

Neurofeedback modulation of insula activity via MEG-based brain-machine interface: A double-blind randomized controlled crossover trial

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

Version 0:

Reviewer comments:

Reviewer #2

(Remarks to the Author)

Wang et al. present a double-blind crossover study using MEG-based neurofeedback to alter pain thresholds. Healthy participants (N = 19) were asked to up- or downregulate activity in the right insular cortex, which was visually displayed to them in real-time. Pain thresholds and insular activity were assessed before and after a 20 minute up- or downregulation period.

The authors find a decrease in insular activity and an increase in pain thresholds after downregulation of insular activity, although with no significant interaction effect. Overall, these results are interesting and might provide a valuable starting point to target chronic pain. However, there are some methodological issues that require clarification.

Major:

- It is not entirely clear whether feedback was obtained from activity specific to the insula. Although source reconstructions were performed, the Insula is over 4 cm deep in the brain, making reconstruction of the signal challenging. Could the authors show a contrast between source activity during up- and downregulation across the entire brain? Such plot would show whether the difference is specific to the insula or involves other brain regions as well.
- No control condition using feedback from a different region was performed, so it remains unclear whether effects are specific to neurofeedback from the insula. Effects could be non-specific and expectation-related. The finding that both up- and downregulation raises pain thresholds reinforces this hypothesis.
- Please provide more information in the main text how insular activity is measured. What exactly is the primary physiological outcome measure? This information is missing from the abstract, figures, and results, but important to increase clarity of presentation.

Minor:

- All figures are missing a unit for the y-axis label.
- A direct comparison between the up- and downregulation conditions should be performed for change in insular activity (Fig. 4) and change in pain thresholds (Fig. 5).
- In the discussion, several studies are cited that use brain stimulation, such as TMS. However, there is a controversy whether such paradigms can directly reach such deep areas (see letters to the editor to Galhardoni et al., e.g.). While non-invasive brain stimulation can be useful, it comes with several challenges (see Nasr et al. 2022, doi:10.1016/j.pneurobio.2022.102311). Combination of neurofeedback with closed-loop approaches (e.g., Haslacher et al. 2023, doi: 10.1016/j.neuroimage.2023.120187), especially those that can reliably target deep brain areas such as ultrasound may offer a promising avenue for more effective and precise interventions. These advanced methods could potentially overcome the limitations of traditional brain stimulation techniques by providing real-time feedback and more accurate targeting.

- Please include information on how the study complies with the “consensus on the reporting and experimental design of clinical and cognitive-behavioural neurofeedback studies” (CRED-nf checklist, <https://pubmed.ncbi.nlm.nih.gov/32176800>).

Reviewer #3

(Remarks to the Author)

This paper investigates the modulation of brain activity in the insular cortex and its effects on pain perception using neurofeedback techniques based on magnetoencephalography (MEG) in healthy individuals. The findings suggest that insula activity can be modulated via Neurofeedback. However, direct evidence for an effect on pain perception was not found.

The study's objective of altering pain perception via neuromodulation is potentially significant. The overall study design appears straightforward and, in principle, sound. However, several critical issues regarding implementation and reporting need to be addressed: Research questions and hypotheses are imprecisely stated, some statistical analyses are flawed, the study appears underpowered, and the descriptions of the methods lack precision and comprehensibility.

Major comments – Introduction:

- The second research question (“...which activity pattern best represents pain perception: oscillatory power or mean cortical excitability evaluated by BOLD signal”) is not addressed by any subsequent hypotheses.
- It is unclear which parts of the study are confirmatory versus exploratory.
- The second hypothesis (“...increase in the pain threshold utilized for neurofeedback to modulate pain”) is unrelated to the research questions and lacks clarity.

Major comments – Results:

- The statistical tests used to assess neurofeedback effects on insular activity during neurofeedback training are invalid. Regression analyses treating different time points as independent samples are flawed. Suggested approach: Analyze changes in insular activity (e.g., second half minus first half of training) at the individual level rather than time points.
- The assessment of neurofeedback effects on resting state insula activity is statistically sound. However, the difference between activity changes between up- and downmodulation training is only borderline significant. Thus, in light of the small sample size, it is questionable how reliable the results are.

Major comments – Methods:

- A priori sample size calculations are missing. Assuming small to medium effect sizes, which are to be expected in translational neuroscience studies like this one, the study is likely underpowered to detect the hypothesized effects. It is strongly recommended that additional data be acquired: $N_{\text{total}} > 40$.
- A description of the exact procedure in which the registration of individual MRI and AAL was achieved is missing
- MEG recordings: The description of the reconstruction of cortical currents in the insula cortex is imprecise. Steps to improve this could include the following: Explain why the BME method was used to compute the spatial filter; provide more methodological detail on spatial filter construction using BME.
- Neurofeedback system: The description of the neurofeedback system is incomprehensible. Steps to improve this could include the following: Provide exact definition of insula activity in mathematical notation; state the physiological rationale for the selected definition of insula activity; provide exact definition of the clamp function in mathematical notation.
- Resting-state data processing: The description of the Resting-state data processing is imprecise. Were artifacts removed by rejecting associated time segments or ICA components? For source reconstruction, minimum norm estimation (MNE) is employed in contrast to BME above. Why this discrepancy? Moreover, the MNE does not necessarily require the computation of a noise covariance matrix. However, the paper uses a version of MNE that requires calculating the noise covariance matrix. Thus, it should be stated more precisely which type of MNE was used and how the noise covariance was estimated precisely.
- Methods/Statistical analysis and reproducibility: This section lacks detail, and there is no information about strategies to ensure the reproducibility of findings.

Major comments – Discussion:

- Lines 218-221: This claim is hardly supported in light of the small sample size and borderline significance of effects.
- Lines 279-280: The claim “...and this is confirmed in the current study” is hardly supported by the data.

Minor comments – Introduction:

- It is currently difficult for the reader to comprehend the precise line of argumentation followed in the introduction. The structure of the introduction should be more stringent, e.g., 1.) Neural correlates of pain processing in the insula cortex and other brain regions. 2.) Behavioral correlates of modulation of insular activity (including those found using neurofeedback techniques in humans). 3.) Neurofeedback as a means to locally modulate brain activity. 4.) Research questions 5.) Study hypotheses 6.) Approach
- Improvements to the language of the introduction are necessary. Things that should be addressed include: Line 42: “...cognitive modulation of processing” -> which processing? ; Line 44: “...and mitigation” -> which mitigation? ; Lines 54-56: Awkward description of the neurofeedback principle. ; Lines 69-70: The formulation of the second hypothesis (or exploratory analysis?) is not semantically sound.

Minor comments – Results:

- Lines 217-218: Reference required.

Minor comments – Discussion:

- Line 224: Unclear what "biomarkers of neural activity" means. Usually, biomarkers indicate a clinically relevant phenomenon, such as the presence and/or degree of a disorder.
- Lines 241-252: Here, the role of the insula in pain is described without links to the study's results. This section should be shortened and/or integrated into the introduction.
- Lines 258-264: The dichotomy "spectral power vs. RMS" is somewhat artificial as the RMS also represents a spectral power but in a broader frequency band.
- Lines 267-278: Here, the role of the insula in chronic pain is described without links to the study's results. This section should be shortened or omitted.

Version 1:

Reviewer comments:

Reviewer #2

(Remarks to the Author)

The authors have addressed most open issues in their revised manuscript. However, there are a few more comments to further improve the presentation and interpretation of the results.

- Do changes in insular activity predict (correlate with) changes in pain thresholds? Showing such a link would reinforce the causal interpretation of the presented results.
- Supplementary Fig. 2 is a visually convincing demonstration that the experimental manipulation was specific to the target region (insula). Consider moving this figure to the main text.
- Please consider using a line plot with variance between subjects indicated by error bars instead of a scatterplot in Fig. 3A, as this is the typical visualization for this type of data.

Reviewer #3

(Remarks to the Author)

The manuscript has undergone revisions; however, substantial concerns remain regarding the clarity and justification of the hypotheses, methodological descriptions (e.g., VBMEG, neurofeedback principles, statistical analyses), and the rationale for the study. While some improvements have been noted, significant revisions are required to address issues of methodological transparency, logical coherence, and alignment with the stated objectives.

Comment 2

- It is hard to believe that hypothesis iii) was defined prior to data analysis. In fact, the phrasing "changes in the mean cortical excitability was (were?) not associated with significant changes in oscillatory power", reads less like a hypothesis but like an observation. Beyond this, the relevance of hypothesis iii) is unclear. I recommend framing this analysis as an exploratory one.

Comment 6

- It should be stated explicitly for which statistical test the sample size calculation was performed. I.e., what was the main analysis?
- The description of the sample size calculation should explicitly state that large effect sizes were assumed. As I pointed out in my review, I consider large effect sizes unrealistic in the given context.
- It should be stated exactly which effect size measure was used.
- "setting the power at 0.7" in the main text is likely an error? The effect size value is 0.7, the power value is 0.8?
- Why is there a sample size calculation for an ANOVA even though no ANOVA was performed in the study?
- Why are two groups mentioned? The distinction between different orders of neurofeedback conditions as different groups is artificial.

Comment 7

- The sentences added to the manuscript do not clarify how the AAL was mapped to each subject's anatomical MRI.

Comment 8

- The revised VBMEG section is still not clear.
- "VBMEG estimated that 4004 single-current dipoles were equidistantly distributed" ...does not make sense. The location and orientation of the 4004 single-current dipoles were likely defined a priori and VBMEG was used estimate their time-varying magnitude?
- How was the inverse filter constructed? Briefly explain the working principle of VBMEG. This is necessary as VBMEG is not a commonly applied method for source reconstruction.
- Were both structural MRI and sensor positions measured before each 10-minute training session?

- What is the meaning of the parameters m_0 and γ_0 ?

Comment 9

- What is meant by "other MEG data" in "The VBMEG was applied to the MEG signals of the resting state with other MEG data of 300-second duration that were recorded without subjects (MEG data of environmental noise)"
- Overall, the description of how insular activity (specifically RMS-values) is computed is clearer now. However, the clearer explanation gives rise to the following concerns: 1.) Averaging cortical signals in 1-second windows effectively eliminates all frequency components above 0.5 Hz. Therefore, the normalization constants μ_A and σ_A are valid only for the frequency range < 0.5 Hz. Is this intended? 2.) Similarly, for the real-time RMS value, averaging cortical signals within 200 ms windows captures only signal components of < 2.5 Hz. This is only a small fraction of the overall frequency spectrum of MEG signals. Therefore, the RMS value reflects low-frequency activity and not broadband activity. Is this intended?

Comment 12

- This is still unclear. I guess, MNE was used first to generate „raw“ source current estimates. In a second step, dSPM was used to standardize these source current estimates?

Comment 13

- The description of the statistical analysis is still insufficient. Which statistical tests were used for which hypotheses? How was Bonferroni correction applied exactly?

Comment 16

- The introduction has improved, but the reasons for conducting the study are still not clear. In particular, the second half of the fourth paragraph (starting with „Similarly, studies in which...“) appears to be at odds with the last sentence of the third paragraph („...although a clear link between neural activity in the insular cortex and pain perception has not been determined“)

- It is not clear what the current study concretely adds beyond the findings of the other insula-neurofeedback studies in pain mentioned in the introduction.

Comment 17

- The description of the neurofeedback principle still lacks the fact that the estimated neural feature (e.g., activity in a certain region) is "fed back" to the participant (e.g., via a visualization on a computer screen).

Comment 21

- Given my explanation above, the way RMS is computed, it mostly reflects very low frequency components (< 2.5 Hz).

Version 2:

Reviewer comments:

Reviewer #2

(Remarks to the Author)

The authors have submitted a thoroughly revised version of their manuscript. However, further analyses have uncovered a critical issue with the current interpretation of the data, specifically regarding the claim that insular neurofeedback in the MEG modulates pain thresholds. As a result, the manuscript should be revised accordingly and clarify that this relationship was not observed.

• A central interpretation of the data suggests that changes in insular activity due to neurofeedback mediate changes in pain thresholds. However, in the latest revision, the authors do not observe any correlation between changes in insular activity and pain threshold changes. Additionally, no difference is found in pain threshold changes between active and control conditions. Consequently, the manuscript presents an interpretation that is not supported by the data. There is no evidence to suggest that neurofeedback influences pain thresholds. The manuscript should be revised accordingly and emphasize that the data do not evidence a direct link between changes in insular activity and pain thresholds.

• The timeseries in Fig. 3A is currently dominated by high-frequency variance, making it difficult to discern low-frequency trends. Please consider smoothing these timeseries prior to plotting.

Reviewer #3

(Remarks to the Author)

I thank the authors for their explanations. The manuscript has improved. However, with reference to my previous comments, the following points need to be addressed:

Comment 9 and comment 21:

- The section "Neurofeedback system" describes how the feedback signal is computed. However, it does not clearly justify why this way of computing the feedback signal adequately reflects insular activity. This must be improved, and potential limitations in terms of capturing insular activity discussed. In particular, it should be justified. In particular, it should be justified, why not simply the signal power across a broad frequency range, say 1 to 100 Hz, was used as the feedback

signal.

Comment 13:

- The explanations in the rebuttal letter should be integrated into the manuscript.

Version 3:

Reviewer comments:

Reviewer #2

(Remarks to the Author)

All remaining issues were well addressed.

Reviewer #3

(Remarks to the Author)

Thank you for your explanations. In my view, the manuscript has improved.

A still unclear detail is why "the standardized currents were time-averaged" to compute the baseline insula activity. By averaging the signal within 1000ms windows, one effectively eliminates many higher frequency components of the signal and, thus, the bulk of signal power.

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Point-by-Point Replies to Reviewers

Neurofeedback modulation of insula activity via MEG-based brain-machine interface: A double-blind randomized controlled crossover trial

We appreciate the reviewers' helpful comments and suggestions. We also appreciate the constructive feedback and believe that it has helped us clarify and improve the manuscript. We have thoroughly revised our manuscript to address all the issues raised by the reviewers. In the revised manuscript, the added or corrected text appears in yellow.

Our point-by-point responses to the comments are listed below, and the reviewers' comments are shown in gray.

Response to Reviewers

Reviewer #2 (Remarks to the Author):

Wang et al. present a double-blind crossover study using MEG-based neurofeedback to alter pain thresholds. Healthy participants (N = 19) were asked to up- or downregulate activity in the right insular cortex, which was visually displayed to them in real-time. Pain thresholds and insular activity were assessed before and after a 20 minute up- or downregulation period.

The authors find a decrease in insular activity and an increase in pain thresholds after downregulation of insular activity, although with no significant interaction effect. Overall, these results are interesting and might provide a valuable starting point to target chronic pain. However, there are some methodological issues that require clarification.

