nature portfolio

Peer Review File

The human claustrum tracks slow waves during sleep

Corresponding Author: Dr Eyiyemisi Damisah

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

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
This is a very interesting study showing that the claustrum is involved in the human sleep. Specifically, the authors recorded claustrum neurons in humans during sleep and showed that claustrum neurons increased their activity and tracked slow waves during NREM sleep. The authors also established some specificity by showing neurons from the amygdala and ACC. As far as I know, this is one of the very first studies showing claustrum recordings at the single-neuron level in humans. Therefore, it is a novel and important study.

I only have a few minor questions for the authors:

What are the operational definitions for SW and SWA? In other words, can the authors clarify the selection/detection procedure of SW and SWA at the beginning of results?

Although the data is very rare, there were only 49 neurons from the CLA, which is on the lower end. If possible, the authors please include more data as a validation of the present findings.

For human single-neuron recordings, it is hard to achieve hour-long stable recordings. Can the authors show that the neural recordings are stable for overnight recordings? Were there any signal dropouts? In other words, it is critical to demonstrate that it is the same neuron that changed firing rate over the hour-long period.

How many sessions did each patient contribute? Are the 49 CLA neurons combined from different sessions of the same patient? If so, the statistics may be inflated, because it is likely the same neuron can be counted in multiple times (given the purported assumption that the neuron will remain stable over a very long time).

Although the SW from different brain regions seem quite similar / correlated (Fig. 1g), can the authors clarify / justify the source of SW to align with spiking activity? For example, why in Fig. 2a, the spiking activity was compared against the right OFC?

What happens to the 3rd unit shown in Fig. 2a that did not increase firing rate for the first SW period?

Is there a direct correlation between CLA and amygdala/ACC activity? It seems the case given that CLA increases and amygdala/ACC decreases the activity.

Does the phase-locking happen in other stages of sleep? This may help to establish some specificity of SW sleep.

A fleshed out / more in-depth discussion is beneficial. The current discussion focuses on a summary of results and some clinical limitations. It will be nice to position the current findings in the broader sleep and functional neural circuit literature.

Signed review from Shuo Wang, Washington University in St. Louis

Reviewer #2

(Remarks to the Author)
In this short paper, Eyiyemisi Damisah and colleagues describe an extraordinarily exciting study on the role that the human

claustrum plays in NREM sleep. This topic is crucially important to our understanding of the human sleep cycle and provides strong evidence of the role that this enigmatic area plays in this process. Beyond these already important findings, the authors also provide completing analyses and comprehensive anatomical localization to bolster their results. Finally, they provide several strong parallels with animal studies (showing, for example, that the majority of CLA neurons increased spiking activity during periods of SWs in NREM sleep) to confirm the generalizability of their findings.

Altogether, I would strongly support publication of this paper. Below are a few comments that could help further strengthen the paper.

First, as the authors appropriately pointed out, the claustrum has a small axial cross-section and lays in close proximity to the insula and striatum. While the authors have done an outstanding job at localizing their microwires, it would be helpful to provide further validation of their localization using other complementary approaches (e.g., LeadDBS). Sometimes even small differences in co-localization or fusion techniques can influence precise localization. Similarly, it would be helpful to provide the estimated distribution and distances of recordings from claustrum axial center.

Second, while the authors use comparisons to other areas such as the ACC to confirm selectivity of their results, most of their results are based on the number of neurons that displayed modulation. It would be helpful to also provide additional metrics for the magnitude of effect (e.g., z-score firing rates) for both modulated and non-modulated neurons as well as across areas.

Third, given the small number of participants, it may be helpful to perform a cross-validation procedure or provide other information about the contribution that different individuals had on the main results. For example, it would be useful to confirm that most of the neurons did not come from a single participant or set of neurons.

Finally, given the long periods of recordings and the potential variability across NREM sleep cycles, it could be helpful to provide some basic model prediction/decoding of population responses. For example, the authors could use an SVM or GLM to quantify the degree to which they are able to predict SWAs in NREM sleep from NREM events not used for model training. Such predictions would provide strong ‘causal’ support that the relation between claustrum activity and NREM is robust.

Reviewer #3

(Remarks to the Author)
This is a timely manuscript showing, for the first time, that human claustrum neurons are associated with NREM sleep. This work follows up on rodent literature, where recent studied have shown a similar finding. I think the work has a lot of potential. However, there are some analytical aspects of the manuscript I would like to see addressed, that will hopefully strengthen the author’s message, and provide further clarity regarding the state-dependent firing of human claustrum cells.

1. I found the Hypnogram in Figure 1G confusing. Wake is indicated by the pink shading, but other sleep states are indicated by the more classical hypnogram. Its confusing because it looks like REM and wakefulness can co-occur. Perhaps I am not reading it right, so please clarify this. Looking at the hypnograms in Fig1 and S. Figure3, participants go from wake to REM. This does not really happen during sleep, so I’m not really sure how their analysis is providing these state transitions.

2. The data presentation in Figure 2C is really not an effective way to convey the message. First, the ACC and CLA are a similar color, so discerning their points on the scatter is visually challenging. Second, I would recommend showing each region separately. These scatters are too crowded. Or alternatively showing a histogram of NREM-notNREM firing rates (which would highlight the difference they want to describe).

3. Following up on this last point, the authors should do a better job comparing the firing rate across states. Currently, the only state comparisons are in figure2C. What about comparing NREM versus REM, or NREM vs wakefulness? These comparisons should be performed to compare with the rodent literature, and for rigor and completeness of the study.

