nature portfolio

Peer Review File

A population of Insula neurons encodes for social preference only after acute social isolation in mice

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REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

The manuscript by Glangetas et al. investigates the role of insula neurons, which project interhemispherically, in socio-emotional processing. The authors first characterize insula interhemispheric neurons anatomically by different viral tracing strategies as well as confirming their interhemispheric projection profile by in vitro electrophysiological recordings. The authors find that a subset of insula layer II/III neurons that or non-GABAergic, project to the contralateral hemisphere as well as to the CeA and the dlBNST, may be characterized by the expression of Satb2 and possess unmyelinated axons. The authors then ablate these neurons and test the functional role of Satb2 neurons in anxiety and social preference tests. The authors try to conclude that it is the interhemispheric projection profile that may be key for the functionality of Satb2+ neurons.

The manuscript is interesting and especially the anatomical characterization is novel. In my opinion, it would be key to characterize this neuronal subset a bit more closely to find out whether all neurons have collaterals to both CeA and dlBNST or whether there is heterogeneity in this subset. This may be particularly important for the functional characterization. I also have major concerns regarding the conclusions about how these neurons may influence social / emotional behavior due to the unspecific neuronal ablation approach.

In the following, I list a few major and minor concerns that I feel should be addressed prior to publication.

Major concerns:

1) The tracing strategy is an integral part of the paper since it lays the foundation for the investigation. For this reason, I would suggest to present the viral approach as part of the main Fig.1 and not as a Suppl.

2) If I understand correctly, neither the anterograde nor the retrograde AAV tracings are ‘monosynaptic’ as written in the manuscript but rather these viruses do not jump a synapse – they only show the axonal projections of the insula neurons either antero- or retrogradely - this would not qualify as transynaptic? Please adapt or explain.

3) The use of Satb2 as molecular marker for this population comes very surprising. How did the authors identify this as a potential marker? Are all layer 2 neurons either Sab2 or Ctip2?

It would be nice to have a more precise characterization of this population. How many of all LII/III neurons express either Satb2 or Ctip2? How many neurons of all LII/III neurons are interhemispheric? Is the Satb2 population heterogenic? This would be particularly important with regards to the later ablation. The authors could express a Cre-dependent AAV in Satb2+ neurons: what would be the projection targets? Only the contralateral hemisphere, CeA and dlBNST – or are there potentially other targets? Does a Satb2+ neuron projecting to the CeA automatically also project interhemisperically and to the dlBNST? I am afraid that without attempting to answer some of these questions the functional ablation experiments are hard to interpret, and also the usefulness of the description of this novel subpopulation, which is very interesting, would not be complete.

4) The authors interpret their genetic ablation as 'split insula' mice. However, I feel that any of the observed effects could be due to the selective ablation of an insula-to-CeA and/ or insula-to-dlBNST projection. One way and potentially the best way would be to perform optogenetic experiments expressing an inhibitory opsin Cre-dependently in Satb2+ insula neurons and then inhibiting the axon terminals either in CeA, dlBNST or in the contralateral insula specifically. I do not see how otherwise the observed effects can be interpreted specifically and the conclusions the authors try to draw could be drawn.

Minor Concerns:

The figure layout seems in places disrupted – potentially by the conversion to PDF? Also labelings are mirrored. Please correct.

Reviewer #2 (Remarks to the Author):

This is an interesting and timely work refining evidence of Insula participation in social behavior. The anatomical and electrophysiological profile of the Insula interhemispheric circuit are well conducted. Mostly for the behavioral part I have some suggestions listed below.

- The title is a bit misleading. If the authors want to push the effect on "social preference" more controls and better investigation on the social behavior should be performed. Moreover, the effects of the lesion seems to be present only after social isolation… thus it

is not a general effect on social preference but something else is going on that with current data is difficult to interpret. Finally, if authors wants to state that these neurons “encode” social preference, in vivo recordings during social explorations must be reported.

- In abstract I would take out “cutting-edge” and give more details on methods used.

- Abstract, point number 3 is too vague and it would be nice to already state here the main results.

- How can authors infer the effects with caspase viral strategy specifically depend only on contralateral insula projections and not also from these neurons’ collaterals to dlBNST and CeA? This should be addressed.

- How can authors infer the effects with caspase viral strategy are selective to insula projections and not compensatory of the altered circuit for such prolonged time? This should be addressed.

- For behavioral experiments, current interpretation is quite difficult. As the lesion is done before the entire test start, and effects are present only after isolation, it might be that there is an effect during the social isolation period and not directly during the 3 chamber test. I would recocmend to add an optogenetics silencing experiment targeting specifically only the time of behavioral testing. In any case, some behavioral observation during the isolation period might also help to better understand these results.

- The reduced interest in social stimulus only after acute social isolation might be a decreased general motivational state after the 24hr acute isolation compared to not-lesioned mice. This should be checked.

- Authors should report motor and other stereotypy (e.g. grooming, thigmotaxic, stretching behaviors etc.) data during the social test, to control for possible confounding factors.

- Discussion, the link with autism is not clear… indeed if that was the case there should be a basal reduction of social interest, not only after single housing.

- Discussion. Not sure from which evidence this statement is supported: “we demonstrated that the interhemispheric neurons occupy a privileged position in synchronizing the activity of the Insula/CeA/dlBNST network in the two hemispheres.”

- This sentence is also misleading and require further experiments to prove this “selectivity”: “all of these data suggest that interhemispheric neurons in the Insula play a specific role for social processing”. It could come from a number of different processes… and if it was selective for social should be evident already at basal condition not only after isolation, in my opinion.

- Some experiments, only one critical one in female mice at this point is due.

Reviewer #3 (Remarks to the Author):

Glegantas et. al., present a manuscript titled “Encoding social preference by interhemispheric neurons in the Insula.” The manuscript explores a unique interhemispheric insular circuit using an array of anatomical tracing techniques, functional in vivo and ex vivo physiological characterization, and behavioral assays to assess the role of this pathway in social and anxiety-like behaviors. The manuscript is well-written, focuses on a novel, understudied circuit using innovative approaches. While the topic is certainly of interest to the broader scientific community, in its current form, the manuscript seems somewhat disjointed and at times the rationale for why certain experiments were performed is not well articulated. To this point, what is missing is how unique this interhemispheric circuit is compared to other insular circuits involved in social behavior. In addition, the authors draw strong conclusions that are not necessarily supported by the viral approaches they used, and the methodology, approach, and statistical comparisons are not clearly articulated or rationalized for several experiments. Given the exciting topic and innovative approach, I am overall enthusiastic about this manuscript, however the experiments need to be fleshed out much more to convince the reader that this circuit is important for regulating social behavior. Detailed comments are below:

1) The tracing data in figure 1 is somewhat over interpreted. With the retrograde-Cre/anterogradeDIO-eYFP approach, the authors are labelling interhemispheric neurons, but fiber expression in the BNST and CeA does not indicate that these neurons are innervating these brain areas. The fluorescence observed could just be fibers passing through this brain areas. To conclude innervation, the authors need to use a strategy that distinguishes terminals from fibers, such as a monosynaptic rabies-based approach or an AAV with a synaptophysin promoter.

a. The use of the word "massively" is a bit strong and subjective. Similar language is used in several places throughout the manuscript (e.g, point 5 below). Magnitude of the effects should be interpreted based on the statistical effect.

2) There are very few details in the methods or results on how projections were quantified (Figure 1g). Was there an arbitrary threshold? It seems unreasonable to quantify a percentage of total insula interhemispheric projections when only three coronal sections of the brain are being analyzed. Similarly, how was colocalization determined in figure 1m-q? Was all fluorescent quantification done manually? More transparency would be useful.

3) A retrograde Cre virus injected into the insula of an ai9 mouse should label all projection cells into the insula. Why does only the contralateral insula express tdtomato when there are several studies documenting insula inputs from sensory cortical areas, for example, which are seen in that same section shown in Figure 1. Showing cell bodies in other known upstream brain areas with insula projections would help validate the viral approach.

a. In line with this, two weeks is a short amount of time to allow for transduction and retrograde transport of a retrograde AAV. Do the authors have any evidence that the viruses have fully reached the target cells by this time? What is the justification for waiting longer for anterograde viruses? Could the lack of expression in other upstream regions be attributed to a lack of full transduction?

