-of-function experiments or rescue studies, it is difficult to conclude that these genes are functionally sufficient to drive modality-specific behavior. This should be tested.

>>>Response: Thank you for the comments. We have been provided supplementary tables showing the top DEGs in the revised manuscript. Fgf2 and Notch2 were chosen based on a combination of significant differential expression, fold change, and their plausible biological roles in neural plasticity. In addition, we have performed a **new experiment** of gain-of-function using agonists for Fgf2 and Notch2 to examine their functional roles in visceral pain and itch. Also, we have performed additional

experiments to increase the sample size to enhance the statistical power and reliability of our findings.

New experiment: To further validate the role of Fgf2 and Notch2 in BLA, we performed agonist experiments. Microinjection of the Heparin (agonist of Fgf2) into the BLA promoted visceral pain in mice, while microinjection of the Jagged-1 (agonist of Notch2) promoted itch.



6. The Introduction and Discussion emphasize that pain and itch often co-occur in clinical conditions such as cholestasis or inflammatory bowel disease. However, the experimental design models the two sensations separately, and the key conclusion is that BLA circuits are segregated. This somewhat contradicts the clinical motivation. To more directly test the hypothesis of convergence in comorbid states, the authors could consider using a disease model in which both symptoms co-occur. Would such a model recruit be overlapping BLA ensembles? This remains unexplored.

>>>Response: Thanks for this exceptionally insightful comment. We fully agree that investigating comorbid states is a crucial next step. We have added a paragraph to the Discussion acknowledging this limitation and proposing that future work employ chronic disease models to directly test for potential circuit-level convergence or interaction between these segregated pathways under pathological conditions (Page 20, Lines 460–465, highlighted in red).

Minor Comments:

1. Several panels (e.g., Figures 1G, 1N, 3H, and 3M) do not specify the number of mice used for calcium imaging, and “trials plot” style is not ideal for interpreting mean population dynamics. Additionally, in Figures 3J and 3N, seven data points are shown despite the legend stating n = 6 mice. This discrepancy should be corrected. Accurate reporting of sample size, error bars, and data distribution is essential.

>>>Response: Thanks for pointing out this error. This has been corrected in the revised legend. The number of mice used for calcium imaging in the mentioned panels has been added to the legends of Figure 1.

2. The manuscript inconsistently states that mice were perfused 2 hours after CRD in some sections and 1.5 hours in others. As c-Fos kinetics are time-sensitive, this inconsistency must be resolved and corrected throughout.

>>>Response: Thanks for pointing out this error. We apologize for the error and have now corrected the perfusion time to 1.5 hours after CRD uniformly across the entire manuscript.

Responses to reviewers' comments

Reviewer #1: This manuscript investigates the neural representation of visceral pain and itch within the BLA. Several fundamental concerns regarding its scientific premise, experimental design, and interpretation significantly limit the impact and validity of the findings.

>>>Response: Thank you for the insightful and constructive comments. In accordance with the suggestions, we have carefully revised the manuscript. The revised sections are highlighted. Our point-by-point responses follow:

1. The core premise of comparing visceral pain and itch at the BLA is not adequately justified. Itch is predominantly a somatosensory sensation, typically originating from the skin. Since itch doesn't occur from internal organs, it is highly unlikely that the neuronal pathways for visceral pain and itch are significantly overlapped. The introduction fails to present a compelling argument for this specific comparison. Existing literature, as even cited by the authors, strongly suggests that comparing somatic pain and itch pathways would be a more scientifically sound and straightforward approach, given their known interactions and shared anatomical substrates in the somatosensory system.

>>>Response: Thank you for this critical point. Our premise is not based on the itch occur from internal organs, but on the following rationales, which we have now more clearly articulated in the revised introduction (Page 3, Lines 51–63):

1. Although the neural mechanisms of somatic pain and itch have been extensively and deeply explored, research on visceral pain and itch remains relatively preliminary and limited. This pronounced disparity reflects the long-term underemphasis on the study of related neural mechanisms in the field of visceral sensation. Therefore, this study focuses on the brain's processing mechanisms for visceral pain and itch information, aiming to provide an in-depth analysis of the underlying neural circuits and molecular underpinnings, with the goal of offering theoretical support and experimental evidence for the development of specific therapeutic strategies targeting visceral sensory abnormalities.

2. From a clinical perspective, certain visceral diseases often present with complex symptoms co-occurring pain and itch, such as primary biliary cholangitis and chronic kidney disease [1-2]. Furthermore, during the treatment of related visceral diseases (e.g., e.g., anti-TNF therapy in IBD patients [3]), intractable secondary itch can also be induced. Since the mechanisms underlying these comorbid or secondary symptoms remain poorly understood, clinical management often lacks specific and effective interventions, imposing a considerable physiological and psychological burden on patients. Therefore, an in-depth exploration of the neural and molecular mechanisms of pain and itch in

visceral diseases is of crucial clinical significance for the development of novel therapeutic approaches.

(1). *Beuers U, Wolters F, Oude Elferink RPJ. Mechanisms of pruritus in cholestasis: understanding and treating the itch. Nat Rev Gastroenterol Hepatol. 2023 Jan;20(1):26-36. doi: 10.1038/s41575-022-00687-7. Epub 2022 Oct 28. PMID: 36307649.*

(2). *Zucker, I., Yosipovitch, G., David, M., Gafter, U. & Boner, G. Prevalence and characterization of uremic pruritus in patients undergoing hemodialysis: uremic pruritus is still a major problem for patients with end-stage renal disease. J Am Acad Dermatol 49, 842-846 (2003).*

(3). *Greuter T, Navarini A, Vavricka SR. Skin Manifestations of Inflammatory Bowel Disease. Clin Rev Allergy Immunol. 2017 Dec;53(3):413-427. doi: 10.1007/s12016-017-8617-4. PMID: 28643285.*

2. The assertion that "visceral pain and itch frequently co-occur in various diseases" is problematic and requires stronger evidence. The provided example of Inflammatory Bowel Disease (IBD) and itch is not generally supported by clinical observations. Itch in IBD patients is more commonly associated with drug-induced skin lesions (e.g., anti-TNF therapy) rather than directly with the bowel inflammation itself. This misrepresentation undermines the translational relevance of the study.

>>>Response: We fully agree with the review. We have been modified the "visceral pain and itch frequently co-occur in various diseases" to "visceral pain and itch frequently co-occur in some diseases" in the revised manuscript (Page 19, Lines 445–447, highlighted in red). Furthermore, we have revised the description of a simple and direct link between IBD and itch (Page 20, Lines 453–455, highlighted in red). Additionally, we have been adopted a more precise clinical context, focusing on conditions like primary biliary cholangitis and chronic kidney disease, where a clear and established link exists between visceral pain and itch[1-2]. This provides a firmer translational foundation for our study.

(1). *Dyson, J. K., Beuers, U., Jones, D. E. J., Lohse, A. W. & Hudson, M. Primary sclerosing cholangitis. Lancet 391, 2547-2559 (2018).*

(2). *Zucker, I., Yosipovitch, G., David, M., Gafter, U. & Boner, G. Prevalence and characterization of uremic pruritus in patients undergoing hemodialysis: uremic pruritus is still a major problem for patients with end-stage renal disease. J Am Acad Dermatol 49, 842-846 (2003).*

3. The use of colorectal distention as a model in the context of comparing visceral pain and itch pathways is unclear and needs substantial justification.

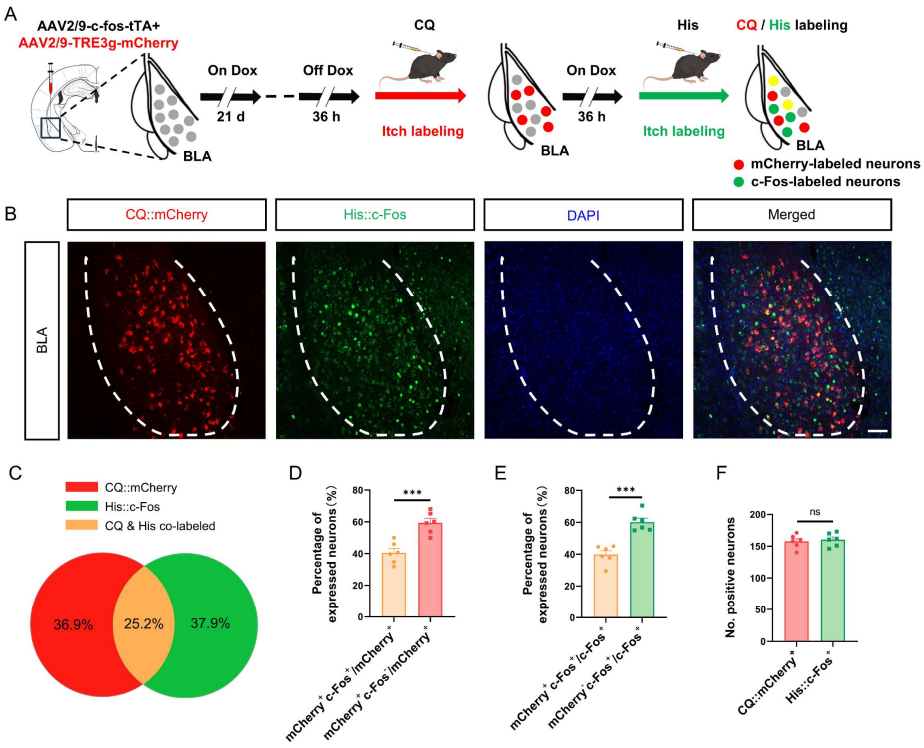
>>>Response: Thank you for this constructive comment and we sincerely apologize for the unclear description. Visceral pain is a broad concept encompassing pain from various internal organs, with irritable bowel syndrome (IBS) representing a key and challenging clinical example. Moreover, Colorectal distention (CRD) is a classic and

reliably induced stimulus, which is commonly used to model visceral pain. Our laboratory has long been dedicated to research on visceral pain. Therefore, we selected CRD to mimic the visceral pain seen in clinical IBS patients. Other types of visceral pain will be explored in our future studies. In addition, we have added this justification in the methods sections, clarifying that our use of "visceral pain" in this study specifically refers to intestinal pain modeled by CRD (Page 24, Line 556, highlighted in red).