4. I am looking at S Figure 5. The red indicates the detected Slow waves. I think the authors have some work to do with regards to their detection algorithm. For example, in the MTG recording, the first detected wave is not a slow wave. It seems like they are missing a lot of slow waves, while also mis-assigning slow waves in some cases. Overall, this is not convincing. But also, why do the authors care about performing this SWs detection? Just calculate the overall power of 1-3Hz activity using a sliding window. Isn’t that good enough for what they want to say here? Don’t overcomplicate an analysis and generate doubt.

5. What about a Figure showing the cross correlations between CLA – ACC, and CLA-BLA spike pairs? And/or showing the multiunit (ensemble) cross correlations between these regions? This would be a first for this type of analysis with claustrum data, and something that would be particularly interesting regarding the ACC correlations. Also, consider showing time-lag correlations to enable the reader to see the temporal relationship between spike-count correlations.

6. Line 79 states CLA neurons increased spiking during SWS. Compared to what? Wakefulness? Whatever you are comparing to, it should be stated here. The same goes for the following sentence when discussing AMY and ACC cells.

What are the SW data compared to , in order to make this claim?

7. I see the paper was originally formatted for a brief comm for NN where there are tight restrictions on word count, and references. I think the reference list could be expanded significantly in most sections of the intro. For example, when discussing NREM importance in consolidation, homeostasis and sleep disorders, only 1 reference I given. The same goes for the discussion. Please take the time to acknowledge the relevant work where appropriate.

8. In line 73 please indicate what brain regions these studies recorded from.

9. I see some CLA cells do not track SWs. Are these cells different from the SW tracking CLA cells in terms of spike width or mean firing rate? Can the authors provide insight into what putative interneurons are doing?

10. All figures showing time series data require time scale bars. Some figures do not currently have these.

11. The writing in the discussion is a little redundant. They re-state their main finding 3 times in a very short amount of space. Consider taking the time to really contextualize the results relative to other studies. Also, some further discussion of the studies limitations (for example, the authors mention there are many cla subtypes. What does this mean for the interpretation of results, or how would be authors suggest overcoming these limitations in the future?)

Reviewer #4

(Remarks to the Author)
Lamsam and colleagues report exceedingly rare data recorded from single neurons in the human claustrum during sleep. Consistent with rodent studies, they find that claustrum neurons show increased firing rates during NREM, while neurons in the amygdala and anterior cingulate cortex showed decreased firing rates. A majority of claustrum neurons showed spiking patterns tied to slow wave activity. Neurons in all three regions showed phase locking to multiple cortical region recorded with macroelectrodes, including orbitofrontal cortex. The authors suggest that these results demonstrate a role for the human claustrum in regulating sleep by coordinating slow wave activity. This is an important and impressive paper.

In addition to the truly unique data reported here from Dr Damisah's group, the brief paper provides a nice confirmation of phenomena observed in rodents. Demonstrating that brain-wide coordinated activity like slow waves are correlated with single neuron firing in the claustrum provides important evidence that this structure is a key part of the cortical architecture for sleep.

MAJOR

1) While the qualitative results and figures are compelling, the paper would benefit from more statistical quantification of results. Throughout the paper, it is important to know to what extent each result is stronger than that expected by chance. There are some results (like the phase-locking findings) where it is not clear whether the relationship is actually real, or whether the consistent phase is just a weak effect of shared noise.
2) What is the justification for relating CLA firing to both SWs and SWA? If I understand correctly, SWA is just normalized delta power, while SWs are the percentage of 10s epochs that have SWA (again, computed using just an arbitrary z-score threshold, rather than a test that accounts for the variance). It seems like SWs are just a more conservative thresholded version of SWA, but it's not clear why both are necessary, other than that SWs are used for the phase-locking analysis.
3) Simply correlating single unit activity with SWA/SWs is potentially misleading for the claim that CLA neurons are uniquely more active. Judging from the raw data presented in Fig. 1 and Fig. S3, there is some variability in the relationship between NREM and CLA. It would be more comprehensive to do something like a classification analysis where it's possible to compute sensitivity and specificity to test whether, during sleep, CLA activity is particularly associated with NREM.
4) Please provide justification for MER placement in claustrum. I assume it is related to the insula placement and trajectory but would be good to make this explicit so readers understand why those were place clinically there.

MINOR

5) There are several places where aspects of the visualization are not explained or quantified. In particular, it is not clear where the gray shading comes from in the UMAP clustering in Fig. 3E. It seems like the points were "manually clustered" (line 333), when they should have performed some kind of quantitative clustering to determine how well the two groups are actually separated (though clustering should not be done in UMAP space). Similarly, they only qualitatively describe the distribution of CLA units in each of the two groups.
6) Another specific place where a lack of quantification is concerning is Fig. 3A-B. If anything, the rasters look like there's a more robust relationship to the SWs for AMY than CLA. And the phase relationship in panel B looks relatively flat. It's certainly possible that this histogram is significantly different from uniform, but this does not appear to have been tested.
7) Fig. 2C is also not quantified sufficiently. The measure of how many units are above and below the diagonal is interesting, however given that most points are on or very close to the unity line, it's not clear how many are actually significantly

different.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The authors have adequately addressed my questions. I congratulate the authors on completing this impactful study.

Reviewer #2

(Remarks to the Author)
Overall, the authors have done a phenomenal job at addressing my prior comments and suggestions. The paper is much improved and provides crucial new insights into the role of the claustrum in sleep/NREM. Specifically, the authors have now used LeadDBS to confirm localization of their electrodes to the claustrum. They have also provided careful new analysis that more directly quantify the magnitude and direction of modulation as well as demonstrated the generalizability of their finding across participants. Finally, they have used a new modeling approach to classify sleep stages and demonstrate that they can be reliably predicted from neural activity.