4) The rationale for performing several experiments could be improved throughout the main text. Specifically, why did the authors perform the experiment to look at colocalization of

interhemispheric insula cells with Satb2 and Ctip2? Is the lack of Ctip2 co-expression unique to this circuit? A negative control experiment showing that this colocalization pattern is unique, and not uniform to the entire insula would be a great addition.

a. Was there layer specificity or differences between insula subregions (anterior, posterior, intermediate?)

5) The authors determine that the insula neurons are “exclusively pyramidal” because there is no colocalization with GAD67 or PV. Without further studies, it is an overstatement to say that this experiment “confirms” that these neurons are pyramidal. The whole cell electrophysiological experiments with channelrhodopsin-assisted circuit mapping start to get at this question, but what are the intrinsic properties of these specific neurons, not just the projection onto general insular neurons. Comparing intrinsic physiological properties of insula-insula cells with non insula-insula cells would not only provide stronger evidence that these neurons are pyramidal, but this comparison could highlight a unique characteristic of these neurons.

6) Why did the authors perform the caspase studies with CeA and insula and not CeA, insula, and BNST? Or just insula? The authors state that they targeted the “two main outputs of insula interhemispheric neurons”, but this is not accurate according to figure 1g. If anything, BNST looks to be a more substantial output than CeA. In addition, with this approach the authors are isolating more than just interhemispheric neurons as you are targeting all insula projections to the CeA and all interhemispheric projections. It would be interesting to see just insula-insula caspase, and even insula-BNST/insula-insula projections too.

7) The statistics and conclusion in figure 3f-m are confusing. In 3j, the authors show significant differences in time spent with the social chamber vs the object chamber in ctrl mice, and differences between caspase and ctrl mice in time spent at the social chamber. This data is then transformed into preference in 3k, but different statistics were run and now the caspase group is not significantly different than the ctrl group? More clarity is needed.

8) The methods for social interaction test state that the group housed mice were subjected to either a juvenile mouse or a chamber filled with lego toys, whereas the isolated mice had

the choice between an empty chamber and one with a juvenile mouse. What is the explanation for this discrepancy?

9) Social isolation generally takes a week or more to have effects on c57 mice. It's possible the impact of this circuit would be more prevalent after prolonged social isolation. It would be interesting to see how insula interhemispheric neurons regulate social interaction after prolonged isolation.

a. While the within subject design is good, the mice are exposed to the social interaction test multiple times within a short period of time. This is a bit of a confound. The authors should examine how social isolation right after surgery would impact social interaction.

10) The major impactful finding of this manuscript is that this circuit plays a role in social interactions, but this conclusion is drawn from one experiment that uses somewhat of a sledgehammer approach with caspase. While I think the model is sound, it would be more impactful to see the opposite- would social interaction be increased with chemogenetic or optogenetic activation of these neurons? This type of experiment could be really interesting in the context of ASD and studies to that seek to increase social interaction.

11) The figure legend provides no information on what the symbols mean. For example, in figure 3, what is the difference between *** and ###?

Point-by-point to the reviewers.

Reviewer #1 (Remarks to the Author):

The authors try to conclude that it is the interhemispheric projection profile that may be key for the functionality of Satb2+ neurons. The manuscript is interesting and especially the anatomical characterization is novel. In my opinion, it would be key to characterize this neuronal subset a bit more closely to find out whether all neurons have collaterals to both CeA and dlBNST or whether there is heterogeneity in this subset.

We are grateful to Reviewer 1 for providing constructive feedback and offering a positive overall evaluation of our work. In response to the important concern regarding the heterogeneity of insula neuronal populations, we conducted intracerebral injections in mice, specifically targeting either the insula contra (INS->INS neuronal population) or the CeA (INS-CeA neuronal population) using a retrograde strategy. For both strategies, the cre-dependent EYFP viruses were injected in the ipsi-Insula. The similarity in the distribution of axonal collaterals among the insula, ovBNST, and CeA implies that the $INS^{->INS}$ and $INS^{->CeA}$ neuronal populations are part of the same neuronal sub-population. Details of these experiments are now reported in Supplemental Figure 3.

I also have major concerns regarding the conclusions about how these neurons may influence social / emotional behavior due to the unspecific neuronal ablation approach.
In the following, I list a few major and minor concerns that I feel should be addressed prior to publication.

Major concerns:
1) The tracing strategy is an integral part of the paper since it lays the foundation for the investigation. For this reason, I would suggest to present the viral approach as part of the main Fig.1 and not as a Suppl.
We have followed the suggestion of reviewer #1. Figure 1 now comprehensively displays all retrograde and anterograde virus-based tracing approaches that specifically target the INS-INS neuronal population.

2) If I understand correctly, neither the anterograde nor the retrograde AAV tracings are 'monosynaptic' as written in the manuscript but rather these viruses do not jump a synapse – they only show the axonal projections of the insula neurons either antero- or retrogradely - this would not qualify as transynaptic? Please adapt or explain.

We appreciate reviewer 1 for bringing up this point. In response, we have provided clarification in the manuscript, specifying that all viral approaches utilized in our study are based on the application of rAAV2-retro, developed by the group of A.Y. Karpova, which enables robust retrograde access to projection neurons. (10.1016/j.neuron.2016.09.021).

The use of Satb2 as molecular marker for this population comes very surprising:

- How did the authors identify this as a potential marker?

SATB2 has been identified as a key regulator of cortical development and is involved in the determination of neuronal identity during brain development. To support our choice to use SATB2 has a potential marker of INS->INS neurons, we have now included relevant citations in the manuscript.

Are all layer 2 neurons either Sab2 or Ctip2?

In response to reviewer 1's question, we conducted new immunohistochemistry experiments with corresponding quantifications to address this point. The findings are detailed in Supplemental Figure 2, revealing that the majority of layer 2 neurons are either SATB2 or CTIP2. However, approximately a

quarter of neurons express both SATB2 and CTIP2, and a small proportion exhibit neither SATB2 nor CTIP2 expression.

- It would be nice to have a more precise characterization of this population. How many of all LII/III neurons express either Satb2 or Ctip2?

We have answered this question in the new experiments reported in supplemental Figure 2. Our quantifications show that 60% of neurons in layers II/III are SATB2 and 50% are CTIP2.

- How many neurons of all LII/III neurons are interhemispheric?

Addressing the question of how many neurons among all LII/III neurons are interhemispheric poses a challenge. Tracing approaches inherently introduce bias, as only neurons labeled by the tracer can be identified as interhemispheric. Since it is not feasible to inject the tracer into the entire insular cortex, the lack of labeling does not necessarily imply that those neurons are not interhemispheric. The limitation lies in the inability to capture all interhemispheric connections due to the selective nature of tracing methods.

- Is the Satb2 population heterogenic? This would be particularly important with regards to the later ablation.

We thank our reviewer 1 for raising this important point. To address the heterogeneity within the Satb2 population, we conducted quantifications of colocalization (confocal imaging) between a retrograde marker (CTb) and Satb2 in layer 2/3 of the insula. This analysis was performed on three groups of mice injected with a retrograde tracer in different regions: the contralateral insula (INS->INS neurons), ipsilateral tail of the striatum (INS->tail of the striatum neurons), or the basolateral amygdala (INS->BLA neurons). The results of these novel experiments, detailed in supplemental Figure 4, led us to the conclusion that SATB2 is not a specific marker for INS-INS neurons. Importantly, this conclusion does not compromise our selective genetic ablation strategy, which is based on the neuronal target rather than the expression of the molecular marker SATB2.

- The authors could express a Cre-dependent AAV in Satb2+ neurons: what would be the projection targets?

  We appreciate the suggestion from the reviewer regarding the expression of a Cre-dependent AAV in Satb2+ neurons. However, as clarified in response to previous comments, while 96% of $INS^{->INS}$ neurons are SATB2 positive (Fig. 2), SATB2 cannot be regarded as an exclusive marker for $INS^{->INS}$ neuronal populations (Supplemental Fig. 4). Consequently, employing a viral strategy specifically targeting SATB2 neurons would lack specificity for targeting the $INS^{->INS}$ neuronal population.