Major:

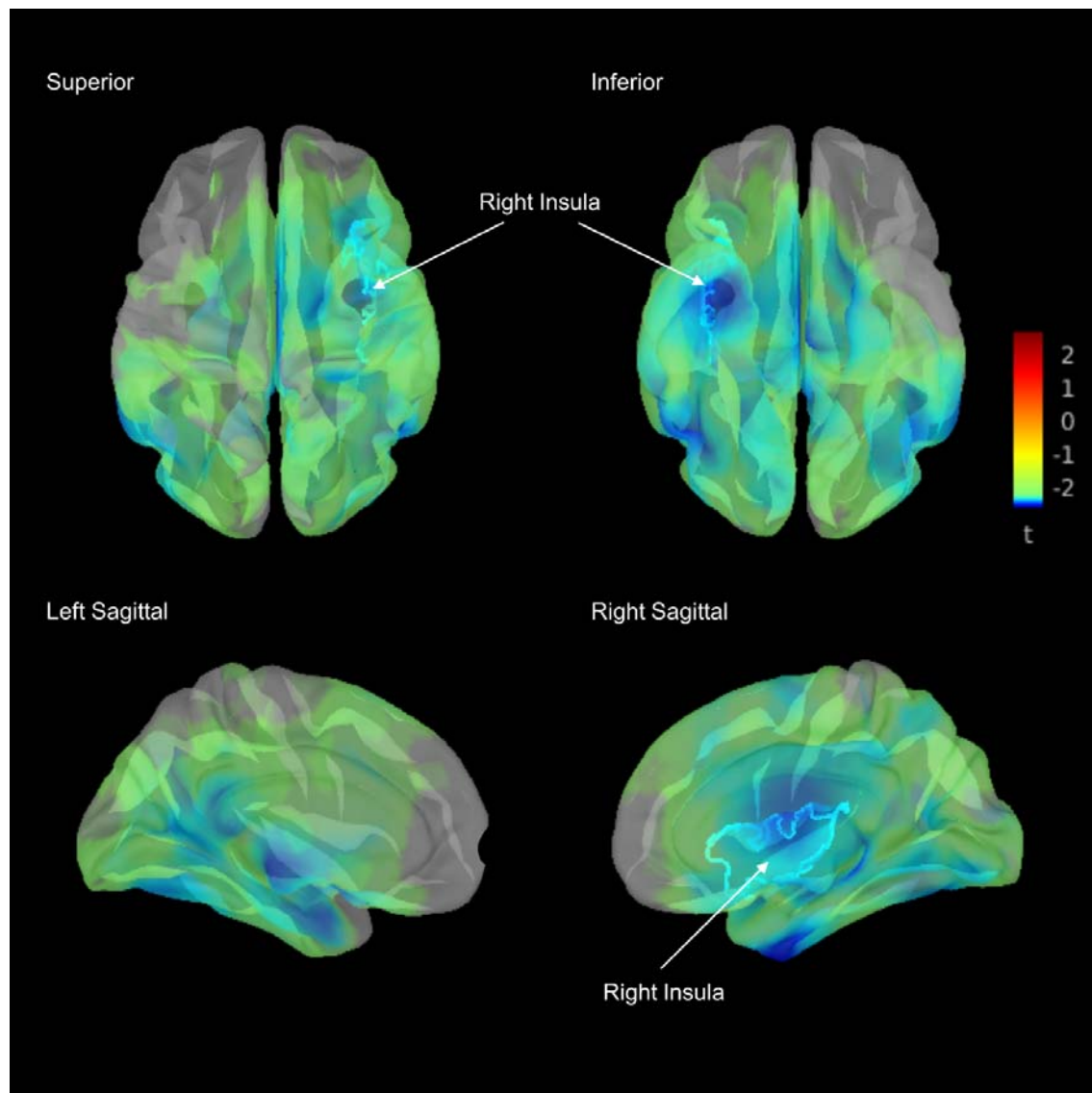
Comment 1

- It is not entirely clear whether feedback was obtained from activity specific to the insula. Although source reconstructions were performed, the Insula is over 4 cm deep in the brain, making reconstruction of the signal challenging. Could the authors show a contrast between source activity during up- and downregulation across the entire brain? Such plot would show whether the difference is specific to the insula or involves other brain regions as well.

Response to comment 1:

Thank you for your valuable feedback regarding the specificity of the feedback obtained from insula activity. We appreciate your concern about the challenges in reconstructing signals from deep brain structures such as the insula.

To address this, we evaluated the whole-brain estimated current during the resting state and compared the cortical current before and after feedback between the two types of feedback. For the estimated whole-brain cortical currents of 300 seconds, we calculated the root mean square (RMS) of the cortical currents and applied a 10 mm Gaussian smoothing kernel to the surface. The difference in the RMS after training was subsequently evaluated for each vertex (posttraining minus pretraining). Finally, we conducted paired t tests on the RMS difference between the two training conditions (downmodulation vs. upmodulation). As shown in Supplementary Figure 2 (below), the RMS difference decreased in the right insular cortex after downmodulation compared with upmodulation. The region with the most significant t value was the right insular cortex, the target of the feedback, although the differences were not statistically significant following correction for multiple comparisons (false discovery rate (FDR), $p > 0.05$). These results suggest that our magnetoencephalography (MEG) neurofeedback altered the targeted region, the right insula, most effectively.



Supplementary Figure 2. Paired t-test results of the comparison of posttraining changes in the whole brain

The color map threshold was set at uncorrected $p < 0.05$. The bright blue line marks the boundary of the right insula.

For the estimated whole-brain cortical currents of 300 seconds during the resting state before and after training, we calculated the root mean square (RMS) of the cortical currents for each vertex over time to represent the average resting-state activity. Additionally, a 10-mm Gaussian smoothing kernel was applied to the cortical surface. The difference in the RMS after training was subsequently evaluated for each vertex (posttraining minus pretraining). Finally, we conducted paired t tests on the RMS difference between the two

training conditions (downmodulation vs. upmodulation). The RMS difference decreased in the right insular cortex after downmodulation compared with upmodulation. The region with the most significant t value was the right insular cortex, the target of feedback, although the differences were not statistically significant following correction for multiple comparisons (false discovery rate (FDR), $p > 0.05$; $N = 19$). These results suggest that our magnetoencephalography (MEG) feedback altered the targeted region, the right insula, most effectively.

Comment 2

- No control condition using feedback from a different region was performed, so it remains unclear whether effects are specific to neurofeedback from the insula. Effects could be non-specific and expectation-related. The finding that both up- and downregulation raises pain thresholds reinforces this hypothesis.

Response to comment 2:

Thank you for your valuable feedback. We appreciate your concern regarding the specificity of the neurofeedback effects on the insula.

As stated in our previous response, we suggest that our neurofeedback intervention had the most significant effect on the targeted insular cortex. Additionally, we employed a fully double-blind randomized controlled crossover design, where neither the operator nor the participants were aware of the feedback type. This design was specifically intended to allow a comparison of the changes in insular activity and pain threshold differences. While insular activity or pain thresholds may be influenced by general factors such as expectations, fatigue, or adaptation, these effects would be equally distributed across both training types. For the observed changes between pre- and posttraining, our design helps mitigate the influence of these overall factors.

To clarify these points, we have revised the discussion as follows:

Lines 217~232: Data from our double-blinded randomized controlled trial suggested that MEG-based neurofeedback using cognitive modulation paradigm training on the basis of the RMS of the estimated insula current is able to decrease insula activity. We compared two types of training, one to increase the RMS of insula cortical currents and one to decrease the RMS of insula cortical currents. A significant decrease in insula activity was associated with a significant increase in the pain threshold, although the specificity of this effect in relation to the control condition could not be demonstrated. Interestingly, these significant changes in insula activity and pain thresholds were not associated with power changes in the theta band^{4,29}, which has been hypothesized to be associated with pain. These results indicate that the insula cortex is susceptible to MEG-based neurofeedback modulation, with a

potential link to the perception of pain and could therefore be a promising tool for repeated neurofeedback training to modulate pain in different contexts.

Comment 3

- Please provide more information in the main text how insular activity is measured. What exactly is the primary physiological outcome measure? This information is missing from the abstract, figures, and results, but important to increase clarity of presentation.

Response to comment 3:

Thank you for your feedback. We apologize for the lack of clarity regarding how insular activity is measured. To address this, we have included additional information in the main text to clarify our methodology.

Line484~489

Insular activity

The insular activity is quantified by computing the root mean square (RMS) value of estimated currents across all vertices within the right insula cortex for each time segment. This RMS value represents the level of insula activity during that specific interval. The formulas are provided below, with symbols defined previously:

$$A(k) = \sqrt{\frac{1}{V} \sum_{v=1}^V (\bar{I}_v(k))^2}$$

For real-time insula activity during training, the current I is defined as the average estimated current for each vertex over 200-ms time windows.

We have added a section to the manuscript where we elaborate on this process. This section includes details about how the RMS value serves as the primary physiological outcome measure for insula activity. We hope this addition increases the clarity of our presentation.

Lines 398~451

Neurofeedback system

Before the neurofeedback training, we recorded the MEG signals for 300 seconds while the subjects remained calm while fixating on the center of a circle presented in front of them. The VBMEG was applied to the MEG signals of the resting state along with corresponding 300-second MEG data recorded without subjects for each participant (environmental noise data). The cortical currents of 4,004 vertices were estimated for 300 seconds (Figure 2b). Among them, we used the cortical currents estimated at the v -th vertices

of the right insula cortex $I_v(t)$ ($v = 1 \dots 225$). For the estimated cortical currents of 300-second duration, we calculated the time average of $I_v(t)$ (μ_v) and standard deviation (σ_v) for each vertex as follows:

$$\mu_v = \frac{1}{T} \sum_{t=1}^T I_v(t), \quad \sigma_v = \sqrt{\frac{1}{T} \sum_{t=1}^T (I_v(t) - \mu_v)^2}$$

Then, using μ_v and σ_v , we obtained the normalized current $\hat{I}_v(t)$ for each v -th vertex at time t as follows:

$$\hat{I}_v(t) = \frac{I_v(t) - \mu_v}{\sigma_v} \quad (t = 0-300 \text{ seconds})$$

The standardized current $\hat{I}_v(t)$ of 300 seconds was segmented into 300 intervals of 1000 ms without overlaps, within which the standardized currents were time averaged to compute the mean values for the k -th interval, $\bar{I}_v(k)$. Then, we computed the root mean square (RMS) value of all vertices within the right insula cortex for the k -th time interval as follows:

$$A(k) = \sqrt{\frac{1}{V} \sum_{v=1}^V (\bar{I}_v(k))^2}$$

The baseline insula activity and its standard deviation across 300 intervals were calculated as follows:

$$\mu_A = \frac{1}{300} \sum_{k=1}^{300} A(k), \quad \sigma_A = \sqrt{\frac{1}{300} \sum_{k=1}^{300} (A(k) - \mu_A)^2}$$

Similarly, we averaged the real-time estimated currents of the v -th vertex in the right insular cortex using a 200-ms time window and subsequently standardized the data by using μ_v and σ_v to compute the real-time insula activity.

The real-time RMS value for insula activity in a 200 ms window was calculated as follows:

$$\hat{A}(t) = \sqrt{\frac{1}{V} \sum_{v=1}^V \left(\frac{1}{N_t} \sum_{t' \in \text{window } t} \frac{I_v(t') - \mu_v}{\sigma_v} \right)^2}$$

During neurofeedback training, the feedback signal was computed differently for upmodulation and downmodulation sessions on the basis of real-time insula activity

$\hat{A}(t)$.

Upmodulation Feedback:

For upmodulation, the aim of feedback was to increase insula activity. The feedback value was calculated as follows:

$$\text{out_vals}_{\text{up}} = \left(\frac{\hat{A}(t) - \mu_A}{8 \cdot \sigma_A} + 0.5 \right) \times 255$$

Downmodulation Feedback:

For downmodulation, the aim of feedback was to decrease insula activity. The feedback value was calculated as follows:

$$\text{out_vals}_{\text{down}} = \left(-\frac{\hat{A}(t) - \mu_A}{8 \cdot \sigma_A} + 0.5 \right) \times 255$$

Clipping and Rounding:

The feedback values `out_vals` were rounded to the nearest integer and clipped to the range [0, 255]:

$$\text{out_vals} = \min(255, \max(0, \text{round}(\text{out_vals})))$$

Notably, we did not remove artifacts to select the noisy time segments for the calculation for neurofeedback training. All the MEG signals obtained in the resting-state and neurofeedback sessions were used for the calculations above.

For both the upmodulation and the downmodulation conditions, the subjects were instructed to intentionally try to increase the size of the black disk on the screen.

Comment 4

Minor:

- All figures are missing a unit for the y-axis label.

Response to comment 4:

Thank you for pointing out the issue with the missing units on the y-axis labels of our figures. We apologize for this. Since the insula activity and spectral power are both standardized values, we did not label the units. We have revised the y-axis to include a description indicating normalization.

We have now added the appropriate units to the y-axis labels as necessary.

Lines 133~146

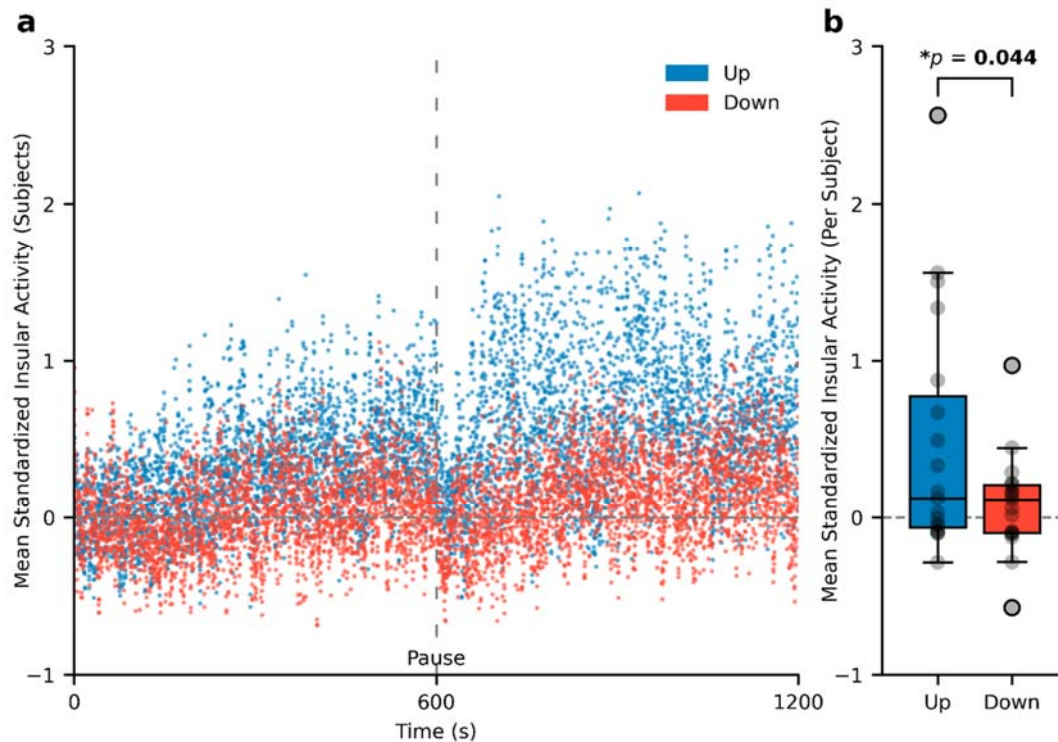


Figure 3. Modulation of insula activity during neurofeedback training.

a) The plot shows the time course of mean standardized insula activity averaged across all subjects during neurofeedback training. Each point represents the group-averaged insula activity (blue for the upmodulation condition, red for the downmodulation condition), with the horizontal axis indicating training time. The vertical dashed line at 600 seconds marks a brief pause between two training sessions.

b) The boxplot compares the mean insula activity during the entire training process for each subject under the upmodulation and downmodulation conditions. Insula activity during upmodulation training was significantly greater than that during downmodulation training (blue for upmodulation training; red for downmodulation training). Two-tailed paired Student's t test, $*p < 0.05$. In the boxplots, centerlines represent medians, the box limits represent the lower and upper quartiles, the whiskers represent 1.5 times the interquartile range, and the circles represent outliers.

Line161~168:

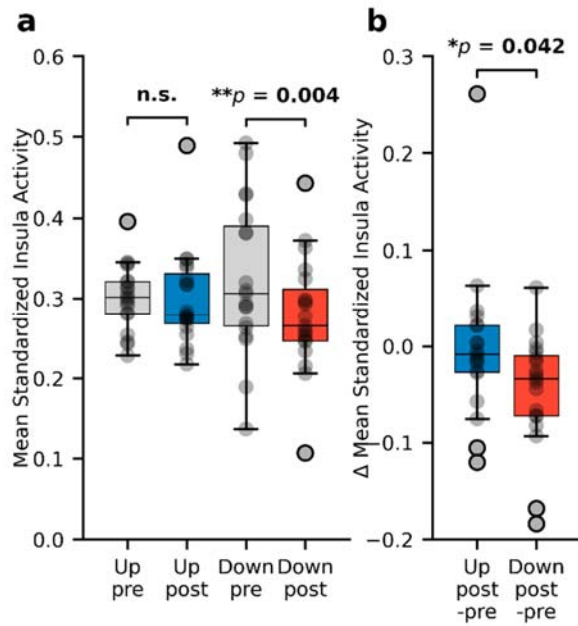


Figure 4. Alterations in insula activity before and after neurofeedback.

a) Mean resting-state insula activity before (pre) and after (post) the upmodulation (blue) and downmodulation (red) training is shown. Paired t-tests were conducted to compare pre and post activity levels within each training condition.

b) Differences in insula activity (post - pre) for the upmodulation (blue) and downmodulation (red) conditions are shown. A paired t-test was conducted to compare these differences between the two conditions.