4. Given that histamine-dependent and histamine-independent itch pathways are distinct, it is crucial to investigate whether these different pruritogenic stimuli activate the same or distinct populations of BLA neurons. The current study does not provide this important distinction.

>>>Response: Thank you for the comment. To address this issue, we conducted a **new experiment** to evaluate the activation status of BLA neurons in response to histamine-dependent and histamine-independent itch. The results are presented in Supplementary Figure 3.

New experiment: To investigate histamine-independent itch, we utilized chloroquine, a well-established pruritogen commonly employed in models of histamine-independent itch. The results demonstrate that histamine-dependent and histamine-independent itch stimuli activate neuronal populations in the BLA with partial overlap of 25.2%.



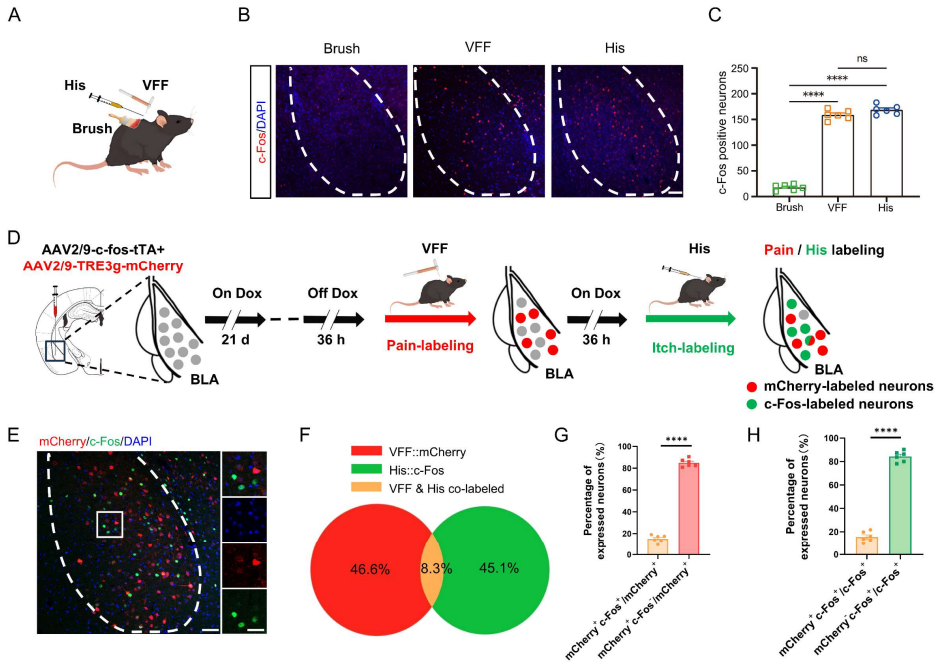
Supplementary Figure 3. Representation of CQ and His-induced itch by distinct neural ensembles in the BLA.

5. The observation that innocuous abdominal pressure activates the BLA raises concerns regarding the specificity of BLA neuron activation. It is possible that the activated BLA neurons are associated with the topological location of the stimulus (i.e., visceral vs. somatic). If so, any observed differences in BLA populations activated by visceral pain versus itch might merely reflect this topographical difference (e.g., back skin vs. internal organ) rather than distinct processing mechanisms for the two sensations.

>>>Response: Thank you for the comment. This was a critical point regarding a potential confound. In response, we conducted a **new experiment**. Since itch doesn't occur from internal organs, it is not feasible to deliver visceral pain and itch stimuli to the same visceral site. Instead, we applied somatic pain and itch stimuli to the same location (the neck skin) to investigate whether these two sensory modalities activate shared or distinct neuronal populations.

New experiment: To determine whether different sensory modalities applied to the same site activate shared or distinct neuronal populations, we delivered somatic pain and itch stimuli to the neck skin by Tet-off system. The results demonstrate that somatic pain and itch stimuli activate neuronal populations in the BLA with a partial overlap of 8.3%, indicating that even sensory stimuli applied to the same location do not recruit identical neuronal ensembles. Although we cannot fully rule out the potential influence

of spatial factors, substantial evidence suggests that the observed differences in BLA populations activated by visceral pain versus itch are primarily due to distinct neural processing mechanisms for these two sensations.

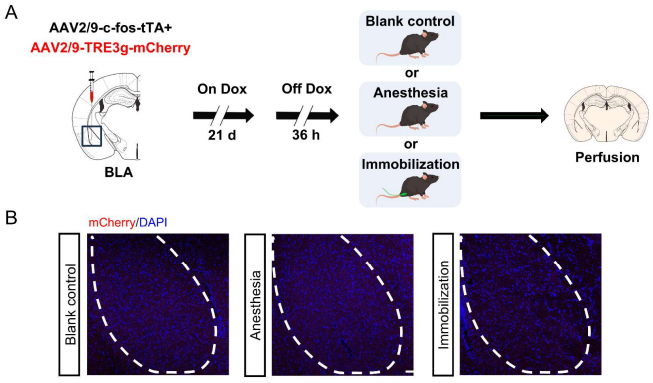


6. The experimental setup involves anesthetized and firmly immobilized mice during visceral pain application. Considering the known role of the CeA in anesthesia, and the potential for BLA activation by stress associated with immobilization, there is a significant risk that BLA activity captured by the Tet-off system is confounded by anesthesia- and immobilization-induced stress responses, rather than being solely attributable to visceral pain.

>>>Response: Thank you for the comment. To address the concern regarding a potential risk that BLA activity could be confounded by anesthesia or immobilization, we have conducted an **additional experiment.** We sincerely apologize for the insufficient methodological detail. To avoid similar misunderstandings among readers, we have explicitly clarified in the revised manuscript's methods section that visceral pain stimuli were applied to awake, partially restrained mice, not completely immobilized or chronically anesthetized animals (Page 24, Lines 560–562, highlighted in red). Isoflurane was used solely during the initial setup phase.

New experiment: To address whether the BLA activation observed in our main experiments could be attributed to procedural stressors rather than the visceral pain, we quantified BLA neuronal activity using Tet-off system in mice exposed only to the transient isoflurane anesthesia (Anesthesia) or partial restraint procedure used

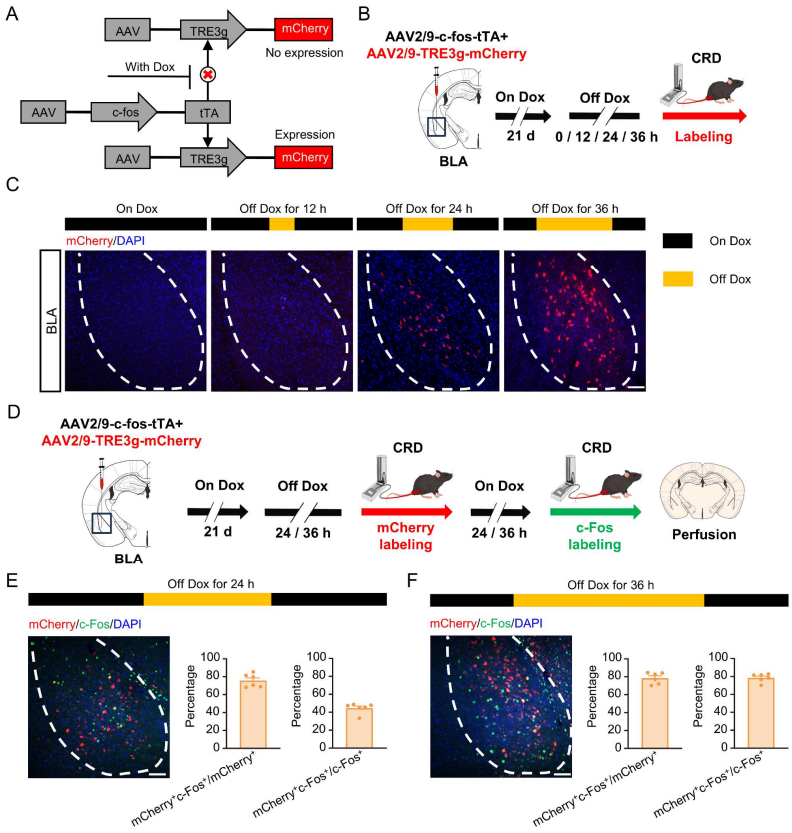
(Immobilization) in our main experimental paradigm. The new results show that neither Immobilization nor Anesthesia meaningfully activates BLA neurons.



7. The 36-hour window for labeling activated neurons via the Tet-off system appears excessively long and could capture neuronal activity unrelated to the acute experimental stimuli. This prolonged window increases the likelihood of capturing baseline activity or activity related to other non-specific stressors.

>>>Response: Thank you for the comment. The 36-hour window was systematically optimized based on our previous work (Neuron, 2024, PMID: 39326407; Neuron, 2025, PMID: 40345185). We performed additional a **new experiment** across multiple time windows (12h, 24h, 36h) and confirmed that the 36-hour window provides the optimal signal-to-noise ratio for activity-dependent labeling in the BLA in our hands. The results are presented in Supplementary Figure 4.

New experiment: To evaluate the optimal drug withdrawal time window for the Tet-off system, we performed new control experiments comparing labeling efficiency and specificity across multiple time windows (12h, 24h, 36h). The new results show that the 36-hour window provides the best balance between the efficiency and specificity.