- Only the contralateral hemisphere, CeA and dlBNST – or are there potentially other targets?

To address this inquiry, we conducted an unbiased whole-brain quantification of all synaptic targets of $INS^{->INS}$ neurons utilizing an anterograde synaptic marker (synaptophysin) (Figure 1 + Supplemental Fig 1). The quantification revealed that the primary synaptic targets of the INS-<INS population include the ipsi and contralateral dorsolateral BNST (JuxtaBNST+ovalBNST), ipsi and contralateral CeA, and contralateral insula (Figure 1). However, our unbiased whole-brain quantification unveiled additional axonal targets, as depicted in Supplemental Figure 1.

- Does a Satb2+ neuron projecting to the CeA automatically also project interhemisperically and to the dlBNST?

We have addressed this significant concern regarding the heterogeneity of insula neuronal populations in the initial comment of this point-by-point letter. The results from our recent experiments, outlined in Supplemental Figure 3, strongly indicate that the $INS^{->INS}$ and $INS^{->CeA}$ neuronal populations are part of the same neuronal sub-population.

I am afraid that without attempting to answer some of these questions the functional ablation experiments are hard to interpret, and also the usefulness of the description of this novel subpopulation, which is very interesting, would not be complete.

We appreciate the reviewer's insightful comments and recognize the importance of addressing the questions raised to enhance the interpretation of our functional ablation experiments. The new set of experiments, as detailed in our responses and supplemental figures, allowed us to demonstrate that: 1) $INS^{->INS}$ neurons have a complex axonal arborization, 2) SATB2 are expressed in almost all $INS^{->INS}$ neurons, and 3) SATB2 could not be considered a marker specific to $INS^{->INS}$ neurons since it was also expressed in other insula populations. We hope these additional experiments will further reinforce the positive impression the reviewer has on our study and contribute to the completeness of the description of this novel subpopulation

The authors interpret their genetic ablation as ‘split insula’ mice. However, I feel that any of the observed effects could be due to the selective ablation of an insula-to-CeA and/ or insula-to-dlBNST projection. One way and potentially the best way would be to perform optogenetic experiments expressing an inhibitory opsin Cre-dependently in Satb2+ insula neurons and then inhibiting the axon terminals either in CeA, dlBNST or in the contralateral insula specifically.

I do not see how otherwise the observed effects can be interpreted specifically and the conclusions the authors try to draw could be drawn.

All the important questions raised by reviewer 1 logically lead to the suggestion of an experiment whose objective would be to target INS-INS neurons with the SATB2 molecular marker and determine which axonal target would support the observed behavioral effects. The conclusions reached by the experiments suggested by reviewer 1 do not detract from the relevance of reviewer 1's suggestion, but they do render the experimental design inapplicable. Firstly, we showed that SATB2 could not be used as a molecular marker to specifically target INS-INS neurons (supplemental figure 4). Secondly, anterograde tracing with synaptophysin highlighted the complexity of axonal arborization in the INS-INS neural population, with 5 major targets having high synapse density and over 15 targets showing low synaptic density. In accordance with the reviewer's suggestion and on the basis of this complex axonal arborization of INS-INS neurons we have removed from the manuscript our interpretation of the selective genetic ablation of INS-INS neurons as "split insula" mice.
These new conclusions obtained on the basis of the experiments suggested by reviewer 1 , confirm the choice of our strategy to target the INS-INS population in its entirety rather than selectively manipulating each axonal target. We have now pointed out in the discussion the limitation in the interpretation we can make of our results concerning the identification of potential axonal targets at the origin of the behavioral effects observed.

All the pertinent questions posed by reviewer 1 logically lead to the suggestion of an experiment aiming to target $INS^{->INS}$ neurons using the SATB2 molecular marker and determine which axonal target supports the observed behavioral effects. The conclusions drawn from the experiments proposed by reviewer 1 do not diminish the relevance of the suggestion, but they render the experimental design too challenging. Firstly, we demonstrated that SATB2 could not serve as a molecular marker to specifically target $INS^{->INS}$ neurons (see supplemental figure 4). Secondly, anterograde tracing with synaptophysin highlighted the complexity of axonal arborization in the $INS^{->INS}$ neural population, revealing five major targets with high synapse density

and over 15 targets exhibiting low synaptic density. In accordance with the reviewer's advice and based on the intricate axonal arborization of $INS^{->INS}$ neurons, we have removed from the manuscript our interpretation of the selective genetic-ablation of INS-INS neurons as "split insula" mice. The new conclusions drawn from the experiments suggested by reviewer 1 affirm our decision to target the entire $INS^{->INS}$ population rather than selectively manipulating individual axonal targets. In the discussion, we have now highlighted the limitation in interpreting our results regarding the identification of potential axonal targets at the origin of the observed behavioral effects.

Minor Concerns:
The figure layout seems in places disrupted – potentially by the conversion to PDF? Also labelings are mirrored. Please correct.

This has been corrected

Reviewer #2 (Remarks to the Author):

This is an interesting and timely work refining evidence of Insula participation in social behavior. The anatomical and electrophysiological profile of the Insula interhemispheric circuit are well conducted. Mostly for the behavioral part I have some suggestions listed below.

We are grateful to Reviewer 2 for providing constructive feedback and offering a positive overall evaluation of our work.

- The title is a bit misleading. If the authors want to push the effect on “social preference” more controls and better investigation on the social behavior should be performed. Moreover, the effects of the lesion seems to be present only after social isolation… thus it is not a general effect on social preference but something else is going on that with current data is difficult to interpret.
Finally, if authors wants to state that these neurons “encode” social preference, in vivo recordings during social explorations must be reported.

We would like to express our gratitude to reviewer 2 for the valuable suggestions provided. We have assessed the impact of genetic ablation of $INS^{->INS}$ neurons on mouse motivation for a natural reward (Supplemental Figure 10) and anxiety state (Supplemental Figure 9). The results from these control experiments indicate that the selective genetic ablation of $INS^{->INS}$ neurons does not influence the animals' motivation for a natural reward, nor does it alter their anxiety state. Additionally, we conducted a fiber photometry experiment, demonstrating that $INS^{->INS}$ neurons undergo activity changes during the discrimination process between an object and a juvenile mouse (Figure 5 and Supplemental Figure 11). Based on these experiments, we propose the following revised title, which we believe is less misleading and accurately reflects our conclusions: "**A population of insular neurons encodes social preference during social isolation.**"

- In abstract I would take out “cutting-edge” and give more details on methods used.

We have followed the reviewer's suggestions.

- Abstract, point number 3 is too vague and it would be nice to already state here the main results.

We have followed the reviewer's suggestions.

- How can authors infer the effects with caspase viral strategy specifically depend only on contralateral insula projections and not also from these neurons’ collaterals to dlBNST and CeA? This should be addressed.

The aspect of collaterals is a particularly important point that was also raised by reviewer 1. We justified, in our responses to reviewer 1's inquiries, that it was crucial to discuss the rationale behind manipulating the entire INS population, considering its comprehensive axonal complexity. Indeed, our anterograde tracing experiment with synaptophysin, coupled with unbiased whole brain mapping, emphasized the complexity of axonal arborization in the $INS^{->INS}$ neural population, revealing five major targets with high synapse density and over 15 targets exhibiting low synaptic density. In the discussion, we have now highlighted the limitation

in interpreting our results concerning the identification of potential axonal targets at the origin of the observed behavioral effects.

- How can authors infer the effects with caspase viral strategy are selective to insula projections and not compensatory of the altered circuit for such prolonged time? This should be addressed.

We thank reviewer 2 for their particularly important suggestion. To address this point, we implemented a chemogenetic experiment to selectively inhibit the $INS^{->INS}$ population, only during the behavioral task (supplemental figure 8). This chemogenetic manipulation proved to have no effect on social preference after isolation. Comparing the results obtained between the selective genetic ablation of the INS-INS population and the chemogenetic manipulation of the same population allows us to conclude that the alterations in the pathway established during the 24 hours of isolation are crucial for controlling social preference.

