nature portfolio

Peer Review File

Processing of pain and itch information by modality-specific neurons within the anterior cingulate cortex in mice

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

The anterior cingulate cortex (ACC) is both implicated in processing pain and itch, but how these two different sensations are processed in the ACC is unclear. This study by Ko et al found that pain and itch information are processed by distinct populations in the ACC. The authors further found that these sensory stimuli ACC neurons receives presynaptic inputs from MD using dual-eGRASP assay. Then, the authors demonstrated that the neuronal populations activated by pain and itch are functional segregated. Inhibition of pain-specific neurons by DREADD did not affect itch sensation, nor did the inhibition of itch-specific neurons affect pain sensation. Overall, the study is rigorous and will be of interest to a broad audience, and provide direct evidence for indicating that pain and itch are processed in the ACC involving modality-specific neuronal populations. However, there are some concerns that need to be addressed.

1. Chemical itch can be classified into two subtypes, histaminergic and nonhistaminergic. The mechanisms underlying histaminergic and nonhistaminergic itch processing might be not identical. The authors manipulated itch using histamine. It is unclear if the nonhistaminergic pruritogens such as 5-HT, chloroquine also encoding distinct neuron populations in the ACC or different presynaptic inputs from MD. What about the other types of algogens such as capsaicin-induced chemical pain or the mechanical pain?

2. In addition to demonstrating the effect of suppressing itch- or pain-specific neurons on pruriception or nociception in Fig 5, performing the same experiments with activating these neurons would be necessary to be able to draw the conclusions.

3. The authors used DREADD to suppress itch- or pain-specific neurons on histamine- or formalin-induced behaviors. it would be desirable to detect the effects on other algogens-induced pain and other non-histaminergic pruritogens-induced itch behaviors.

4. The authors showed that suppressing itch- or pain specific neurons on histamine or formalin-induced behavior, it would be helpful to detect the effects on the other algogens-induced pain and other pruritogens-induced itch behaviors.

5. The authors should provide functional evidence for manipulating itch- or pain-specific neurons in Fig 5. For example, fiber photometry or electrophysiological data to monitor neurons' activity.

6. ACC is composed of excitatory pyramidal neurons and inhibitory GABAergic neurons. What about the neuronal cell types of these itch- and pain-specific neurons? Double immunofluorescence staining for c-fos and neuronal markers will be helpful.

7. For formalin test, it contains two sessions. An initial acute phase (phase 1) is a relatively short period (1-10min), followed by a prolonged tonic response (phase 2, 10-60 min). Which phase did the author monitor? Did author find anything different between these phases, regarding neuronal activities or behaviors?

Reviewer #2 (Remarks to the Author):

In this study, the investigative team identified distinct neuronal populations related to pain and itch processing in layer II/III of the ACC. Using the dual-eGRASP technique, the study team found that pain- and itch-specific neurons from the ACC preferentially receive synaptic connections from mediodorsal thalamic neurons activated by pain and itch stimuli, respectively. Using an inhibitory DREADD approach, they found that while suppressing itch- or pain-specific neurons reduced pruriception or nociception, such inhibition was specific for the sensory modality. This is an important study, as distinct cortical mechanisms for pain and itch are not well understood, and the study also employs very novel technical approaches to address their hypothesis. I have the following comments/questions:

1) The data regarding the sensory modality-specific activation of ACC is very interesting and well supported. However, it should be noted that although the study team is able to show there is consistent and relatively specific activation of a small number of neurons in response to either itch or noxious stimulus, there could be additional contextual cues that may have given rise to such consistency. Other studies have shown that neurons in the PL or ACC may not show consistent activation in response to noxious inputs, and that overall connectivity is what drives overall population response (Li et al. Cell Reports, 2021; Acuna et al., PNAS 2023; Liu et al., Neuron, 2023). The authors may thus want to discuss these differences further. It is possible that the criteria for characterizing neuronal response varies depending on different experimental techniques, and that different layers may have specific effects. It is also possible that a combination of cell-specific and population responses operate at different scales in the cortex.

2) Please elaborate on the measures for nociceptive assays in Fig 5.

3) Please consider CPA/CPP assays for assessing pain, especially as the ACC is well-known for pain-aversive processing.

4) Please elaborate more clearly the subregion of ACC under investigation.

Reviewer #3 (Remarks to the Author):

The manuscript by Ko et al. describes overlap between neurons in the mouse ACC which respond to pain and itch. Many sophisticated approaches are used to label these two populations. The major conclusion is that these are largely separate populations, which make separable contributions to behavior. The manuscript contains an impressive range of sophisticated approaches for labeling, imaging, and manipulating ACC neuronal populations. However, when I examine the data, I unfortunately do not see support for the major conclusion of this study, as expressed by the title. I think the data could end up being valuable but would need to be analyzed and interpreted in very different ways, leading to a fundamentally different manuscript.

1. The results depend critically on the validity cfos based methods for labeling specific populations are valid. This raises several questions – first, how exactly is the chance level calculated in Fig 2d, f, and why does it appear to be systematically different for the 6d vs. the 3h interval? Presumably this reflect different absolute numbers of cells being recruited by the same stimuli at these two intervals? The total number of cells, and the number activated by pain and itch should be provided to confirm that this is the case.

2. Related to the preceding, it does not appear that the level of overlap between itch & pain cells for the 3d interval is significantly different from the level of overlap between the itch & itch cells for the same interval. A statistical test should be performed to directly compare these two levels of overlap. If these two levels of overlap are not different, then this does not support the idea that itch and pain information are carried by distinct populations.

3. Also related to this, it appears that the level of overlap between itch & pain cells at the 6h interval might actually be higher than that between itch & itch cells at the same interval. At the very least, there does not appear to be higher overlap between itch & itch cells. Again, if this is the case, I don't understand how the authors can argue that itch and pain signals are being carried by distinct neuronal populations?

4. These cfos labeling experiments should include a negative control, in which no stimulus is delivered at one of the timepoints, in order to quantify the degree of overlap that occurs simple as a result of spontaneous neural activity.

5. A similar concern relates to the imaging-derived data in Fig. 3d,f. Once again, the level of overlap between itch and pain cells appears to be higher at a 6h interval than the corresponding level of overlap between itch and itch cells. And the levels of overlap between itch and pain cells at the 3d interval appear very similar to the levels of overlap between itch and itch cells at the same interval. Again, this seems to directly contradict the idea that these are distinct neuronal populations.

6. How was the registration of the calcium imaging movies performed? How was the accuracy of cell registration validated? Again, there should be a negative control in which no stimulus is delivered at one timepoint to quantify the degree of overlap that is attributable to just spontaneous activity.

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

The anterior cingulate cortex (ACC) is both implicated in processing pain and itch, but how these two different sensations are processed in the ACC is unclear. This study by Ko et al found that pain and itch information are processed by distinct populations in the ACC. The authors further found that these sensory stimuli ACC neurons receives presynaptic inputs from MD using dual-eGRASP assay. Then, the authors demonstrated that the neuronal populations activated by pain and itch are functional segregated. Inhibition of pain-specific neurons by DREADD did not affect itch sensation, nor did the inhibition of itch-specific neurons affect pain sensation. Overall, the study is rigorous and will be of interest to a broad audience, and provide direct evidence for indicating that pain and itch are processed in the ACC involving modality-specific neuronal populations. However, there are some concerns that need to be addressed.

1. Chemical itch can be classified into two subtypes, histaminergic and nonhistaminergic. The mechanisms underlying histaminergic and nonhistaminergic itch processing might be not identical. The authors manipulated itch using histamine. It is unclear if the nonhistaminergic pruritogens such as 5-HT, chloroquine also encoding distinct neuron populations in the ACC or different presynaptic inputs from MD. What about the other types of algogens such as capsaicin-induced chemical pain or the mechanical pain?