Lines 183~190:

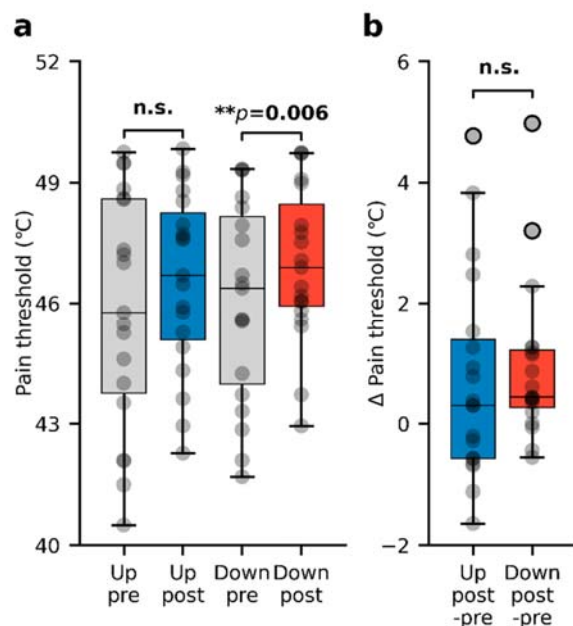


Figure 5. Alterations in pain thresholds before and after neurofeedback training.

a) Pain thresholds before (pre) and after (post) the upmodulation (blue) and downmodulation (red) training are shown. Paired t-tests were conducted to compare pre and Pain thresholds within each training condition.

b) Differences in Pain thresholds (post - pre) for the upmodulation (blue) and downmodulation (red) conditions are shown. A paired t-test was conducted to compare these differences between the two conditions.

All the data were analyzed by two-tailed paired Student's t tests with Bonferroni correction. Asterisks (*) denote statistically significant differences, $*p < 0.05$, $**p < 0.01$. In the boxplots, dots represent individual data points, centerlines represent medians, box limits represent lower and upper quartiles, whiskers represent 1.5 times the interquartile range, and circles represent outliers.

Lines 210~217:

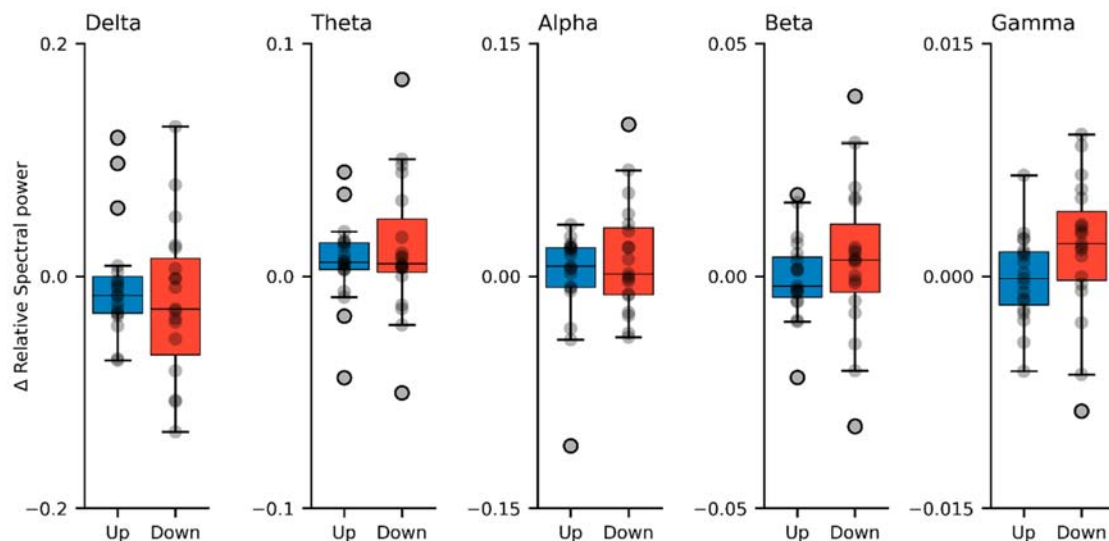


Figure 6. Comparing the band power between the pretraining and posttraining resting states.

We observed no significant difference ($n=19$, two-tailed paired Student's t test, Bonferroni correction) in the spectral power changes (posttraining minus pretraining) after training. In the boxplots, dots represent individual data points, centerlines represent medians, box limits represent lower and upper quartiles, whiskers represent 1.5 times the interquartile range, and circles represent outliers.

Comment 5

- A direct comparison between the up- and downregulation conditions should be performed for change in insular activity (Fig. 4) and change in pain thresholds (Fig. 5).

Response to comment 5:

Thank you for your feedback. We apologize for any confusion caused by the figure presentations. We have included t tests between the differences (posttraining–pretraining) of the up- and downmodulation conditions in the figures. Specifically, these t tests are shown in the rightmost subplots of Figures 4 and 5. We hope this addresses your concerns and provides the clarity you were seeking.

Line161~168:

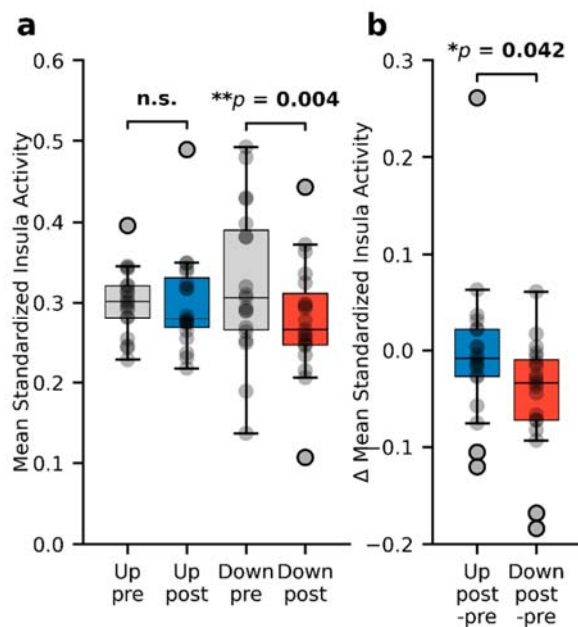


Figure 4. Alterations in insula activity before and after neurofeedback.

a) Mean resting-state insula activity before (pre) and after (post) the upmodulation (blue) and downmodulation (red) training is shown. Paired t-tests were conducted to compare pre and post activity levels within each training condition.

b) Differences in insula activity (post - pre) for the upmodulation (blue) and downmodulation (red) conditions are shown. A paired t-test was conducted to compare these differences between the two conditions.

Lines 183~190

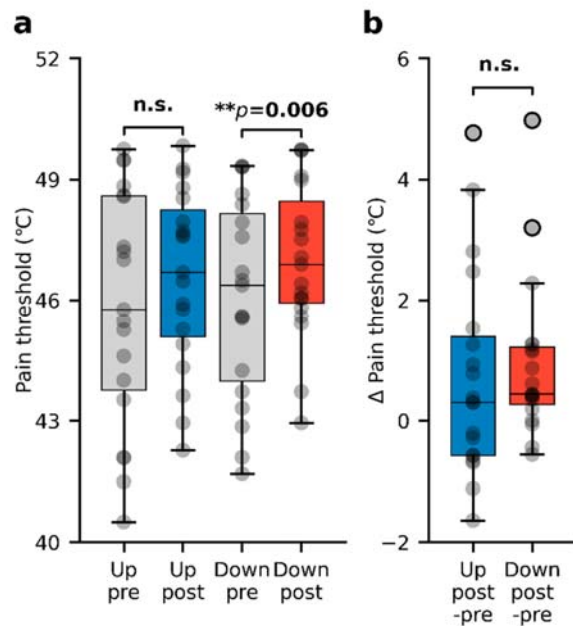


Figure 5. Alterations in pain thresholds before and after neurofeedback training.

a) Pain thresholds before (pre) and after (post) the upmodulation (blue) and downmodulation (red) training are shown. Paired t-tests were conducted to compare pre and Pain thresholds within each training condition.

b) Differences in Pain thresholds (post - pre) for the upmodulation (blue) and downmodulation (red) conditions are shown. A paired t-test was conducted to compare these differences between the two conditions.

All the data were analyzed by two-tailed paired Student's t tests with Bonferroni correction. Asterisks (*) denote statistically significant differences, $*p < 0.05$, $**p < 0.01$. In the boxplots, dots represent individual data points, centerlines represent medians, box limits represent lower and upper quartiles, whiskers represent 1.5 times the interquartile range, and circles represent outliers.

Comment 6

- In the discussion, several studies are cited that use brain stimulation, such as TMS. However, there is a controversy whether such paradigms can directly reach such deep areas (see letters to the editor to Galhardoni et al., e.g.). While non-invasive brain stimulation can be useful, it comes with several challenges (see Nasr et al. 2022, doi:10.1016/j.pneurobio.2022.102311). Combination of neurofeedback with closed-loop approaches (e.g., Haslacher et al. 2023, doi: 10.1016/j.neuroimage.2023.120187), especially those that can reliably target deep brain areas such as ultrasound may offer a promising avenue for more effective and precise interventions. These advanced methods

could potentially overcome the limitations of traditional brain stimulation techniques by providing real-time feedback and more accurate targeting.

Response to comment 6:

Thank you for your valuable comment and insightful suggestions. In accordance with the reviewer's comments, we added a discussion at Lines 274~286 as follows:

Brain stimulation techniques such as TMS have been employed in several studies to modulate brain activity in deep regions such as the insula. However, there is ongoing debate regarding whether these methods can effectively reach such deep areas owing to limitations in the depth of stimulation. This controversy highlights the challenges inherent in noninvasive brain stimulation techniques. Nasr *et al.*¹ discussed the challenges of traditional noninvasive brain stimulation techniques, particularly their limited precision and potential side effects when targeting deep brain regions. They emphasize the need for more advanced approaches that can overcome these limitations. The combination of neurofeedback with closed-loop approaches², especially those that can reliably target deep brain areas, such as ultrasound, could offer a promising avenue for more effective and precise interventions. Neurofeedback combined with these advanced methods could overcome the limitations of traditional brain stimulation techniques by providing real-time feedback and more accurate targeting.

Comment 7

Please include information on how the study complies with the “consensus on the reporting and experimental design of clinical and cognitive-behavioural neurofeedback studies” (CRED-nf checklist, <https://pubmed.ncbi.nlm.nih.gov/32176800>).

Response to comment 7:

Thank you for this comment. We have addressed this comment by generating the requested document, which has been included as an attachment to this submission.

Reviewer #3 (Remarks to the Author):

This paper investigates the modulation of brain activity in the insular cortex and its effects on pain perception using neurofeedback techniques based on magnetoencephalography (MEG) in healthy individuals. The findings suggest that insula activity can be modulated via Neurofeedback. However, direct evidence for an effect on pain perception was not found.

The study's objective of altering pain perception via neuromodulation is potentially significant. The overall study design appears straightforward and, in principle, sound. However, several critical issues regarding implementation and reporting need to be

addressed: Research questions and hypotheses are imprecisely stated, some statistical analyses are flawed, the study appears underpowered, and the descriptions of the methods lack precision and comprehensibility.

Comment 1

Major comments – Introduction:

- The second research question ("...which activity pattern best represents pain perception: oscillatory power or mean cortical excitability evaluated by BOLD signal") is not addressed by any subsequent hypotheses.

Response to comment 1:

Thank you for pointing out this issue with respect to the second research question. To answer the second research question, we compared the band power between the pre- and posttraining resting states, as shown in Figure 6. Although the root mean square (RMS) of the cortical currents, which corresponds to the mean cortical excitability, significantly differed between the two training conditions in association with the pain threshold changes, there were no significant changes in frequency band powers. This result suggests that, in our study, the mean cortical excitability evaluated by magnetoencephalography (MEG) was better correlated with the pain threshold than with the oscillatory power. Our intent was to explore feedback methods that are based on mean cortical excitability, such as functional magnetic resonance imaging (fMRI) blood oxygen level-dependent (BOLD) signals or the mean cortical current magnitude estimated from MEG or electroencephalography (EEG). The previous description, "mean cortical excitability evaluated by BOLD signal", might be misleading. Therefore, we have revised the manuscript to clarify this, and the research question now reads as follows:

Lines 67~68:

"...and ii) which activity pattern best represents pain perception: oscillatory power or mean cortical excitability

Lines 210~217:

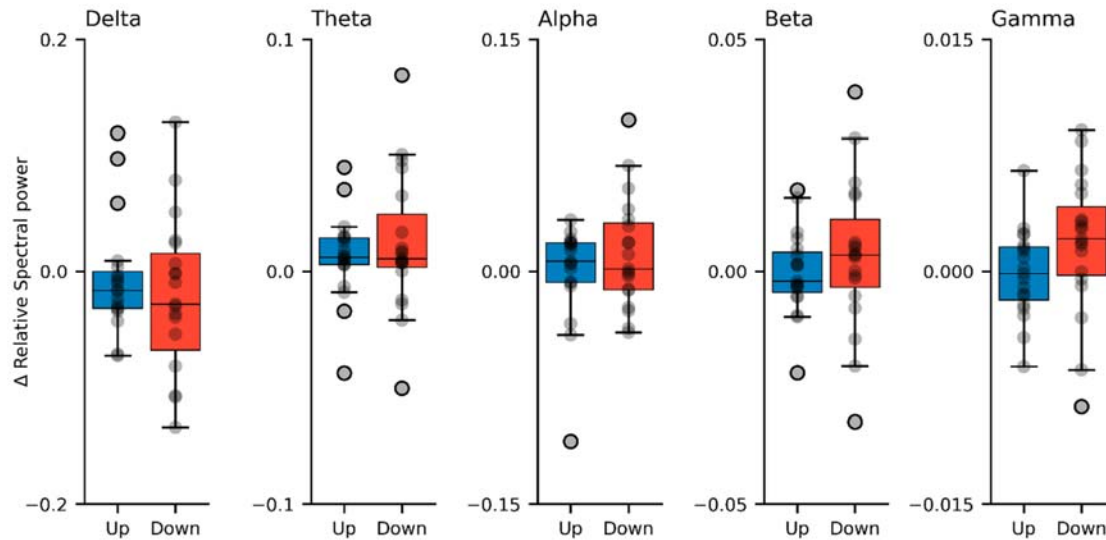


Figure 6. Comparing the band power between the pretraining and posttraining resting states.

We observed no significant difference ($n=19$, two-tailed paired Student's t test, Bonferroni correction) in the spectral power changes (posttraining minus pretraining) after training. In the boxplots, dots represent individual data points, centerlines represent medians, box limits represent lower and upper quartiles, whiskers represent 1.5 times the interquartile range, and circles represent outliers.

Comment 2

- It is unclear which parts of the study are confirmatory versus exploratory.

Response to comment 2:

Thank you for pointing this out. We apologize for the lack of clarity in our original writing. We would like to clarify that both hypotheses in the study are confirmatory. We have revised the second hypothesis for better clarity.

Lines 74~75: ii) Downmodulating the mean cortical excitability of insula activity through neurofeedback could increase the pain threshold

Comment 3

- The second hypothesis ("...increase in the pain threshold utilized for neurofeedback to modulate pain") is unrelated to the research questions and lacks clarity.

Response to comment 3:

Thank you for your feedback. We acknowledge that the original phrasing was not precise. Our intended hypothesis was that downmodulating the mean cortical excitability of insula

activity could increase the pain threshold. If this hypothesis is confirmed, it would help address our second research question by determining whether mean cortical excitability, as opposed to spontaneous oscillatory activity, better represents pain perception.

We have revised the manuscript as follows.

Lines 65~69: Although these results have suggested a strong relationship between insula activity and pain perception, it is unknown i) to what extent insula activity is under cognitive control such that it is susceptible to neurofeedback, ii) whether changes in insula activity are associated with changes in pain perception, and iii) which activity pattern is associated with changes in pain perception: oscillatory power or mean cortical excitability.