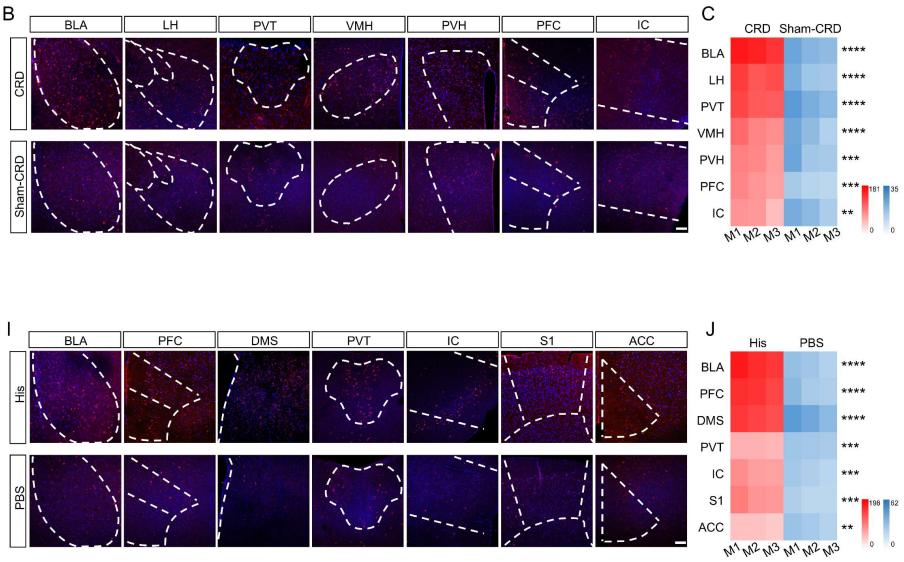


Supplementary Figure 4. Optimization of the time window for signal detection using the Tet-off system.

8. Crucial control groups are missing. The study should include a control group for the intra-dermal (id) injection of PBS (for itch controls) and a control group for the insertion of the pressure machine for CRD without increasing pressure. These controls are essential to differentiate specific neuronal activation related to the stimuli from non-specific responses to injection or mechanical presence.

>>>Response: We fully agree with the comment. We have now included the essential control groups for intradermal PBS injection and CRD device insertion without distension (Sham-CRD). These data have been incorporated into Figures 1B-1C and 1I-1J, confirming that the observed neuronal activation is stimulus-specific.

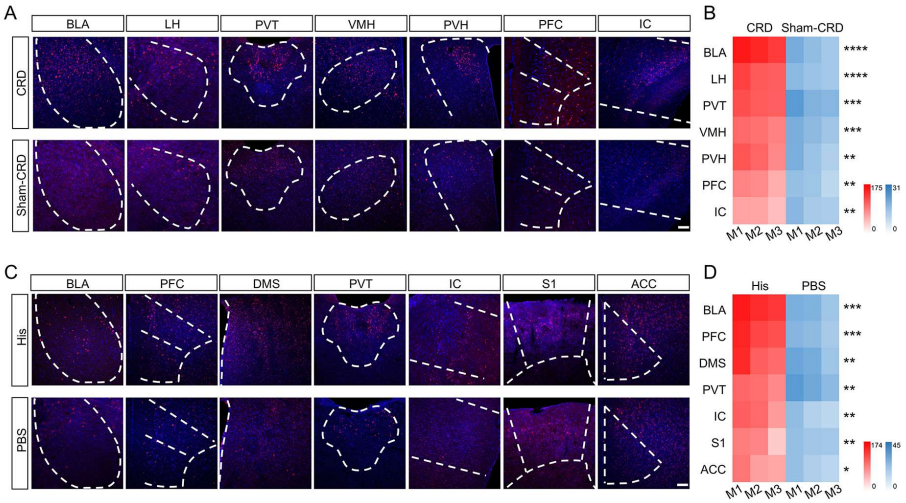
New experiment: To rigorously control for potential nonspecific neuronal activation from procedural artifacts, we have conducted new experiments including intradermal PBS injection and Sham-CRD. These new data, now presented in Figures 1B-1C and 1I-1J, show that the observed neuronal activation is stimulus-specific.



9. The justification for using only male mice to "avoid hormonal variability" is weak and insufficient. It is imperative to acknowledge the importance of sex as a biological variable and to include female mice or provide a more robust scientific rationale for their exclusion, particularly given the known sex differences in pain and itch processing.

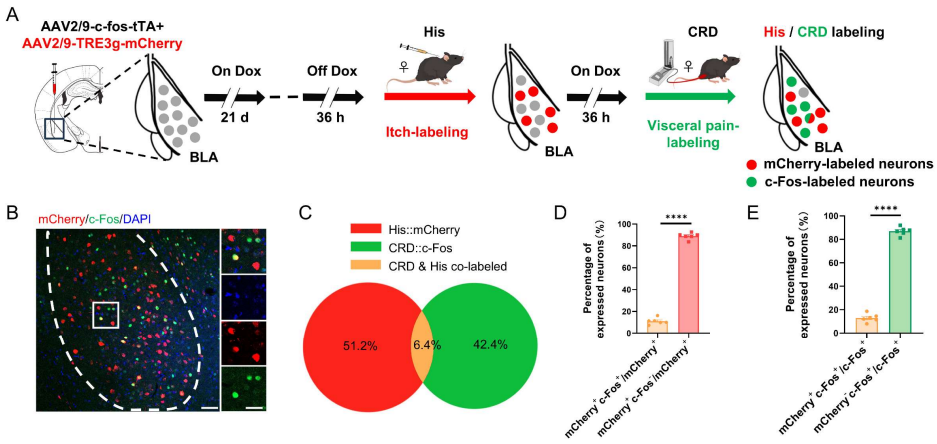
>>>Response: Thanks for your constructive comment. In response, we have conducted **four (4) additional experiments** in female mice to validate our core findings, and we have explicitly clarified in the revised manuscript's methods section (Page 22, Lines 501–502, highlighted in red):

New experiment 1: To further confirm the role of the BLA in female mice, we assessed the expression of CRD- and His-induced c-Fos in the BLA. As shown below, c-Fos induced by CRD or His was primarily expressed in the BLA. These data are consistent with the results from the male mice and have been incorporated into Supplementary Figure 1 of the revised manuscript.



Supplementary Figure 1. Expression of CRD- and His-induced c-Fos in the BLA in female mice.

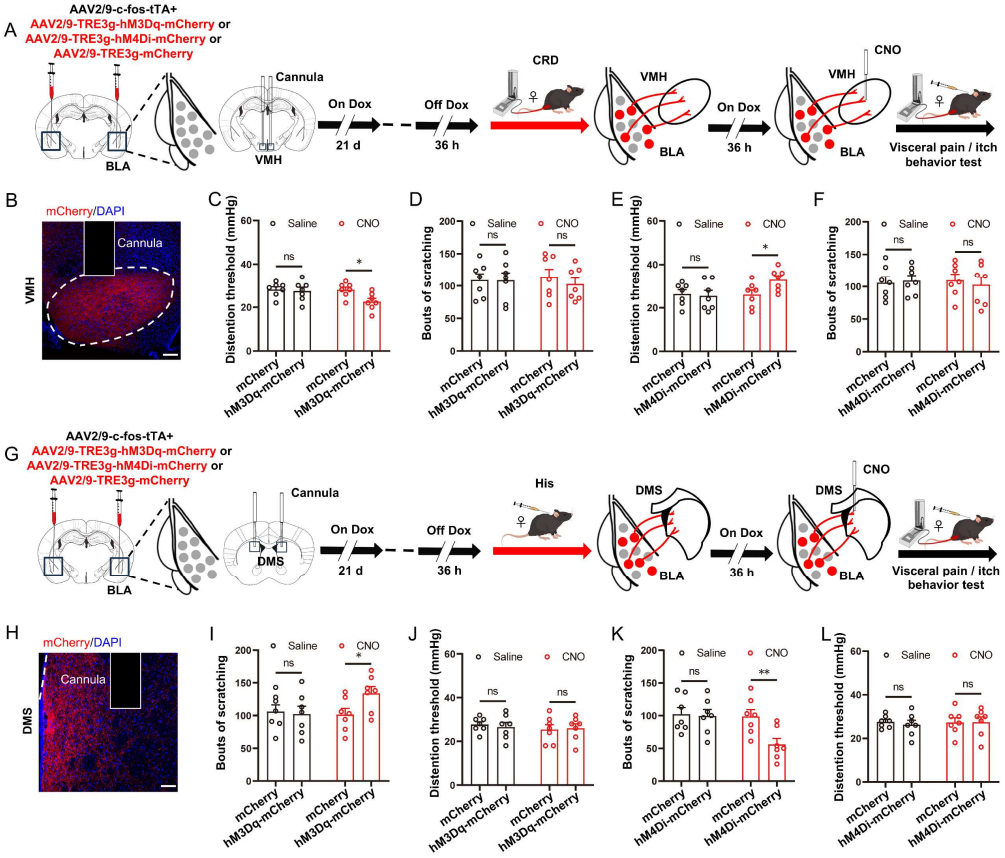
New experiment 2: To investigate the representation of neural ensembles activated by visceral pain and itch in the BLA in female mice, Tet-off system combined with c-Fos staining was utilized. We mapped the distribution of BLA neural ensembles activated by His and CRD using mCherry expression and c-Fos staining. Quantitative analysis showed that the percentage of double-positive neurons for mCherry and c-Fos was significantly lower than single-positive neurons expressing either mCherry or c-Fos alone. As shown below, these data are consistent with the results from the male mice and included in Supplementary Figure 6 of the revised manuscript.



Supplementary Figure 6. His and CRD activated neurons are two distinct ensembles in BLA in female mice.