Response: **To address this question, we adopt the experimental scheme used in Extended Data Fig. 8 of the original manuscript. In this experiment, the ACC of TRAP2 mice was injected with an AAV mixture expressing CaMKII::DIO-tdTomato, Fos::rtTA, and TRE3G::mEmeraldNuc. Under 4-OHT, neurons activated by the first stimulus, either histamine (His) or capsaicin (CAP), were labeled with tdTomato. Under doxycycline (Dox), histamine-injected mice received a non-histaminergic second stimulus, chloroquine (CQ), while CAP-injected mice received formalin. Contrary to our expectations, the percentage of overlapping neurons was not higher when the mice received two stimuli of different modalities. We assume that although pain- and itch-specific neuron populations are distinct within the ACC, the activation characteristics of neurons responsible for each modality are highly variable. Our behavioral data further support this, showing that inhibiting pain-specific neurons does not influence pruriception, and inhibiting itch-specific neurons does not influence nociception. This suggests that the processing of pruriceptive or nociceptive stimulus within the ACC is managed by neurons specific to each modality. Moreover, connectivity within each modality-specific neuronal population seems to be crucial. As mentioned in the discussion session, neurons within each modality-specific population may also have a functional hierarchy. This is consistent with the significance of connectivity within modality-specific neuron populations. For more details on the connectivity of these neurons, please refer to reviewer #2's first comment. We have**

incorporated this information into the main text and Extended Data Fig. 11.

2. In addition to demonstrating the effect of suppressing itch- or pain-specific neurons on pruriception or nociception in Fig 5, performing the same experiments with activating these neurons would be necessary to be able to draw the conclusions.

Response: **We also agree on the need for gain-of-function experiments for itch- or pain-specific neurons as you suggested. To investigate alterations in pruriceptive or nociceptive responses by activating these neurons, we used a DREADD-mediated neuronal activation system combined with IEG promoter-based expression. We injected AAV mixtures (fos::rtTA and TRE3G::hM3Dq-mCherry) into the ACC of wildtype mice. Under doxycycline, hM3Dq-mCherry was selectively expressed in neurons activated by histamine or formalin. This allowed us to activate these itch- or pain-specific neurons via CNO injection. Initially, we measured the scratching bouts on the nape as a pruriceptive response 30 min following CNO injection in the absence of histamine injection. However, we did not observe any difference between the vehicle and CNO groups. When we measured the duration of licking, lifting, and flinching responses of the foot as a nociceptive response 30 min following CNO injection without formalin, we also did not observe any difference between the groups. However, whether scratching behavior can be regarded as a pruriceptive response and licking behavior as a nociceptive response in the absence of specific stimuli remains questionable. This is because mice did not exhibit these behaviors immediately after entering the chamber for behavioral recording 30 min following CNO administration; they only showed these behaviors after adapting to the chamber to some extent. Additionally, these behaviors are typical grooming behaviors in rodents. Therefore, we examined how mice responded to mild algogen or pruritogen when ACC modality-specific neurons were activated. The results showed that the activation of itch-specific neurons decreased rather than increased scratching bouts induced by a low histamine concentration. Activation of pain-specific neurons did not induce any significant change in the nociceptive response induced by a low formalin concentration. However, these results should not be interpreted as suggesting that activating itch-specific neurons reduces pruriception. This is because we observed an increase in freezing-like behavior in mice when activating either itch- or pain-specific neurons in the ACC. Without any stimuli, freezing-like behavior significantly increased in the group with activated pain-specific neurons. Injecting weak histamine or formalin resulted in a significant increase in freezing-like behavior in the group where itch- or pain-specific neurons were activated. This increase in freezing does not seem to be a side effect of CNO because CNO administration did not increase freezing in the hM4Di experiment (Extended Data Fig 10e and g). In summary, these findings suggest that itch- or pain-specific neurons in the ACC contribute to evoking anxiety or negative emotions related to itch or pain rather than directly influencing nociception or pruriception. We have included these findings in the main text and Fig. 6.**

3. The authors used DREADD to suppress itch- or pain-specific neurons on histamine- or formalin-induced behaviors. it would be desirable to detect the effects on other algogens-induced pain and other non-histaminergic pruritogens-induced itch behaviors.

Response: **To address this question, we again employed DREADD-mediated chemogenetics combined with IEG promoter-based hM4Di expression. We injected an AAV mixture (fos::rtTA and TRE3G::hM4Di-mCherry) into the ACC of wildtype mice. Under doxycycline, hM4Di-mCherry was selectively expressed in neurons activated by histamine or formalin. Furthermore, 30 min after CNO injection to suppress itch- or pain-specific neurons, chloroquine as a non-histaminergic pruritogen and capsaicin as another algogen were administered, respectively, and behavioral changes were monitored. We found that specifically inhibiting neurons previously activated by formalin reduced capsaicin-induced nociceptive responses. Scratching responses induced by chloroquine were suppressed by specifically inhibiting only the neurons previously activated by histamine. These findings suggest that the specificity of ACC neurons to pain and itch modalities does not differentiate within each modality. We have included these findings in the main text and Extended Data Fig. 10.**

4. The authors showed that suppressing itch- or pain specific neurons on histamine or formalin-induced behavior, it would be helpful to detect the effects on the other algogens-induced pain and other pruritogens-induced itch behaviors.

Response: **We believe we have addressed this comment in our response to your #3 comment above.**

5. The authors should provide functional evidence for manipulating itch- or pain-specific neurons in Fig 5. For example, fiber photometry or electrophysiological data to monitor neurons' activity.

Response: **We appreciate the suggestion of the reviewer for fundamental evidence to support our behavioral experiments. To validate the functionality of the DREADD system, we measured the number of action and resting membrane potentials in neurons expressing hM3Dq or hM4Di after bath application of CNO. Our results confirmed that the DREADD system effectively increased and decreased the activities of specific ACC neurons expressing hM3Dq and hM4Di, respectively. These data have been included in the main text and Extended Data Fig. 9.**

6. ACC is composed of excitatory pyramidal neurons and inhibitory GABAergic neurons. What about the neuronal cell types of these itch- and pain-specific neurons? Double immunofluorescence staining for c-fos and neuronal markers will be helpful.

Response: **As suggested by the reviewer, we conducted double immunofluorescence staining**

for c-fos and CaMKII, or c-fos and GABA, in the ACC of mice injected with formalin or histamine. We observed that most fos (+) cells are excitatory neurons that are co-stained with CaMKII. These findings have been included in the main text and Fig. 1g-j.

7. For formalin test, it contains two sessions. An initial acute phase (phase 1) is a relatively short period (1-10min), followed by a prolonged tonic response (phase 2, 10-60 min). Which phase did the author monitor? Did author find anything different between these phases, regarding neuronal activities or behaviors?