Comment 4

Major comments – Results:

- The statistical tests used to assess neurofeedback effects on insular activity during neurofeedback training are invalid. Regression analyses treating different time points as independent samples are flawed. Suggested approach: Analyze changes in insular activity (e.g., second half minus first half of training) at the individual level rather than time points.

Response to comment 4:

Thank you for your valuable feedback regarding the statistical analysis we used to assess the neurofeedback effects on insular activity during training. We agree with your point that treating different time points as independent samples in the regression analysis is not appropriate. We have deleted the regression analysis in Figure 3.

On the basis of your suggestion, we compared insular activity between the first and second halves of the training at the individual level. The results of this analysis revealed no significant differences ($p = 0.145 > 0.05$, paired t test, $n = 19$). However, we found a significant difference in insular activity at the individual level between the two training conditions ($p = 0.044 < 0.05$, paired t test, $n = 19$), suggesting that the training was effective in changing insular activity, but the activity changes over time were not prominent. We have revised Figure 3 accordingly.

Lines 134~147:

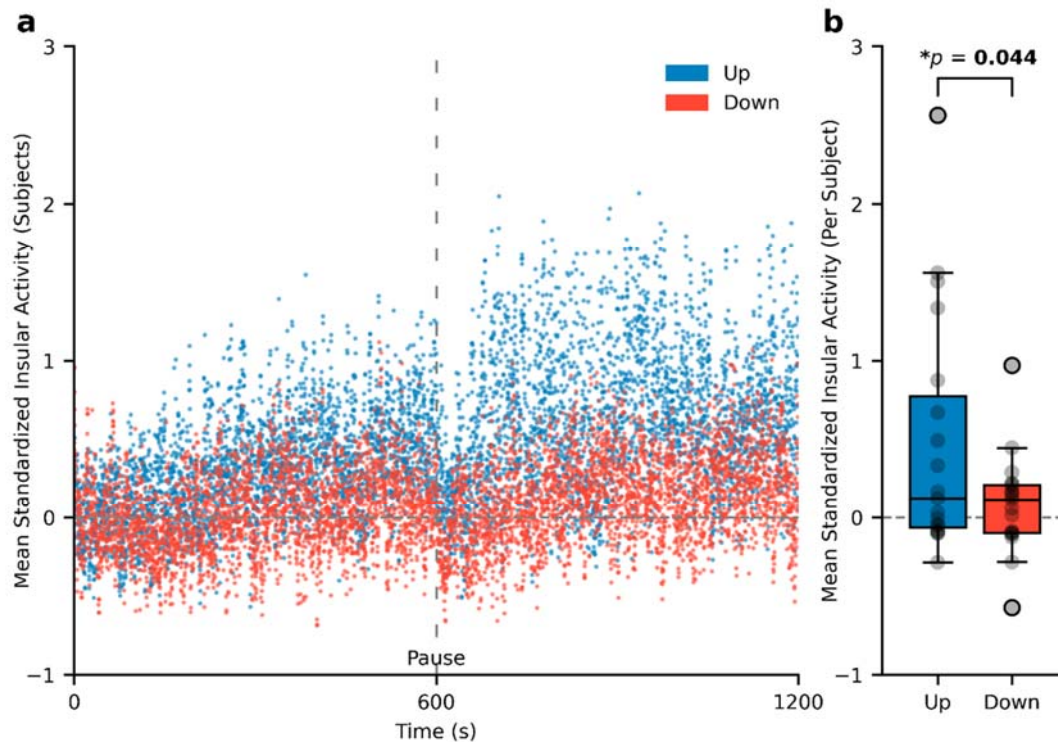


Figure 3. Modulation of insula activity during neurofeedback training.

a) The plot shows the time course of mean standardized insula activity averaged across all subjects during neurofeedback training. Each point represents the group-averaged insula activity (blue for the upmodulation condition, red for the downmodulation condition), with the horizontal axis indicating training time. The vertical dashed line at 600 seconds marks a brief pause between two training sessions.

b) The boxplot compares the mean insula activity during the entire training process for each subject under the upmodulation and downmodulation conditions. Insula activity during upmodulation training was significantly greater than that during downmodulation training (blue for upmodulation training; red for downmodulation training). Two-tailed paired Student's t test, $*p < 0.05$. In the boxplots, centerlines represent medians, the box limits represent the lower and upper quartiles, the whiskers represent 1.5 times the interquartile range, and the circles represent outliers.

Comment 5

- The assessment of neurofeedback effects on resting state insula activity is statistically sound. However, the difference between activity changes between up- and downmodulation training is only borderline significant. Thus, in light of the small sample size, it is questionable how reliable the results are.

Response to comment 5:

Thank you for your thoughtful comments regarding the assessment of neurofeedback effects on resting-state insula activity. We acknowledge your concern about the borderline significance of the difference between activity changes during up- and downmodulation training, particularly given the small sample size. We agree that the limited sample size may affect the reliability of the results, and we have now emphasized this limitation in the discussion section of the manuscript. We also acknowledge that the borderline significance suggests that these findings should be interpreted with caution and that further research with a larger sample size is needed to confirm these effects.

Lines 232~235

We acknowledge the limitations of our study, particularly the relatively small sample size and the borderline significance of the results, which may affect the reliability and generalizability of our findings. Future studies with larger sample sizes are needed to validate these observations and further investigate the underlying effects.

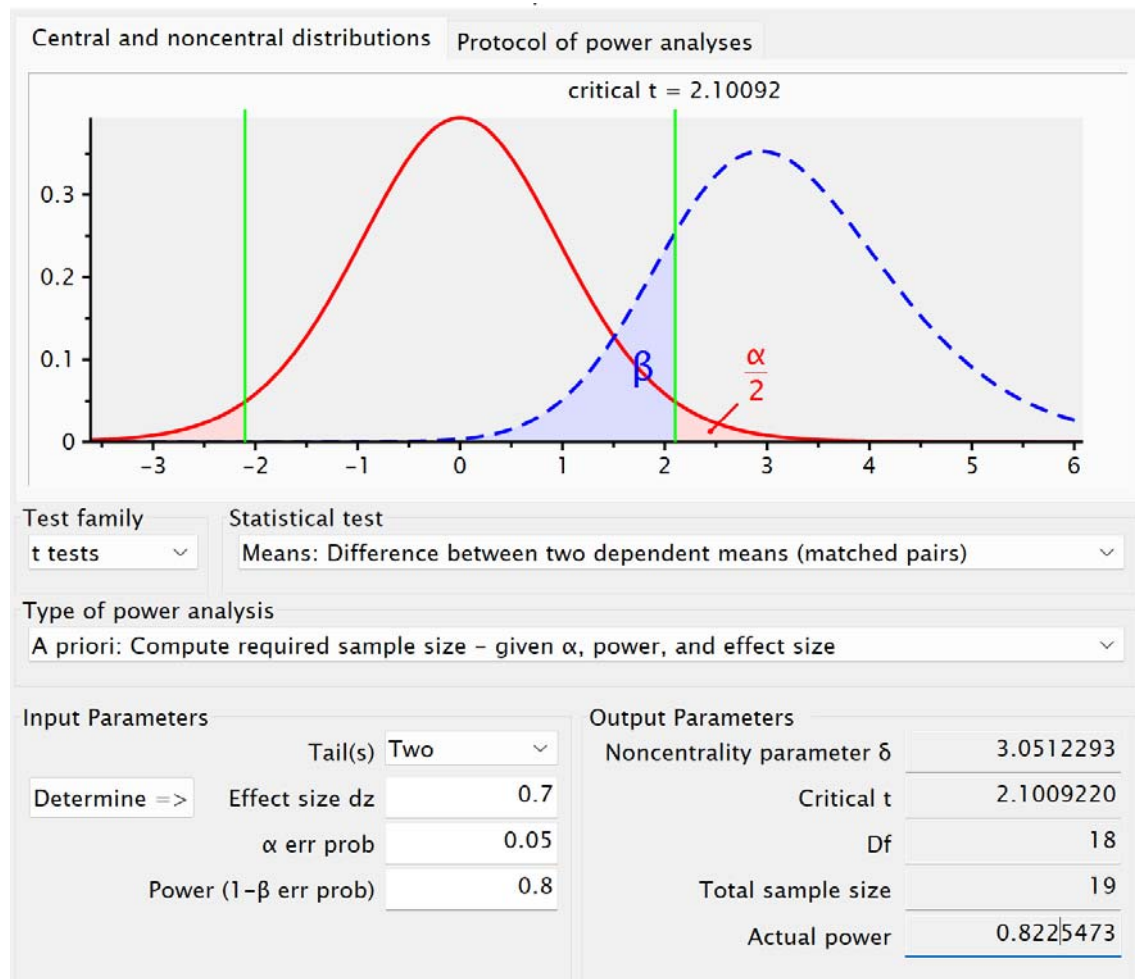
Comment 6

Major comments – Methods:

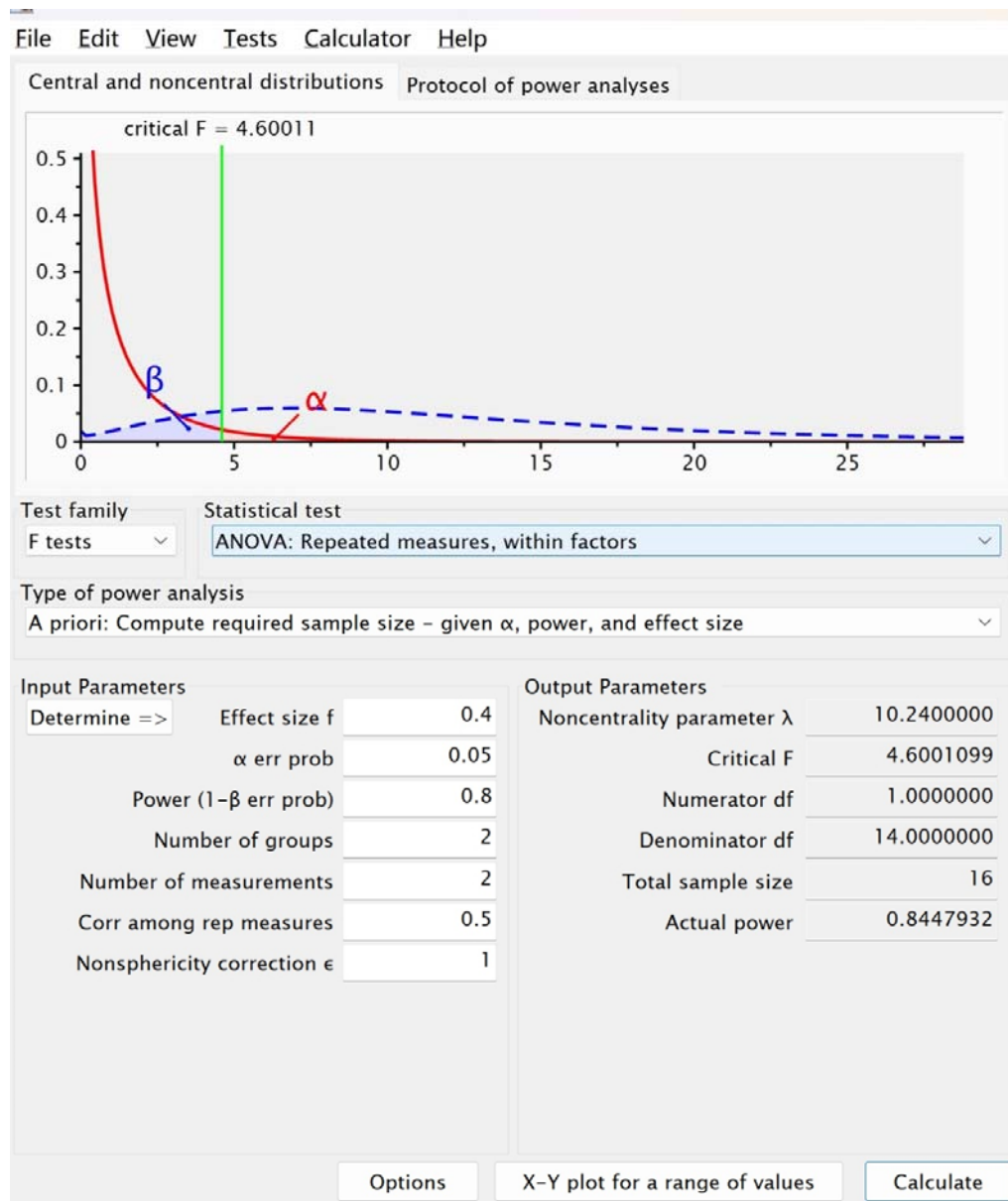
- A priori sample size calculations are missing. Assuming small to medium effect sizes, which are to be expected in translational neuroscience studies like this one, the study is likely underpowered to detect the hypothesized effects. It is strongly recommended that additional data be acquired: $N_{total} > 40$.

Response to comment 6:

Thank you for your valuable feedback regarding the sample size calculation. We apologize for not providing detailed information in the manuscript about how we determined the sample size. Initially, we assumed a larger effect size for the study. We used G*Power 3.1 to calculate the required sample size for a t test, setting the power at 0.7 and the significance level at 0.05, which indicated that 19 participants would be necessary for the study.



For the comparison of pre- and posttraining pain thresholds between the two groups, we assumed an effect size of 0.4, with a power of 0.8, an alpha level of 0.05, and a correlation of 0.5. From this, the calculated sample size was 16 participants.



Taking these considerations into account, we initially set the sample size to 20 participants, based on the assumption of a large effect size in our calculations. We acknowledge that this relatively small sample size may have limited our ability to detect effects in the study. While we understand your suggestion that acquiring additional data could enhance the statistical power of our findings, our study was designed as a double-blind randomized crossover trial, and the data collection has already been completed and unblinded. Collecting new data at this stage would compromise the integrity of the study design and introduce inconsistencies in external factors, such as experimental conditions and the overall time span, potentially affecting the reliability and comparability of the results. To address this limitation, we plan to increase the sample size in future experiments to ensure more robust and statistically reliable

results.

To explain how the sample size was calculated, we added a section on sample size calculation at Lines 498~505, in the Methods section.

Sample size calculation

We used G*Power 3.1 to calculate the required sample size for a t test, setting the power at 0.7 and the significance level at 0.05, and the results indicated that 19 participants would be necessary for the study. For the comparison of pre- and posttraining pain thresholds between the two groups, we assumed an effect size of 0.4, with a power of 0.8, an alpha level of 0.05, and a correlation of 0.5. From this, the calculated sample size was 16 participants. Taking these considerations into account, we initially set the sample size to 20 participants.

Comment 7

- A description of the exact procedure in which the registration of individual MRI and AAL was achieved is missing

Response to comment 7:

Thank you for your valuable comments, and we apologize for the incomplete description of the registration. We constructed a polygon model of the cortical surface from structural T1-weighted magnetic resonance imaging (MRI) by using FreeSurfer software (Martinos Center for Biomedical Imaging, Massachusetts General Hospital, Charlestown, MA, USA). The extracted individual cortical surface model was projected into default Montreal Neurological Institute (MNI) normalized ICBM152/ FreeSurfer 15000 vertices anatomy. The normalized brain was subsequently matched to the automated anatomical labeling 2 (AAL2) parcellation. Finally, we extracted the estimated source currents for the insula using the vertex numbers corresponding to the right insula region defined by the AAL2 atlas in the normalized brain. To clarify these steps, we added the following sentences to the Methods section:

Line404~406:

Among them, we used the cortical currents estimated at the v -th vertices of the right insula cortex $I_v(t)$ ($v = 1 \dots 225$) using the AAL2 parcellation provided in VBMEG.

Line473~476:

Then, the individual source was projected into default MNI ICBM152/FreeSurfer 15000 vertices anatomy. The time course of the source activity was extracted from the right insular cortex using the AAL2 template provided in Brainstorm.

Comment 8

- MEG recordings: The description of the reconstruction of cortical currents in the insula cortex is imprecise. Steps to improve this could include the following: Explain why the BME method was used to compute the spatial filter; provide more methodological detail on spatial filter construction using BME.