Taken together, the findings and the rarity of recordings making this paper truly remarkable and will likely generate intense interest within both the scientific and lay community. I have no further comments and would strongly support publication of this study.

Reviewer #3

(Remarks to the Author)
The authors have addressed the majority of my concerns and the manuscript is considerably improved. I have some remaining questions/clarifications that should be addressed before publication.

Discussion:
The use of SWB could be replaced with the more frequently used SWA
The authors state that the claustrum has functional gradients across subregions. I don't believe there is strong evidence for functional gradients, but anatomical gradients are well described. I would recommend adding citations for function and/or anatomical gradients.

Methods:
How many interictal epileptiform events were detected and removed? Please provide the average/session.
The jittering of the spike times for the surrogate cross correlations is not well described. Please elaborate more. Currently it says 20ms uniform distribution. Does this mean that they were jittered by a distribution of times centered on 20ms? This seems awfully short. Why not jitter by random values the up to the full length of the recording. In any case, more detail is required for the correlation analysis. I find it strange that this threshold enhancement is required, why not just take the distribution of surrogate values and define a threshold based on these?

Figures:
In Figure 3b, the y-axis is "FR in not NREM" – this is a little confusing. In other parts of the paper WREM is used. Is WREM the same 'not NREM'. If so, please pick a convention.

Also in Figure 3b, please show the p-values for the AMY and ACC as is done for the CLA

In Figure 4b I was initially very confused because the counts (n) are so high. However, I realized it was because the n is for each cell-LFP channel pair. It would be helpful to state somewhere in the figure the n's for the cells and n's for channels to avoid reader confusion. This would also help understand why there are negative correlations in the positive group, and likewise for the positive correlations in the negative group.

In Figure 4e, the firing rate for each decile is supposed to be shown. Decile implies 10 bins, but there are clearly >10 points on each plot. Please clarify and/or revise. Also, I believe in the methods there is mention of linear fitting, when the fits shown are not linear. I see that the LoESS was used for fitting, but unless there is some meaning to these curves, I recommend showing the linear fits.

Please add scale bars to all MRI images in the manuscript and supplementary data

Reviewer #4

(Remarks to the Author)
The authors have addressed all of my comments. This is a very interesting and important paper on the neuronal physiology

of the human claustrum.

Reviewer #1 (Remarks to the Author):

This is a very interesting study showing that the claustrum is involved in the human sleep. Specifically, the authors recorded claustrum neurons in humans during sleep and showed that claustrum neurons increased their activity and tracked slow waves during NREM sleep. The authors also established some specificity by showing neurons from the amygdala and ACC. As far as I know, this is one of the very first studies showing claustrum recordings at the single-neuron level in humans. Therefore, it is a novel and important study.

I only have a few minor questions for the authors:

What are the operational definitions for SW and SWA? In other words, can the authors clarify the selection/detection procedure of SW and SWA at the beginning of results?

We thank the reviewer for their helpful comments and for pointing out this oversight – we have now added these operational definitions near the beginning of the results section on lines 84 - 88 in addition to the full descriptions in the methods section. In brief, we used a slow-wave detection algorithm from the YASA Python library written by Raphael Vallat at UC Berkeley, which had been previously validated for scalp EEG (see reference 54). One optional procedure in this algorithm is to normalize the data before SW detection, which we did to better adapt it to our intracranial EEG data. The full operational definition of SWs is detailed in the "Slow-Wave Detection" section of the Methods (lines 290 - 301). The operational definition of SWA is the z-score of the log-normalized delta band power.

Although the data is very rare, there were only 49 neurons from the CLA, which is on the lower end. If possible, the authors please include more data as a validation of the present findings.

We agree with the reviewer that data from additional neurons can add to the robustness of results. However, our ability to collect additional data is dependent entirely on clinical opportunity, which is exceedingly rare in the case of the claustrum. Furthermore, only a subset of those opportunities results in successful placement of the microwires into the claustrum. Although the absolute number is limited, the quality and consistency of our recordings allowed for robust analyses that yielded significant results on claustral function. Future studies should include additional neurons if possible and examine ways to pool data across experiments.

For human single-neuron recordings, it is hard to achieve hour-long stable recordings. Can the authors show that the neural recordings are stable for overnight recordings? Were there any signal dropouts? In other words, it is critical to demonstrate that it is the same neuron that changed firing rate over the hour-long period.

We thank the reviewer for their comment and agree that ensuring that we are recording from the same neuron is important to support our claims. To address this, we have now added additional analyses (**Supplementary Figure 3**) to better assess unit stability over the many hours of our sleep recordings. First, we have plotted and quantified the cumulative spike distribution for each

unit over the full recording. Second, we have randomly sampled 1000 spikes from each single unit, and we used them to calculate several waveform metrics (coefficient of variance for maximum amplitude, full width at half maximum, area under the curve, Euclidean distance from mean waveform, and the L-Ratio) to assess waveform stability across the length of a sleep recording. The results of both analyses show small amounts of variation over time, suggesting that the recordings were stable.

How many sessions did each patient contribute? Are the 49 CLA neurons combined from different sessions of the same patient? If so, the statistics may be inflated, because it is likely the same neuron can be counted in multiple times (given the purported assumption that the neuron will remain stable over a very long time).

We thank the reviewer for their comment. The 49 units are derived from both subjects. Patient A contributed one sleep session (16 CLA units), and Patient B contributed three sleep sessions (12, 11, and 10 CLA units, respectively). It is possible that some of the units from the three sequential sleep sessions in Patient B are identical. Qualitatively, we find that it is unlikely that we record from the same units across multiple days.