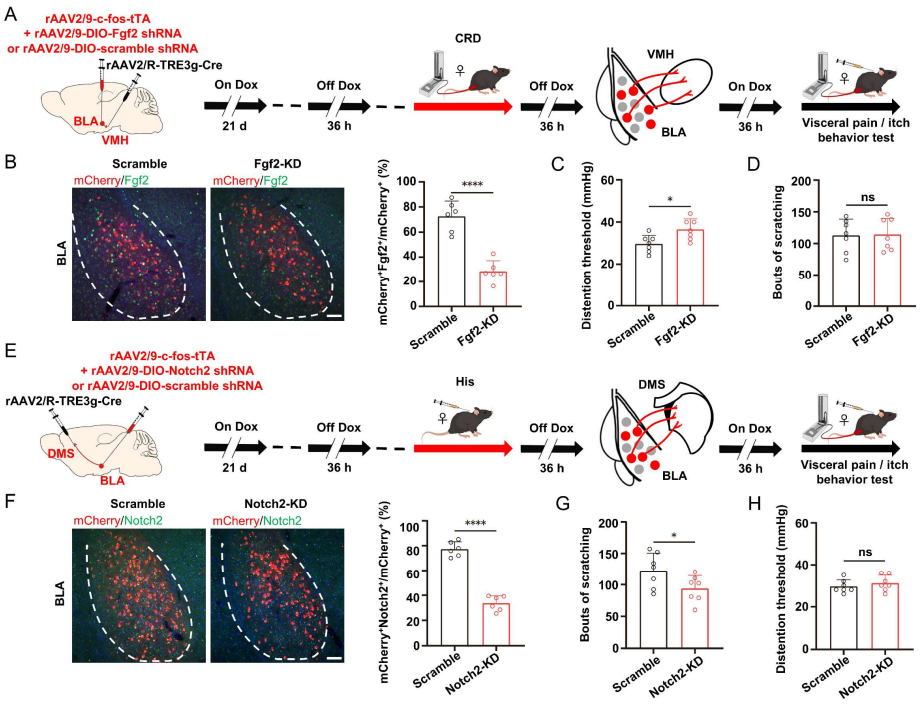
New experiment 3: To further investigate the function of the neural circuits involving CRD-labeled BLA neurons to the VMH and His-labeled BLA neurons to the

DMS, chemogenetic manipulations were conducted. The results showed that manipulation of the projections of CRD-labeled BLA neural ensembles to the VMH specifically modulates visceral pain, whereas manipulation of the projections of His-labeled BLA neural ensembles to the DMS specifically modulates itch. These results are consistent with the results from the male mice and have been incorporated into Supplementary Figure 7 of the revised manuscript.



Supplementary Figure 7. Manipulation of the projections from CRD-labeled BLA neurons to VMH or His-labeled BLA neurons to DMS specifically modulates visceral pain or itch behaviors in female mice.

New experiment 4: To further explore the role of Fgf2 in regulating visceral pain through the BLA-VMH circuit and Notch2 in regulating itch through the BLA-DMS circuit, we selectively knocked down Fgf2 expression in CRD-labeled BLA neurons projecting to VMH and Notch2 expression in His-labeled BLA neurons projecting to DMS. The results showed that knockdown of Fgf2 in CRD-labeled BLA neurons projecting to VMH attenuates visceral pain whereas knockdown of Notch2 in His-labeled BLA neurons projecting to DMS inhibits itch. These results are consistent with the results from the male mice and have been incorporated into Supplementary Figure 8 of the revised manuscript.



Supplementary Figure 8. Knockdown of Fgf2 in CRD-labeled BLA neurons projecting to VMH attenuates visceral pain whereas knockdown of Notch2 in His-labeled BLA neurons projecting to DMS inhibits itch in female mice.

10. Several statements throughout the manuscript are overstatements. Examples include: "The present study provides compelling evidence that the basolateral amygdala (BLA) serves as a sorting center for the processing of visceral pain and itch." This conclusion is not supported given the limitations discussed above. "Various abdominal organ diseases - including hepatobiliary pancreatic disorders, renal diseases, and inflammatory bowel diseases - frequently present with concurrent visceral pain and itch through distinct yet overlapping pathophysiological mechanisms." This broad generalization is unsubstantiated and misrepresents the clinical reality, particularly regarding the co-occurrence of primary visceral itch.

>>>Response: Thank you for the comments. We have thoroughly checked and toned down all instances of overinterpretation or overly broad claims throughout the manuscript. In revised manuscript, we revised " The present study provides compelling evidence that the basolateral amygdala (BLA) serves as a sorting center for the processing of visceral pain and itch." to a more measured and accurate description: " The present study provides evidence that the BLA plays a role in distinguishing and integrating the sensory components of visceral pain and itch." (Page 16, Lines 358–359, highlighted in red). In addition, we revised " Various abdominal organ diseases - including hepatobiliary pancreatic disorders, renal diseases, and inflammatory bowel diseases - frequently present with concurrent visceral pain and itch through distinct yet

overlapping pathophysiological mechanisms." to " In clinical practice, visceral pain and itch frequently co-occur in some diseases-including primary biliary cholangitis, chronic kidney disease, and inflammatory bowel diseases." (Page 19, Lines 445–447, highlighted in red).

Minor concerns:

1. The methods section lacks critical details regarding the delivery of CNO via cannula. This includes:

-The timing of cannula implantation relative to the experimental procedures.

- Specific stereotaxic coordinates for cannula placement.

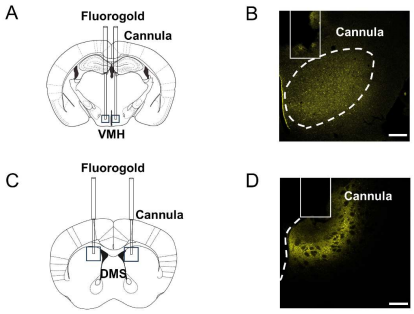
- The size and type of cannula used.

- The precise timing and duration of CNO delivery.

- Confirmation of whether an infusion cannula was indeed implanted.

>>>Response: Thank you for the comments. In response, we have incorporated these essential details into the methods section (Pages 27-28, Lines 640–647, Lines 650–652, highlighted in red). Figure 4 presents representative fluorescent images illustrating the implantation of the infusion cannula.

Furthermore, to ensure the accuracy of targeting, we performed a co-injection of Fluorogold alongside CNO as shown below. Specifically, we administered an identical volume of 300 nL of Fluorogold into the VMH or the DMS via the implanted cannulas and subsequently quantified its dissemination.



2. In Figure 5M, the Notch2 signal shown next to the CRD-labeled image appears considerably weaker compared to the Notch signal adjacent to the histamine-labeled image. This visual discrepancy needs to be addressed or explained.

>>>Response: Thank you for the comments. We apologize for the prior selection of an inappropriate representative image and have replaced it with a more suitable one to prevent any potential misinterpretation.

Reviewer #2: In the manuscript titled "The basolateral amygdala decodes visceral pain and itch via distinct circuits and molecules," Zhao et al. demonstrated that distinct neuronal groups within the BLA are responsible for the regulation of visceral pain and itch, respectively. The neurons activated by pain projected to the VMH, while those activated by itch projected to the DMS. They also screened for gene specificity and identified that Fgf2 was highly selectively expressed in BLA-VMH projecting neurons, whereas Notch2 was selectively expressed in BLA-DMS projecting neurons. Finally, through functional gene knockdown experiments, the regulatory effects of Fgf2 and Notch2 on visceral pain and itch were identified. Overall, this work is interesting and presents significant novelties. The experiments were well conducted, and the results are reliable. However, there are several concerns that need to be addressed to further improve the quality of the work.

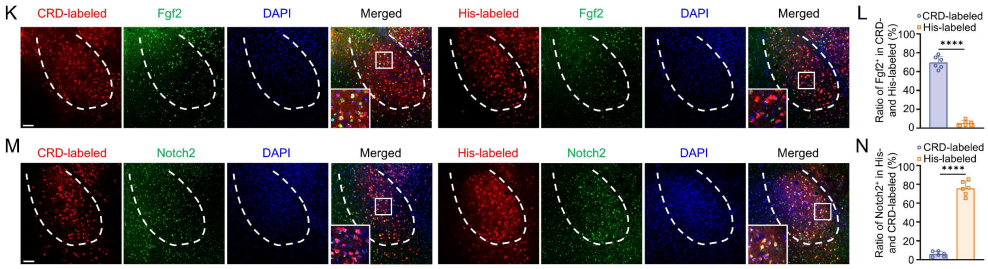
>>>Response: Thank you very much for the positive comments and constructive suggestions.

1. The sample size of behavioral animals in each group in Figure 4 is relatively small. In fact, I found that the number of animals in each behavioral test is six, which may increase the uncertainty of the data. It is suggested to conduct repeated experiments to supplement the data.

>>>Response: Thanks for the constructive comments. We have performed additional experiments to increase the sample size for the key behavioral tests in Figures 4C-4F and 4I-4L, enhancing the statistical power and reliability of our conclusions.

2. There is a lack of basal expression levels of Fgf2 and Notch2 in the sham groups in Figures 5K-M. Additionally, it is recommended to include high-magnification images to better illustrate the co-localization of Fgf2 and Notch2 with FOS cells in different groups.

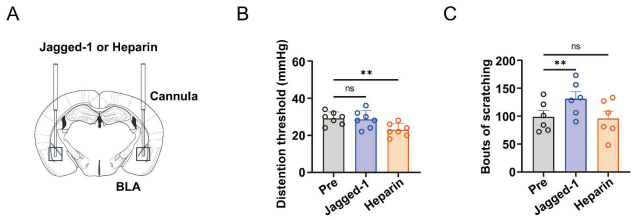
>>>Response: Thank you for the comment. The primary objective of this study is to determine whether Fgf2 and Notch2 expression is predominantly localized to neurons activated by specific stimuli (visceral pain or itch), not to compare whether Fgf2 and Notch2 expression is altered. We sincerely apologize for the visual confusion caused by the color scheme choice in Figures 5L and 5N, and we have modified the color scheme in the revised manuscript. Additionally, as suggested, we have provided high-magnification images (Figures 5K and 5M) to more clearly demonstrate the colocalization of Fgf2 and Notch2 with mCherry-positive cells across experimental groups.