- For behavioral experiments, current interpretation is quite difficult. As the lesion is done before the entire test start, and effects are present only after isolation, it might be that there is an effect during the social isolation period and not directly during the 3 chamber test. I would recocmend to add an optogenetics silencing experiment targeting specifically only the time of behavioral testing.

We thank reviewer 2 for this comment, which aligns with the previous feedback. Given the relatively slow temporal dynamics (on the order of tens of seconds to a minute) of events in the three-chamber test, we chose to implement a chemogenetic strategy to inhibit the $INS^{->INS}$ population during the behavioral testing (supplemental figure 10). As mentioned above, the difference in results between the chemogenetic experiment and the selective genetic ablation of the the $INS^{->INS}$ population allows us to conclude that this pathway is affected during the 24 hours of isolation. This crucial point is now addressed in our discussion.

In any case, some behavioral observation during the isolation period might also help to better understand these results.

We thank reviewer 2 for their suggestion. We quantified grooming, rearing, and spontaneous locomotion behaviors during the isolation period. The results, reported in Supplemental Figure 6, allowed us to highlight an increase in grooming time in mice isolated for 24 hours, confirming the hypothesis suggested by reviewer 2, namely, a specific involvement of the $INS^{->INS}$ population during the 24 hours of isolation in maintaining social preference in isolated animals.

- The reduced interest in social stimulus only after acute social isolation might be a decreased general motivational state after the 24hr acute isolation compared to not-lesioned mice. This should be checked.

We thank Reviewer 2 for this suggestion. To perform this important control experiment, we have evaluated the effects of genetically ablating INS-INS neurons on mouse motivation for a natural reward obtained in an operant paradigm (Supplemental Figure 10). The results from these control experiments show that the selective genetic ablation of INS-INS neurons does not affect the animals' motivation for a natural reward.

- Authors should report motor and other stereotypy (e.g. grooming, thigmotaxic, stretching behaviors etc.) data during the social test, to control for possible confounding factors.

We followed the suggestion of Reviewer 2 and quantified grooming and rearing behaviors during the social test. The quantified results show no difference between the control groups and the caspase groups during the social interaction time and are reported in Figure 4.

- Discussion, the link with autism is not clear… indeed if that was the case there should be a basal reduction of social interest, not only after single housing.

We agree with Reviewer 2 and have removed the link with autism from our discussion.

- Discussion. Not sure from which evidence this statement is supported: "we demonstrated that the interhemispheric neurons occupy a privileged position in synchronizing the activity of the Insula/CeA/dlBNST network in the two hemispheres."

We agree with Reviewer 2. This sentence was clearly an over-statement that has been removed from discussion.

- This sentence is also misleading and require further experiments to prove this "selectivity": "all of these data suggest that interhemispheric neurons in the Insula play a specific role for social processing". It could come from a number of different processes… and if it was selective for social should be evident already at basal condition not only after isolation, in my opinion.

We appreciate the comment from Reviewer 2. In response, we conducted a new experiment, demonstrating that the selective genetic ablation of $INS^{->INS}$ neurons does not affect the anxious state of the mice (supplemental figure 9). This finding confirms the selectivity of the observed effect on social preference. We have now discussed this selective aspect of social preference, which becomes evident only after a 24-hour isolation, in reference to the study by the K. Tye group (DOI: 10.1016/j.cell.2015.12.040). Their research showed comparable behavioral effects in a similar neural circuit.

- Some experiments, only one critical one in female mice at this point is due.

We have followed this important suggestion and conducted our selective genetic ablation experiment of INS->INS neurons on social behavior, along with the novel chemogenetic and fiber photometry experiments, in females and males.

Reviewer #3 (Remarks to the Author):

The manuscript explores a unique interhemispheric insular circuit using an array of anatomical tracing techniques, functional in vivo and ex vivo physiological characterization, and behavioral assays to assess the role of this pathway in social and anxiety-like behaviors. The manuscript is well-written, focuses on a novel, understudied circuit using innovative approaches. While the topic is certainly of interest to the broader scientific community, in its current form, the manuscript seems somewhat disjointed and at times the rationale for why certain experiments were performed is not well articulated. To this point, what is missing is how unique this interhemispheric circuit is compared to other insular circuits involved in social behavior. In addition, the authors draw strong conclusions that are not necessarily supported by the viral approaches they used, and the methodology, approach, and statistical comparisons are not clearly articulated or rationalized for several experiments. Given the exciting topic and innovative approach, I am overall enthusiastic about this manuscript, however the experiments need to be fleshed out much more to convince the reader that this circuit is important for regulating social behavior. Detailed comments are below:

We would like to express our sincere gratitude for the constructive feedback provided by the reviewer 3. We appreciate the careful consideration of our manuscript and the positive overall evaluation of our work. We have addressed the concerns raised, specifically focusing on providing a more cohesive narrative. We have incorporated nine new experiments into our study, enabling us to better structure the various sections of our manuscript, and thus ensuring robust methodology, approach, and statistical justifications.

The tracing data in figure 1 is somewhat over interpreted. With the retrograde-Cre/anterogradeDIO-eYFP approach, the authors are labelling interhemispheric neurons, but fiber expression in the BNST and CeA does not indicate that these neurons are innervating these brain areas. The fluorescence observed could just be fibers passing through this brain areas. To conclude innervation, the authors need to use a strategy that distinguishes terminals from fibers, such as a monosynaptic rabies-based approach or an AAV with a synaptophysin promoter.

The reviewer makes a very accurate comment here that led us to design a new viral approach for anterograde tracing. We have now performed an unbiased whole-brain quantification of all synaptic targets of $INS^{->INS}$ neurons using an anterograde synaptic marker (synaptophysin). Those new results are displayed in Figure 1 and in Supp Fig 1.

a. The use of the word “massively” is a bit strong and subjective. Similar language is used in several places throughout the manuscript (e.g, point 5 below). Magnitude of the effects should be interpreted based on the statistical effect.

We agree with the reviewer's comment, and have now removed these superlative adjectives from our manuscript.

2) There are very few details in the methods or results on how projections were quantified (Figure 1g). Was there an arbitrary threshold? It seems unreasonable to quantify a percentage of total insula interhemispheric projections when only three coronal sections of the brain are being analyzed. Similarly, how was colocalization determined in figure 1m-q? Was all fluorescent quantification done manually? More transparency would be useful.

Data in Figure 1g has now been replaced by the synaptophysin experiment. The methods used to quantify colocalization are now clarified in the Methods section.

3) A retrograde Cre virus injected into the insula of an ai9 mouse should label all projection cells into the insula. Why does only the contralateral insula express tdtomato when there are several studies documenting insula inputs from sensory cortical areas, for example, which are seen in that same section shown in Figure 1. Showing cell bodies in other known upstream brain areas with insula projections would help validate the viral approach.

The reviewer is correct, and have now illustrated the presence of certain cell bodies in the somatotopic sensory cortex contralateral to the injection site of retroAAV, as depicted in Supplemental Figure 2A.

a. In line with this, two weeks is a short amount of time to allow for transduction and retrograde transport of a retrograde AAV. Do the authors have any evidence that the viruses have fully reached the target cells by this time? What is the justification for waiting longer for anterograde viruses? Could the lack of expression in other upstream regions be attributed to a lack of full transduction?

The reviewer raises a point here that we had not previously addressed. In response to this comment, we injected new Ai9 mice with the retroAAV virus and sacrificed these mice after 4 weeks of expression. As illustrated in Supplemental Figure 2, the obtained labeling is similar after 2 weeks or 4 weeks of neuronal infection by the virus.

4) The rationale for performing several experiments could be improved throughout the main text. Specifically, why did the authors perform the experiment to look at colocalization of interhemispheric insula cells with Satb2 and Ctip2? Is the lack of Ctip2 co-expression unique to this circuit? A negative control experiment showing that this colocalization pattern is unique, and not uniform to the entire insula would be a great addition.

We thank reviewer 3 for this comment and have followed his/her suggestion. We verified the selectivity of the expression pattern of the molecular markers CTIP2 and SATB2 by targeting two other neuronal populations in the insula: $INS^{->tail\ of\ the\ striatum}$ neurons and $INS^{->BLA}$ neurons. The results of this experiment are presented in Supplementary Figure 4, notably showing that the $INS^{->BLA}$ neuron population exhibits an expression pattern of CTIP2 and SATB2 distinct from the other two populations.

a. Was there layer specificity or differences between insula subregions (anterior, posterior, intermediate?) .