Although the SW from different brain regions seem quite similar / correlated (Fig. 1g), can the authors clarify / justify the source of SW to align with spiking activity? For example, why in Fig. 2a, the spiking activity was compared against the right OFC?

We thank the reviewer for their comment. To address this ambiguity, we have now modified the language of the relevant figure captions (panel four of Fig. 1g, now **Fig. 2a**; upper right panel of Fig. 2a, now **Fig. 3a**; Fig. 3a-b, now **Fig. 6a-b**). The individual regions that we chose for visualization of SWA in the figures were simply illustrative examples, although as you pointed out, they were representative of the overall SWA. First, for these illustrative examples, we have performed additional analyses to assess the statistical significance of the relationships that we had initially observed in a qualitative manner (**Fig. 3b, Fig. 4d-e**). Pre-operative tractographic data may have identified the subset of cortical contacts with the highest structural connectivity to the subregion of the claustrum that our microwires were sampling. This may have better directed our analyses and given us a basis for narrowing in our analysis on the subset of most relevant contacts. We were unable to collect this data, and therefore we had to consider all contacts in our statistical analysis (**Fig. 4a**).

What happens to the 3rd unit shown in Fig. 2a that did not increase firing rate for the first SW period?

We thank the reviewer for their careful observation and agree with their assessment – there is a minimal if any increase in the firing rate during the first SW period for this claustrum unit. One finding that is beyond the scope of our current research questions is the heterogeneity in claustrum unit spiking behavior. While the spiking activity of most CLA units was highly positively correlated with SWA and other measures of SWS sleep, their spiking pattern over the course of the sleep recording was not always identical. It is possible that the behavior of CLA

neurons evolves over the course of a night of sleep (i.e. it may respond more strongly in subsequent NREM sleep cycles). However, we do not have enough sleep sessions recorded to pursue this question with sufficient statistical rigor.

Is there a direct correlation between CLA and amygdala/ACC activity? It seems the case given that CLA increases and amygdala/ACC decreases the activity.

We thank this reviewer for their comment and for asking this important question. We have now run a cross-correlation analysis, the results of which are now displayed in **Fig. 3c-d**. Our findings show that, while there was some cross-correlation between units in the same region (especially in the amygdala), we did not find significant correlation in spiking activity between our CLA units and spiking activity with AMY or ACC.

Does the phase-locking happen in other stages of sleep? This may help to establish some specificity of SW sleep.

We thank the reviewer for this excellent suggestion. We have now incorporated this analysis into our results (**Fig. 6)**. In particular, we assessed phase-locking with the slow-wave band (SWB, 0.3 – 1.5 Hz) during W/REM, NREM, and high-delta-NREM (DREM) sleep. Phase-locking with algorithmically detected morphological slow waves was removed due to concerns from Reviewers #3 and 4. We defined DREM sleep as periods of NREM sleep in which the SWA power is $\geq 75^{th}$ percentile of NREM SWA power. We then narrowed down our final phase-locking results by displaying only phase-locking relationships that were significant in DREM sleep but not in W/REM sleep (**Fig. 6f-g**). This was to ensure that we only reported phase-locking relationships that were unique to DREM sleep, which we used as an indicator for slow-wave sleep.

A fleshed out / more in-depth discussion is beneficial. The current discussion focuses on a summary of results and some clinical limitations. It will be nice to position the current findings in the broader sleep and functional neural circuit literature.

We thank the reviewer for the suggestion, and they are correct that we had truncated our manuscript due to space limitations. We have now significantly increased the number of citations and our discussion of the broader sleep literature in the discussion section (lines 175 - 202).

Signed review from Shuo Wang, Washington University in St. Louis

Reviewer #2 (Remarks to the Author):

In this short paper, Eyiyemisi Damisah and colleagues describe an extraordinarily exciting study on the role that the human claustrum plays in NREM sleep. This topic is crucially important to our understanding of the human sleep cycle and provides strong evidence of the role that this

enigmatic area plays in this process. Beyond these already important findings, the authors also provide completing analyses and comprehensive anatomical localization to bolster their results. Finally, they provide several strong parallels with animal studies (showing, for example, that the majority of CLA neurons increased spiking activity during periods of SWs in NREM sleep) to confirm the generalizability of their findings.

Altogether, I would strongly support publication of this paper. Below are a few comments that could help further strengthen the paper.

First, as the authors appropriately pointed out, the claustrum has a small axial cross-section and lays in close proximity to the insula and striatum. While the authors have done an outstanding job at localizing their microwires, it would be helpful to provide further validation of their localization using other complementary approaches (e.g., LeadDBS). Sometimes even small differences in co-localization or fusion techniques can influence precise localization. Similarly, it would be helpful to provide the estimated distribution and distances of recordings from claustrum axial center.

We thank the reviewer for their positive feedback and helpful suggestions. We have now repeated the localization of our CLA microwires using LeadDBS and confirm that they remain in the claustrum **(Supplementary Table 1, Supplementary Fig. 2)**. We have also now included measurements of the perpendicular axial distance from the microwire tip to the axial centerline of the claustrum for all microwires, all of which are sub-millimetric **(Supplementary Table 1)**.

Second, while the authors use comparisons to other areas such as the ACC to confirm selectivity of their results, most of their results are based on the number of neurons that displayed modulation. It would be helpful to also provide additional metrics for the magnitude of effect (e.g., z-score firing rates) for both modulated and non-modulated neurons as well as across areas.