3. In Figure 6, the authors demonstrated that knockdown of Fgf2 in CRD-labeled BLA neurons projecting to VMH attenuates visceral pain, while knockdown of Notch2 in His-labeled BLA neurons projecting to DMS inhibits itch. Has the author considered examining the impact of the overexpression of Fgf2 and Notch2 on visceral pain and itch? Furthermore, similar to the suggestion for Figure 4, it is recommended to increase the number of animal experiments.

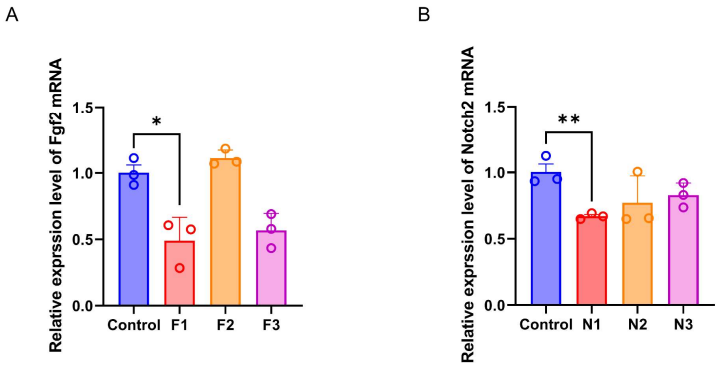
>>>Response: Thank you for the comments. Given that the Notch2 is too large to be packaged into an adenoviral vector, we used agonists to achieve gain-of-function for both targets for consistency. Also, we have performed additional experiments to increase the sample size to enhance the statistical power and reliability of our findings.

New experiment: To further validate the role of Fgf2 and Notch2 in BLA, we performed agonist experiments. The data show that microinjection of the Heparin (agonist of Fgf2) into the BLA promoted visceral pain, while the Jagged-1 (agonist of Notch2) promoted itch.



4. How did the authors select the most efficient sequence of the shRNA for knocking down the expression of Fgf2 and Notch2? The knockdown efficacy for different candidates should be demonstrated.

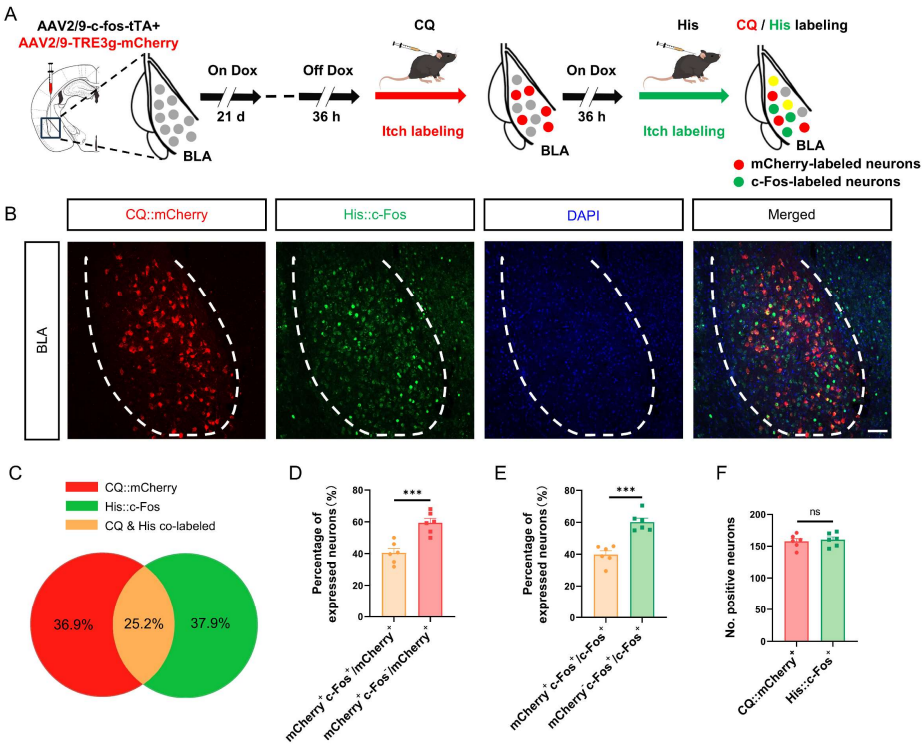
>>>Response: Thank you for the comment. We have provided **additional data** demonstrating the knockdown efficacy and specificity of the selected Fgf2 and Notch2 shRNA sequences used in this study. Representative qPCR results are shown below. The candidate knockdown sequences have been added in the revised manuscript (Page 23, Lines 526–530, highlighted in red). Based on the qPCR results, we selected the one with the highest knockdown efficiency.



5. What about the FOS expression induced by CQ and His stimulation in the BLA? Is the same group of neurons responding to CQ and His stimulation?

>>>Response: Thank you for the comment. To elucidate this issue, **a new experiment** was conducted to evaluate the activation of BLA neurons in response to both histamine-dependent and histamine-independent itch. The corresponding data are presented in Supplementary Figure 3.

New experiment: To investigate histamine-independent itch, we utilized chloroquine, a well-established pruritogen commonly employed in models of histamine-independent itch. The results demonstrate that histamine-dependent and histamine-independent itch stimuli activate neuronal populations in the BLA with partial overlap of 25.2%.



Supplementary Figure 3. Representation of CQ and His-induced itch by distinct neural ensembles in the BLA.

6. The authors divided BLA neurons into pain-activated neural ensembles and itch-activated neural ensembles. How should we understand the statement, "The results revealed that 47.6% of BLA neural ensembles only expressed itch-induced mCherry, while 46.5% only expressed visceral pain-activated c-Fos, and 5.9% co-expressed both itch-induced mCherry and visceral pain-activated c-Fos" (page 8, line 162)? In this sentence, it seems that 'neural ensembles' may represent the total neurons within the BLA. The authors should specify the definition of 'neural ensembles'.

>>>Response: We apologize for the lack of clarity. In our manuscript, "neural ensembles" here refer to functionally defined populations of BLA neurons activated by either visceral pain (c-Fos^{+}) or itch (mCherry^{+}), including non-overlapping and overlapping subsets, and not to the total neurons within the BLA.

7. The article's expression is not entirely accurate and contains some grammatical errors in English. It would be beneficial to have a native English speaker meticulously proofread and refine it.

>>>Response: We thank the reviewer for pointing out the need for language polishing. The manuscript has been thoroughly proofread and revised by a native English speaker to correct grammatical errors and improve overall expression and accuracy.

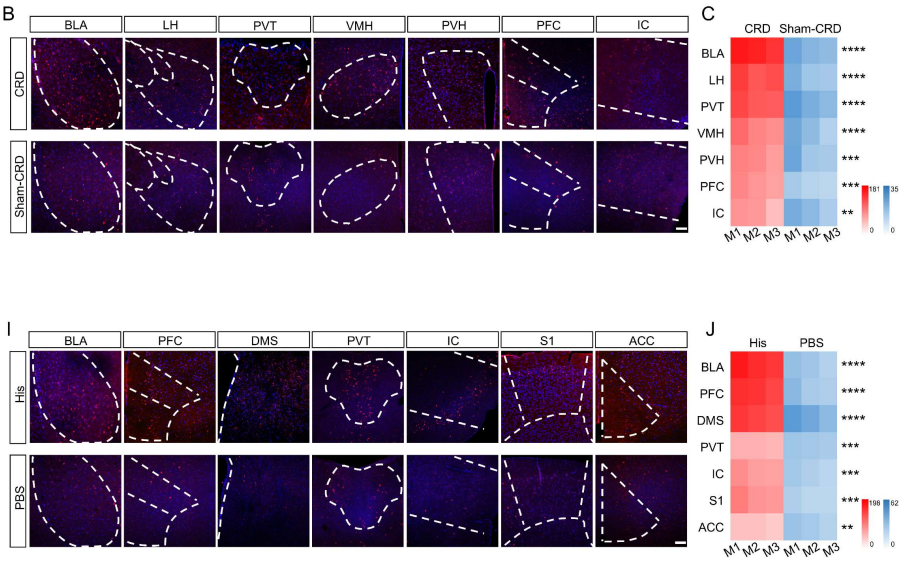
Reviewer #3: This manuscript presents an extensive and technically sophisticated investigation into how the basolateral amygdala (BLA) decodes visceral pain and itch via distinct glutamatergic neuronal ensembles. The authors employ a wide range of cutting-edge tools-including activity-dependent viral labeling (Tet-off), projection-specific chemogenetics, fiber photometry, and function-based single-cell transcriptomics (fLCM-seq)-to identify two parallel circuits: a BLA-VMH pathway for visceral pain and a BLA-DMS pathway for itch. They further show that Fgf2 and Notch2 are selectively expressed in these respective ensembles and functionally contribute to modality-specific behaviors. The central claims are conceptually interesting and the data are largely convincing. The demonstration of circuit-level and molecular segregation in the BLA is of potential importance for understanding the central processing of visceral sensory signals. However, several key aspects of the experimental design, data interpretation, and anatomical precision need to be clarified or strengthened. In particular, control data are missing in critical figures, circuit specificity may be overstated without further anatomical validation, and the rationale for molecular selection is unclear.

>>>Response: Thank you for the insightful comments and positive assessment of our work's technical sophistication.

1. Figure 1 lacks appropriate controls to confirm that c-Fos expression in the BLA is specifically induced by CRD or histamine. Without a baseline group (e.g., unstimulated or saline-injected animals), it is not possible to conclude that the observed activation is stimulus-specific. Given that c-Fos is used to label and define functional ensembles throughout the study, the absence of a control group substantially weakens this foundation. The authors must include data from control animals and provide a discussion of basal Fos expression in BLA under resting conditions.

>>>Response: We fully agree with the comment. We have now included the essential control groups for intradermal PBS injection and CRD device insertion without distension (Sham-CRD). These data have been incorporated into Figures 1B-1C and 1I-1J, confirming that the observed neuronal activation is stimulus-specific.

New experiment: To rigorously control for potential nonspecific neuronal activation from procedural artifacts, we have conducted new experiments including intradermal PBS injection and Sham-CRD. These results have been incorporated into Figures 1B-1C and 1I-1J of the revised manuscript.