Response to comment 8:

Thank you for your valuable comments, and we apologize for the incomplete description of Variational Bayesian Multimodal Encephalography (VBMEG). We have added the methodological details on the spatial filter construction to the Methods section, and we added an explanation of why the BME method was used in our online neurofeedback experiment. We developed our MEG neurofeedback system on the basis of the VBMEG source reconstruction method. We confirmed the efficacy of the system in estimating cortical currents online in our previous studies^{3,4}. Therefore, we constructed our neurofeedback system with VBMEG. We have revised the VBMEG section in the Methods section as follows:

Lines 380~396:

Online cortical current estimation via variational Bayesian multimodal encephalography

For online estimation of the cortical currents in the insular cortex from MEG data, we used Variational Bayesian Multimodal Encephalography (VBMEG)^{5,6} (ATR Neural Information Analysis Laboratories, Kyoto, Japan) in MATLAB (version R2013). First, a polygon model of the cortical surface was constructed on the basis of structural MRI data via FreeSurfer software (Martinos Center for Biomedical Imaging, Massachusetts General Hospital, Charlestown, MA, USA). Then, VBMEG estimated that 4,004 single-current dipoles were equidistantly distributed and perpendicular to the cortical surface. An inverse filter was used to estimate the cortical current of each dipole using MEG signals. We computed the lead field by applying the boundary element method to the coregistered sensor positions and polygon model on the basis of data collected immediately before each 10-minute training session. We used the cortical currents estimated from the MEG signals of the 110 selected sensors (Supplementary Figure 3). The hyperparameters m_0 and γ_0 were set to 100 and 10, respectively. We used this online cortical current estimation because we confirmed that the system can effectively evaluate motor cortical responses via neurofeedback^{3,4}.

Comment 9

- Neurofeedback system: The description of the neurofeedback system is incomprehensible. Steps to improve this could include the following: Provide exact definition of insula activity in

mathematical notation; state the physiological rationale for the selected definition of insula activity; provide exact definition of the clamp function in mathematical notation.

Response to comment 9:

We apologize for the incomplete descriptions of the neurofeedback process. Following the suggestions of reviewer 2, we have thoroughly revised the Methods section to provide an exact definition of insula activity in mathematical notation, to state the physiological rationale for the selected definition of insula activity, and to provide an exact definition of the clamp function in mathematical notation.

Lines 398~451:

Neurofeedback system

Before the neurofeedback training, we recorded the MEG signals for 300 seconds while the subjects remained calm while fixating on the center of a circle presented in front of them. The VBMEG was applied to the MEG signals of the resting state with other MEG data of 300-second duration that were recorded without subjects (MEG data of environmental noise). The cortical currents of 4,004 vertices were estimated for 300 seconds (Figure 2b). Among these currents, we used the cortical currents estimated at the v -th vertices of the right insula cortex $I_v(t)$ ($v = 1 \dots 225$). For the estimated cortical currents of 300 seconds, we calculated the time average of $I_v(t)$ (μ_v) and standard deviation (σ_v) for each vertex as follows:

$$\mu_v = \frac{1}{T} \sum_{t=1}^T I_v(t), \quad \sigma_v = \sqrt{\frac{1}{T} \sum_{t=1}^T (I_v(t) - \mu_v)^2}$$

Then, using μ_v and σ_v , we obtained the normalized current $\hat{I}_v(t)$ for each v -th vertex at time t as follows:

$$\hat{I}_v(t) = \frac{I_v(t) - \mu_v}{\sigma_v} \quad (t = 0-300 \text{ s})$$

The normalized current $\hat{I}_v(t)$ of 300-second duration was segmented into 300 intervals of 1000 ms without overlaps, within which the normalized currents were time averaged to compute the mean values for the k -th interval, $\overline{I}_v(k)$. Then, we computed the root mean square (RMS) value of all vertices within the right insula cortex for the k -th time interval as follows:

$$A(k) = \sqrt{\frac{1}{V} \sum_{v=1}^V (\overline{I}_v(k))^2}$$

The baseline insula activity and its standard deviation across 300 intervals were calculated as follows:

$$\mu_A = \frac{1}{300} \sum_{k=1}^{300} A(k), \quad \sigma_A = \sqrt{\frac{1}{300} \sum_{k=1}^{300} (A(k) - \mu_A)^2}$$

Similarly, we averaged the real-time estimated currents of the v -th vertex in the right insular cortex using a 200-ms time window and subsequently normalized the data by using μ_v and σ_v to compute the real-time insula activity.

The real-time RMS value for insula activity in a 200 ms window was calculated as follows:

$$\hat{A}(t) = \sqrt{\frac{1}{V} \sum_{v=1}^V \left(\frac{1}{N_t} \sum_{t' \in \text{window } t} \frac{I_v(t') - \mu_v}{\sigma_v} \right)^2}$$

During neurofeedback training, the feedback signal was computed differently for upmodulation and downmodulation sessions on the basis of real-time insula activity $\hat{A}(t)$.

Upmodulation Feedback:

For upmodulation, the aim of feedback was to increase insula activity. The feedback value was calculated as follows:

$$\text{out_vals}_{\text{up}} = \left(\frac{\hat{A}(t) - \mu_A}{8 \cdot \sigma_A} + 0.5 \right) \times 255$$

Downmodulation Feedback:

For downmodulation, the aim of feedback was to decrease insula activity. The feedback value was calculated as follows:

$$\text{out_vals}_{\text{down}} = \left(-\frac{\hat{A}(t) - \mu_A}{8 \cdot \sigma_A} + 0.5 \right) \times 255$$

Clipping and Rounding:

The feedback values out_vals were rounded to the nearest integer and clipped to the range [0, 255]:

$$\text{out_vals} = \min(255, \max(0, \text{round}(\text{out_vals})))$$

Notably, we did not remove artifacts to select the noisy time segments for the calculation for neurofeedback training. All the MEG signals obtained in the resting-state and neurofeedback sessions were used for the calculations above.

For both the upmodulation and the downmodulation conditions, the subjects were instructed to intentionally try to increase the size of the black disk on the screen.

Comment 10

- Resting-state data processing: The description of the Resting-state data processing is imprecise. Were artifacts removed by rejecting associated time segments or ICA components?

Response to comment 10:

We appreciate this comment and apologize for the incomplete description in our previous manuscript. For the calculation of the resting state for the baseline of the neurofeedback experiments, we did not remove the artifacts. On the other hand, for the offline analysis of the resting state, artifacts were removed through visual inspection and by applying the signal space separation (SSS) method.

To clarify this point, we have revised the text as follows:

Lines 461~464: Artifacts were removed through visual inspection and by applying the signal space separation (SSS) method. Cardiac and ocular artifacts were further minimized by detecting and removing signal segments corresponding to heartbeats and eye movements via event-based artifact rejection techniques.

Comment 11

For source reconstruction, minimum norm estimation (MNE) is employed in contrast to BME above. Why this discrepancy?

Response to comment 11:

Thank you for asking this question. We used two types of source reconstruction methods to increase the reproducibility of our analysis and to achieve high-performance neurofeedback. Although we believe that Variational Bayesian Multimodal Encephalography (VBMEG) is a good tool for reconstructing cortical currents, we know that VBMEG is rather uncommon. On the other hand, minimum-norm estimation (MNE) in Brainstorm is a widely used method that is supported by user-friendly and flexible software. Therefore, we selected the MNE method to evaluate the MEG signals in the offline analysis so that the results can be easily reproduced with our data. On the other hand, Brainstorm is not suitable for the online data processing that is required in neurofeedback experiments. We have developed our neurofeedback system by using VBMEG, which has many useful functions for real-time data processing. To clarify these points, we have added some comments on the methods at Lines 482~489, as follows:

It should be noted that we used two source reconstruction methods to ensure reproducibility and achieve high-performance neurofeedback. MNE in Brainstorm was applied for offline analysis of MEG signals during the resting state, while VBMEG was used for online

neurofeedback experiments. MNE was chosen for offline analysis because it is a commonly used method, facilitating easier reproduction of our results compared to VBMEG. On the other hand, VBMEG can estimate cortical currents with high accuracy in real time, and its efficacy has been validated in our previous studies^{3,4}. therefore, we constructed our neurofeedback system with VBMEG.

Comment 12

Moreover, the MNE does not necessarily require the computation of a noise covariance matrix. However, the paper uses a version of MNE that requires calculating the noise covariance matrix. Thus, it should be stated more precisely which type of MNE was used and how the noise covariance was estimated precisely.

Response to comment 12:

Thank you for your comment. We applied dynamic statistical parametric mapping (dSPM)⁷, which is derived from the default minimum-norm estimation (MNE) and normalized via the noise covariance matrix. When we reconstruct the current during a certain task, such as hand movements, we can estimate the cortical currents by contrasting the magnetoencephalography (MEG) signals during resting and moving the hands. However, when we reconstruct the currents from resting-state data in Brainstorm, we need to use the MEG signals from empty-room data that are recorded prior to each experiment. To clarify these points, we revised the Methods section as follows:

Lines 467~478:

Source reconstruction was performed using the minimum-norm estimation (MNE) method. We applied the dynamic statistical parametric mapping (dSPM)⁷, which is derived from the default MNE and standardized via the noise covariance matrix. The resulting dSPM maps represent a set of z scores, expressed in unitless "z" values. Therefore, we used the noise covariance matrix, which was generated from the MEG signals of 5 minutes of empty-room data, to the source reconstruction in Brainstorm.

Comment 13

- Methods/Statistical analysis and reproducibility: This section lacks detail, and there is no information about strategies to ensure the reproducibility of findings.

Response to comment 13:

Thank you for pointing this out. To ensure the reproducibility of the findings, we have added relevant formulas and more detailed software information. In addition, we uploaded the raw data for all the results so that everyone can reproduce our findings from the data. We believe these efforts will increase the reproducibility of our findings. To clarify these points, we revised the Methods/Statistical analysis and reproducibility section as follows:

Lines 507–511

Statistical analysis was performed via Python 3.8.5 with the scipy package v.1.5.2⁸. Two-sided statistical tests were performed for all the subjects. Adjusted p values <0.05 were considered significant. Bonferroni correction was applied for multiple comparisons. All raw data used in the statistical analysis are available in ***.

Comment 14

Major comments – Discussion:

- Lines 218-221: This claim is hardly supported in light of the small sample size and borderline significance of effects.

Response to comment 14:

Thank you for your comments. Following the suggestion of the reviewer, we have revised the sentence as follows:

Lines 220~222

Data from our double-blinded randomized controlled trial suggested that MEG-based neurofeedback using cognitive modulation paradigm training on the basis of the RMS of the estimated insula current is able to decrease insula activity.

Comment 15

- Lines 279-280: The claim "...and this is confirmed in the current study" is hardly supported by the data.

Response to comment 15:

Thank you for your comments. Following the suggestion of the reviewer, we have revised the sentence as follows:

Lines 290~292:

First, it is necessary to show that insula activity is susceptible to neurofeedback control, and this is suggested in the current study.

Comment 16

Minor comments – Introduction:

- It is currently difficult for the reader to comprehend the precise line of argumentation followed in the introduction. The structure of the introduction should be more stringent, e.g., 1.) Neural correlates of pain processing in the insula cortex and other brain regions. 2.) Behavioral correlates of modulation of insular activity (including those found using neurofeedback techniques in humans). 3.) Neurofeedback as a means to locally modulate brain activity. 4.) Research questions 5.) Study hypotheses 6.) Approach

Response to comment 16:

Thank you for your comments. Following the reviewer's comments, we have thoroughly revised the introduction section as follows:

Lines 37~76

Pain perception is determined not only by external stimuli but also by internal states related to perceptual sensitivity. Various neuroimaging studies have demonstrated that pain perception is regulated by a broad cerebral network^{9,10}, which includes the S1, S2, insular cortex, anterior cingulate cortex, prefrontal cortex and thalamus¹¹.

The insular cortex is an important hub involved in pain perception and is associated with nociceptive processing and cognitive modulation of nociceptive processing¹²⁻¹⁸. Research in animal models has shown that manipulating neural activity in the insula significantly affects pain perception^{19,20}. Multiple human studies have shown that the insular cortex is activated following exposure to noxious stimuli^{9,15,21-26}. In addition, insula activity has been reported to be associated with chronic pain states²⁷, such as those experienced by patients with fibromyalgia^{28,29}, and complex regional pain syndrome³⁰. Electrical stimulation of the anterior insular cortex results in an increased thermal pain threshold in epileptic patients³¹. These findings suggest that the insular cortex could be a good target for neuromodulation, although a clear link between neural activity in the insular cortex and pain perception has not been determined.

One strategy to modulate region-specific neural activity is neurofeedback³². This approach was previously developed as a method to drive pain reduction by changing cortical activities³³. For example, functional magnetic resonance images were used to estimate the activity in the anterior cingulate cortex (ACC) in real time so that the subject could consciously gain control over this output, i.e., the activity strength of the ACC, to modify the brain processes in the ACC that affect the pain perception of the subject. Similarly, studies in which neurofeedback is used to modulate electroencephalogram (EEG) oscillation power have demonstrated that the theta power in the insular cortex is related to alterations in pain perception¹². In addition, alterations in blood oxygen level-dependent (BOLD) signals in the insular cortex, as evaluated by functional magnetic resonance imaging (fMRI), have been shown to alter pain processing^{34,35}.

Although these results suggest a strong relationship between insula activity and pain perception, it is unknown i) to what extent insula activity is under cognitive control such that it is susceptible to neurofeedback, ii) whether changes in insula activity are associated with changes in pain perception, and iii) which activity pattern is associated with changes in pain perception: oscillatory power or mean cortical excitability.

To answer the above question, we developed a neurofeedback model using estimated cortical currents in the insula cortex via magnetoencephalography (MEG). Specifically, we hypothesized that i) the mean cortical excitability, or root mean square (RMS) of the cortical

currents, of insula activity can be downmodulated through neurofeedback, ii) downmodulating the mean cortical excitability of insula activity through neurofeedback can increase the pain threshold, and iii) the changes in the mean cortical excitability are not associated with significant changes in oscillatory power.

To test our hypothesis, we conducted a double-blind randomized controlled crossover trial involving 19 healthy subjects. The aim of this study was to assess the modulation of insula activity and pain thresholds through the control of right insular cortex activity via neurofeedback training. We employed an MEG-based neurofeedback system to extract the RMS values of estimated currents in insular cortex vertices as a representation of insula activity. This insula activity was then translated into the size of a circular black disk displayed on a screen, with participants instructed to willfully enlarge the disk as a means to modulate insula activity. We measured thermal pain thresholds before and after the sessions using the method of limits.

Comment 17

- Improvements to the language of the introduction are necessary. Things that should be addressed include: Line 42: "...cognitive modulation of processing" -> which processing? ; Line 44: "...and mitigation" -> which mitigation? ; Lines 54-56: Awkward description of the neurofeedback principle. ; Lines 69-70: The formulation of the second hypothesis (or exploratory analysis?) is not semantically sound.

Response to comment 17:

Thank you for the valuable comments. We have revised the sentences to improve readability.

Line 42: "...cognitive modulation of processing" ->

The insular cortex is an important hub involved in pain perception and is associated with nociceptive processing and cognitive modulation of nociceptive processing

Line 44: "...and mitigation" -> which mitigation? ;

We removed "mitigation" as follows:

Research in animal models has shown that manipulating neural activity in the insula significantly affects pain perception

Lines 53~59: Awkward description of the neurofeedback principle.

We have revised this part to explain the neurofeedback principle as follows:

One strategy to modulate region-specific neural activity is neurofeedback³². This approach was previously developed as a method to drive pain reduction by changing cortical activities³³.

For example, functional magnetic resonance images were used to estimate the activity in the anterior cingulate cortex (ACC) in real time so that the subject could consciously gain control over this output, i.e., the activity strength of the ACC, to modify the brain processes in the ACC that affect the pain perception of the subject.

Lines 65~69: The formulation of the second hypothesis (or exploratory analysis?) is not semantically sound.