We thank the reviewer for their comment and for pointing this out. We have now included additional analyses that more extensively quantify the magnitude and direction of modulation of our three single unit response types (positive, none, and negative) across regions. First, **Fig. 4b** builds on the heatmap in Fig. 4a by showing the distribution of Spearman's Rho across unit-channel pairs stratified by unit response type and unit region. Second, **Fig. 4d** shows how spiking activity changes between sleep stages for the same strata. Third, **Fig. 4e** plots the spiking activity across deciles of global SWA. The results of all three panels better quantify the modulation of spiking activity across sleep sessions and further validate the classification of unit response types.

Third, given the small number of participants, it may be helpful to perform a cross-validation procedure or provide other information about the contribution that different individuals had on the main results. For example, it would be useful to confirm that most of the neurons did not come from a single participant or set of neurons.

We thank the reviewer for this comment. Patient A contributed one sleep session (16 CLA units), and Patient B contributed three sleep sessions (12, 11, and 10 CLA units, respectively). The results of each subject, when considered separately, independently support our claims. However, this is a fundamental limitation of our experiment, and it is difficult to overcome given the extremely rare clinical opportunity of claustrum microwire recordings. We have added this as a limitation in the discussion section.

Finally, given the long periods of recordings and the potential variability across NREM sleep cycles, it could be helpful to provide some basic model prediction/decoding of population responses. For example, the authors could use an SVM or GLM to quantify the degree to which they are able to predict SWAs in NREM sleep from NREM events not used for model training. Such predictions would provide strong 'causal' support that the relation between claustrum activity and NREM is robust.

We thank the reviewer for their helpful suggestion. We have now implemented SVM models that use dimensionally reduced population activity to classify sleep stage and regress SWA, respectively **(Fig. 5b-d)**. We found that the population activity of all unit regions and response types can be used to accurately classify sleep stage and regress SWA. We suspect that these results are due to all neurons being fundamentally modulated by (or modulating) sleep cycles. Thus, although CLA and AMY units generally behave in opposite manners during NREM sleep, they both contain information coded in their spiking activity that is highly reflective of sleep stage, SWA, and other measures of sleep (**Fig. 5b-d**, lines 131 - 139).

Reviewer #3 (Remarks to the Author):

This is a timely manuscript showing, for the first time, that human claustrum neurons are associated with NREM sleep. This work follows up on rodent literature, where recent studied have shown a similar finding. I think the work has a lot of potential. However, there are some analytical aspects of the manuscript I would like to see addressed, that will hopefully strengthen the author's message, and provide further clarity regarding the state-dependent firing of human claustrum cells.

1. I found the Hypnogram in Figure 1G confusing. Wake is indicated by the pink shading, but other sleep states are indicated by the more classical hypnogram. Its confusing because it looks like REM and wakefulness can co-occur. Perhaps I am not reading it right, so please clarify this. Looking at the hypnograms in Fig1 and S. Figure3, participants go from wake to REM. This does not really happen during sleep, so I'm not really sure how their analysis is providing these state transitions.

We thank the reviewer for raising this concern. Upon our review of the hypnogram (first panel of Fig. 1g, now **Fig. 2a**), we realized that the line break in the y-axis label of "W/REM" was confusing and we agree with their assessment. We have now changed this to clearly indicate that the Wake and REM states have been grouped together. Our hypnogram is based on scalp EEG,

and without reliable EOG/EMG, we were unable to accurately differentiate between Wake and REM states. Thus, we performed manual review of the audiovisual recordings to classify time periods in which the subjects were moving or otherwise demonstrating wakeful activity. These results are indicated by the red and blue background coloring on the hypnogram (**Fig. 2a**, top panel), and they provide a limited but useful way to differentiate wakeful periods from potential REM sleep periods.

2. The data presentation in Figure 2C is really not an effective way to convey the message. First, the ACC and CLA are a similar color, so discerning their points on the scatter is visually challenging. Second, I would recommend showing each region separately. These scatters are too crowded. Or alternatively showing a histogram of NREM-notNREM firing rates (which would highlight the difference they want to describe).

We thank the reviewer for their comment and pointing out this limitation in our figure presentation. We have now split Fig. 2c (now **Fig. 3b**) by unit region to visually disentangle the crowded points on the original scatterplot. In addition, we have changed the color scheme for unit region across all figures so that the colors are maximally dissimilar in order to better differentiate unit region.

3. Following up on this last point, the authors should do a better job comparing the firing rate across states. Currently, the only state comparisons are in figure2C. What about comparing NREM versus REM, or NREM vs wakefulness? These comparisons should be performed to compare with the rodent literature, and for rigor and completeness of the study.

We thank the reviewer for this helpful suggestion. We have now better characterized the firing rate across sleep stages and deciles of SWA in **Fig. 4d-e** and **Fig. 7d**. Due to a lack of reliable EOG/EMG data, we were unable to differentiate Wakeful and REM sleep states, so we had to group these in our analyses.

4. I am looking at S Figure 5. The red indicates the detected Slow waves. I think the authors have some work to do with regards to their detection algorithm. For example, in the MTG recording, the first detected wave is not a slow wave. It seems like they are missing a lot of slow waves, while also mis-assigning slow waves in some cases. Overall, this is not convincing. But also, why do the authors care about performing this SWs detection? Just calculate the overall power of 1-3Hz activity using a sliding window. Isn't that good enough for what they want to say here? Don't overcomplicate an analysis and generate doubt.

We thank the reviewer for their comment and helpful suggestion, which is aligned with Major Comment #2 from Reviewer #4. We have now revamped our analysis to focus on SWA (z-score of log-normalized delta band power) instead of detected SWs. We retained SW detection in a few portions of the analysis (**Fig. 1f**, **Fig. 4c, Fig. 6a**), but most of the results regarding SWs have now been removed from both the main figures and supplementary figures (e.g., **Fig. 2a-b** and **Fig. 3a-b** now have SW-related results removed).