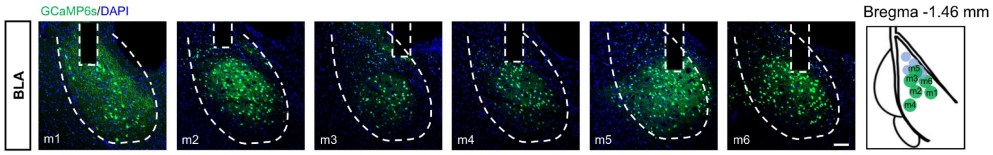
2. In Figure 3B, viral expression appears to extend well beyond the BLA into the central amygdala (CeA). Given the proximity of these nuclei and the known roles of CeA in pain, itch, and emotional processing, this raises the possibility that some of the observed behavioral and circuit effects may result from unintended manipulation of CeA neurons. The authors must provide the following for each mouse: Injection volumes for each virus; Raw images showing viral spread, optical fiber placements, and cannula placements; Summary maps (per group) showing targeting success and spatial coverage. Particular attention should be paid to delineating BLA from neighboring CeA in all datasets. Without such anatomical precision, the specificity of the conclusions is in question. For experiments involving local infusion of CNO into VMH or DMS, no data are provided on the extent of spread. The authors should report how far CNO diffuses from the injection site (e.g., through co-injection of dye or prior validation) to confirm that the effects are due to local modulation of axon terminals and not off-target actions.

>>>Response: Thank you for emphasizing anatomical precision.

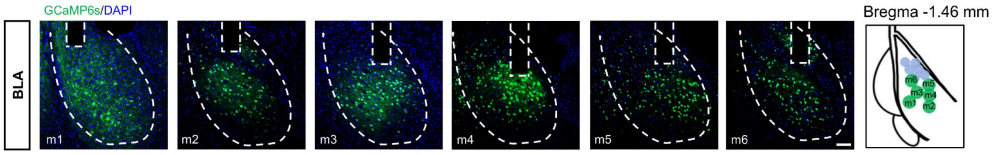
(1) In our study, only samples with accurate injection sites were included in the analysis. We regret the prior selection of an inappropriate representative image and have replaced it with a more suitable one to prevent any potential misinterpretation (Figure 3B). In addition, the injection volumes for each virus have been provided in the methods section (Page 23, Line 535, highlighted in red).

(2) The following raw images of each mouse have been provided to validate the specificity of viral targeting in BLA.

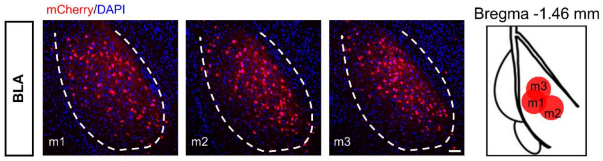
Raw images of 6 mice (m1-m6) for Figure 1F, along with summary places of fiber tips (shown by blue dots) and virus injection sites (shown by green dots) in the BLA.



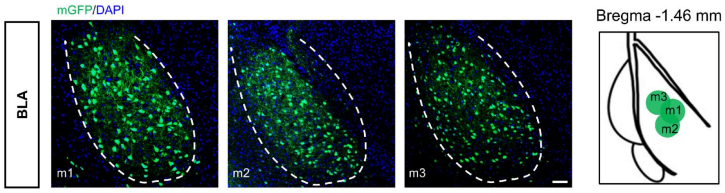
Raw images of 6 mice (m1-m6) for Figure 1M, along with summary places of fiber tips (shown by blue dots) and virus injection sites (shown by green dots) in the BLA.



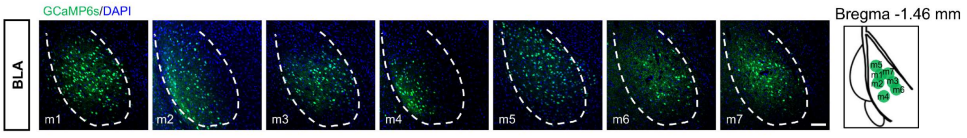
Raw images of 3 mice (m1-m3) for Figure 2B and the summary map of virus injection sites (shown by red dots) in the BLA.



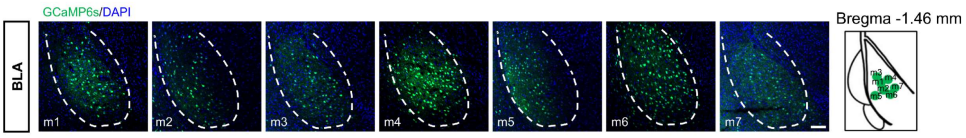
Raw images of 3 mice (m1-m3) for Figure 3B and the summary map of virus injection sites (shown by green dots) in the BLA.



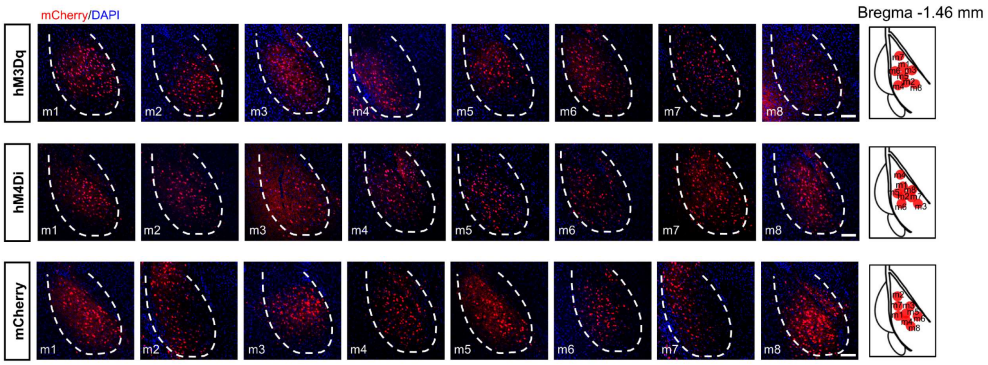
Raw images of 7 mice (m1-m7) for Figure 3I and the summary map of virus injection sites (shown by green dots) in the BLA.



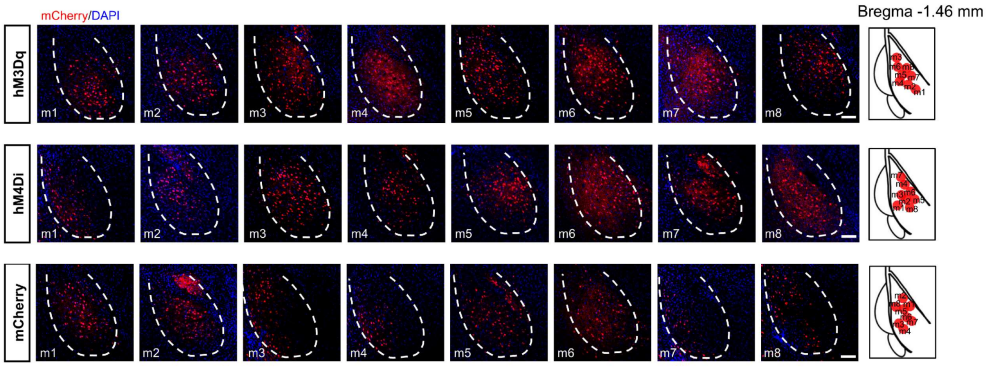
Raw images 7 mice (m1-m7) for Figure 3N and the summary map of virus injection sites (shown by green dots) in the BLA.



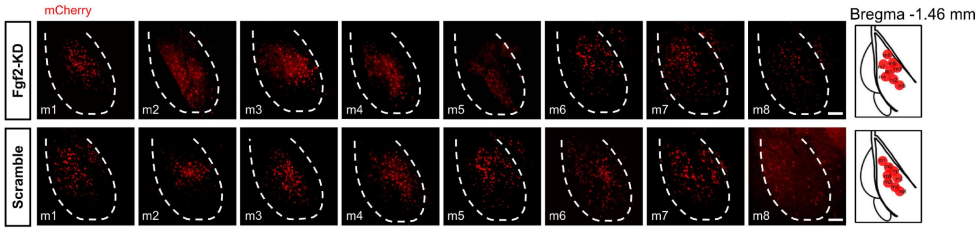
Raw images for Figure 4A of three groups (8 mice per group, m1-m8) and the summary map of virus injection sites (shown by red dots) in the BLA.



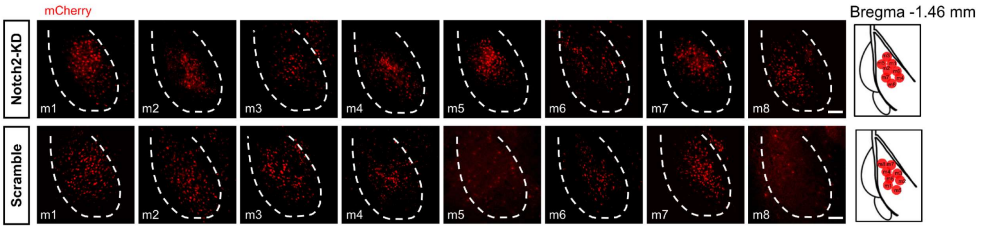
Raw images for Figure 4G of three groups (8 mice per group, m1-m8) and the summary map of virus injection sites (shown by red dots) in the BLA.



Raw images for Figure 6A of two groups (8 mice per group, m1-m8) and the summary map of virus injection sites (shown by red dots) in the BLA.

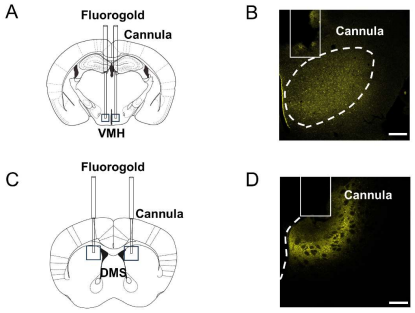


Raw images for Figure 6H of two groups (8 mice per group, m1-m8) and the summary map of virus injection sites (shown by red dots) in the BLA.