We have revised the sentences concerning the hypothesis by breaking down the second hypothesis into two sentences as follows:

Although these results suggest a strong relationship between insula activity and pain perception, it is unknown i) to what extent insula activity is under cognitive control such that it is susceptible to neurofeedback, ii) whether changes in insula activity are associated with changes in pain perception, and iii) which activity pattern is associated with changes in pain perception: oscillatory power or mean cortical excitability.

Comment 18

Minor comments – Results:

- Lines 217-218: Reference required.

Response to comment 18:

Thank you for pointing out the need for a reference at Lines 217–218. We have reviewed the text and added the appropriate citation to support the statement.

Revised text

Lines 226~228

Interestingly, these significant changes in insula activity and pain thresholds were not associated with power changes in the theta band^{12,36}, which has been hypothesized to be associated with pain.

Comment 19

Minor comments – Discussion:

- Line 224: Unclear what "biomarkers of neural activity" means. Usually, biomarkers indicate a clinically relevant phenomenon, such as the presence and/or degree of a disorder.

Response to comment 19:

Thank you for pointing out the potential ambiguity in our use of the term "biomarkers of neural activity." We agree that this term may suggest direct clinical relevance, which is not our intention in this context. We have revised the text to use "indicators of neural activity strength"

instead. This term better reflects our intent to describe the relationship between the estimated current RMS and the corresponding neural activity strength without implying direct clinical significance.

Comment 20

- Lines 241-252: Here, the role of the insula in pain is described without links to the study's results. This section should be shortened and/or integrated into the introduction.

Response to comment 20:

Thank you for your feedback regarding the detailed description of the role of the insula in pain perception. On the basis of your suggestion, we have significantly condensed this section to focus on the most relevant points while ensuring that the essential information is retained.

Lines 256~264:

Revised text

The diverse functions of the insula include assessing the emotional aspects of pain, modulating cognitive processes related to pain anticipation and attention, regulating physiological responses (e.g., heart rate and blood pressure), perceiving internal body states, and adjusting pain thresholds on the basis of the sensory input context³⁵⁻⁴⁰. The successful modulation of insular activity in our research may impact pain perception across these dimensions. Further explorations of how neurofeedback may modulate pain thresholds could be performed in future studies by focusing on specific functions of the insula, thereby increasing our understanding of and ability to manage pain.

Comment 21

- Lines 258-264: The dichotomy "spectral power vs. RMS" is somewhat artificial as the RMS also represents a spectral power but in a broader frequency band.

Response to comment 21:

Thank you for your valuable feedback on the distinction between spectral power and the root mean square (RMS). We agree that the RMS can indeed be considered a form of spectral power that represents a broader frequency band. We have revised the manuscript to clarify that the aim of our comparison was to emphasize the direct correspondence of the RMS with the overall neural activity strength, in contrast to spectral power analysis, which often focuses on specific frequency bands. The revised text now better reflects the rationale behind the use of the RMS in our study, and the presentation of an artificial dichotomy between the two concepts is avoided. We appreciate your insight and believe that the revision has improved the clarity and focus of our discussion.

Lines 265~273:

In previous studies, brain spectral power adjustments have predominantly been used to modulate insula activity³⁷. While some research has indicated potential alterations in pain discrimination¹², robust double-blind controlled studies demonstrating that changes in insula brain spectral power within specific frequency bands can modify pain perception or thresholds are lacking. In our study, we did not observe significant changes in spectral power within these specific bands that correlated with alterations in insula activity or changes in pain thresholds, suggesting that the overall neural activity in the insula might be more relevant to pain and could be a more appropriate target for neurofeedback.

Comment 22

- Lines 267-278: Here, the role of the insula in chronic pain is described without links to the study's results. This section should be shortened or omitted.

Response to comment 22:

Thank you for your constructive feedback regarding the role of the insula in chronic pain. We agree that this section could be more concise and directly linked to the results of the study. In response to your suggestion, we have shortened and streamlined this section to better align it with our findings, ensuring that the discussion remains focused and relevant.

Revised text

Lines 287~298

Although our research was focused primarily on acute pain induced by thermal stimulation, the insula is known to play a role in both the modulation of pain perception and the development of chronic pain³⁸. Neurofeedback treatments that modulate insula activities are therefore a plausible clinical option for patients with chronic pain. Two key steps are required to achieve this goal. First, it is necessary to show that insula activity is susceptible to neurofeedback control, and the findings of the current study suggest that this is the case. The second step is to show that training can have an impact on pain perception. For most neuromodulatory strategies, including neurofeedback, regular, repeated training sessions are required. The increased pain threshold we observed after a single session of neurofeedback is in keeping with this prediction, but for robust demonstration against the control condition, multiple sessions are likely required.

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Point-by-Point Replies to Reviewers

Neurofeedback modulation of insula activity via MEG-based brain-machine interface: A double-blind randomized controlled crossover trial

We appreciate the reviewers' helpful comments and suggestions. We also appreciate the constructive feedback and believe that it has helped us clarify and improve the manuscript. We have thoroughly revised our manuscript to address all the issues raised by the reviewers. In the revised manuscript, the added or revised text appears in **yellow**.

Our point-by-point responses to the comments are listed below, and the reviewers' comments are shown in gray.

Response to Reviewers

Response to Reviewer #2

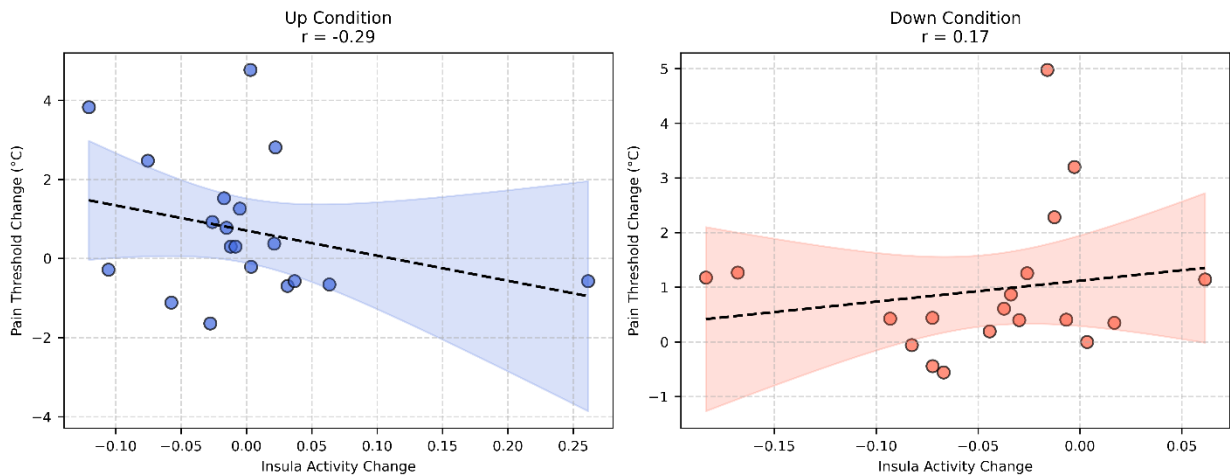
Comment 1. "Do changes in insular activity predict (correlate with) changes in pain thresholds? Showing such a link would reinforce the causal interpretation of the presented results."

Response:

We thank the reviewer for raising this important point. In response, we conducted correlation analyses between insular activity changes and pain threshold changes in both the Up and Down conditions. Specifically, we found that the Pearson correlation coefficient in the Up condition was $r = -0.292$, whereas in the Down condition, it was $r = 0.171$. To further examine whether these two correlations differed, we employed a bootstrap approach (10,000 iterations) at the subject level to preserve within-subject pairing. The observed difference in correlations was $\Delta r = -0.464$ (with a 95% CI of $[-0.979, 0.100]$) and was not statistically significant ($p = 0.532$).

Thus, while there appears to be some variation in the direction and magnitude of correlations between conditions, the evidence does not support a robust or statistically significant prediction of changes in pain thresholds by changes in insular activity within this sample.

Compare Correlations via Bootstrap
 Up-Down diff = -0.464, $p = 0.532$
 95% CI = (-0.979, 0.100)



Supplementary Figure 2. Correlation analyses between insular activity changes and pain threshold changes

Scatter plots illustrating the relationship between insular activity changes and pain threshold changes in two conditions: *Up* (left panel) and *Down* (right panel). Each panel displays individual participant data points ($n=19$) alongside a simple linear regression line (dashed) with the corresponding 95% confidence interval (shaded region). The difference in these two correlation coefficients ($\Delta r = r_{Up} - r_{Down}$) was tested via a bootstrap approach

Comment 2. “Supplementary Fig. 2 is a visually convincing demonstration that the experimental manipulation was specific to the target region (insula). Consider moving this figure to the main text.”

Response:

Thank you for your acknowledgment. As suggested, we have moved the previous Supplementary Fig. 2 into the main text (now Fig. 5).

Lines172~192:

Addressing Whole-Brain Activity and Insula Specificity

To ensure that our feedback was indeed specific to insular activity despite the inherent challenges of reconstructing signals from this deep cortical structure, we performed a whole-brain analysis of source activity during both upmodulation and downmodulation training. Specifically, we estimated cortical current distributions across the entire brain for a 300-second resting period, computed the root mean square (RMS) of these currents, and then applied a 10-mm Gaussian smoothing kernel to the cortical surface. We subsequently evaluated the RMS differences (posttraining minus pretraining) between the two feedback conditions by conducting paired t tests on each vertex (downmodulation vs. upmodulation). As shown in Figure 5 (below), the RMS difference decreased in the right insular cortex after downmodulation compared with upmodulation. The region with the most significant t value was the right insular cortex, the target of the feedback, although the differences were not statistically significant after correction for multiple comparisons (false discovery rate (FDR), $p > 0.05$). The result suggests that our magnetoencephalography (MEG) neurofeedback altered the targeted region, the right insula, most effectively.

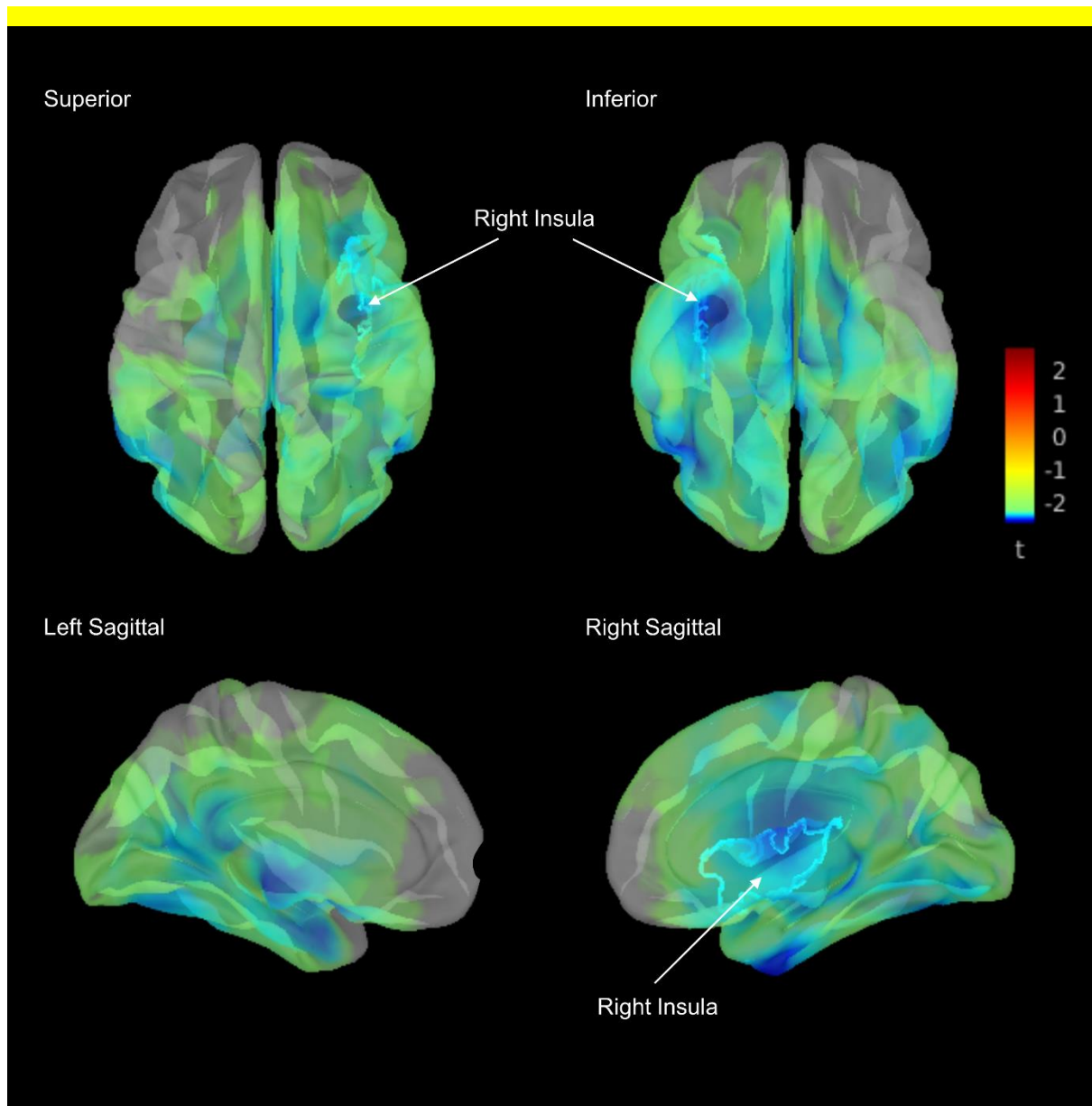


Figure 5. Paired t test results of the comparison of posttraining changes in the whole brain

The color map threshold was set at uncorrected $p < 0.05$. The bright blue line marks the boundary of the right insula. N=19, uncorrected.

Comment 3. "Please consider using a line plot with variance between subjects indicated by error bars instead of a scatterplot in Fig. 3A, as this is the typical visualization for this type of data."

Response:

We appreciate this recommendation for improved clarity. We have replaced the

scatterplot in Fig. 3A with a line plot showing the mean values and standard errors across participants for each time point.

Lines131~136

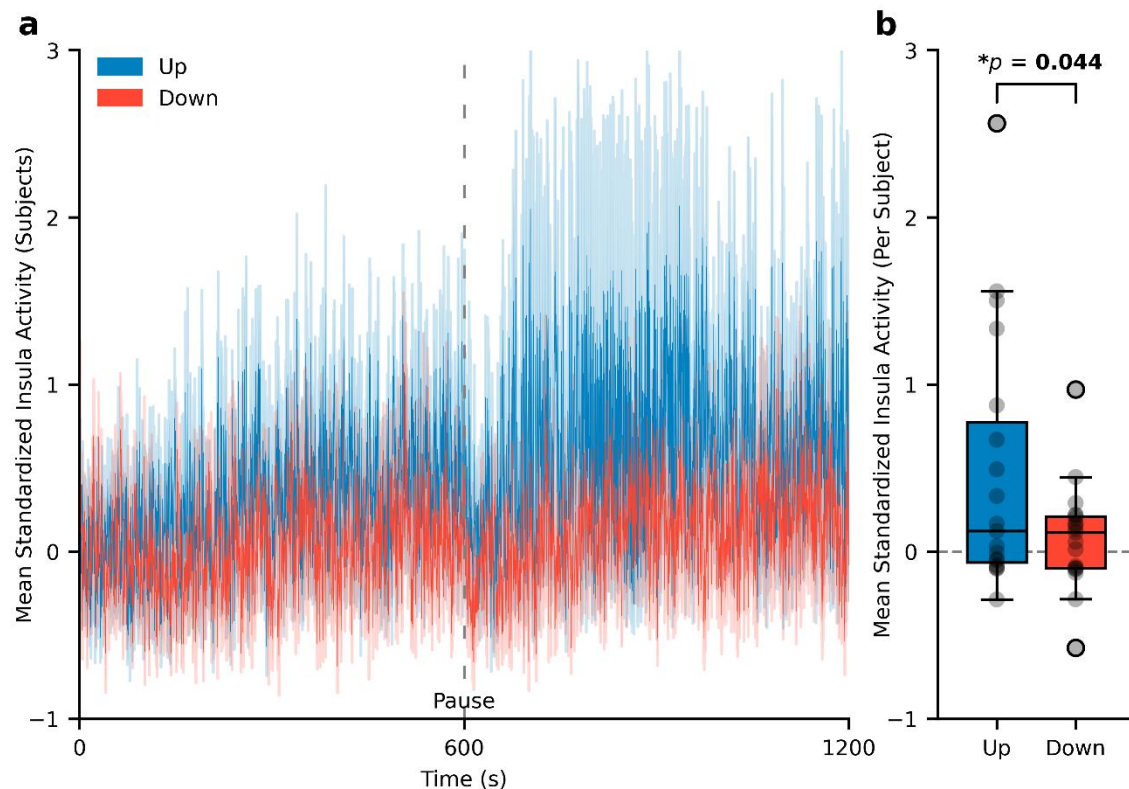


Figure 3. Modulation of insula activity during neurofeedback training.

a) Temporal dynamics of mean standardized insula activity during upregulation (blue) and downregulation (red) conditions. The solid lines represent the mean activity across subjects, and the shaded areas indicate the standard error of the mean (SEM).