5. What about a Figure showing the cross correlations between CLA – ACC, and CLA-BLA spike pairs? And/or showing the multiunit (ensemble) cross correlations between these regions? This would be a first for this type of analysis with claustrum data, and something that would be particularly interesting regarding the ACC correlations. Also, consider showing time-lag correlations to enable the reader to see the temporal relationship between spike-count correlations.

We thank this reviewer for their comment and for asking this important question. We have now run a cross-correlation analysis, the results of which are now displayed in **Fig. 3c-d**. Our findings show that, while there are some intraregional single unit pairs with high levels of cross-correlation, we did not find significant correlation in spiking activity between our CLA units and spiking activity in AMY or ACC.

6. Line 79 states CLA neurons increased spiking during SWS. Compared to what? Wakefulness? Whatever you are comparing to, it should be stated here. The same goes for the following sentence when discussing AMY and ACC cells. What are the SW data compared to , in order to make this claim?

We thank the reviewer for pointing out this ambiguity in our description. This sentence referred to CLA increasing spiking activity during periods of high SW presence compared to periods of low SW presence. To implement your previous suggestion to focus on SWA instead of detected SWs, we have removed this part of the analysis. However, we have maintained the spirit of your suggestion in the modified sentence that has now replaced it regarding NREM sleep compared to W/REM sleep (lines 107 - 110).

7. I see the paper was originally formatted for a brief comm for NN where there are tight restrictions on word count, and references. I think the reference list could be expanded significantly in most sections of the intro. For example, when discussing NREM importance in consolidation, homeostasis and sleep disorders, only 1 reference I given. The same goes for the discussion. Please take the time to acknowledge the relevant work where appropriate.

We thank the reviewer for their comment and agree with their suggestions. We have now expanded both the introduction and discussion sections and added many new references in order to properly cite the broader literatures on sleep and the claustrum.

8. In line 73 please indicate what brain regions these studies recorded from.

We thank the reviewer for their comment. We have now specified that these studies recorded local field potentials (LFPs) from the prefrontal cortex and retrosplenial cortex, respectively, in rodents (line 101).

9. I see some CLA cells do not track SWs. Are these cells different from the SW tracking CLA

cells in terms of spike width or mean firing rate? Can the authors provide insight into what putative interneurons are doing?

We thank the reviewer for their comment and suggestion. We have now better characterized differences in firing rate and spike width, in addition to other waveform metrics, among strata of unit response types and region in **Fig. 7a-c**. Furthermore, we assessed differences in spiking activity across sleep stages of the interneurons compared to pyramidal cells (**Fig. 7d**). While we did not have a sufficient sample of putative interneurons to make a firm conclusion, we found that all CLA interneurons decreased spiking activity in NREM sleep compared to WREM sleep. This contrasts with CLA pyramidal cells, almost all of which had the opposite pattern of spiking activity (**Fig. 7d**, lines 157 - 160).

10. All figures showing time series data require time scale bars. Some figures do not currently have these.

We thank the reviewer for their observation. We have now added time scale bars to all relevant figure panels (**Fig. 2a-b, 3a, 5a** and **Supplementary Fig. 5, 6, 7, 9**).

11. The writing in the discussion is a little redundant. They re-state their main finding 3 times in a very short amount of space. Consider taking the time to really contextualize the results relative to other studies. Also, some further discussion of the studies limitations (for example, the authors mention there are many cla subtypes. What does this mean for the interpretation of results, or how would be authors suggest overcoming these limitations in the future?)

We thank the reviewer and agree with their suggestion. We have now significantly expanded the discussion section and reduced its redundancy. In addition, we have significantly expanded the discussion of limitations. We also specifically addressed your question regarding the functional gradient across claustrum subregions and its implications for our results (lines 191 - 200). In brief, because all of our microwires sampled the mid-claustrum, we were unable to generalize our results to the entire claustrum. Future studies that simultaneously sample multiple subregions of the claustrum may extend our results.

Reviewer #4 (Remarks to the Author):

Lamsam and colleagues report exceedingly rare data recorded from single neurons in the human claustrum during sleep. Consistent with rodent studies, they find that claustrum neurons show increased firing rates during NREM, while neurons in the amygdala and anterior cingulate cortex showed decreased firing rates. A majority of claustrum neurons showed spiking patterns tied to slow wave activity. Neurons in all three regions showed phase locking to multiple cortical region recorded with macroelectrodes, including orbitofrontal cortex. The authors suggest that these results demonstrate a role for the human claustrum in regulating sleep by coordinating slow wave activity. This is an important and impressive paper.

In addition to the truly unique data reported here from Dr Damisah's group, the brief paper provides a nice confirmation of phenomena observed in rodents. Demonstrating that brain-wide coordinated activity like slow waves are correlated with single neuron firing in the claustrum provides important evidence that this structure is a key part of the cortical architecture for sleep.

MAJOR

1) While the qualitative results and figures are compelling, the paper would benefit from more statistical quantification of results. Throughout the paper, it is important to know to what extent each result is stronger than that expected by chance. There are some results (like the phase-locking findings) where it is not clear whether the relationship is actually real, or whether the consistent phase is just a weak effect of shared noise.

We thank the reviewer for this helpful suggestion. We have now significantly expanded our statistical analyses throughout the manuscript (now 7 main figures). To address this reviewer's question regarding the phase-locking findings, we have changed our methodology. This analysis now uses permutation tests to better isolate true phase-locking findings from noise. In brief, we generate surrogate data via bootstrap resampling (n = 1000) of original phase data which has been jittered. The z-statistics from these surrogates are used to calculate the p-value of the original data's phase-locking relationship, then FDR-correction is applied, and finally we remove significant phase-locking relationships that are also present in W/REM sleep to isolate relationships unique to NREM sleep.