Response: **In the case of neuronal activities monitored by *in vivo* free-moving miniscope imaging (Fig. 3), we assessed Ca^{2+} transients in the ACC for 10 min following formalin injection. Thus, we could not directly compare the acute phase with the tonic phase. However, in the behavioral experiment, we were able to reanalyze the test results by phase. We observed that suppressing ACC neurons previously activated by formalin decreased nociceptive responses only during the tonic phase, not during the acute phase. We replaced the original Fig. 5h and j with reanalyzed data. The mechanism of nociception induced by formalin differs between the acute and tonic phases. Unfortunately, the role of neurons activated during the acute phase following formalin injection, as observed in miniscope imaging, remains unclear, and further experiments are needed to clarify this point. However, we believe this is beyond the scope of this study owing to limited interest in this aspect.**

Reviewer #2 (Remarks to the Author):

In this study, the investigative team identified distinct neuronal populations related to pain and itch processing in layer II/III of the ACC. Using the dual-eGRASP technique, the study team found that pain- and itch-specific neurons from the ACC preferentially receive synaptic connections from mediodorsal thalamic neurons activated by pain and itch stimuli, respectively. Using an inhibitory DREADD approach, they found that while suppressing itch- or pain-specific neurons reduced pruriception or nociception, such inhibition was specific for the sensory modality. This is an important study, as distinct cortical mechanisms for pain and itch are not well understood, and the study also employs very novel technical approaches to address their hypothesis. I have the following comments/questions:

1. The data regarding the sensory modality-specific activation of ACC is very interesting and well supported. However, it should be noted that although the study team is able to show there is consistent and relatively specific activation of a small number of neurons in response to either itch or noxious stimulus, there could be additional contextual cues that may have given rise to such consistency. Other studies have shown that neurons in the PL or ACC may not show consistent activation in response to noxious inputs, and that overall connectivity is what drives overall population response (Li et al. Cell

Reports, 2021; Acuna et al., PNAS 2023; Liu et al., Neuron, 2023). The authors may thus want to discuss these differences further. It is possible that the criteria for characterizing neuronal response varies depending on different experimental techniques, and that different layers may have specific effects. It is also possible that a combination of cell-specific and population responses operate at different scales in the cortex.

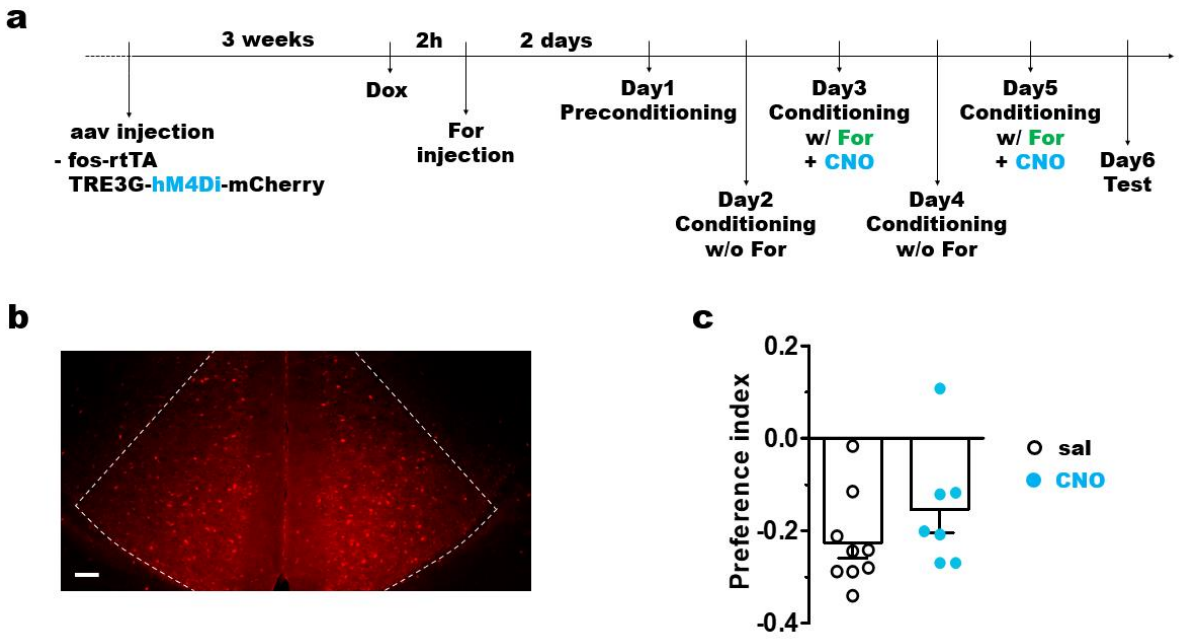
Response: **We completely agree with this comment and appreciate your crucial insight into our main question. We were puzzled by the low percentage of overlapping cells in the experiments using TetTag mice and miniscope, even when the mice were given the same stimuli. This seems contradictory to the results of our behavioral experiments. Despite the extremely low reactivation to the same stimulus, inhibiting neurons previously activated by an algogen suppressed only nociception, not pruriception, and this was similar for itch-specific neurons in the ACC. These findings were consistent even in additional experiments using other types of algogen or pruritogen. To interpret these seemingly conflicting results, we mentioned in the discussion session that a functional hierarchy might exist among modality-specific neurons, with a small number of core hub neurons variably recruiting neurons that process the same modality. However, these conflicting results may also suggest that, as you mentioned, overall connectivity within a modality-specific neuronal population is significant for processing stimuli of a specific modality. Based on your suggestion, we have further discussed these points in the discussion section.**

2. Please elaborate on the measures for nociceptive assays in Fig 5.

Response: **In the formalin test, we measured the duration of licking, flinching, and lifting of the formalin-injected foot as anti-nociceptive responses. As you know, the formalin-induced nociceptive response is divided into early (0–10 min) and late (10–60 min) phases. We reanalyzed the formalin test results and updated Fig. 5h and j accordingly. This reanalyzed data indicates that pain-specific neurons in the ACC are involved only in the late phase of formalin-induced pain, not the acute phase.**

3. Please consider CPA/CPP assays for assessing pain, especially as the ACC is well-known for pain-aversive processing.

Response: **Agreeing with your opinion, we conducted the CPA assay to further validate our behavioral experiment results. We slightly modified the CPA assay used in rats, as described by Sarah Jarin et al., Front. Behav. Neurosci., 2020, and integrated it with a chemogenetic method to assess whether inhibiting itch- or pain-specific neurons could reverse CPA. Below is the experimental scheme and preliminary results.**



We designed behavioral experiments combining the CPA assay with inhibitory hM4Di expression selectively in pain-specific neurons in the ACC (Fig. a above). hM4Di-mCherry was well expressed in the ACC (Fig. b above); however, despite a tendency we did not observe significant evidence that suppression of pain-specific neurons (CNO group) reverses the conditioned aversion to the formalin-paired chamber (Fig. c above). While increasing the number of samples would be ideal, we consider our chemogenetic inhibition experiments using other algogen and pruritogen (Extended Data Fig. 10) and chemogenetic activation experiments (Fig. 6) sufficient to strengthen our findings. Additionally, conducting more experiments with additional AAVs is time-consuming owing to the nature of behavioral experiments, which require extensive preparation and execution. Therefore, we appreciate your understanding that further experiments cannot be conducted within this revision time frame.

4. Please elaborate more clearly the subregion of ACC under investigation.

Response: **We did not consider the subregions identified as cg1 and cg2 in all experiments targeting ACC. However, in experiments using TetTag mice, layers I/II were analyzed separately from other layers, as stated in the manuscript. For experiments involving miniscopes, eGRASP, and chemogenetics that require AAV injection, it is challenging to target specific layers within the ACC. Therefore, specific layers were not considered in these experiments.**

Reviewer #3 (Remarks to the Author):

The manuscript by Ko et al. describes overlap between neurons in the mouse ACC which respond to pain and itch. Many sophisticated approaches are used to label these two populations. The major

conclusion is that these are largely separate populations, which make separable contributions to behavior. The manuscript contains an impressive range of sophisticated approaches for labeling, imaging, and manipulating ACC neuronal populations. However, when I examine the data, I unfortunately do not see support for the major conclusion of this study, as expressed by the title. I think the data could end up being valuable but would need to be analyzed and interpreted in very different ways, leading to a fundamentally different manuscript.