(3) For CNO infusion experiments, we performed a **new experiment** co-injecting Fluorogold to visualize spread. The data confirm that the diffusion was localized to the target region (VMH/DMS), supporting circuit-specific effects.

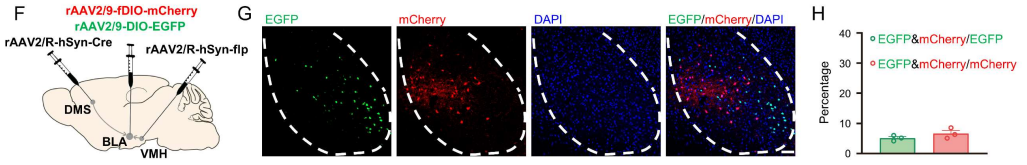
New experiment: To evaluate the extent of CNO diffusion following local infusion, we performed experiments as suggested by the reviewer. Accordingly, we injected an equal volume (300 nl) of Fluorogold into the VMH or DMS via the implanted cannulas and quantified its spread.



3. The conclusion that pain and itch ensembles in the BLA project exclusively to VMH and DMS, respectively, is a central claim. However, the current tracing methods do not definitively rule out collateral projections. The degree of exclusivity in projection patterns is not entirely clear. Do any BLA neurons collateralize to both VMH and DMS? If not, were intersectional approaches used to test this? Some clarification or quantification here would be valuable. Dual retrograde tracing (e.g., CTB-488/594 injected into VMH and DMS) or single-neuron projection analysis could help determine whether BLA neurons send axon collaterals to both targets. The manuscript would benefit from a brief discussion of other potential downstream targets involved in processing visceral signals, such as the central amygdala, PAG, or parabrachial nucleus, and how they might interact with the circuits described.

>>>Response: Thank you for the constructive comments. To further verify whether the neural ensembles projecting to VMH and DMS are distinct populations, a dual retrograde tracing strategy was employed. In addition, we have expanded the discussion section to include an analysis of downstream targets (Page 19, Lines 427–432, highlighted in red).

New experiment: To directly address the question of projection specificity, we have performed the retrograde tracing experiment, injecting mixed AAV2/9-DIO-EGFP and AAV2/9-fDIO-mCherry viruses into the BLA and AAV2/R-hSyn-Cre and AAV2/R-hSyn-flp into DMS and VMH, respectively, revealed a low co-localization rate of retrogradely labeled BLA neurons from VMH and DMS. These results have been incorporated into Figures 3F-3H of the revised manuscript.



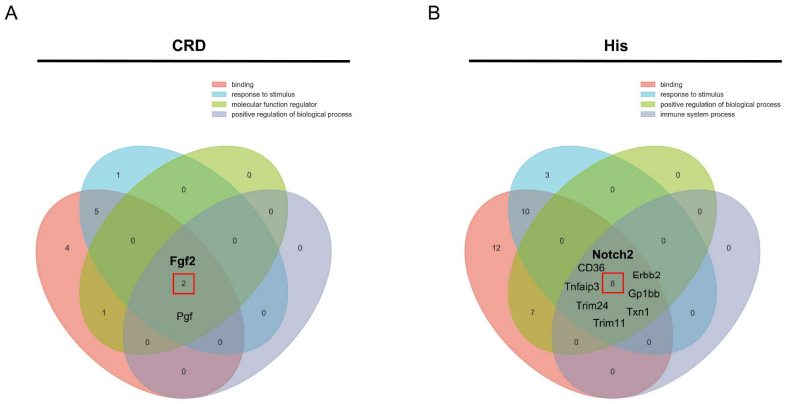
4. The authors characterize the outputs of the BLA ensembles in detail but provide little information about their upstream inputs. Understanding what drives the selective activation of these ensembles is important for interpreting their role in the broader circuit. Are these ensembles recruited by distinct ascending sensory pathways, or are they selected locally within the BLA based on convergent inputs? Some mention of how thalamic, cortical, or brainstem afferents may contribute to the specificity of pain vs. itch responses would strengthen the Discussion.

>>>Response: Thank you for the constructive comments. While the current study focused on outputs, we have now expanded the discussion to include a paragraph speculating on potential distinct upstream pathways (e.g., thalamic, cortical) that might drive the selective activation of these BLA ensembles in revised manuscript (Page 17, Lines 386–390, highlighted in red).

5. While the use of fLCM-seq is technically impressive, the rationale for focusing on Fgf2 and Notch2 is not clear. Were these the most significantly differentially expressed genes? If not, why were they prioritized over top-ranking candidates? A table or supplementary figure showing the top DEGs would help readers understand the selection process. Moreover, whether other highly enriched genes also contribute functionally remains an open question. Knockdown of Fgf2 and Notch2 produced modest effects. Without gain-of-function experiments or rescue studies, it is difficult to conclude that these genes are functionally sufficient to drive modality-specific behavior. This should be tested.

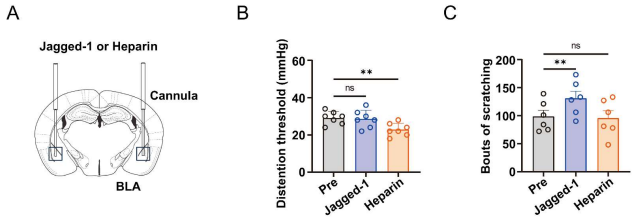
>>>Response: Thank you for your comments. In the revised manuscript, we have included Supplementary Table S1, which presents a core set of DEGs. As indicated in the table, Fgf2 and Notch2 are ranked among the top 30 DEGs for CRD-labeled and His-labeled neurons, respectively.

To functionally characterize these DEGs, we conducted a GO analysis. By examining the overlap across four key biological process terms, we identified Fgf2 from the CRD-labeled group and Notch2 from the His-labeled group for further investigation. A Venn diagram illustrating the selection is provided below.



In addition, we have performed a **new experiment** of gain-of-function using agonists for Fgf2 and Notch2 to examine their functional roles in visceral pain and itch. Also, we have performed additional experiments to increase the sample size to enhance the statistical power and reliability of our findings.

New experiment: To further validate the role of Fgf2 and Notch2 in BLA, we performed agonist experiments. Microinjection of the Heparin (agonist of Fgf2) into the BLA promoted visceral pain in mice, while microinjection of the Jagged-1 (agonist of Notch2) promoted itch.



6. The Introduction and Discussion emphasize that pain and itch often co-occur in clinical conditions such as cholestasis or inflammatory bowel disease. However, the experimental design models the two sensations separately, and the key conclusion is that BLA circuits are segregated. This somewhat contradicts the clinical motivation. To more directly test the hypothesis of convergence in comorbid states, the authors could consider using a disease model in which both symptoms co-occur. Would such a model recruit be overlapping BLA ensembles? This remains unexplored.

>>>Response: Thanks for this exceptionally insightful comment. We fully agree that investigating comorbid states is a crucial next step. We have added a paragraph to the

Discussion acknowledging this limitation and proposing that future work employ chronic disease models to directly test for potential circuit-level convergence or interaction between these segregated pathways under pathological conditions (Page 20, Lines 461–465, highlighted in red).

Minor Comments:

1. Several panels (e.g., Figures 1G, 1N, 3H, and 3M) do not specify the number of mice used for calcium imaging, and “trials plot” style is not ideal for interpreting mean population dynamics. Additionally, in Figures 3J and 3N, seven data points are shown despite the legend stating n = 6 mice. This discrepancy should be corrected. Accurate reporting of sample size, error bars, and data distribution is essential.

>>>Response: Thank you for highlighting this error. We have made the necessary corrections to the figure legends. Specifically, we have included the number of mice used for calcium imaging in the legends of Figures 1G, 1N, 3H, and 3M. Additionally, we have replaced the "trials plot" style in Figures 1F and 1M with plots that more effectively represent the mean population dynamics and variability. We sincerely apologize for the inconsistency between the data points and the legends in Figures 3J and 3N. The legends for Figures 3J and 3N have been updated to reflect n = 7 mice.

2. The manuscript inconsistently states that mice were perfused 2 hours after CRD in some sections and 1.5 hours in others. As c-Fos kinetics are time-sensitive, this inconsistency must be resolved and corrected throughout.

>>>Response: Thanks for pointing out this error. We apologize for the error and have now corrected the perfusion time to 1.5 hours after CRD uniformly across the entire manuscript.

Responses to reviewers' comments

Reviewer #1: I appreciate the authors' efforts in performing additional experiments in response to prior comments. However, my major concerns have not been sufficiently addressed.

>>>Response: Thank you for the insightful and constructive comments. We have carefully considered all the comments and revised the manuscript accordingly. The modifications have been highlighted in the revised version.

1. Although the neural mechanisms underlying somatic pain and itch have been extensively investigated, it remains unclear whether distinct neuronal subpopulations within the BLA differentially mediate these two modalities. One of the newly added experiments provides important evidence that somatic pain and itch stimuli activate partially overlapping neuronal populations in the BLA (8.3% overlap), as demonstrated using the Tet-off system. These findings are valuable and should be incorporated into the manuscript. Furthermore, the authors should expand the discussion to address whether these results suggest a potential topographical organization versus distinct processing mechanisms for pain and itch within the BLA.

>>>Response: Thanks for the constructive suggestions. As suggested, the partial overlap (8.3%) between neuronal populations activated by somatic pain and itch in the BLA has been incorporated into a new figure (**Supplementary Figure 8**, Page 9, lines 203~209). The expanded discussion was highlighted in red (Pages 17~18, lines 391~409).

2. The revised Introduction places strong emphasis on the clinical relevance of visceral pain and non-cutaneous itch. However, the present study primarily examines neuronal mechanisms of acutely induced cutaneous itch rather than non-cutaneous itch. This conceptual discrepancy should be clarified and better aligned throughout the manuscript.