2) What is the justification for relating CLA firing to both SWs and SWA? If I understand correctly, SWA is just normalized delta power, while SWs are the percentage of 10s epochs that have SWA (again, computed using just an arbitrary z-score threshold, rather than a test that accounts for the variance). It seems like SWs are just a more conservative thresholded version of SWA, but it's not clear why both are necessary, other than that SWs are used for the phase-locking analysis.

We thank and agree with the reviewer for these comments and suggestion, which is aligned with Comment #4 from Reviewer #3. SWA is indeed the z-score of the log-normalized delta band power, whereas SWs were algorithmically detected grapho-elements on the low-passed EEG time series. Both were binned into epochs – average power for SWA and the percent of the epoch during which a SW was present for SWs. We have now revamped our analysis to focus on SWA, and most results relating to SWs have been removed from the main figures and supplementary figures.

3) Simply correlating single unit activity with SWA/SWs is potentially misleading for the claim that CLA neurons are uniquely more active. Judging from the raw data presented in Fig. 1 and Fig. S3, there is some variability in the relationship between NREM and CLA. It would be more comprehensive to do something like a classification analysis where it's possible to compute

sensitivity and specificity to test whether, during sleep, CLA activity is particularly associated with NREM.

We thank the reviewer for their suggestion and have now implemented modeling analyses for the classification of sleep stage and regression of SWA, respectively, using population activity (**Fig. 5b-d**). We found that positive-responding CLA units can classify and regress both dependent variables with a high degree of accuracy. We also found that other response-region combinations had similar performance, suggesting that for the purposes of modeling, many unit populations have information encoded in their spiking activity that reflects sleep stage and SWA (though in an opposite direction, when considering the cases of AMY and ACC). We have also added analyses that partially address this suggestion. These include (1) comparison of firing rates in W/REM versus NREM sleep stratified by unit response and region (**Fig. 4d**) and cell type (**Fig. 7d**) with statistical analysis with FDR-corrected Wilcoxon signed-ranked tests and (2) calibration analysis of firing rate versus global SWA (divided into deciles) stratified by unit response and region and quantified by R^2 statistics (**Fig 4e**).

4) Please provide justification for MER placement in claustrum. I assume it is related to the insula placement and trajectory but would be good to make this explicit so readers understand why those were place clinically there.

We thank the reviewer for making this important assumption and have now made it explicit that we were able to sample the claustrum using depth electrodes that were placed into the middle insula via an oblique trajectory for clinical purposes. We have now added this explanation to the introduction section (lines 61 - 63).

MINOR

5) There are several places where aspects of the visualization are not explained or quantified. In particular, it is not clear where the gray shading comes from in the UMAP clustering in Fig. 3E. It seems like the points were “manually clustered” (line 333), when they should have performed some kind of quantitative clustering to determine how well the two groups are actually separated (though clustering should not be done in UMAP space). Similarly, they only qualitatively describe the distribution of CLA units in each of the two groups.

We thank the reviewer for this important suggestion. We have now implemented Hierarchical Density-Based Spatial Clustering of Applications with Noise (HDBSCAN) to cluster units in an unsupervised manner (**Fig. 5a**, left panel). For each cluster, we have quantified the proportion of CLA units present (**Fig. 5a**, middle panel).

6) Another specific place where a lack of quantification is concerning is Fig. 3A-B. If anything, the rasters look like there’s a more robust relationship to the SWs for AMY than CLA. And the phase relationship in panel B looks relatively flat. It’s certainly possible that this histogram is significantly different from uniform, but this does not appear to have been tested.

We thank the reviewer for this comment and upon further review, we have realized that these panel (**Fig. 6a-c**) were presented in a confusing manner. The results in Fig. 3a-b were illustrative examples taken from the analysis shown by Fig. 3d. In this analysis, we tested the phase-locking relationship of all units with all contacts using Rayleigh's Test, which had to be corrected for the false-discovery rate (FDR) given the large number of comparisons. The examples in Fig. 3a-b were picked from among the unit-contact pairs that demonstrated statistically significant phase-locking after FDR correction.

To your point regarding Fig. 3a, in which the AMY-SW relationship appears stronger than the CLA-SW relationship, we agree with this reviewer's assessment. In this example, the amygdala was selected as a positive control and was therefore expected to have a stronger phase-locking relationship (**Fig. 6a, f**). Specifically, for these analyses we aligned amygdala units to amygdala field potentials, which we expect to demonstrate strong phase-locking. In contrast, the claustrum units were being compared to remote slow waves in the orbitofrontal cortex, as we did not record from macroelectrodes in the CLA. Our discussion of this point in the results section has been clarified, as it was ambiguous in our original manuscript (lines 167 - 170).

7) Fig. 2C is also not quantified sufficiently. The measure of how many units are above and below the diagonal is interesting, however given that most points are on or very close to the unity line, it's not clear how many are actually significantly different.

We thank the reviewer for pointing this out. We have improved the visualization of the results in Fig. 2c (now **Fig. 3b**) for clarity, which are also accompanied by p-values from Chi-Square tests. More important, we further quantify these results with additional analyses in **Fig. 4d-e**, which are accompanied by p-values from Wilcoxon signed-rank tests (FDR-corrected) and R^2 values.

Reviewer #1 (Remarks to the Author):

The authors have adequately addressed my questions. I congratulate the authors on completing this impactful study.

We thank you for your helpful comments – they have improved the manuscript greatly!