1. The results depend critically on the validity cfos based methods for labeling specific populations are valid. This raises several questions – first, how exactly is the chance level calculated in Fig 2d, f, and why does it appear to be systematically different for the 6d vs. the 3h interval? Presumably this reflect different absolute numbers of cells being recruited by the same stimuli at these two intervals? The total number of cells, and the number activated by pain and itch should be provided to confirm that this is the case.

Response: **The chance levels were calculated as follows:**

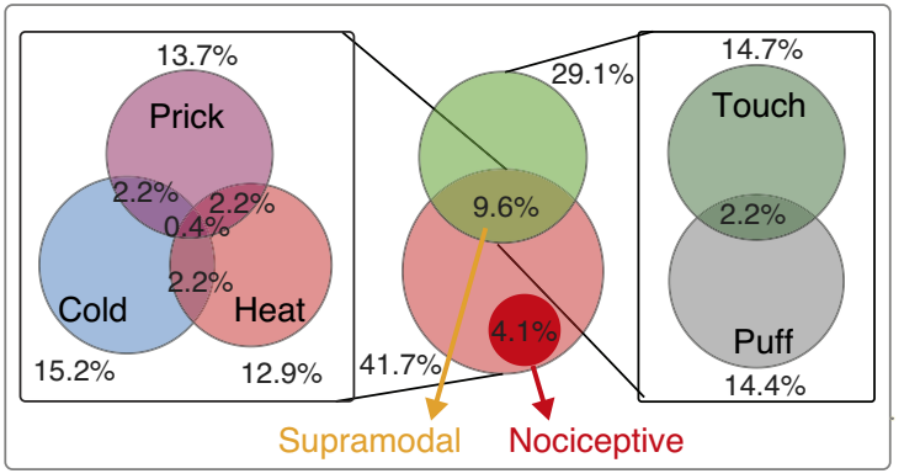
Chance level = ((number of H2BGFP+ cells) / (number of DAPI+ cells)) × ((number of Fos+ cells) / (number of DAPI+ cells)).

When we compared the chance level between the 6 h and 3 days groups, no significant difference was observed (unpaired t-test; Itch → Pain, $t_{15} = 1.819$, $p > 0.05$; Itch → Itch, $t_{12} = 1.210$, $p > 0.05$). Moreover, although H2BGFP (+) cells (% DAPI) tended to be higher in the 6 h group than in the 3 days group in the "Itch → Pain" experiment, no significant differences exist in H2BGFP (+) cells (% DAPI) or Fos (+) cells (% DAPI) between the 6 h and 3 days groups in both "Itch -> Pain" and "Itch → Itch" experiments (Extended Data Fig. 2). Even if the absolute numbers of cells recruited by the same stimuli differ between these two intervals, we believe it is not crucial. Increased absolute numbers of recruited cells also raise the chance level. That is why our study and many previous studies using TetTag mice consider the chance level in labeling experiments.

2. Related to the preceding, it does not appear that the level of overlap between itch & pain cells for the 3d interval is significantly different from the level of overlap between the itch & itch cells for the same interval. A statistical test should be performed to directly compare these two levels of overlap. If these two levels of overlap are not different, then this does not support the idea that itch and pain information are carried by distinct populations.

Response: **Unfortunately, we could not compare the "Itch & Pain 3d interval" and "Itch & Itch 3d interval" groups because the "Itch & Pain" and "Itch & Itch" experiments were conducted independently. As the reviewer noted, it seems that the overlap level in the "Itch & Itch 3d interval" group is not as high as we anticipated. This is partly owing to the low absolute number of overlapping cells. However, our findings align with previous research findings, indicating that**

at the single cell level, stimulus representation by ACC neurons is dynamic rather than fixed over time (Acuna et al., PNAS 2023. see the Fig below).



The same neurons are not activated whenever the corresponding stimuli are applied; however, we do not believe these experimental results disapprove of the existence of a modality-specific, distinct neuronal population. Our behavioral data strongly supports the existence of such modality-specific, distinct neuronal populations. As discussed in the manuscript, a functional hierarchy might occur among modality-specific neurons or specific patterns of population coding or connectivity that are critical for stimulus processing in the ACC. This view is supported by recent investigations performed in the prefrontal cortex (Li et al., Cell Reports, 2021; Liu et al., Neuron, 2023). For a more detailed explanation, please refer to our response to Reviewer #2's first comment above.

3. Also related to this, it appears that the level of overlap between itch & pain cells at the 6h interval might actually be higher than that between itch & itch cells at the same interval. At the very least, there does not appear to be higher overlap between itch & itch cells. Again, if this is the case, I don't understand how the authors can argue that itch and pain signals are being carried by distinct neuronal populations?

Response: **A low absolute number of overlapping cells may raise concerns. However, we believe that not all neurons involved in pain or itch processing are consistently activated by pain or itch stimuli. That is, when a pain stimulus occurs, not all pain-specific neurons respond; instead, only some pain-specific neurons are activated in a fixed manner. Despite this, our eGRASP and behavioral experiments support the existence of distinct neuronal populations responsible for pain and itch, respectively. These populations receive distinct presynaptic MD inputs, and suppressing them reduces the processing of stimulus only within their specific modality. For further details, please refer to our response to Reviewer #2's first comment.**

4. These cfos labeling experiments should include a negative control, in which no stimulus is delivered at one of the timepoints, in order to quantify the degree of overlap that occurs simple as a result of spontaneous neural activity.

Response: **We deeply appreciate your comment. Therefore, we conducted extensive handling steps in our labeling experiment using TetTag mice (7 days). While TetTag mice are commonly used for labeling neurons activated by two stimuli at intervals, they come with limitations. The time window during which the labeling is available cannot be precisely controlled, making them more suitable for brain regions with low spontaneous or background activity, such as the hippocampus. However, their application is limited in brain areas with high spontaneous or background activity, such as the ACC. This led us to perform miniscope experiments, where we analyzed neurons activated only after a stimulus and observed results similar to those of experiments using TetTag mice. Producing TetTag mice is time-consuming, and we believe that some of your concerns have been addressed through the miniscope experiment. We hope you understand that we are unable to conduct the specific experiment you requested.**

5. A similar concern relates to the imaging-derived data in Fig. 3d,f. Once again, the level of overlap between itch and pain cells appears to be higher at a 6h interval than the corresponding level of overlap between itch and itch cells. And the levels of overlap between itch and pain cells at the 3d interval appear very similar to the levels of overlap between itch and itch cells at the same interval. Again, this seems to directly contradict the idea that these are distinct neuronal populations.

Response: **We believe that this is also partially owing to a low percentage of overlapping cells. For further details, please see our response to your third comment above.**

6. How was the registration of the calcium imaging movies performed? How was the accuracy of cell registration validated? Again, there should be a negative control in which no stimulus is delivered at one timepoint to quantify the degree of overlap that is attributable to just spontaneous activity.

Response: **We provided a more detailed description of how we conducted the registration of calcium imaging movies and validated the accuracy of cell registration, in addition to the methods section. However, we have some reservations regarding your point about the negative control. This is because we classified cells into three types and conducted overlapping analysis exclusively with neurons that were activated only after a stimulus. This addresses your concern that the overlapping neuronal population may include neurons activated solely owing to spontaneous activity. Furthermore, we included absolute cell numbers and ratios for the three classes of neurons in Fig. 3c.**

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

The authors' responses are generally thorough, and we are satisfied with the changes made in the manuscript and figures.

Reviewer #2 (Remarks to the Author):

The authors addressed most of my concerns.

Reviewer #3 (Remarks to the Author):

In my original comments 2 and 4, I wrote that the authors should compare overlap between itch & pain cells to overlap between itch & itch cells, because if the itch-itch overlap is not significantly greater than itch-pain overlap, then this would contradict the idea that itch & pain are encoded by distinct populations. The authors first say they cannot do this because itch & pain and itch & itch overlap was assessed in different experiments. This is no way precludes a statistical comparison, and this is in fact the entire point of using statistics which tests whether differences observed between two independent samples is meaningful vs. simply reflects chance variation. This statistical comparison is essential because if there is no meaningful difference (as appears to be the case), this would contradict the central claim of this manuscript.