We thank reviewer 3 for their comment. We have conducted new quantifications to compare the expression of CTIP2 and SATB2 in the different layers of the insula. These results are now reported in

Supplementary Figure 2. We also quantified the repartition of INS-INS neurons in all the layers and in the different subregions of the insula (anterior intermediate and posterior). These data are now reported in Figure 1 e-f.

5) The authors determine that the insula neurons are “exclusively pyramidal” because there is no colocalization with GAD67 or PV. Without further studies, it is an overstatement to say that this experiment “confirms” that these neurons are pyramidal. The whole cell electrophysiological experiments with channelrhodopsin-assisted circuit mapping start to get at this question, but what are the intrinsic properties of these specific neurons, not just the projection onto general insular neurons. Comparing intrinsic physiological properties of insula-insula cells with non insula-insula cells would not only provide stronger evidence that these neurons are pyramidal, but this comparison could highlight a unique characteristic of these neurons.

This comment from Reviewer 3 is particularly important. In response, we conducted patch-clamp experiments on two distinct populations of the insula, differentiated by their projection site. Consequently, we confirmed that all recorded $INS^{->INS}$ neurons exhibited an anatomical and electrophysiological profile characteristic of glutamatergic pyramidal neurons. We did not identify a specific electrophysiological signature for the $INS^{->INS}$ neuronal population. These results are shown in Supplementary Figure 5.

6) Why did the authors perform the caspase studies with CeA and insula and not CeA, insula, and BNST? Or just insula? The authors state that they targeted the “two main outputs of insula interhemispheric neurons”, but this is not accurate according to figure 1g. If anything, BNST looks to be a more substantial output than CeA. In addition, with this approach the authors are isolating more than just interhemispheric neurons as you are targeting all insula projections to the CeA and all interhemispheric projections. It would be interesting to see just insula-insula caspase, and even insula-BNST/insula-insula projections too.

We thank Reviewer 3 for these comments. Our unbiased whole-brain quantification of synaptophysin labeling in $INS^{->INS}$ neurons allowed us to highlight that the insula, the CeA, and the BNST are the three major axonal projection sites of this neuronal population (Supplementary Figure 1). Additionally, we compared viral strategies targeting specifically $INS^{->INS}$ or $INS^{->CeA}$ neuronal populations, and the pattern of anterograde labeling distribution from these two populations strongly suggests that they belong to the same neuronal population. The strategic decision to target both the CeA and the insula was made to increase the number of neurons in the insula, and this is now discussed in the manuscript. It is worth noting that we have abandoned our initial efforts to specifically target the juxta and oval nuclei of the BNST. Due to their small size in mice and their "core/shell" organization, these nuclei are extremely challenging to target.

7) The statistics and conclusion in figure 3f-m are confusing. In 3j, the authors show significant differences in time spent with the social chamber vs the object chamber in ctrl mice, and differences between caspase and ctrl mice in time spent at the social chamber. This data is then transformed into preference in 3k, but different statistics were run and now the caspase group is not significantly different than the ctrl group? More clarity is needed.

We appreciate Reviewer 3's feedback on our statistics, which we have now clarified in the manuscript.

We have also added six new animals to our study to enhance the statistical power of our analyses. These additional animals have enabled us to convert a trend in the ratios into significant results. These new experiments and analyses are reported in Figure 4.

8) The methods for social interaction test state that the group housed mice were subjected to either a juvenile mouse or a chamber filled with lego toys, whereas the isolated mice had the choice between an empty chamber and one with a juvenile mouse. What is the explanation for this discrepancy?

We thank Reviewer 3 for this comment. We have now clarified this apparent discrepancy in the manuscript. The reason for using an empty chamber or chambers filled with different objects (different lego toys) was to vary the nature of the objects presented and avoid presenting the same object in two consecutive trials. In this way, we could each time compare a novel object versus a novel social juvenile mouse.

9) Social isolation generally takes a week or more to have effects on c57 mice. It's possible the impact of this circuit would be more prevalent after prolonged social isolation. It would be interesting to see how insula interhemispheric neurons regulate social interaction after prolonged isolation.

Reviewer 3 raised a particularly important point that gave us the opportunity to explore the impact of 15-day isolation on social preference. The results of this new experiment are reported in Supplementary Figure 7, confirming the point raised by Reviewer 3, namely, that an extended isolation time affects the social preference of both control and $INS^{->INS}$ neuronal population-lesioned mice without difference between groups.

a. While the within subject design is good, the mice are exposed to the social interaction test multiple times within a short period of time. This is a bit of a confound. The authors should examine how social isolation right after surgery would impact social interaction.

We believe that our experimental procedure is designed to allow multiple repetitions of the social interaction test, involving the presentation of a new object and a new juvenile each time. Additionally, isolating the animals after surgery would require a total of 6 weeks (4 weeks for the virus and 2 weeks for behavior). However, this prolonged isolation time is incompatible with the ethical guidelines of our animal facilities. Furthermore, we find it interesting to replicate the protocol from the seminal paper by Dr. K. Tye's group (DOI: 10.1016/j.cell.2015.12.040), and this aspect is now discussed in the manuscript.

10) The major impactful finding of this manuscript is that this circuit plays a role in social interactions, but this conclusion is drawn from one experiment that uses somewhat of a sledgehammer approach with caspase. While I think the model is sound, it would be more impactful to see the opposite- would social interaction be increased with chemogenetic or optogenetic activation of these neurons? This type of experiment could be really interesting in the context of ASD and studies to that seek to increase social interaction.

We thank Reviewer 3 for this suggestion. We have implemented a new experimental group in which chemogenetic activation of the INS->INS neuronal population did not significantly impact behavior. These results are presented in Supplementary Figure 6.

11) The figure legend provides no information on what the symbols mean. For example, in figure 3, what is the difference between *** and ###?

This has been corrected

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REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

The authors have addressed all my concerns and in my opinion also concerns of the other reviewers. I therefore recommend publication of the study.

Reviewer #2 (Remarks to the Author):

I really like the story and the several new experiments reported. Usually, I don't ask more in the second revision, and I am sorry about this, but I just want to help to better interpret the data. Indeed, with the new experiments I have even more problems on the strong conclusions on the specific involvement of this circuit in social preference. In particular, in light of no effect in grouped housed, no effect following chronic social isolation, no effect for the chemogenetic silencing, no effects in females, no selective effect in social exploration with fiber photometry, the conclusions on social preference are an overstatement. I believe it remains a very interesting and novel study, but the claims on social preference are difficult. Here I report few specific considerations:

- The new chemogenetic experiments are important and should be moved to the main figures. Moreover, to support now their data and conclusions, they should provide the same chemogenetic experiment, but performed during the 24hr isolation period. Otherwise, as I was stating previously, their effects might be more interpreted as compensatory mechanisms on other circuits after the altered circuit for such prolonged time? I think s this could conclude the study nicely. But I understand the effort, so it is up to the authors.

- Title, maybe better "A population of insular neurons encodes for social preference ONLY AFTER ACUTE social isolation IN MALES"?

- In the introductions, it remains unclear and does not follow previous statements this statement: “As alteration in interhemispheric communication is associated with a social deficit17–20, we postulated that Insula interhemispheric communication is essential to develop adaptive reactions when facing novel social cues or threatening situations.” I would suggest rephrasing it. From where is it coming the focus on novel social cues or threatening situations?

- The fiber photometry experiments are an important addition. However, they again support the conclusion that INS-INS connections are not important for social preference per se. Indeed, they are equally active in grouped and socially-isolated mice during social interaction. What seems to be changing is their activity towards objects, and only after social isolation.

- Figure 5f traces are representative of the entire population? It looks like objects/social explorations are switched by the group housing condition. Isosbestic or other control channel should be reported as well. It is not the most correct way to subtract the control channel, see for some reference for example review by Siciliano and Tye in 2019, or more recent work from the Sabatini lab.

- This sentence should be supported by a reference: “Since rodents are innately pro-social species, social isolation represents an aversive experience.” I am actually not convinced this is true for 24hr isolation.