Response to Reviewer #3

We are grateful for your careful reading and detailed comments. We have addressed

each point below and have revised the manuscript to improve its methodological transparency, logical coherence, and alignment with the stated objectives.

Comment 2

"It is hard to believe that hypothesis iii) was defined prior to data analysis... the relevance of hypothesis iii) is unclear. I recommend framing this analysis as an exploratory one."

Response: We appreciate the reviewer's feedback. In response, we have revised our approach so that only two key hypotheses remain. The analysis of the associations between changes in cortical excitability and oscillatory power has been framed as an exploratory component rather than a primary hypothesis.

Lines 72~77

To address these questions, we proposed the following hypotheses: i) the mean cortical excitability, or root mean square (RMS) of the cortical currents, of insula activity can be downmodulated through neurofeedback; and ii) downmodulating the mean cortical excitability of insula activity through neurofeedback can increase the pain threshold. Additionally, as an exploratory analysis, we examined whether changes in mean cortical excitability were associated with changes in oscillatory power.

Comment 6

"It should be stated explicitly for which statistical test the sample size calculation was performed. I.e., what was the main analysis?"

Response: We apologize for the unclear description in our previous manuscript. In our revised manuscript, we explicitly state that the sample size was calculated on the basis of a paired t test framework, as our main analyses focus on within-subject comparisons (insula activity changes during training and pre/post changes in insula activity and pain thresholds) in a crossover design. We have revised the sample size calculation section in accordance with your suggestions.

Lines 532~537:

Sample size calculation

We used G*Power 3.1 to calculate the required sample size for a paired t test for

within-subject comparisons, specifically for changes in insula activity during training and pre/post changes in insula activity and pain thresholds. The effect size (Cohen's d^1) was set to 0.7 (large effect), with a power of 0.8 and a significance level of 0.05. The results indicated that at least 19 participants were required for the study.

The description of the sample size calculation should explicitly state that large effect sizes were assumed. As I pointed out in my review, I consider large effect sizes unrealistic in the given context.

Response:

We thank the reviewer for prompting us to clarify our assumption regarding both sample size and effect size.

We based our target sample size in part on previous fMRI- and MEG-based neurofeedback studies, which often enroll 10–20 participants²⁻¹⁰.

Because our feedback approach differs from those used in previous studies, we lacked precisely comparable effect sizes. We therefore hypothesized that there would be moderate-to-substantial neurofeedback effects (Cohen's $d \sim 0.5-0.7$). Using $d = 0.7$ in the power calculation led us to recruit 20 participants, slightly exceeding the suggested sample size of ~ 19 participants. This choice reflected both prior studies' sample ranges and our estimation.

Post Hoc Effect Size Calculations

To further explore the effect sizes observed in our study, we conducted a post hoc analysis of Cohen's d for three key paired t test comparisons ($n=19$) via the following standard formula:

$$d = |t|/\sqrt{n}$$

where t is the paired t statistic and n is the sample size.

Insula Activity (Up vs. Down) During Training

$t = 2.168$, Cohen's $d = 0.497$

Resting-State Insula Activity (Pre- vs. Posttraining Difference, Up vs. Down)

$t = 2.191$, Cohen's $d = 0.503$

Pain Threshold (Pre- vs. Posttraining Difference, Up vs. Down)

$t = 0.474$, Cohen's $d = 0.109$

The results indicate that insula activity measures during neurofeedback training and resting-state changes exhibited moderate effect sizes (0.497 and 0.503, respectively). However, the effect size for pain threshold changes was substantially smaller (0.109), suggesting that our initial estimate of $d = 0.7$ may have been overly optimistic.

During the planning phase, our sample size estimation was influenced by many similar

studies that employed 10–20 participants, which affected our judgment of the expected experimental effect. We now recognize that our estimated effect size was overly optimistic, and we will increase the sample size in future studies to increase the reliability of our findings. We thank the reviewer for pointing this out.

We have revised the sample size calculation section on the basis of your suggestions.

Lines 532~546:

Sample size calculation

We used G*Power 3.1 to calculate the required sample size for a paired t test for within-subject comparisons, specifically for changes in insula activity during training and pre/post changes in insula activity and pain thresholds. The effect size (Cohen's d^1) was set to 0.7 (large effect), with a power of 0.8 and a significance level of 0.05. The results indicated that at least 19 participants were required for the study. On the basis of this calculation, we initially set the sample size to 20 participants. Although relatively small, this sample size ($n = 20$) is consistent with previous fMRI and MEG decoded neurofeedback studies, which typically include 10–20 participants²⁻¹⁰.

Post hoc effect size calculations indicate that insula activity measures during neurofeedback training and resting-state changes exhibited moderate effect sizes (0.497 and 0.503, respectively). However, the effect size for pain threshold changes was substantially smaller (0.109), suggesting that our initial estimate of 0.7 may have been overly optimistic. To improve the reliability of our findings, we plan to increase the sample size in future studies.

"It should be stated exactly which effect size measure was used."

Response:

Thank you for pointing this out. We used Cohen's d as the effect size measure, and we have now included this information in the revised manuscript.

Lines 532~537:

Sample size calculation

We used G*Power 3.1 to calculate the required sample size for a paired t test for within-subject comparisons, specifically for changes in insula activity during training and pre/post changes in insula activity and pain thresholds. The effect size (Cohen's d^1) was set to 0.7 (large effect), with a power of 0.8 and a significance level of 0.05. The results indicated that at least 19 participants were required for the study.

“‘setting the power at 0.7’ in the main text is likely an error? The effect size value is 0.7, the power value is 0.8?”

Response:

Thank you for pointing this out. You are correct; this was a typographical error. The power value is 0.8. We have corrected this in the revised manuscript.

“Why is there a sample size calculation for an ANOVA even though no ANOVA was performed in the study?”

Response:

Thank you for pointing this out.

We initially considered using repeated-measures ANOVA for a multisession comparison of pain measurement results, which explains our reference to ANOVA. However, after further refining our data analysis approach, we opted for paired tests instead.

We apologize for any confusion caused by the previous mention of ANOVA. We have now removed references to ANOVA where it is no longer applicable.

“Why are two groups mentioned? The distinction between different orders of neurofeedback conditions as different groups is artificial.”

Response:

Thank you for your comment. The two groups mentioned in our study refer to the two sequence orders in the crossover design—one group receiving up modulation first, followed by down modulation, and the other group receiving down modulation first, followed by up modulation. This randomization of sequence orders ensures counterbalancing to eliminate order effects (such as learning or fatigue), with a two-week washout period between the training sessions to prevent carryover effects.

We used block randomization with block sizes of 2 and 4 to ensure equal group sizes and minimize any risk of imbalance if participants dropped out. This approach ensured that the groups remain balanced, even with potential dropout, thereby reducing any bias.

By randomizing the sequence order, we ensured that the training sessions are compared in a balanced, unbiased manner. This improved the statistical power of the

study, controls for individual differences, and strengthens the robustness of the results.

We hope this explanation clarifies the rationale for the two groups in our study design.

Comment 7

"The sentences added to the manuscript do not clarify how the AAL was mapped to each subject's anatomical MRI."

Response:

We appreciate the reviewer's comment and have revised the manuscript to provide a clearer and more precise description of how the AAL right insular cortex was mapped to each subject's anatomical MRI. The updated text explicitly describes the standard VBMEG workflow, ensuring methodological transparency.

Revised Manuscript Text

Line410~420

To localize the right insular cortex, as defined in the AAL atlas, in each participant's anatomical MRI, the individual MRI was spatially normalized to the MNI152 template space using SPM8 (Wellcome Trust Centre for Neuroimaging, University College London, UK). This process generated a deformation field and an affine transformation matrix that mapped the subject space to MNI space. Following normalization, the inverse transformation was applied to the AAL atlas, originally defined in MNI space, to warp it into each participant's native anatomical space. The right insular cortex label was then projected onto the subject's cortical surface model, ensuring anatomical alignment with the individual's brain structure. The transformed region was used as the region of interest for source localization and neurofeedback training within the VBMEG system.

Comment 8

- The revised VBMEG section is still not clear.
- "VBMEG estimated that 4004 single-current dipoles were equidistantly distributed" ...does not make sense. The location and orientation of the 4004 single-current dipoles were likely defined a priory and VBMEG was used estimate their time-varying magnitude?
- How was the inverse filter constructed? Briefly explain the working principle of VBMEG. This is necessary as VBMEG is not a commonly applied method for source

reconstruction.

- Were both structural MRI and sensor positions measured before each 10-minute training session?
- What is the meaning of the parameters m_0 and γ_0 ?

"VBMEG estimated that 4004 single-current dipoles were equidistantly distributed" ...
does not make sense...

Response:

Thank you for your insightful feedback regarding the description of the VBMEG methods. We agree that the original wording was unclear. We have revised the Methods section to explicitly state that the 4,004 dipoles are predefined in location and orientation on the cortical surface and that VBMEG was used to estimate the time-varying activity (current magnitudes) at these fixed dipoles.

Lines 404~410:

First, cortical segmentation was performed on T1-weighted MR images via FreeSurfer software (Martinos Center for Biomedical Imaging, Massachusetts General Hospital, Charlestown, MA, USA), generating a subject-specific cortical surface model. On this cortical surface, 4,004 current dipoles were predefined, each positioned at a fixed location covering the entire cortex and oriented perpendicular to the local cortical surface. VBMEG was then used to estimate the time-varying current amplitude at each dipole by applying an MEG/EEG inverse solution incorporating fMRI-informed priors.

"How was the inverse filter constructed? Briefly explain the working principle of VBMEG."

Response:

We appreciate the reviewer's request for clarification. The inverse filter in VBMEG is constructed via a variational Bayesian approach to solve the ill-posed MEG inverse problem. The process consists of three main steps:

1. Leadfield Calculation: A forward model (leadfield matrix) is computed on the basis of individual MRI-derived cortical surface models via boundary element modeling (BEM). This defines how source currents contribute to MEG sensor measurements.
2. Source Variance Estimation: VBMEG employs a hierarchical Bayesian model,

where each source dipole current is assumed to follow a zero-mean Gaussian distribution with an unknown variance. These variances are estimated iteratively via a variational Bayes (VB) algorithm, which alternates between updating the estimated cortical source activity and refining its variance priors. This allows for automatic relevance determination (ARD), where sources that do not contribute significantly to explaining the sensor data are suppressed, improving spatial localization. If fMRI priors are available, they are incorporated as soft constraints on the source variance distribution.

3. Inverse Filter Construction: Once the source variances are estimated, VBMEG constructs an optimal linear inverse operator (K) to map MEG signals to cortical source activity. This filter is computed as:

$$K = C_J L^T (L C_J L^T + C_n)^{-1}$$

where C_J is the diagonal matrix of estimated source variances, L is the leadfield matrix, and C_n is the noise covariance matrix. This formulation ensures that the inverse filter adapts to the spatial structure of neural activity while accounting for measurement noise. The computed K matrix is then applied to the MEG signals to reconstruct the cortical current time series.

This Bayesian approach allows VBMEG to achieve spatially sparse and physiologically informed source reconstructions, improving localization accuracy compared with traditional minimum-norm or beamforming methods.

"Were both structural MRI and sensor positions measured before each 10-minute training session?"

Response:

Thank you for your question. All the participants had structural MR images acquired prior to the experiment, as they had previously participated in other fMRI studies. Sensor positions were measured at the beginning of each MEG recording session.

"What is the meaning of the parameters m_0 and γ_0 ?"

Response:

In VBMEG, m_0 and γ_0 are hyperparameters that define the prior distribution of source current variances in the Bayesian estimation framework.

m_0 (variance magnification parameter) determines the expected amplitude of the

source currents by scaling the prior variance. A larger m_0 allows greater flexibility in estimating stronger neural currents.

γ_0 (confidence parameter) controls how strictly the algorithm adheres to the prior variance distribution. A higher γ_0 enforces stronger confidence in prior knowledge (e.g., from baseline noise or fMRI priors), leading to a more constrained estimation.

These parameters help balance data-driven inference with prior constraints, ensuring biologically plausible source reconstructions while maintaining sensitivity to MEG signals¹¹⁻¹³.

Comment 9

- What is meant by “other MEG data” in “The VBMEG was applied to the MEG signals of the resting state with other MEG data of 300-second duration that were recorded without subjects (MEG data of environmental noise)”

- Overall, the description of how insular activity (specifically RMS-values) is computed is clearer now. However, the clearer explanation gives rise to the following concerns: 1.) Averaging cortical signals in 1-second windows effectively eliminates all frequency components above 0.5 Hz. Therefore, the normalization constants μ_A and σ_A are valid only for the frequency range < 0.5 Hz. Is this intended? 2.) Similarly, for the real-time RMS value, averaging cortical signals within 200 ms windows captures only signal components of < 2.5 Hz. This is only a small fraction of the overall frequency spectrum of MEG signals. Therefore, the RMS value reflects low-frequency activity and not broadband activity. Is this intended?

Response:

Thank you for your question. Since VBMEG requires environmental noise data, we recorded MEG data without the subject present before the experiment began each day. We have made the following revision to enhance clarity:

Lines 431~438

" Before the neurofeedback training session, MEG signals were first recorded for 300 seconds without the subject in the scanner. This environmental noise data was used to characterize baseline noise for signal preprocessing and to refine the VBMEG reconstruction filters. The neurofeedback training session began with a 300-second resting-state MEG recording, during which participants remained calm and fixated on the center of a disk presented in front of them. VBMEG was then applied to the resting-state MEG data to estimate the cortical currents at 4,004 vertices over the 300-second period (Figure 2b). "

Concerns 1,2

Our experiment focused on modulating long-term mean cortical excitability, and we did not specifically target low-frequency activity. Since this was a neurofeedback experiment, we needed to estimate the mean cortical excitability and provide participants with stable and timely feedback. The choice of 1-second and 200-ms time windows was based on the practical considerations of modifying our existing neurofeedback system used in previous studies, as well as the parameters selected during our system tuning process.

Comment 12

"It is still unclear how MNE and dSPM were used. I guess MNE was used first to generate 'raw' source estimates, then dSPM was used to standardize?"

Response:

Yes, as the reviewer correctly surmised, we first computed the minimum norm estimate (MNE) for each cortical vertex and then applied the dynamic statistical parametric mapping (dSPM) approach¹⁴ to standardize these source estimates. Specifically, MNE provides the initial "raw" source current distribution, whereas dSPM normalizes this distribution via the noise covariance derived from baseline or empty-room measurements. This two-step procedure is standard in many MEG source reconstruction pipelines and ensures more robust and comparable source estimates across participants.

Comment 13

"Which statistical tests were used for which hypotheses? How was Bonferroni correction applied exactly?"

Response:

To test Hypothesis 1 (comparison of insula activity pre- vs. posttraining), we conducted a paired t test.

To test Hypothesis 2 (correlation between insula activity and pain thresholds), we also conducted a paired t test.

For exploratory analyses (oscillatory power across frequency bands), we also conducted paired t tests.