The authors additionally respond to this by saying that there is no meaningful itch-itch overlap in either cfos or imaging experiments, because the neurons responding to a specific stimulus represent a dynamic subset within larger populations that are nonetheless modality-specific and distinct ("We assume that although pain- and itch specific neuron populations are distinct within the ACC, the activation characteristics of neurons responsible for each modality are highly variable.... functional hierarchy might exist among modality-specific neurons, with a small number of core hub neurons variably recruiting neurons that process the same modality... at the single cell level, stimulus representation by ACC neurons is dynamic rather than fixed over time..."). The problem with this assertion is that it is mathematically implausible. For example, Fig. 2 shows that the chance level of overlap for itch-itch and itch-pain cells is ~1%. Assume this means that about

10% of cells are activated by each stimulus. The authors imply that within a larger population of itch cells, a random subset is activated by each different itch stimulus. Assume that itch cells represent ~30% of the total population of neurons, and that 1/3 of this population is activated by each itch stimulus. Then the itch-itch overlap should be about 11% of the total neuronal population. You can work this out with other higher or lower percentages – assume 50% of the population represents potential itch neurons. Then 1/5 of this population would be activated by each stimulus, meaning that the itch-itch overlap would be about 2%. If the itch population represents 20% of the total neuron population, then ½ of this population would be activated by each stimulus, so the itch-itch overlap should be 5%. This illustrates why it is so important to provide actual numbers which the author have not done for Figure 2. Based on the calculations outlined above, it seems like to observed the levels of overlap the authors found, the majority of ACC neurons may need to be part of the potential itch population. This either undercuts the argument that there are distinct subsets of ACC neurons which encode itch vs. pain, or shows that the method that is being using to label these cells is unreliable, which calls into question Figure 2 as well as later figures.

For Figure 3, the authors observe an overlap of ~5% of the population. They do not explicitly state how this is calculated (is it based on the neurons which were only activated after the injections and the total number of neurons?) or specify the total number of neurons (including unactivated neurons). Furthermore, the numbers they provide do not make sense – based on Fig. 3g there were a total of 473+128 = 601 itch-activated neurons and 131 + 385 = 516 pain-activated neurons, but in the legend they state that there were a total of 2006 neurons that were activated 'only after' the stimuli were delivered. If the authors really want to claim that the neurons encoding a specific modality represent a dynamic subset within a larger modality-specific population, then they need to provide enough information to test whether that claim is mathematically plausible – specifically the total number of neurons imaged, as well the numbers that fall into each category (itch or pain responsive neurons). And they need to do appropriate negative controls to estimate the overlap due to spontaneous activity. In declining to do negative controls, the authors wrote: "This led us to perform miniscope experiments, where we analyzed neurons activated only after a stimulus and observed results similar to those of experiments using TetTag mice. Producing TetTag mice is time-consuming, and we believe that some of your concerns have been addressed through the miniscope experiment." Indeed, the authors did see similar results using the miniscope experiment and the TetTag mice – namely that the level of overlap was similar for itch-itch and itch-pain cells. But this does not "address" my concerns, rather it reinforces my concern that the populations of potential itch and pain cells must be so large that they are highly overlapping and do not represent distinct modality-specific populations as the title of the paper claims.

Unfortunately, I cannot endorse publication without providing the cell counts and negative controls which would support the conclusion that is explicitly stated in the title.

REVIEWER COMMENTS

Reviewer #3 (Remarks to the Author):

In my original comments 2 and 4, I wrote that the authors should compare overlap between itch & pain cells to overlap between itch & itch cells, because if the itch-itch overlap is not significantly greater than itch-pain overlap, then this would contradict the idea that itch & pain are encoded by distinct populations. The authors first say they cannot do this because itch & pain and itch & itch overlap was assessed in different experiments. This is no way precludes a statistical comparison, and this is in fact the entire point of using statistics which tests whether differences observed between two independent samples is meaningful vs. simply reflects chance variation. This statistical comparison is essential because if there is no meaningful difference (as appears to be the case), this would contradict the central claim of this manuscript.

Response #1: **We apologize if our responses to points 2 and 4 of your original comment gave the impression that we were refuting your views. We completely agree that a comparison between the "Itch & Pain" and "Itch & Itch" groups is necessary. However, as mentioned in our original response, the "Itch & Pain 3-day interval" and "Itch & Itch 3-day interval" groups cannot be statistically compared because the "Itch & Pain" and "Itch & Itch" experiments were conducted independently. In the experiments using TetTag mice (Fig. 2), the conditions for immunolabeling (such as antibody batch, serum, etc.) and imaging (e.g., scan speed, master gain, digital gain, etc.) were not identical between the "Itch & Pain 3-day interval" and "Itch & Itch 3-day interval" groups. Therefore, the percentage of H2BGFP+Fos cells (%DAPI) cannot be directly compared between these two groups.**

However, we deeply agree with your point about the importance of comparing these groups, and after much consideration, we determined that the ratio of 'P(H2BGFP+Fos cells)/P(chance)' can be compared. This is because H2BGFP+Fos cells (%DAPI) and chance levels were calculated within the same brain slice, allowing P(chance) to be used to normalize differences in experimental conditions. Based on this normalization, we observed that the overlap/chance ratio in the "Itch & Itch 3-day interval" group is significantly higher than in the "Itch & Pain 3-day interval" group. This suggests that when two stimuli are given with a sufficient time interval (3 days), only neurons specific to each corresponding stimulus are activated. Specifically, when itch and pain stimuli are given at a 3-day interval, the neurons responding to both stimuli do not differ from the chance level, indicating that the two events are independent. In contrast, when two itch stimuli are given at a 3-day interval, the activation probability of anterior cingulate cortex (ACC) neurons is higher than the chance level, supporting the existence of itch-specific neurons.

In the case of the 6-h interval, the overlap/chance ratio did not show any significant difference between the "Itch & Pain" and "Itch & Itch" groups. However, since both groups displayed

significantly higher overlap than the chance level, this suggests that the activation of neurons for the first and second stimuli is not independent, indicating a dependency. For the "Itch & Itch" condition, this dependency can be easily explained by assuming the existence of itch-specific neurons. However, the identity of neurons responding to both itch and pain stimuli remains somewhat puzzling. One explanation is that previously activated neurons maintain high excitability and can be reactivated by a consecutive stimulus, regardless of its type. Another possibility is that this class of neurons could function as gatekeepers in gate control theory, meaning these neurons are activated by an itch stimulus and can be reactivated only when a pain stimulus follows within a short time window, but not by the same itch stimulus. In this case, the identity of reactivated neurons in the "Itch & Pain 6-h interval" group would differ from those in the "Itch & Itch 6-h interval" and "Itch & Itch 3-day interval" groups. Therefore, we do not think that the overlap in the "Itch & Itch 6-hour interval" group should be higher than in the "Itch & Pain 6-hour interval" group simply due to an additive effect. What is more important in experiments using TetTag mice is whether the overlap probability is significantly higher than the chance level, as this tells us whether the activity of neurons for two stimuli given with a time interval is independent or related. We included this result in new Fig. 2f. We included this result in the new Fig. 2f. At the same time, Figs. 2e and 2g from the previous version were deleted, as their information has been integrated into the new Fig. 2f.

