nature portfolio

Peer Review File

Thalamic feedback shapes brain responses evoked by cortical stimulation in mice and humans

Corresponding Author: Dr Irene Rembado

This manuscript has been previously reviewed at another journal. This document only contains information relating to versions considered at Nature Communications.

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)
In this paper Russa et al. address the involvement of thalamocortical loops in responses to cortical singe pulse stimulation. Single pulse stimulation is a method in clinical practice and in human research. The authors suggest based on their presented work that the late component of these evoked responses might be related to thalamic repolarization.
To achieve this, they combined human experiments with TMS (in a rather small sample of n=12 subjects), optogenetic experiments in rodents and simulations.

They suggest that the cortical stimulation engages cortico-thalamo-cortical circuits highly preserved across different species and stimulation modalities and that intrinsic thalamic currents as well as cortical and thalamic GABAergic neurons contribute to this response profile.

This is nice work that demonstrates the benefit of combining human, rodent and simulation work to better understand cross-species mechanisms of neuronal processing, in this case cortico-thalamo-cortico interactions and the role of the thalamus. The technological approach in rodents is well established and applied for this specific question. The behavioral target is motor behavior, in humans distal skilled hand functions were chosen in rodents rather gait/running, one would probably have expected the choice of tasks more comparable in the two species.

However, there are a couple of open questions, suggestions, clarifications to address.

Methods:
Behavioural task in humans and rodents: in humans a skilled distal upper extremity task was chosen (squeezing a ball), in rodents a running/gait task. The choice of these tasks is not very clear for me. The authors stated that they want to determine cross-species mechanisms, then it is surprising that they used such fundamentally different tasks in the 2 species. Skilled hand functions is something very human typical, which not very comparable to any motor task in rodents especially not to running. Probably food pellet retrieval would have been much closer. Also, in terms of underlying networks and interactions in cortical, subcortical and cortico-cortical and cortico-subcortical networks is different for a skilled distal upper extremity task and a gait or running task. Could the authors provide additionally evidence that the findings are comparable if they use more comparable tasks, e.g. in humans a TMS study with rhythmic leg movements like cycling (e.g., Zhang et al. 2024), or at least a more proximal upper extremity task or alternatively e.g., a pellet retrieval task?
Human experiments: In the human experiments, if I understand well, the TMS pulses during the behavioral motor task (squeezing a ball) were randomly applied. This is in my view somehow problematic as it has been demonstrated in different studies that the application of TMS pulses and its evoke potential depends strongly on the stage of the motor act (e.g. preparatory phase, movement phase etc.) intrahemispherically (e.g., O'Shea et al. 2007 EJN, Heise et al. 2013 J Neuroscience), as well as interhemispherically (e.g. Murase et al. 2004 Ann Neurol; Liuzzi et al. 2019 Cerebral Cortex; Liuzzi et al. 2014 Neurology; Lazzari et al.2022 Nat Comm). Were TMS pulses only applied in specific windows of the movement process? How much did the task performance (strength of squeezing impact on these parameters? How well was the strength controlled? how did these windows compare to the stimulation windows in the running tasks?

Intensities of Stimulation Human rodents (page 6): Could the authors comment on the applied intensities in humans and rodents. How comparable are there? Subthreshold, suprathreshold, adjustment to motor threshold in humans, in the animal model are the intensities of stimulation also adjusted to a motor threshold?

Rodent exps: Rest vs running was defined by a threshold of wheel speed greater or smaller 0.1 cm/sec. Is this an arbitrary definition? Is this an established definition? How do the results change if the threshold would be 1.5cm/sec or 0.5 cm/sec?

Control-Exp. represented in Fig2Sc: This experiment is done in n=4 subjects. In my view this does not provide reliable evidence in one or the other direction, as can be seen already in the individual data heterogeneity. As the sample of the study is already rather small with n=12 subjects, the control exps should also be done in n=12 subjects, ideally the same ones.

Minor:
- Ref 35 is missing
- Sometimes data for analyses is z-scored sometimes not, can the authors provide rational for it.