When multiple statistical tests were performed on related outcome variables (e.g., different frequency bands in the exploratory analysis), we applied a Bonferroni

correction to control for multiple comparisons. Specifically, we adjusted the significance threshold ($\alpha = 0.05$) by dividing it by the number of comparisons (n). In other words, for each set of multiple comparisons, the corrected significance threshold was $0.05/n$.

Comment 16

- The introduction has improved, but the reasons for conducting the study are still not clear. In particular, the second half of the fourth paragraph (starting with „Similarly, studies in which...“) appears to be at odds with the last sentence of the third paragraph („...although a clear link between neural activity in the insular cortex and pain perception has not been determined“)
- It is not clear what the current study concretely adds beyond the findings of the other insula-neurofeedback studies in pain mentioned in the introduction.

Response:

Thank you for your question. We have revised the text to clarify that while previous studies have linked insular activity to pain modulation (for example, via fMRI and EEG neurofeedback findings), these studies were largely correlational and did not establish a definitive causal link between insula activity and pain perception. In the revised introduction, we explicitly note that prior research suggests a strong relationship while not clarifying whether insula activity can be voluntarily controlled to causally influence pain.

Lines 42~86:

Among these regions, the insular cortex serves as a key hub in pain perception, contributing to both nociceptive processing and its cognitive modulation.¹⁵⁻²¹. Research in animal models has shown that manipulating neural activity in the insula significantly affects pain perception^{22,23}. Multiple human studies have shown that the insular cortex is activated following exposure to noxious stimuli^{18,24-30}. Additionally, insula activity has been reported to be associated with chronic pain states³¹, such as those experienced by patients with fibromyalgia^{32,33}. and complex regional pain syndrome³⁴. Notably, direct electrical stimulation of the human anterior insula can increase pain thresholds³⁵, suggesting that engaging this region can causally influence pain perception. However, despite these associations, it remains unclear whether insular cortex activity can be deliberately controlled and, if so, whether such control has a direct causal effect on pain perception. In other words, a definitive link between insula modulation and changes in pain experience has yet to be established, thus highlighting the need for studies that directly test insula-focused neuromodulation in humans.

One promising strategy to modulate region-specific neural activity is neurofeedback³⁶. In a typical neurofeedback system, neural activity is continuously recorded, processed, and displayed in real time. Participants interact with the feedback, learning to regulate their brain activity toward a target state, thereby facilitating adaptive neuroplasticity⁹. Previous neurofeedback studies have provided preliminary support for targeting the insula in pain modulation. For example, EEG-based neurofeedback training has shown that changes in insular theta-band oscillatory power correlate with alterations in pain perception¹⁵. Similarly, real-time fMRI studies reported that modulating blood-oxygen-level dependent (BOLD) signals in the insula can lead to changes in pain processing^{8,37}. These prior findings suggest a strong relationship between insula activity and pain modulation. Nevertheless, they do not conclusively demonstrate a direct causal link, as they primarily show correlations or group differences rather than providing proof that an individual's deliberate control of insular activity drives pain changes. Key questions remain: i) To what extent is insula activity under cognitive control such that it is susceptible to neurofeedback? ii) Are changes in insula activity associated with changes in pain perception?

To address these questions, we proposed the following hypotheses: i) the mean cortical excitability, or root mean square (RMS) of the cortical currents, of insula activity can be downmodulated through neurofeedback; and ii) downmodulating the mean cortical excitability of insula activity through neurofeedback can increase the pain threshold. Additionally, as an exploratory analysis, we examined whether changes in mean cortical excitability were associated with changes in oscillatory power.

To test our hypothesis, we developed a magnetoencephalography (MEG)-based neurofeedback approach that enables participants to modulate their right anterior insula activity in real time. We then conducted a double-blind, randomized controlled crossover trial. Our system was specifically designed to extract the root mean square (RMS) of the insular cortical current—a measure of mean cortical excitability—from the MEG data. By using the RMS of estimated insula currents as the feedback signal, we targeted the overall amplitude of insular activity rather than focusing on a single frequency band. This approach differs from previous insula neurofeedback studies, which primarily targeted oscillatory power (e.g., theta rhythm) or BOLD activity.

Comment 17

"The description of the neurofeedback principle still lacks the fact that the estimated neural feature is 'fed back' to the participant."

Response:

We appreciate the reviewer's comments.

Our study employed a widely used neurofeedback system paradigm, as illustrated in the figure below (adapted from the cited literature³⁸). This closed-loop, real-time brain training technique involves neural signal acquisition, real-time neural signal analysis, graphical representation of processed neural activity, and active participant engagement. The system continuously records neural activity, processes it in real time, and presents the processed data to participants as a dynamic visual representation. During the visual feedback process, participants actively interact with the system by attempting to modulate their brain activity through cognitive strategies and subjective experience. They observe the ongoing relationship between their conscious states and fluctuations in neural activity, learning to control the feedback display. Through repeated attempts, they refine their ability to regulate neural activity toward a target state, thereby inducing neurophysiological changes.

To enhance clarity, we have incorporated the following description into the Introduction section:

Lines 57~60:

In a typical neurofeedback system, neural activity is continuously recorded, processed, and displayed in real time. Participants interact with the feedback, learning to regulate their brain activity toward a target state, thereby facilitating adaptive neuroplasticity.

Comment 21

"The RMS computed mostly reflects very low frequency components (<2.5 Hz)."

Response:

Thank you for your insightful comment.

Our primary objective was to modulate the mean cortical excitability rather than specifically targeting low-frequency components. Since neurofeedback operates as a closed-loop system, it inherently requires an update rate that provides participants with timely feedback. The chosen feedback rate determines the frequency range of the extracted neural features rather than an intentional focus on low-frequency components.

We appreciate this perspective and will consider the potential role of low-frequency components in neurofeedback in future studies.

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Point-by-Point Responses to Reviewers

Neurofeedback modulation of insula activity via a MEG-based brain-machine interface: A double-blind randomized controlled crossover trial

We appreciate the reviewers' helpful comments and suggestions. We also appreciate the constructive feedback and believe that it has helped us clarify and improve the manuscript. We have thoroughly revised our manuscript to address all the issues raised by the reviewers. In the revised manuscript, the added or revised text appears in **yellow**.

Our point-by-point responses to the comments are listed below, and the reviewers' comments are shown in gray.

Response to Reviewers

Response to Reviewer #2

A central interpretation of the data suggests that changes in insular activity due to neurofeedback mediate changes in pain thresholds. However, in the latest revision, the authors do not observe any correlation between changes in insular activity and pain threshold changes. Additionally, no difference is found in pain threshold changes between active and control conditions. Consequently, the manuscript presents an interpretation that is not supported by the data. There is no evidence to suggest that neurofeedback influences pain thresholds. The manuscript should be revised accordingly and emphasize that the data do not evidence a direct link between changes in insular activity and pain thresholds.

Response:

Response:

Thank you for this valuable suggestion. As recommended, we have now clarified and emphasized throughout the manuscript that our data do not provide direct evidence for a causal relationship between the modulation of insula activity and changes in pain thresholds.

Abstract: Line34~36:

However, the present findings do not provide direct evidence of a causal link between modulation of insula activity and changes in pain thresholds.

Discussion: Line258~266:

Although downmodulation of insula activity was accompanied by a significant increase in pain thresholds, the specificity of this effect compared with the upmodulation control condition was not confirmed. These findings suggest that insula activity is amenable to modulation via MEG-based neurofeedback and highlight its potential as a therapeutic target for pain management through repeated neurofeedback training. However, further evidence is needed. Due to the lack of a significant interaction effect between training type and pain threshold changes, these findings must be interpreted cautiously and do not establish a direct causal relationship between insula modulation and altered pain perception.

The timeseries in Fig. 3A is currently dominated by high-frequency variance, making it difficult to discern low-frequency trends. Please consider smoothing these timeseries prior to plotting.

Response:

We appreciate this suggestion. We smoothed the time series data presented in Fig. 3A to clearly illustrate the low-frequency trends in insula activity during neurofeedback training. The updated figure is now included in the revised manuscript.

Lines 145~146:

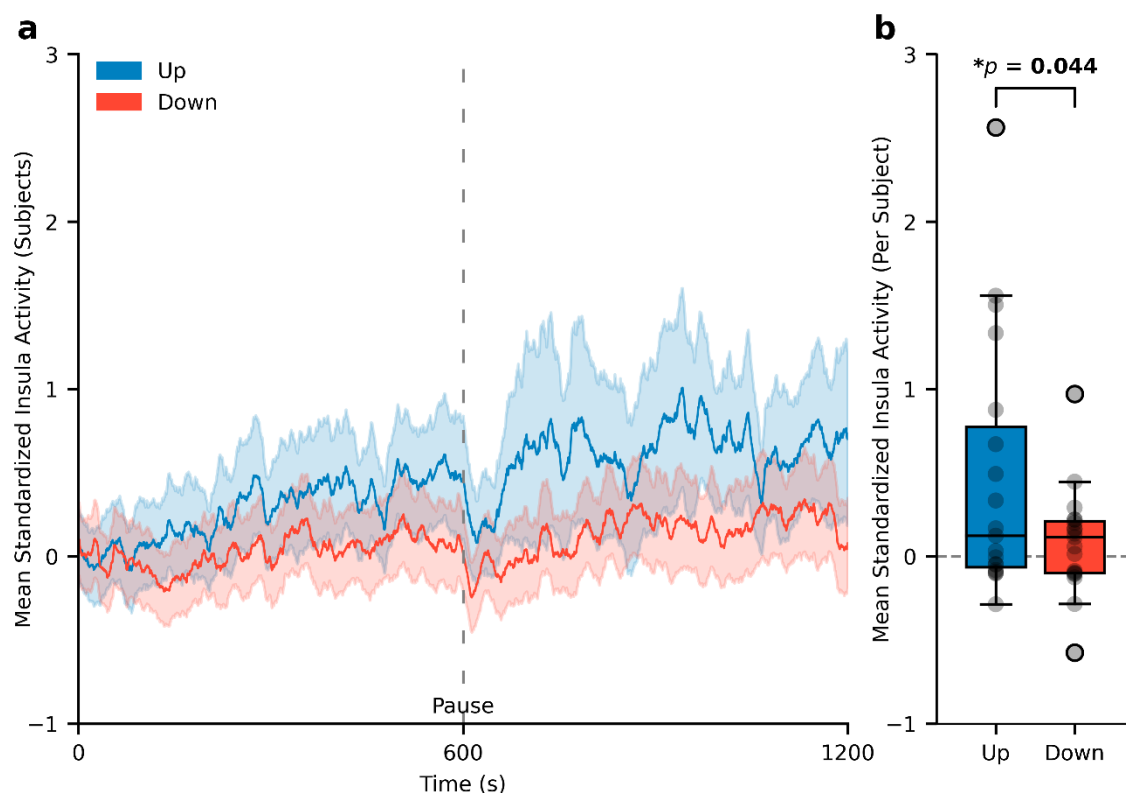


Figure 3. Modulation of insula activity during neurofeedback training.

a) Temporal dynamics of mean standardized insula activity during upregulation (blue) and downregulation (red) conditions. The solid lines represent the mean activity across subjects, and the shaded areas indicate the standard error of the mean (SEM). All curves in this figure have been smoothed using a 100-point (20-s) moving average to clearly illustrate the neurofeedback modulation trends. The vertical dashed line at 600 seconds marks a brief pause between two training sessions.

b) Boxplot comparing the mean insula activity during the entire training process for each subject under the upmodulation and downmodulation conditions. Insula activity during upmodulation training was significantly greater than that during downmodulation training (blue for upmodulation training; red for downmodulation training). Two-tailed paired Student's t test, $*p < 0.05$. In the boxplots, centerlines represent medians, the box limits represent the lower and upper quartiles, the whiskers represent 1.5 times the interquartile range, and the circles represent outliers.

Line 131~133

Time-series data were smoothed using a moving average filter with a window of 100 data points (corresponding to 20 s). This smoothing was performed to better illustrate the trends of neurofeedback-induced modulation of neural activity

Response to Reviewer #3

We are grateful for your careful reading and detailed comments. We address each point below and have revised the manuscript to improve its methodological transparency, logical coherence, and alignment with the stated objectives.

Comment 9 and comment 21:

- The section "Neurofeedback system" describes how the feedback signal is computed. However, it does not clearly justify why this way of computing the feedback signal adequately reflects insular activity. This must be improved, and potential limitations in terms of capturing insular activity discussed. In particular, it should be justified. In particular, it should be justified, why not simply the signal power across a broad frequency range, say 1 to 100 Hz, was used as the feedback signal.

Response: Thank you for highlighting this important point. We have clarified our rationale for using RMS (root mean square) values as feedback signals in the "Neurofeedback system" subsection. Specifically, we emphasized that the RMS provides a broad-band measure of cortical excitability that does not assume

importance for any specific frequency band. We also discussed the potential limitations associated with this choice.

Lines 515~529:

We chose the RMS value as the feedback signal because it provides a single scalar measure of overall insular cortical activity, capturing broadband changes without focusing on a specific frequency band. In contrast to using a band-limited power measure (e.g., total power in the range of 1–100 Hz), the RMS of the time-domain insula signal reflects combined activity across the spectrum and can be computed rapidly in real time. This approach allowed us to provide immediate feedback on the basis of the general level of insula activation, leveraging the full bandwidth of MEG signals and avoiding assumptions about which oscillatory component might be most relevant. However, a potential limitation of using an RMS-based feedback signal is that it does not isolate particular frequency bands or network-specific activity. Thus, any subtle changes confined to a narrow frequency range (for instance, pain-related theta oscillations) would be blended into the overall measure and not specifically highlighted for the participant. Despite this limitation, the simplicity and inclusiveness of the RMS metric make it a suitable choice for capturing insula activity in our neurofeedback paradigm, given our goal to modulate the general excitability of the insular cortex.

Comment 13:

The explanations in the rebuttal letter should be integrated into the manuscript.

Response: Thank you for this suggestion. We have now integrated the explanations provided in our rebuttal letter into the Statistical analysis and reproducibility section of the manuscript, as follows:

Lines 617~628:

To test Hypothesis 1 (pre- versus posttraining changes in insula activity), we performed a paired t test to compare insula activity measured before and after neurofeedback training. To evaluate Hypothesis 2 (changes in pain thresholds associated with modulation of insula activity), we similarly employed a paired t test on pain threshold data measured before and after training. Exploratory analyses assessing oscillatory power changes across multiple frequency bands were also conducted using paired t tests.

To control for multiple comparisons arising from related statistical tests (e.g., analyses of multiple frequency bands in exploratory testing), we applied Bonferroni correction by adjusting the original significance threshold ($\alpha = 0.05$) according to the number of

comparisons performed (n). Specifically, the adjusted threshold for statistical significance was set as $\alpha = 0.05/n$ for each set of related comparisons.

Point-by-Point Responses to Reviewers

Neurofeedback modulation of insula activity via MEG-based brain-machine interface: A double-blind randomized controlled crossover trial

We appreciate the reviewers' helpful comments and suggestions. We also appreciate the constructive feedback and believe that it has helped us clarify and improve the manuscript.

Our point-by-point responses to the comments are listed below, and the reviewers' comments are shown in gray.

Response to Reviewers

Response to Reviewer #3

A still unclear detail is why "the standardized currents were time-averaged" to compute the baseline insula activity. By averaging the signal within 1000ms windows, one effectively eliminates many higher frequency components of the signal and, thus, the bulk of signal power.

Response:

We sincerely appreciate your careful reading of the manuscript and your thoughtful comments, which have helped improve the clarity of our work.

We used a 1000 ms time window to average the standardized currents in order to obtain a stable estimate of baseline insula activity. Our primary goal was to capture the tonic level of cortical excitability during the baseline period, rather than fast, transient fluctuations that may reflect noise or irrelevant dynamics.

Averaging over this time scale reduces high-frequency components, but in doing so, it enhances the signal-to-noise ratio and emphasizes the sustained features of neural activity that are most relevant to our study. Since we are not analyzing event-related responses or oscillatory components tied to specific frequencies, the attenuation of fast-changing components does not compromise the validity of our baseline measure. We believe this method provides a robust and meaningful index of baseline insular activity appropriate for assessing changes in cortical excitability.

We appreciate the opportunity to clarify this point.