Just in case you are concerned, the results of the miniscope experiment in Fig.3 make it logically impossible to compare the "Itch & Pain 3d interval" and "Itch & Itch 3d interval" groups. While it might seem reasonable to expect the overlap in the "Itch & Itch 3-day interval" group to be higher than in the "Itch & Pain 3-day interval" group, we do not have information on the absolute size or activation properties of the neuronal populations processing itch and pain stimuli. For example, if the neuronal population responding to pain stimuli were much larger than that responding to itch stimuli, the overlap in the "Itch & Pain 3-day interval" group should be higher than in the "Itch & Itch 3-day interval" group. Alternatively, if higher concentrations of histamine recruit more ACC neurons, using a high dose of histamine could result in the overlap being higher in the "Itch & Itch 3-day interval" group than in the "Itch & Pain 3-day interval". While this might seem plausible, we are skeptical about whether these results would be scientifically sound.

At this point, you might ask how the comparison between the "Itch & Itch 3-day interval" and "Itch & Pain 3-day interval" groups was possible in the TetTag mice experiment. In the TetTag mice experiment, it was possible to normalize for the variables mentioned above using the chance level, but such normalization is impossible with the miniscope due to the nature of the experiment. The miniscope data cannot provide the actual number of whole cells, making it impossible to calculate the chance level. For further details, please refer to response #3 below.

The authors additionally respond to this by saying that there is no meaningful itch-itch overlap in either

cfos or imaging experiments, because the neurons responding to a specific stimulus represent a dynamic subset within larger populations that are nonetheless modality-specific and distinct ("We assume that although pain- and itch specific neuron populations are distinct within the ACC, the activation characteristics of neurons responsible for each modality are highly variable…. functional hierarchy might exist among modality-specific neurons, with a small number of core hub neurons variably recruiting neurons that process the same modality... at the single cell level, stimulus representation by ACC neurons is dynamic rather than fixed over time…"). The problem with this assertion is that it is mathematically implausible. For example, Fig. 2 shows that the chance level of overlap for itch-itch and itch-pain cells is ~1%. Assume this means that about 10% of cells are activated by each stimulus. The authors imply that within a larger population of itch cells, a random subset is activated by each different itch stimulus. Assume that itch cells represent ~30% of the total population of neurons, and that 1/3 of this population is activated by each itch stimulus. Then the itch-itch overlap should be about 11% of the total neuronal population. You can work this out with other higher or lower percentages – assume 50% of the population represents potential itch neurons. Then 1/5 of this population would be activated by each stimulus, meaning that the itch-itch overlap would be about 2%. If the itch population represents 20% of the total neuron population, then ½ of this population would be activated by each stimulus, so the itch-itch overlap should be 5%. This illustrates why it is so important to provide actual numbers which the author have not done for Figure 2. Based on the calculations outlined above, it seems like to observed the levels of overlap the authors found, the majority of ACC neurons may need to be part of the potential itch population. This either undercuts the argument that there are distinct subsets of ACC neurons which encode itch vs. pain, or shows that the method that is being using to label these cells is unreliable, which calls into question Figure 2 as well as later figures.

Response #2**: We apologize for any misunderstanding regarding our explanation. To explain in more detail, we assume that if events A and B occur independently, the joint probability is given by $P(A \cap B) = P(A) \times P(B)$, which represents the chance level. Here, event A is the neuronal activation induced by the first stimulus (histamine), and event B is the neuronal activation induced by the second stimulus (histamine or formalin). Therefore:**

- ***P(A∩B)* = % of H2BGFP+Fos double (+) cells (%DAPI)**
- ***P(A)* = % of H2BGFP (+) cells (%DAPI)**
- ***P(B)* = % of Fos (+) cells (%DAPI)**

If *P(A∩B)* is significantly higher than the chance level, it indicates that events A and B are not independent. Conversely, if *P(A∩B)* is not significantly higher than the chance level, it suggests that events A and B are independent. In Fig. 2d, H2BGFP+Fos cells (%DAPI) at the 3-day interval did not show a significant difference compared to the chance level. This indicates that when pain and itch stimuli are administered with a sufficient time interval (3 days), ACC neurons are activated independently for the two different stimuli. Combined with our behavioral experiments (Fig. 5, where inhibition of pain-specific neurons did not affect itch sensation, and vice versa),

we conclude that this independence is due to the involvement of distinct neuronal populations. The existence of modality-specific distinct neuronal populations seems to be the best way to interpret the results of the behavioral experiments in Fig.5, which show that inhibition of neurons activated by itch or pain stimuli does not affect each other's behavioral output.

At the 6-h interval, H2BGFP+Fos cells (%DAPI) were higher than the chance level. This suggests that neuronal activation induced by the itch stimulus influences the subsequent neuronal activation induced by the second pain stimulus. We interpret this result as evidence that previously activated neurons can be more easily reactivated when a second stimulus occurs within a short interval, regardless of the stimulus type.

In Fig. 2e, two consecutive itch stimuli resulted in values higher than the chance level, regardless of the time interval between stimuli. Even with a sufficient interval of 3 days, the fact that H2BGFP+Fos cells (%DAPI) remained higher than the chance level indicates that the neuronal populations responding to the two itch stimuli are not independent. This can be interpreted as evidence of an itch-specific neuronal population in the ACC.

This probability-based approach using TetTag mice has been applied in many studies since 2007 [1–11]. Additionally, to clarify our interpretation and facilitate understanding, we have updated the actual values related to Fig. 2 in the Source Data file. Lastly, we would like to carefully point out an error in your probability calculations. It appears that your calculation is based on conditional probability. It seems you already assumed that event A could influence event B and calculated $P(A \mid B) = P(A \cap B) / P(B)$, rather than $P(A \cap B)$. We think that it should not presuppose that the activation of neurons depends on their previous activation history or apply conditional probability without solid evidence.

For Figure 3, the authors observe an overlap of ~5% of the population. They do not explicitly state how this is calculated (is it based on the neurons which were only activated after the injections and the total number of neurons?) or specify the total number of neurons (including unactivated neurons).

Response #3**: We apologize for any misunderstanding caused by the lack of detailed explanation. In *in vivo* live Ca^{2+} imaging, it is impossible to determine the actual number of neurons, including unactivated neurons, within the imaging field because only neurons that exhibit fluorescence, reflecting Ca^{2+} transients, can be detected. Therefore, we calculated the overlapping population as follows:**

Overlapping (%reactivated) = C/ (A+B) X 100 %

Where:

A = number of neurons activated only after the 1st stimulus (Histamine)

B = number of neurons activated only after the 2nd stimulus (Histamine or Formalin)
C = number of neurons activated after both the 1st and 2nd stimuli

Thus, in Figs. 3e and 3g, the Y-axis is labeled as "Overlapping population (% reactivated)," which is different from the Y-axis title in Figs. 2d and 2e.

Furthermore, the numbers they provide do not make sense – based on Fig. 3g there were a total of 473+128 = 601 itch-activated neurons and 131 + 385 = 516 pain-activated neurons, but in the legend they state that there were a total of 2006 neurons that were activated 'only after' the stimuli were delivered. If the authors really want to claim that the neurons encoding a specific modality represent a dynamic subset within a larger modality-specific population, then they need to provide enough information to test whether that claim is mathematically plausible – specifically the total number of neurons imaged, as well the numbers that fall into each category (itch or pain responsive neurons).