(Remarks on code availability)

Reviewer #2

(Remarks to the Author)
This is an exemplary study demonstrating similar features of cortically evoked activity in mice and humans using a variety of methods and modelling. Since the two species display similar features and the mechanisms of cortico-thalamic influence can be studied in detail in mice the authors may draw clinically relevant inferences on the human condition.
I have the following advice to improve the presentation:
Major:
1) The impact of corticothalamic afferents on thalamic networks has been investigated in detail in the past decades (E.g. Kalil and Chase 1970 J. Neurophysiol; Deschenes and Hu, 1990 EJN or Olsen et al., 2012 Nature). More specifically, the early and late component of the response to cortical stimulation together with the long-lasting inhibition and its state dependency have also been described using in vivo intracellular recordings and modelling (For a review see e.g Destexhe 2000 J. Physiol Paris). The direct impact of cortex on RTN has also been studied (Fuentealba et al., 2004 EJN). The authors have to make it clear which of these early data have been confirmed by their novel approach and which must be revised.
2) The exact nuclear localization of thalamic recording electrodes has to be described in detail and should be demonstrated in a figure. The impact of cortex on thalamic activity depends hugely on nuclear identity and ranges from negligible to very strong (see e.g Diamond et al., 1992 JCN but also Guo et al., 2017 Nature). The authors implicate cortical disfacilitation during the long thalamic inhibition before the rebound response. If this interpretation is correct the activity in first and higher order nuclei (see Sherman and Guillery's work) is expected to differ during the inhibitory phase of the cortically induced activity.
3) The impact and significance of the study could be greatly enhanced by the inclusion of spontaneous activity that evokes a pattern of response similar to cortical stimulation. The most plausible would be e.g. interictal activity in epileptic patients with intracranial electrodes.
4) The cortically induced responses seem to show large intertrial variability even in the same behavioral conditions. The authors may characterize this variability and could provide a hypothesis for its source using the mouse data.
5) The early response component in the thalamus may reflect both ortodromic as well as antidromic activation. Since these two options result in different recruitment of RTN (cortical vs thalamic) it should be clarified. Using peristimulus time histograms, the variability of the response latency and possibly collision tests in case of individual neurons orto- vs. antidromic activation of thalamic neurons could be differentiated.
6) Were low intensity stimulations used in humans as well? If yes, could the omission of the second component be demonstrated in case of low intensity stimulations?
7) The experimental data in mice clearly demonstrate a major role of the evoked RTN inhibition in the pause-rebound response and less support for the cortical disfacilitation hypothesis (there is very little thalamic rebound after cortical inhibition alone, the timing of thalamic but not cortical inhibition alters cortical rebound). These data are supported by the earlier results as well. In contrast the modelling data describe that RTN inhibition is dispensable and place more emphasis on cortical disfaciliation. Discussion is quite ambiguous on this matter. Please clarify exactly what supports the role of cortical vs thalamic inhibition in timing the rebound and examine the discrepancies between experimental and in silico results.
8) Along the same argument the difference between the unit response to optogenetic inhibition of MO vs SM-Th (Fig S8C) should be better quantified.
9) It is unclear what is exactly shown in FigS2C and the corresponding rodent Fig S6 what is the rationale and exactly what these experiments want to demonstrate. Were all the recordings made in MO or also in the stimulated cortices as well? What EEG refers to in Fig S6a? Both the rectified amplitude and the PLF show very different shape in case of the posterior cortical stimulations (FigS2C) and the rebound response differs also from MO in Fig S6B as well. The early and late components are unclear in these examples. Why we expect a rebound in MO if posterior cortices are stimulated, when rebound is generated by topographically organized TC cells, which project back to posterior cortex? Does the pause-rebound response have cortical region specificity. I.e. if parietal cortex is the stimulation and recording site as well do we have the same two

components as in MO? What is exactly meant by “control” cortices? Are posterior cortices are not involved in movements? Movement is thought to involve and modulate all cortical areas.
10) It would be important to state whether increasing stimulation intensity can turn LF cells to HF cells or these are two distinct cell types recruited by different intensities.
11) It would be nice to directly show that synchrony between LF cells, as opposed to HF cells decrease during movements e.g. by analyzing cross correlograms.

Minors
a) The author write „As opposed to the late component, the early component of the EP (3-50 ms from stimulus onset) showed a reduction in amplitude, but not in phase locking…….. Figure S2A)”.
b) Both the early and the late components show a reduction in amplitude thus „As opposed to…” is disturbing here.
c) In Suppl Fig 1C please indicate the cut-off value for the low vs high signal-to-noise ratio and plot the same results showing the used, high signal-to-noise data points. In this dataset no correlation is expected.
d) In Fig S10, there is large difference between the two subjects in thalamic responses while cortical and RTN responses are similar. How this interindividual variability can be explained.
e) Since the reference of Sinkkonen et al (1995) is not so well known and the phase locking factor (PLF) is not described, as such, in this reference but PLF is a highly important measure here please describe it in a bit more detail, how it is calculated and what it is sensitive for etc.
f) Please indicate significance with stars above the lines in the figures rather than in the text, it is simply faster to read.
g) Abbreviation for the reticular thalamic nucleus (RTN vs TRN) seems to be inconsistent throughout the ms.
h) Please indicate in what state (move or rest) the optogenetic inferences have been delivered.
i) In Suppl F10A clearly label Ctx and TRN.
j) Please provide a reference for the similarity of Ih and It between primates and rodents.