>>>Response: Thank you for the insightful comment. As suggested, these descriptions have been clarified and better aligned throughout the manuscript. The present study primarily examined neuronal mechanisms of acutely induced cutaneous itch and visceral pain, thus establishing a fundamental paradigm for future investigations into clinically relevant diseases (Pages 3~4, lines 53~66, highlighted in red).

3. In addition, the use of irritable bowel syndrome mouse model is not fully justified, as this condition is not typically associated with pruritus.

>>>Response: Thank you for the comment. We would like to clarify that the irritable bowel syndrome was not mentioned in our study. To avoid any potential confusion, we

have further refined the Methods to ensure that the experimental procedures are stated even more clearly (Page 25, lines 592~594, highlighted in red).

4. Finally, references 9 and 10 do not appear to discuss inflammatory bowel disease as implied in the text and should be corrected or replaced.
>>>Response: Thank you for the comment. The relevant sentences and citations have been removed.

Reviewer #2: The authors have satisfactorily addressed all my concerns with additional experiments. I have no more questions for the work.
>>>Response: We appreciate the constructive comments provided by the reviewer.

Reviewer #3: Authors have addressed all major concerns from the first round with detailed, point-by-point responses, complemented by key additional experiments, refined experimental design, revised overinterpreted claims and supplemented critical methodological details. These revisions markedly improved the scientific rigor, validity and reproducibility of the study; supplementary data and analytical logic are consistent with the standards of neural circuit research. A small number of core aspects still lack sufficient elaboration and could be further improved to enhance the study's comprehensiveness and translational relevance.

Responses to major first-round concerns and supplementary experiments are all valid and meet research requirements:

Study rationale and clinical evidence are now well-supported—authors revised the description of visceral pain-itch comorbidity, focused on clinically validated conditions (primary biliary cholangitis, chronic kidney disease) with appropriate citations, and clearly articulated the research gap in visceral sensory neural mechanisms relative to somatic pain/itch.

Experimental model and stimulus specificity are well-established: CRD is clearly defined as a classic model for intestinal visceral pain with the scope of "visceral pain" specified; PBS injection and sham-CRD controls are added to confirm stimulus-specific neuronal activation; mouse experimental conditions are corrected to awake with partial restraint, and additional controls rule out BLA activation by anesthesia/immobilization-induced stress.

Neuronal coding specificity for pain and itch is convincingly demonstrated: additional experiments show only 25.2% overlap of BLA neuronal populations activated by histamine-dependent vs. independent itch, and 8.3% overlap for somatic pain/itch stimuli at the same neck skin site—directly confirming that BLA discriminates pain and itch independent of stimulus topographical location.

Experimental techniques are well-optimized: Tet-off system time window optimization (12/24/36 h) validates 36 h as the optimal window for BLA activity-dependent labeling; four core experiments replicated in female mice yield consistent results with males, fully accounting for sex as a biological variable; methodological details (cannula implantation, viral injection, CNO delivery) are completed, and Fluorogold tracing verifies local CNO diffusion at target sites.

Result interpretation is appropriately tempered: overstated claims (e.g., "sorting center") are revised to descriptions consistent with study findings, and clinical disease references are refined for accuracy.

Two minor points could be further elaborated to add depth and translational value to the study:

Given the study is motivated by clinical visceral pain-itch comorbidity, preliminary exploration of a comorbidity model (rather than only modeling the two sensations separately) would better bridge experimental findings with clinical scenarios and strengthen translational relevance.

BLA activated neurons are currently classified only as glutamatergic or GABAergic. Further subtyping of the dominant glutamatergic population (e.g., via neuropeptide or transcription factor markers) and brief investigation of the potential modulatory role of GABAergic neurons would refine neuronal characterization and add mechanistic depth.

Overall, authors' responses to first-round major concerns are fully satisfactory, with supplementary experiments effectively supporting the study's core conclusions and significantly improving manuscript quality. Addressing the above two points—either via targeted minor experimental supplementation or detailed textual elaboration—would further enhance the study's comprehensiveness. The manuscript will be acceptable for publication following these minor revisions.

>>>Response: We sincerely thank the reviewer for the constructive comments.

1. Given the study is motivated by clinical visceral pain-itch comorbidity, preliminary exploration of a comorbidity model (rather than only modeling the two sensations separately) would better bridge experimental findings with clinical scenarios and strengthen translational relevance.

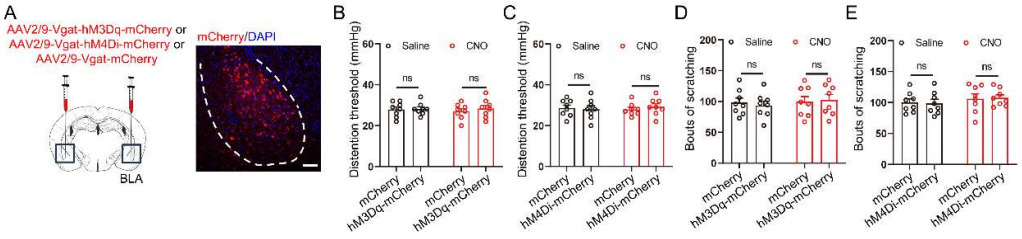
>>>Response: We fully agree with this insightful comment that incorporating comorbidity models would substantially enhance the translational relevance of the study. The present work primarily focused on elucidating how the BLA differentially encodes and processes visceral pain and itch at both the neural circuit and molecular levels. Given that chronic pain and chronic itch coexist in clinical conditions and involve complex, experience-dependent neuroplasticity, future studies incorporating such comorbidity models will be essential to bridge the gap between basic mechanistic

insights and translational application. This discussion has been incorporated into the revised manuscript (Page 21, lines 491~497, highlighted in red).

2. BLA activated neurons are currently classified only as glutamatergic or GABAergic. Further subtyping of the dominant glutamatergic population (e.g., via neuropeptide or transcription factor markers) and brief investigation of the potential modulatory role of GABAergic neurons would refine neuronal characterization and add mechanistic depth.
>>>Response: Thank the reviewer for the insightful comments.

As suggested, we have refined the characterization of neurons in the BLA by incorporating molecular markers identified through our sequencing data. Specifically, glutamatergic neurons in the BLA activated by visceral pain are labeled as BLA^{Fgf2}, while those activated by itch are labeled as BLA^{Notch2}.

To address the concern regarding the potential modulatory role of GABAergic neurons in BLA, we have conducted a **new experiment (Supplementary Figure 4,** see figures below**)**. Using chemogenetic manipulation (Fig. S4, A), we selectively inhibited or activated GABAergic neurons in BLA. Chemogenetic activation of GABAergic neurons in BLA did not alter the distention threshold or bouts of scratching (Fig. S4, B and D). Similarly, chemogenetic inhibition of GABAergic neurons in BLA did not affect the distention threshold and bouts of scratching (Fig. S4, C and E). These findings suggest that BLA GABAergic neurons do not play a role in the regulation of these two sensory modalities. These new findings (Pages 7~8, lines 157~167) and the corresponding discussion have been incorporated into the revised manuscript (Page 17, lines 381~384).



Authors have addressed all major concerns from the first round with detailed, point-by-point responses, complemented by key additional experiments, refined experimental design, revised overinterpreted claims and supplemented critical methodological details. These revisions markedly improved the scientific rigor, validity and reproducibility of the study; supplementary data and analytical logic are consistent with the standards of neural circuit research. A small number of core aspects still lack sufficient elaboration and could be further improved to enhance the study's comprehensiveness and translational relevance.

Responses to major first-round concerns and supplementary experiments are all valid and meet research requirements:

Study rationale and clinical evidence are now well-supported—authors revised the description of visceral pain-itch comorbidity, focused on clinically validated conditions (primary biliary cholangitis, chronic kidney disease) with appropriate citations, and clearly articulated the research gap in visceral sensory neural mechanisms relative to somatic pain/itch.

Experimental model and stimulus specificity are well-established: CRD is clearly defined as a classic model for intestinal visceral pain with the scope of "visceral pain" specified; PBS injection and sham-CRD controls are added to confirm stimulus-specific neuronal activation; mouse experimental conditions are corrected to awake with partial restraint, and additional controls rule out BLA activation by anesthesia/immobilization-induced stress.

Neuronal coding specificity for pain and itch is convincingly demonstrated: additional experiments show only 25.2% overlap of BLA neuronal populations activated by histamine-dependent vs. independent itch, and 8.3% overlap for somatic pain/itch stimuli at the same neck skin site—directly confirming that BLA discriminates pain and itch independent of stimulus topographical location.

Experimental techniques are well-optimized: Tet-off system time window optimization (12/24/36 h) validates 36 h as the optimal window for BLA activity-dependent labeling; four core experiments replicated in female mice yield consistent results with males, fully accounting for sex as a biological variable; methodological details (cannula implantation, viral injection, CNO delivery) are completed, and Fluorogold tracing verifies local CNO diffusion at target sites.

Result interpretation is appropriately tempered: overstated claims (e.g., "sorting center") are revised to descriptions consistent with study findings, and clinical disease references are refined for accuracy.

Two minor points could be further elaborated to add depth and translational value to the study:

Given the study is motivated by clinical visceral pain-itch comorbidity, preliminary exploration of a comorbidity model (rather than only modeling the two sensations

separately) would better bridge experimental findings with clinical scenarios and strengthen translational relevance.

BLA activated neurons are currently classified only as glutamatergic or GABAergic. Further subtyping of the dominant glutamatergic population (e.g., via neuropeptide or transcription factor markers) and brief investigation of the potential modulatory role of GABAergic neurons would refine neuronal characterization and add mechanistic depth.

Overall, authors' responses to first-round major concerns are fully satisfactory, with supplementary experiments effectively supporting the study's core conclusions and significantly improving manuscript quality. Addressing the above two points—either via targeted minor experimental supplementation or detailed textual elaboration—would further enhance the study's comprehensiveness. The manuscript will be acceptable for publication following these minor revisions.