Reviewer #2 (Remarks to the Author):

Overall, the authors have done a phenomenal job at addressing my prior comments and suggestions. The paper is much improved and provides crucial new insights into the role of the claustrum in sleep/NREM. Specifically, the authors have now used LeadDBS to confirm localization of their electrodes to the claustrum. They have also provided careful new analysis that more directly quantify the magnitude and direction of modulation as well as demonstrated the generalizability of their finding across participants. Finally, they have used a new modeling approach to classify sleep stages and demonstrate that they can be reliably predicted from neural activity.

Taken together, the findings and the rarity of recordings making this paper truly remarkable and will likely generate intense interest within both the scientific and lay community. I have no further comments and would strongly support publication of this study.

Thank you for your suggestions, particularly regarding LeadDBS, and we hope this manuscript will indeed generate interest as you predict!

Reviewer #3 (Remarks to the Author):

The authors have addressed the majority of my concerns and the manuscript is considerably improved. I have some remaining questions/clarifications that should be addressed before publication.

Discussion:
The use of SWB could be replaced with the more frequently used SWA

Thank you for pointing out this ambiguity. We use the term “slow-wave band (SWB)” to refer to the lower frequency portion (0.3 – 1.5 Hz) of “slow-wave activity (SWA)” (0.3 – 4 Hz). While we defined this in the Methods section, it was not clear in the main text. We have now added this specification to properly differentiate the two terms in the main text.

The authors state that the claustrum has functional gradients across subregions. I don’t believe there is strong evidence for functional gradients, but anatomical gradients are well described. I would recommend adding citations for function and/or anatomical gradients.

Thank you for this excellent point. Indeed, evidence for a functional gradient in the claustrum is only recently emerging and not well-established. We have now rephrased this portion of the discussion to emphasize this point and to also to be better aligned with the current terminology of “functional module”. References 44 and 45 address functional modules in the claustrum, and references 46 and 47 address the anatomical gradient in the claustrum.

Methods:

How many interictal epileptiform events were detected and removed? Please provide the average/session.

We thank the reviewer for making us aware of this omission. The number of interictal epileptiform discharges (IEDs) that were detected in at least one channel were 83, 796, 1407, and 1130 for the four recordings of 2, 9.7, 10.6, and 10.4 hours, respectively. This is now included in the methods.

The jittering of the spike times for the surrogate cross correlations is not well described. Please elaborate more. Currently it says 20ms uniform distribution. Does this mean that they were jittered by a distribution of times centered on 20ms? This seems awfully short. Why not jitter by random values the up to the full length of the recording. In any case, more detail is required for the correlation analysis.

Thank you for highlighting this ambiguity and for the excellent methodological point. The jittering for cross-correlation is performed randomly within a time window centered around the original time point. The width of this time window is 20ms. We agree that there are likely to be many valid widths for the time window. In fact, one could perform surrogate data generation using time window widths up to the length of the recording, as the reviewer has suggested. It is possible, however, that longer time windows increase the likelihood of Type I errors. Thus, our time window width represents a conservative parameter setting that may reduce the incidence of false positive results in our multiple comparisons.

I find it strange that this threshold enhancement is required, why not just take the distribution of surrogate values and define a threshold based on these?

We thank the reviewer for this point of clarification. We used the threshold-free cluster enhancement (TFCE) procedure as an intermediate step to more clearly identify statistically significant cross-correlations. Certainly, as you suggest, we could also perform the cross-correlation without this intermediate procedure; but we feel that this additional measure increases the robustness of our findings.

Figures:

In Figure 3b, the y-axis is “FR in not NREM” – this is a little confusing. In other parts of the paper WREM is used. Is WREM the same ‘not NREM’. If so, please pick a convention.

Thank you for pointing out this ambiguity. The “Not in NREM” y-axis label has now been changed to “in W/REM”.

Also in Figure 3b, please show the p-values for the AMY and ACC as is done for the CLA

Thank you for raising this important point. There are no equivalent p-values for AMY or ACC, because the p-values specified in the figure reflects testing of the CLA as compared against the pooled AMY and ACC units.

In Figure 4b I was initially very confused because the counts (n) are so high. However, I realized it was because the n is for each cell-LFP channel pair. It would be helpful to state somewhere in the figure the n's for the cells and n's for channels to avoid reader confusion. This would also help understand why there are negative correlations in the positive group, and likewise for the positive correlations in the negative group.

We thank the reviewer for this suggestion. The total sample sizes for units (n = 122) and channels (n = 64 unique, repeating across recordings) represented in this plot are now specified in the caption for this figure.

In Figure 4e, the firing rate for each decile is supposed to be shown. Decile implies 10 bins, but there are clearly >10 points on each plot. Please clarify and/or revise.

Thank you for bringing this to our attention. Multiple points are plotted per decile since each of the four sleep recordings is plotted separately. Thus, there will be four data points for each decile. This has now been clarified in the caption.

Also, I believe in the methods there is mention of linear fitting, when the fits shown are not linear. I see that the LoESS was used for fitting, but unless there is some meaning to these curves, I recommend showing the linear fits.

Thank you for this suggestion. The LOESS fits have now been changed to linear fits (Fig. 4e).

Please add scale bars to all MRI images in the manuscript and supplementary data

Thank you for pointing out the omission of scale bars in the MR images. Scale bars are now added to all MR images (Fig. 1b, Sup. Fig. 1, Sup. Fig. 2).

Reviewer #4 (Remarks to the Author):

The authors have addressed all of my comments. This is a very interesting and important paper on the neuronal physiology of the human claustrum.

Thank you for your guidance through this revision process. Your suggestions have distinctly improved our manuscript.