(Remarks on code availability)
N/A

Reviewer #3

(Remarks to the Author)
In this paper, Russo and colleagues explore the mechanisms underlying an early and late component of the cortical population response to stimulation in humans and mice. For both species and independently of the stimulation method, they find both an early and late component of the cortical population response, with the late component being modulated by behavioral state. Recordings from thalamus and biophysical modelling reveal that the late component originates from rebound spiking mediated by thalamic hyperpolarization, likely through activation of both cortical and thalamic (TRN) inhibition.

While the results are not surprising to researchers working on thalamocortical circuits, they constitute important interpretative guidelines for clinical results involving cortical stimulation and are therefore a significant contribution to the field. I only have a few suggestions for further enhancing the clarity of the manuscript.

MAJOR:
- Please add page and line numbers to facilitate the communication about the manuscript.

- The merged manuscript file does not contain the figure captions for the supplementary figures (they only appear in the separate Supplemental information file). This made more difficult than usual it to assess the supporting information.

- I would like to ask the authors to be less specific about the interpretation of the active condition. In the abstract, they refer to the manipulation by “behavioral state”; later on, they frequently refer to the condition as “movement” condition. In my view, active state / active condition describes the condition more appropriately. To understand whether movement was indeed required, one would need to do control experiments, in which the human subjects or animals would be aroused or in an activated behavioral state but without movement. The passive movement condition could also induce higher arousal than rest, so the results obtained in this control would not contradict the sentence “The modulation observed during passive movement suggests that peripheral inputs are sufficient to change the state of the thalamus.” Given what we know about modulations of thalamic activity, the described mechanism would not require movement, but would also work with state-dependent modulation of thalamus. Hence, I would suggest to consistently use the term active condition / state.

I am not convinced that the interpretation “progressively delayed both thalamic and cortical units’ responses” is correct. An alternative interpretation that is fully in line with the authors’ conclusion could be that the strong activation of TRN will strongly hyperpolarize the thalamus, which will then respond with rebound activity at the offset. This would not be a “response” to the cortical ES, but a mechanism that would override the native response to ES, and elicit its own rebound through strong hyperpolarization and rebound excitation. I would therefore suggest to avoid the term “delay the responses”.

I have a few questions regarding the placement of electrodes: For the iEEG experiments, in which layer were the two adjacent contacts used for iEEG stimulation? How did the response pattern look like at more distant electrodes? For the experiments in mice, please give a few more details on how the units were assigned to thalamic nuclei and TRN. Please

also give the stereotaxic coordinates for the TRN fibre implant.

For the comparisons of latency across SM-TH and MO units, does the difference also hold when matching the firing rates in the two structures? Maybe this is already answered in Figure S7B, but given that these are simultaneous recordings in cortex and thalamus during the mouse experiments, could the authors please do the comparisons of latencies on a session-by-session basis?

I cannot follow the argument: "…evoked firing rate in MOs and SM-TH units was not modulated by movement, unlike EEG, LFP, and CSD responses. Therefore, MOs and SM-TH firing rate changes did not explain the movement-related modulation of the EPs (Figures, 3A, S9, S10 p=0.03, rest lower than movement)". Why is p=0.03 not considered significant? Why is it not taken into account that the baseline firing rate is enhanced during movement (Figure 3A)? If this was the case, wouldn't the modulation be even further enhanced? I also do not understand the subsequent argument: in Figure 3E, it seems like there is a reduction in the HF response frequency with movement without a concomitant decrease in HF ROV; at the same time there is an increase in LF ROV, but no change in response frequency. I therefore cannot fully follow why ROV should underlie the changes in response frequency.

Regarding the introduction of the two thalamic response modes, I do not understand why the authors chose to first say "we identified two identical thalamic cell-types" before then saying that the "two subsets reflected two different response patterns to the cortical stimulation rather than two different cell types". I would suggest to rephrase the first statement to avoid sending the reader on the wrong track. For non-expert readers, I would suggest to also add a note about connectivity here - according to the authors own models, this is the reason why the two different "response patterns" to cortical stimulation arise.

I would suggest to move the figure panels with thalamic optogenetic stimulation alone and cortical stimulation alone to the main manuscript. This would give a chance to give some more space to the relative role of both sources of thalamic hyperpolarization.