- Discussion. I would eliminate this sentence: “After this aversive event, control mice developed adaptive behavior that favoured social interactions compared to object interactions thereby restoring social homeostasis.” First of all, 24hr isolation is not clear or evident that it is aversive… it is more aversive indeed the chronic social isolation (in which authors don’t see effects). Moreover, looking at figure 4 and figure 5, it is evident that acute social isolation does not trigger more social exploration as social exploration is identical between grouped and acutely isolated mice.

Reviewer #3 (Remarks to the Author):

I commend the authors on the significant amount of work done to improve the manuscript. I am very impressed with the responses to my and the other reviewers' comments (in my opinion). The manuscript is much improved in terms of the defined goals, and data to support the conclusions. However, seemingly important details and rationale for some of the experiments is still missing, making it difficult to interpret the major outcome and conclusions from several experiments and how they relate to each other. For example, why some experiments were run in both sexes, but some were only in males should be discussed. Despite the significant overall improvement, I still have some specific concerns, mostly minor, that should be addressed prior to publication.

1) The addition of the synaptophysin tracing is excellent and the authors added data regarding my original concern with the lack of heterotopic inputs in figure 1a. But the results are still not in agreement with the whole mouse brain insula input/output mapping study by the Gogolla lab (Gehrlach et al., 2020 eLife). They showed nearly equal, if not slightly more, input from the sensory and motor cortex compared to the contralateral insula. This is not to say the data is inaccurate, but potential reasons for these discrepancies should be discussed, and the paper should be cited. Or perhaps Figure 1B is an exemplary image and representative of the other 3 brains? In which case the other 3 brains should be shown in the supplement.

a. Please list the N and individual data points for figure 1i as is done in figure 1a-f.

2) The in vivo (fig 2c, 3m) and ex vivo (fig 3i) electrophysiology conclusions, interpretations, and how the data fit with one another is unclear. Better rationale for this experiment, and more description of the methodology and outcome measurements would be very helpful, particularly for the ex vivo studies (lines 208-215).

a. For the ChR2 experiment, did 100% of cells recorded from respond to light stimulation? But then only 32.73% of the cells recorded in vivo responded to contralateral stimulation (figure 3m-s)? I think defining the orthodromic response better in the figure, and what a "no response" cell is may help this confusion. As presented, it seems like a no response cell still responds by producing an AP after electrical stimulation, but it is not orthodromic?

b. Wouldn't the electrical simulation in the contralateral side also be stimulating the terminals of the neurons being recorded from contralaterally? Can this be controlled for?

3) The DREADD studies in Supplemental figure 9 are quite interesting, even with mostly negative results. Given the amount of text dedicated to this experiment, it's unclear why some of these data are not included in the main figures. Regardless, there are several major issues that need to be addressed prior to drawing the conclusion that this pathway has no impact on anxiety-like behavior, or as the authors conclude, the effects are specific to social behavior. Specifically:

a. There are no experimental details. For example, when was CNO given? What dose? What is the control group? There should be multiple controls (CNO in mice without DREADD, saline in DREADD mice). It's unclear which of these controls was used but the other should be included. This is particularly important given the use of CNO (as opposed to C21 or DCZ) and off target effects with the conversion to clozapine.

b. The figure panels are not presented in the order they are mentioned in the text.

c. There is a significant effect of hM3D in the light dark test, but the results say "there was similar performance" between groups.

d. Why were only EPM and open field tested for the hM4D experiment? Especially considering the significant effect with light dark in the hM3D experiment.

e. The hM4D experiment was conducted in males and females, but the hM3D was only run in males?

f. The ephys recordings in panel g are very small and this experiment and outcome measures is not explained at all in the figure legend, results, or methods.

g. One of my original comments suggested an experiment to chemogenetically or optogenetically activate this pathway to see if it decreases social interaction. The response by the authors indicates this data is in supplemental figure 6 but it is not. Including this would significantly enhance the overall impact, as it could implicate this pathway in ASD.

4) The fiber photometry experiment is a great addition. A few minor things:

a. The x's above the interactions are very small and difficult to see. There is also a gray x with no explanation. The green line and red dashed line are not defined in the figure legend. I am assuming they represent the spike threshold and width, but this should be clarified in the figure legend and results section.

b. Are there any changes in the calcium signal time locked to the initiation of an interaction (social or object)? Or any differences in the signal at the onset of the first interaction versus a later interaction?

Point-by-point to the reviewers.

Reviewer #1 (Remarks to the Author):

The authors have addressed all my concerns and in my opinion also concerns of the other reviewers. I therefore recommend publication of the study.

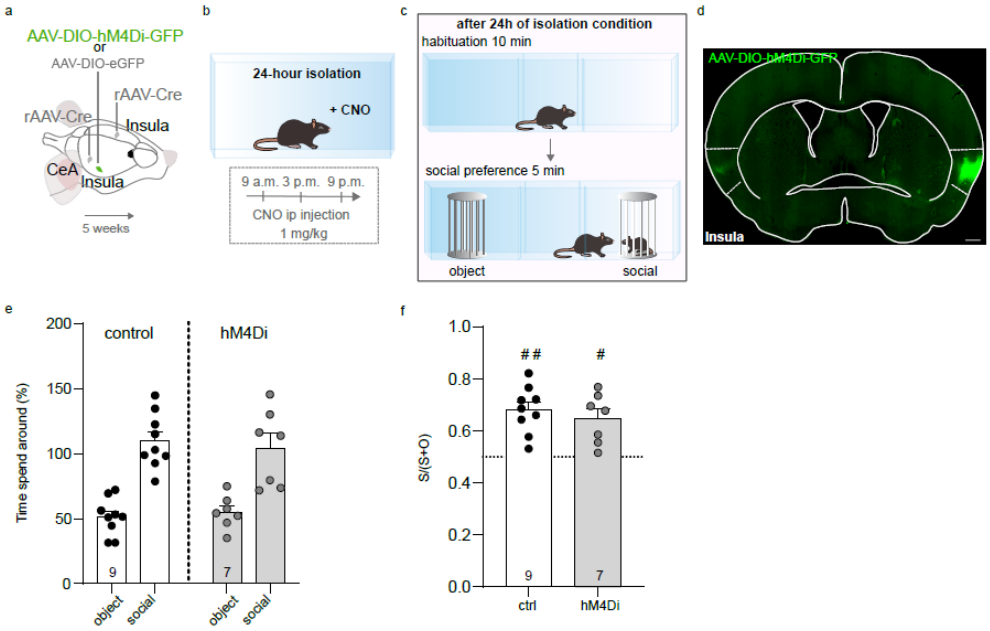
Thank you sincerely for your thoughtful review in the first round of revision and your positive feedback on our manuscript. We are delighted to hear that you feel we have successfully addressed not only your concerns but also those of the other reviewers. We are thankful for your time, expertise, and support throughout this process.

Reviewer #2 (Remarks to the Author):

I really like the story and the several new experiments reported. Usually, I don't ask more in the second revision, and I am sorry about this, but I just want to help to better interpret the data. Indeed, with the new experiments I have even more problems on the strong conclusions on the specific involvement of this circuit in social preference. In particular, in light of no effect in grouped housed, no effect following chronic social isolation, no effect for the chemogenetic silencing, no effects in females, no selective effect in social exploration with fiber photometry, the conclusions on social preference are an overstatement. I believe it remains a very interesting and novel study, but the claims on social preference are difficult. Here I report few specific considerations:

- The new chemogenetic experiments are important and should be moved to the main figures. Moreover, to support now their data and conclusions, they should provide the same chemogenetic experiment, but performed during the 24hr isolation period. Otherwise, as I was stating previously, their effects might be more interpreted as compensatory mechanisms on other circuits after the altered circuit for such prolonged time? I think s this could conclude the study nicely. But I understand the effort, so it is up to the authors.