Response #4**: We apologize for any confusion caused by the previous version of our analysis. We discovered that some mice were inadvertently omitted from the machine learning and frequency analysis sections (Fig. 3h, Extended Data Fig. 7: Machine Learning Analysis, Extended Data Fig. 6: Frequency Analysis) when using miniscope data, with the exception of the overlap analysis. To address this, we have conducted additional analyses and updated the corresponding figures accordingly. We regret any confusion this oversight may have caused and appreciate the opportunity to correct it.**

And they need to do appropriate negative controls to estimate the overlap due to spontaneous activity. In declining to do negative controls, the authors wrote: "This led us to perform miniscope experiments, where we analyzed neurons activated only after a stimulus and observed results similar to those of experiments using TetTag mice. Producing TetTag mice is time-consuming, and we believe that some of your concerns have been addressed through the miniscope experiment." Indeed, the authors did see similar results using the miniscope experiment and the TetTag mice – namely that the level of overlap was similar for itch-itch and itch-pain cells. But this does not "address" my concerns, rather it reinforces my concern that the populations of potential itch and pain cells must be so large that they are highly overlapping and do not represent distinct modality-specific populations as the title of the paper claims. Unfortunately, I cannot endorse publication without providing the cell counts and negative controls which would support the conclusion that is explicitly stated in the title.

Response #5**: Following your suggestion, we conducted additional analyses of the miniscope data to estimate spontaneous activity. We initially focused on neurons activated 'only before' the stimulus, which respond to the chamber where the mice were located during *in vivo* live imaging. Our findings showed that the overlap at a 6-h interval was significantly higher than at a 3-day interval, regardless of the type of two consecutive stimuli. This suggests that certain**

ACC neurons previously activated by the environment become more readily activated when re-exposed to the same environment within a short time window (Extended Data Fig. 5a-c).

Next, we identified neurons activated 'both before and after' the stimulus as exhibiting spontaneous activity, as these neurons continued to activate irrespective of the stimulus. We observed that the reactivation ratio of neurons activated by histamine remained consistent, regardless of the type or interval of the subsequent stimulus (Extended Data Fig. 5d-f). More importantly, including 'only after' neurons (considered stimulus-specific) in the analysis of 'both before and after' neurons did not yield the same overlap results as when analyzing 'only after' neurons alone (Extended Data Fig. 5g-i). This indicates that neurons showing spontaneous activity are not part of the population that responds to both stimuli of different modalities within a short time interval (6 h).

Finally, based on your feedback and our further analysis, we have updated the title from "Cortical processing of pain and itch information by distinct neuronal populations" to "Independent processing of pain and itch information in the anterior cingulate cortex." We believe that the term 'independent' more accurately reflects the findings of our study.

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REVIEWER COMMENTS

Reviewer #3 (Remarks to the Author):

In Response #1 the authors state that the overlap between Itch & Itch is higher than between Itch & Pain at 3-day intervals but not at 6-hr intervals. Furthermore, at short time intervals the encoding of itch and pain are more overlapping than expected by chance. These findings contradict the title "Independent processing of pain and itch information in the anterior cingulate cortex" as they directly shows that this processing is more overlapping than expected by chance, and hence not independent. More than not independent, they are actually indistinguishable at short intervals using the authors' methodologies. These findings also contradict the section of the Abstract which reads "Here we identified distinct neuronal populations related to pain and itch processing in layer II/III of the ACC. These include neurons activated by both itch and pain stimuli separated by a short time interval and modality-specific neurons activated only by either itch or pain stimuli regardless of the interval between them."

Later in Response #1, the authors write that for miniscope experiments, "we do not have information on the absolute size of activation properties of the neuronal populations processing itch and pain stimuli." The authors are using the "only after" neurons to identify neurons which respond to each stimulus. The authors seem to imply that the existence of cells which are not active during the experiment makes any sort of calculation impossible, but this is not correct. It is true that one cannot compute the chance level of overlap, but one can still compute the level of overlap to see if it is different between the itch & itch vs. itch & pain cases. The authors suggest that this would be problematic if the size of the neuronal population responding to pain is much greater than that which responds to itch, e.g., possibly because they are using inappropriate doses of histamine or formalin. But the authors have already shown that this is not the case because similar numbers of neurons respond (based on their definition of respond) to their histamin vs. formalin injections based on the Fig. 3 legend (69.1 vs. 82.5 neurons per session). Furthermore, the authors find almost exactly the same numbers of neurons are active "both before and after" in both sets of experiments (35 vs. 33 neurons/session), which suggests that the total number of neurons being imaged is relatively stable across sessions. In fact, if the authors are extremely concerned about the unknown number of total neurons being potentially variable they could use the number of "both before and after neurons" to normalize the other counts on a per session basis.

Later in their response #2 the authors state that they believe I made a calculation error by calculating conditional probabilities. The authors are incorrect -- my calculations were done the way the authors are saying they should be done. Specifically, I stated that if the overlap of itch-itch and itch-pain cells is 1%, then about 10% of the population might be activated by each stimulus. This is because 0.1 * 0.1 = 0.01. This assumes independence not conditional probabilities. Similarly if the total pool of potential itch responsive cells is 50% and 1/5 are activated by each stimulus,

then the fraction within this pool that is repeatedly activated by two stimuli would be 1/5 * 1/5 = 1/25 = 4% of this pool = 2% of the total neuronal population. Again this is assuming independence not conditional probabilities. I wrote this quickly so I apologize if there were minor typos / some of the text was ambiguous or unclear. These are relatively simple calculations so hopefully the basic idea is clear.

Finally, in Response #5, the authors seem to be saying that analyzing the “both before and after” + “only after” cells does not show a difference between activity at 6h vs. 3d whereas examining just “only after” does show a difference. Based on this they conclude that neurons showing spontaneous activity are not part of the population that responds to both modalities within 6h. I don’t really understand this argument because spontaneous activity might occur sparsely in time, i.e., it need not occur during both the before and after period. Regardless, I don’t see how this would mitigate my concern that 1) overlap between itch + pain is higher than chance in Figure 2f, 2) overlap between itch + pain is not significantly different from overlap between itch + itch in Figure 2f, 3) overlap between itch + pain is actually higher than overlap between pain + itch in Figure 3e,g. Even if one supposes that pain activates slightly more neurons than itch based on the slightly larger size of the ‘only after’ population for formalin (82.5 neurons/session) vs. histamine (69.1 neurons/session), the pain-activated population is only ~19% larger for pain than itch. Given the much larger overlap (7%) for itch + pain in Figure 3e compared to the level (4%) for itch + itch in Figure 3g, this is still inconsistent with the idea that itch and pain are encoded by largely distinct populations.

REVIEWER COMMENTS

Reviewer #3 (Remarks to the Author):

In Response #1 the authors state that the overlap between Itch & Itch is higher than between Itch & Pain at 3-day intervals but not at 6-hr intervals. Furthermore, at short time intervals the encoding of itch and pain are more overlapping than expected by chance. These findings contradict the title “Independent processing of pain and itch information in the anterior cingulate cortex” as they directly shows that this processing is more overlapping than expected by chance, and hence not independent. More than not independent, they are actually indistinguishable at short intervals using the authors' methodologies. These findings also contradict the section of the Abstract which reads “Here we identified distinct neuronal populations related to pain and itch processing in layer II/III of the ACC. These include neurons activated by both itch and pain stimuli separated by a short time interval and modality-specific neurons activated only by either itch or pain stimuli regardless of the interval between them.”