Thank you for your feedback and thoughtful consideration. We appreciate your support and understand your concerns regarding the interpretation of the data, especially concerning the conclusions on social preference. We thank you for your suggestion and agree with your comment suggesting that the ideal experiment would be to inhibit our circuit of interest through a chemogenetic approach during the 24 hours of isolation. A crucial point for this experimental intervention is that the activity of our circuit of interest is not blocked during social behavior. After careful consideration, we have conducted the experiment you suggested, and to maintain optimal activation of the inhibitory Dreadds, we repeated the intraperitoneal injections of CNO three times during the 24-hour isolation period with an interval of 6 hours (which has been described as the presumed effective duration of action of CNO). The results of this experiment are reported below.



Effect of partial chemogenetic inhibition of Ins^{Ins} during the 24 hours of social isolation on social interaction test in male mice. a-c. Experimental design for the viral injection (a) and behavioral assay (b,c). **d.** Example of a histological control of AAV9-DIO-hM4Di-GFP injection in the Insula (green labeling) taken at epifluorescence microscope (scale bar: 500 µm). **e.** Quantification of the time spent around the object and social enclosures in the three-chamber test after 24 hours of social isolation in Insula control and Insula hM4Di groups pre-treated with intraperitoneal injection of CNO. (Two-Way repeated measure Anova, no interaction $F(1,4)=0.4087, p>0.05$, main effect of "enclosure " $F(1,8)=56.62, p<0.05$) **f.** Social preference ratio in the control group and hM4Di group after 24-hour of social isolation. *# is used to mention a difference with a social preference ratio equal to 0.5.*

As illustrated in the figure above, the result of this experiment shows no difference between the grouped animals and the 24h-isolated animals. However, we find the interpretation of this negative result to be complicated, since for experimental reasons (notably the multiple injections of CNO), we cannot blocked our circuit of interest (Insula^{INS} neurons) for the entire 24 hours. Additionally, this strategy involves another confounding factor (three IP injections in animals), which in itself may constitute a stress factor. For these reasons, and because we have not succeeded in proposing an effective strategy capable of reliably blocking our circuit of interest for 24 hours, we have decided to not include this experiment in the manuscript, thinking that it would introduce confusion that disrupts the main message.

However, in this new version of our manuscript, we propose a new electrophysiology experiment that partially addresses this important point raised by reviewer 2. By identifying our circuit of interest through a retrograde approach (Insula^{INS} neurons), we were able to demonstrate that a 24-hour isolation episode increases the excitability of Insula^{INS} neurons, thus demonstrating the specificity of this isolation procedure. These results are now reported in Figure 6.

- Title, maybe better "A population of insular neurons encodes for social preference ONLY AFTER ACUTE social isolation IN MALES"?

We have modified the title of our manuscript and mentioned in the abstract that the effect on social preference was only visible in males

- In the introductions, it remains unclear and does not follow previous statements this statement: "As alteration in interhemispheric communication is associated with a social deficit17–20, we postulated that Insula interhemispheric communication is essential to develop adaptive reactions when facing novel social cues or threatening situations." I would suggest rephrasing it. From where is it coming the focus on novel social cues or threatening situations?

We agree with the reviewer comment and performed the changes in the introduction.

- The fiber photometry experiments are an important addition. However, they again support the conclusion that INS-INS connections are not important for social preference per se. Indeed, they are equally active in grouped and socially-isolated mice during social interaction. What seems to be changing is their activity towards objects, and only after social isolation.

We appreciate your insight into our fiber photometry experiments. We agree that it is indeed the activity of INS^{INS} neurons that appears to decrease when mice explore the object in the three-chamber social interaction test. What we are now reporting in the manuscript is that the activity of the INS^{INS} neurons reflects the change in discrimination between the object and the social, which materializes as a difference in signal polarity when the mouse explores the social or the object

- Figure 5f traces are representative of the entire population? It looks like objects/social explorations are switched by the group housing condition. Isosbestic or other control channel should be reported as well. It is not the most correct way to subtract the control channel, see for some reference for example review by Siciliano and Tye in 2019, or more recent work from the Sabatini lab.

We have changed the trace and now reported the isobestic signal in figure 6

The way we analyzed the fiber photometry signal is not the most common analysis, though was carefully chosen. The classical analysis by Lerner et al., 2015 (DOI:https://doi.org/10.1016/j.cell.2015.07.014was not sufficient to account for the combination of motion-related artifacts and baseline drifting of the signal, especially in GFP-control animal. Only applying a linear fit to the control channel suggests that all non-calcium-related changes in fluorescence are of similar amplitudes and that the isobestic channel follows similar kinetics than the reference channel. As reviewed by many https://doi.org/10.1016/j.alcohol.2018.05.013 ,https://doi.org/10.1016/j.neuron.2023.11.016), the isobestic channel is not a perfect control and can exhibit different photobleaching rates than the calcium-dependent channel, can shift with changes in intracellular pH (https://doi.org/10.1371/journal.pone.0170934), and more importantly, the isosbestic channel can display a negative correlation to the calcium-dependent channel (https://doi.org/10.1016/j.neuron.2023.11.016).
To palliate those caveats, we thus used the method detailed by Martianova et al. 2019 (doi: 10.3791/60278) to reduce motion-related artifacts by 1) removing baseline drifting of the signal and 2) remove motion-related artifacts by normalizing both signals.
We further confirmed that our methodology removed properly artefactual signals, as peak detections in the GFP control signal were rarely present. The same peak detection analysis using the analysis described by Lerner et al., 2015 increased false positives, with multiple wrongful peak detections in GFP mice (Data not shown).

- This sentence should be supported by a reference: “Since rodents are innately pro-social species, social isolation represents an aversive experience.” I am actually not convinced this is true for 24hr isolation.

We agree with the reviewer and removed the sentence.

- Discussion. I would eliminate this sentence: “After this aversive event, control mice developed adaptive behavior that favoured social interactions compared to object interactions thereby restoring social homeostasis.” First of all, 24hr isolation is not clear or evident that it is aversive… it is more aversive indeed the chronic social isolation (in which authors don’t see effects). Moreover, looking at figure 4 and figure 5, it is evident that acute social isolation does not trigger more social exploration as social exploration is identical between grouped and acutely isolated mice.

We thank the reviewer and changed the discussion accordingly.

Reviewer #3 (Remarks to the Author):

I commend the authors on the significant amount of work done to improve the manuscript. I am very impressed with the responses to my and the other reviewers’ comments (in my opinion). The manuscript is much improved in terms of the defined goals, and data to support the conclusions. However, seemingly important details and rationale for some of the experiments is still missing, making it difficult to interpret the major outcome and conclusions from several experiments and how they relate to each other. For example, why some experiments were run in both sexes, but some

were only in males should be discussed. Despite the significant overall improvement, I still have some specific concerns, mostly minor, that should be addressed prior to publication.

Thank you very much for your kind words and appreciation of our efforts to improve the manuscript. We have now addressed, in the discussion section as a limitation, the fact that all experiments were not systematically conducted in both males and females.

1) The addition of the synaptophysin tracing is excellent and the authors added data regarding my original concern with the lack of heterotopic inputs in figure 1a. But the results are still not in agreement with the whole mouse brain insula input/output mapping study by the Gogolla lab (Gehrlach et al., 2020 eLife). They showed nearly equal, if not slightly more, input from the sensory and motor cortex compared to the contralateral insula. This is not to say the data is inaccurate, but potential reasons for these discrepancies should be discussed, and the paper should be cited. Or perhaps Figure 1B is an exemplary image and representative of the other 3 brains? In which case the other 3 brains should be shown in the supplement.

We thank the reviewer for their positive appreciation of our synaptophysin experiment. We have now discussed our results by referencing the seminal paper from the Gogolla lab and addressing the apparent differences between our study and theirs. These differences may account for distinct retrograde viral approaches used between studies.

Gogolla and colleagues performed whole-brain mapping of Insula retrograde labelling in ipsilateral regions by using a rabies virus approach in CAMKII-cre mouse, whereas our study analyzed Insula retrograde labelling in contralateral cortical regions by using a rAA2-retro-CAG-cre monosynaptic retrograde virus strategy in Ai9-dTomato mouse. The study by Gehrlach et al., 2020 in eLife is now cited. Regarding Figure 1b, we have followed the reviewer's suggestion and have now included the injection sites for the 4 mice in the manuscript to complete the histological cartography (Figure 1b-c + Supplementary Figure 1h).

a. Please list the N and individual data points for figure 1i as is done in figure 1a-f.
Done

2) The in vivo (fig 2c, 3m) and ex vivo (fig 3i) electrophysiology conclusions, interpretations, and how the data fit with one another is unclear. Better rationale for this experiment, and more description of the methodology and outcome measurements would be very helpful, particularly for the ex vivo studies (lines 208-215).

We thank the reviewer for these comments. We have revised the in vivo electrophysiology figure to make it more didactic regarding the orthodromic response. We have provided additional details on the ex vivo procedure. The rationale and methodology have been clarified.

a. For the ChR2 experiment, did 100% of cells recorded from respond to light stimulation?

We thank the reviewer for their comment and have now clarified this point in the results section. Regarding the ex vivo electrophysiology experiment, indeed, 100% of patched neurons responded to optogenetic stimulation. Those neurons have been targeted and recorded in the area of the insula rich in axonal terminals expressing the CHR2 protein. The difference in the percentage of responsive cells between the ex vivo and in vivo experiments is due to the fact that the in vivo approach does not allow us to specifically target the recording area receiving the maximum excited axons in response to stimulation of the contralateral insular cortex. The 100% corresponds to the total neurons recorded in the insula.

I think defining the orthodromic response better in the figure, and what a “no response” cell is may help this confusion. As presented, it seems like a no response cell still responds by producing an AP after electrical stimulation, but it is not orthodromic?

We thank the reviewer for providing us with the opportunity to clarify this methodological point and add this information to the material and methods. What is referred to as a "no response cell" is a neuron that does not emit

an action potential in response to stimulation of the insula, specifically within the time window ranging from 0 to 25 ms after stimulation. Baseline activity was computed for each peri-stimulus time histogram (PSTH) during the 500 ms preceding stimulation to generate a Z-score for each responsive neuron. A "no response cell" is a neuron with a Z-score < 1.96, meaning a probability value (p) > 0.05 of emitting an action potential within the time window of a monosynaptic transmission between the ipsilateral and contralateral insula (i.e., between 0 and 25 ms).

b. Wouldn't the electrical simulation in the contralateral side also be stimulating the terminals of the neurons being recorded from contralaterally? Can this be controlled for?

This is an important point raised by the reviewer. We have now clarified in the results section of the manuscript that none of the neurons responding with an orthodromic response to insula stimulation also exhibited an antidromic response. These two distinct responses could have been distinguished based on their latency and the phenomenon of "collision" (see Figure 2D).

3) The DREADD studies in Supplemental figure 9 are quite interesting, even with mostly negative results. Given the amount of text dedicated to this experiment, it's unclear why some of these data are not included in the main figures.

We have followed the reviewer's suggestion. We have now moved the inhibitory dreadd experiment to the main figure (Figure 5), as also suggested by reviewer 2. However, for the sake of clarity, we have kept the results regarding the excitatory dreadd in Supplementary (now in Supplementary Figure 8).

Regardless, there are several major issues that need to be addressed prior to drawing the conclusion that this pathway has no impact on anxiety-like behavior, or as the authors conclude, the effects are specific to social behavior. Specifically:

a. There are no experimental details. For example, when was CNO given? What dose? What is the control group? There should be multiple controls (CNO in mice without DREADD, saline in DREADD mice). It's unclear which of these controls was used but the other should be included. This is particularly important given the use of CNO (as opposed to C21 or DCZ) and off target effects with the conversion to clozapine.

We have now specified in the methods section of the manuscript the experimental procedures for CNO (namely, a concentration of 1 mg/kg, administered intraperitoneally 30 minutes before the behavior). Regarding the specificity control of this experiment, we chose to use as the control situation the most stringent condition, namely the injection of CNO in animals not expressing the Dreadd. This choice has allowed us to reduce the number of animals used without compromising the interpretation of our results, which we believe is an ethically valid decision. Moreover, since CNO is given to all the groups, this experimental design allows us to control for any potential nonspecific effects that could be induced by the "off-target" effects of CNO with the conversion to clozapine. Furthermore, the low dose of CNO that we used and the absence of repeated injections of CNO for a given behavioral situation greatly limit the risk of off-target effects of CNO.

b. The figure panels are not presented in the order they are mentioned in the text.
Thanks. This is now corrected.

c. There is a significant effect of hM3D in the light dark test, but the results say "there was similar performance" between groups.

This is a valid point raised by the reviewer. We have now adjusted the overall conclusion of this series of experiments by stating that the effects of hM3D do not induce major changes in anxiety levels across all behavioral tests used.

d. Why were only EPM and open field tested for the hM4D experiment? Especially considering the significant effect with light dark in the hM3D experiment.

We agree with the reviewer that it would have been preferable to conduct the Light-dark experiment. However, due to organizational reasons and the long delay between the different experimental phases, we were unable to carry out this experiment.

e. The hM4D experiment was conducted in males and females, but the hM3D was only run in males?

Male and female comparisons of the "key experiments" were requested by our reviewers in the first round of review. Since our main effect on social preference was observed only in males, we decided to conduct the control HM3D experiment only in males.

f. The ephys recordings in panel g are very small and this experiment and outcome measures is not explained at all in the figure legend, results, or methods.

Corrected

g. One of my original comments suggested an experiment to chemogenetically or optogenetically activate this pathway to see if it decreases social interaction. The response by the authors indicates this data is in supplemental figure 6 but it is not. Including this would significantly enhance the overall impact, as it could implicate this pathway in ASD.

As requested by reviewers 1 and 2, we will no longer refer to a potential impact of our results on ASD in this manuscript. We agree that activating this pathway is a promising topic that we are ready to explore in future studies, particularly in investigating the involvement of this pathway in an experimental model of ASD.

4) The fiber photometry experiment is a great addition. A few minor things:
a. The x's above the interactions are very small and difficult to see. There is also a gray x with no explanation. The green line and red dashed line are not defined in the figure legend. I am assuming they represent the spike threshold and width, but this should be clarified in the figure legend and results section.

We thank the reviewer for these suggestions. We have now completely reorganized the fiber photometry figure (now in Figure 6) and notably illustrated and clarified in the methods and legend how we detected the events.

b. Are there any changes in the calcium signal time locked to the initiation of an interaction (social or object)?

Our behavioral apparatus does not allow us to distinguish between social contacts and social investigation (Adult mouse contact with the social enclosure). The enclosures used to place the objects and juvenile stimulus mouse are opaque and prevent visual feedback on the position of the juvenile mouse. Thus, time-locked analysis of the signal would have provided insight only into the activity of these neurons when the mouse is entering the social enclosure area but not specifically at the initiation of direct contact with the juvenile mouse.
However, as these contacts can only occur when the mouse is investigating the social enclosure, we report global changes of the fiber photometry signal within the different social or non-social enclosures.

Or any differences in the signal at the onset of the first interaction versus a later interaction?

To determine whether there are any changes between early versus later interactions in the activity of INS^{INS} neurons, we averaged the first 15 seconds versus the last 15 seconds of the z-scored signal when the animal was within the social enclosure area. Those Data are now reported in Supplementary figure 10.

REVIEWERS' COMMENTS

Reviewer #2 (Remarks to the Author):

The authors adequately addressed all my previous concerns.

Just minor, they mentioned in the rebuttal letter that “mentioned in the abstract that the effect on social preference was only visible in males”. However, I cannot see it in the abstract.

Reviewer #3 (Remarks to the Author):

The authors have adequately addressed my remaining concerns. I recommend the publication of the manuscript in its current form. Kudos to all authors on an exciting and impactful study.

A point-by-point response to the reviewers' comments

REVIEWERS' COMMENTS

Reviewer #2 (Remarks to the Author):

The authors adequately addressed all my previous concerns.
Just minor, they mentioned in the rebuttal letter that “mentioned in the abstract that the effect on social preference was only visible in males”. However, I cannot see it in the abstract.

We thank Reviewer 2 to help us to improve the quality of our manuscript. We have now corrected the abstract.

Reviewer #3 (Remarks to the Author):

The authors have adequately addressed my remaining concerns. I recommend the publication of the manuscript in its current form. Kudos to all authors on an exciting and impactful study.

We thank Reviewer 3 for all the concerns raised during this reviewing process which enabled us to upgrade our manuscript.