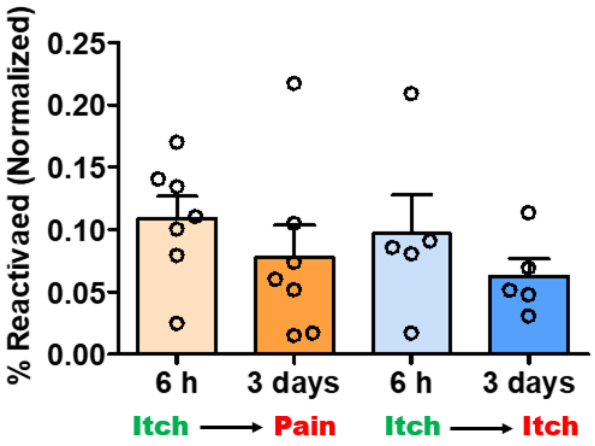
Response #1**: We apologize for any confusion caused by our inaccurate wording. In response to your comments, we have removed the sentence you pointed out in the Abstract, as it was not necessary for the main narrative of our study. Our study, which includes GRASP and behavioral experiments, focuses on modality-specific neurons, not on neurons activated by both itch and pain stimuli separated by a short time interval. Consequently, we have also modified the title from “Independent processing of pain and itch information in the anterior cingulate cortex” to “Processing of pain and itch information by modality-specific neurons in the anterior cingulate cortex.”**

We are currently investigating neurons activated by two different stimuli when presented in close succession as part of a separate project. As such, this manuscript specifically addresses neurons that are either pain- or itch-specific. In Fig. 2d, we show that the overlap between itch- and pain-specific neurons at a 3-day interval does not exceed chance level, supporting the existence of distinct itch-specific and pain-specific neurons. This assumption is further reinforced by Fig. 2e, which demonstrates that the overlap between itch-specific neurons over a 3-day interval is above chance level.

Moreover, our behavioral experiments provide critical evidence for the specificity of pain- and itch-related neurons. We demonstrated that inhibiting ACC neurons activated by algogens reduced pain without affecting itch, and inhibiting ACC neurons activated by pruritogens reduced itch without impacting pain (see Fig. 5 and Extended Data Fig. 11).

Later in Response #1, the authors write that for miniscope experiments, “we do not have information on the absolute size of activation properties of the neuronal populations processing itch and pain stimuli.” The authors are using the “only after” neurons to identify neurons which respond to each stimulus. The authors seem to imply that the existence of cells which are not active during the experiment makes any sort of calculation impossible, but this is not correct. It is true that one cannot compute the chance level of overlap, but one can still compute the level of overlap to see if it is different between the itch & itch vs. itch & pain cases. The authors suggest that this would be problematic if the size of the neuronal population responding to pain is much greater than that which responds to itch, e.g., possibly because they are using inappropriate doses of histamine or formalin. But the authors have already shown that this is not the case because similar numbers of neurons respond (based on their definition of respond) to their histamin vs. formalin injections based on the Fig. 3 legend (69.1 vs. 82.5 neurons per session). Furthermore, the authors find almost exactly the same numbers of neurons are active “both before and after” in both sets of experiments (35 vs. 33 neurons/session), which suggests that the total number of neurons being imaged is relatively stable across sessions. In fact, if the authors are extremely concerned about the unknown number of total neurons being potentially variable they could use the number of “both before and after neurons” to normalize the other counts on a per session basis.

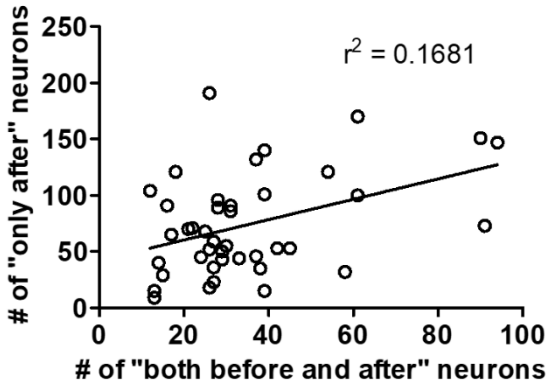
Response #2**: As you mentioned, the total number of neurons being imaged remains relatively stable across sessions. Thus, we normalized overlapping population (% reactivated) by the number of “both before and after neurons” per session following your suggestion (Please see the graph below).**



However, we are concerned that this may not be a universally applicable approach. We found no references to this type of normalization in in vivo miniscope imaging studies, and we question the validity of using the number of "both before and after neurons" count as a reliable normalizing factor. Unlike studies with TetTag mice, in miniscope experiments, not all neurons within the imaging field express GCaMP, making it challenging to assert that the number of "both before and after neurons" truly represents the unknown total number of neurons.

Even if all neurons within the imaging field expressed GCaMP, there is no evidence that an

increase in "both before and after" neurons correlates with an increase in "only after" neurons. In fact, a correlation analysis of the number of "both before and after" and "only after" neurons per session showed no significant relationship between them (see the graph below). We believe that this lack of correlation contributed to the large variation observed in the "overlapping population (% reactivated)" presented above.



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Response #3**: We apologize if we misunderstood your comment. Your probability calculations appear correct. In Extended Data Fig. 2 and Source Data Excel file, we presented the actual numbers of H2BGFP(+) cells and Fos (+) cells (%DAPI). If the overlap of "itch-itch" cells is 1%, then theoretically, approximately 10% of the population should be activated by each itch stimulus. However, in the TetTag mouse system, the expression of H2BGFP in response to the first stimulus is typically lower than the endogenous Fos expression in response to the second stimulus. As a result, this TetTag-based labeling system should be used minimally only to determine the dependence of two events. In other words, it is challenging to use this TetTag mice system to calculate the absolute probability of occurrence of an individual event.**

Finally, in Response #5, the authors seem to be saying that analyzing the "both before and after" + "only

after" cells does not show a difference between activity at 6h vs. 3d whereas examining just "only after" does show a difference. Based on this they conclude that neurons showing spontaneous activity are not part of the population that responds to both modalities within 6h. I don't really understand this argument because spontaneous activity might occur sparsely in time, i.e., it need not occur during both the before and after period. Regardless, I don't see how this would mitigate my concern that 1) overlap between itch + pain is higher than chance in Figure 2f, 2) overlap between itch + pain is not significantly different from overlap between itch + itch in Figure 2f, 3) overlap between itch + pain is actually higher than overlap between pain + itch in Figure 3e,g. Even if one supposes that pain activates slightly more neurons than itch based on the slightly larger size of the 'only after' population for formalin (82.5 neurons/session) vs. histamine (69.1 neurons/session), the pain-activated population is only ~19% larger for pain than itch. Given the much larger overlap (7%) for itch + pain in Figure 3e compared to the level (4%) for itch + itch in Figure 3g, this is still inconsistent with the idea that itch and pain are encoded by largely distinct populations.

Response #4**: We agree with your comments that spontaneous activity might occur sparsely in time, meaning it need not occur during both the before and after periods. However, it seems reasonable that the "only after" group of neurons would include a larger number of pure stimulus-responsive neurons compared to those in the "both before and after" + "only after" groups combined.**

Regarding your concerns 1) and 2), we explored methods to normalize groups that were initially non-comparable, allowing us to analyze them as per your suggestion. We had no prior bias before the reanalysis shown in Fig. 2F; rather, we aligned with your perspective and found a method for a meaningful comparison.

On concern 3), we addressed this in our initial point-by-point response. "*Part of this discrepancy can be attributed to the low absolute number of overlapping cells. However, our findings are consistent with prior research, which suggests that ACC neurons represent stimuli dynamically over time, rather than in a fixed manner; see Acuna et al., PNAS, 2023*." This dynamic representation of stimuli was also confirmed in additional experiments (Extended Data Fig. 12).

We believe that even if modality-specific neurons exist, the absolute percentage of overlap is not particularly meaningful if only a small fraction of neurons exhibit a dynamic activation pattern upon stimulus. Instead, the key evidence supporting the conclusion that itch and pain are encoded by largely distinct neural populations comes from our behavioral experiments. As stated in Response #1, we observed that inhibiting ACC neurons activated by formalin specifically reduced formalin-induced pain without affecting histamine-induced itch. Likewise, inhibiting neurons activated by histamine reduced itch without affecting pain (see Fig. 5). To interpret these results, we find no better explanation than to postulate that itch and pain are encoded by distinct neuronal populations.