MINOR:
- 50 ms later,between -> blank missing
- "These factors were modulated by their baseline firing rate" -> unclear, please rewrite. In particular, it is difficult to understand what "THEIR" refers to
- "response patterns to the cortical stimulation rather than two different cell types" -> please say even more clearly that this is expected given a topographic connectivity and is not related to intrinsic factors that might still determine "response patterns"
- "(Elbow method, Calinski- Harabasz score, and Davies-Bouldin score; metrics.calinski_harabasz_score and metrics.davies_bouldin_score functions from sklearn - Python)" too much detail in the results, consider replacing by (see Methods) if wanted
- "leading to an increased onset variability of LF units" - I don't think 'leading' is appropriate, given that these are correlational analyses
- "Our results show that the animal's state (Figures 1, 3) and the intensity of the ES (Figure S4) modulate the late EP component via their effects on thalamic bursting frequency and synchronicity, rather than cortical and thalamic evoked firing rate (Figure 3, S9)." Is it possible to make such a strong statement? Isn't the thalamic evoked firing rate directly related to depolarization and thus to bursting frequency?
- Some typos in y-axis labels of Figure S8C
- Maybe I missed it, but I could not find the list of thalamic nuclei in the methods and how contacts were assigned to individual nuclei. Could the authors please provide a more explicit reference to the position within the article ("sensorimotor-related thalamic nuclei (SM-TH; see full list of thalamic nuclei in the Methods)")

(Remarks on code availability)
I have not tried to install and run the code, but I browsed the repository. It seems like the readme file will provide enough details for installation, but it contains only limited instructions which files are associated with which analyses. In addition, I would urge the authors to provide a cleaned-up version of their code. For instance, folder names like "IreneScripts", "Anecdotic_Opto", should be renamed to more professional names. To reproduce the in silico results, the code for the thalamo-cortical model should also be made public (apologies if I couldn't identify the relevant parts of the code from browsing the folder structure).

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
Congratulations to the authors. They have addressed all my comments in an exemplary manner. I have no further remarks.

(Remarks on code availability)
na

Reviewer #2

(Remarks to the Author)
Congratulations for the authors they have thoroughly addressed all my questions and comments. I have no more question. Maybe one more suggestion.
Concerning the response to my point 3. I understand why the authors hesitate to include this nice figure to the present manuscript. However, the mechanisms of spike and wave discharges is still heavily debated, and the role of thalamus is usually underestimated. The present data provide compelling evidence for the role of thalamus in the wave component. Indeed, it may not be wise to bury the SWD data shown as a supplementary figure of this paper, but I strongly recommend the authors to consider a separate brief study on the similarities between evoked activity and spontaneous SWDs in humans.

(Remarks on code availability)

Reviewer #3

(Remarks to the Author)
In this revised study, the authors investigated the circuit and network mechanisms underlying the late component evoked through cortical stimulation. They show that this late component depends on the thalamus and is affected by behavioral state. Through experiments and modelling the authors show that both thalamic intrinsic currents and cortical and thalamic GABAergic neurons are involved in the late responses to cortical stimulation in both mice and humans.

I still have some issues with the clarity of the text, partly overlapping with my previous concerns.
More importantly, as mentioned in my previous review: The results are not surprising at all to researchers working on cortico-thalamo-cortical circuits; they seem to, however, constitute important interpretative guidelines for clinical results involving cortical stimulation. The editors would have to decide if the advance here is large enough to warrant publication in Nature Communications.

MINOR:

- l. 214: consider rephrasing (on the other hand is not preceded by on the one hand, and it is unclear what exactly is contrasted to what on the other hand; however)
- l. 226: similarly to what?
- l. 227: consider moving the reference to Fig. 3 after “active state” to avoid confusion
- l. 227: is rebound rate here the same as late component?
- l. 230-234: give a bit of intuition why this analysis was pursued
- l. 236: how can it be known that these are relay neurons as compared to local inhibitory neurons etc.
- l. 250: can the authors please at this point already explain a bit more what is entailed by “connectivity profile” here. I assume it could be how the thalamic neurons are connected to the specific site of cortical stimulation, assuming some topography between the cortex and the thalamus (as later illustrated for the model). Or do the authors mean that there is a specific class that is more strongly connected in general to cortex, as potentially implied in l. 244 (“class of more effectively modulated neurons”)
- l. 439: I would suggest to phrase this and the previous paragraph more carefully; the corticothalamic pathways in other systems has been widely investigated, also in awake animals

(Remarks on code availability)
The code repo is revised according to my suggestions

Reviewer #1 (Remarks to the Author):

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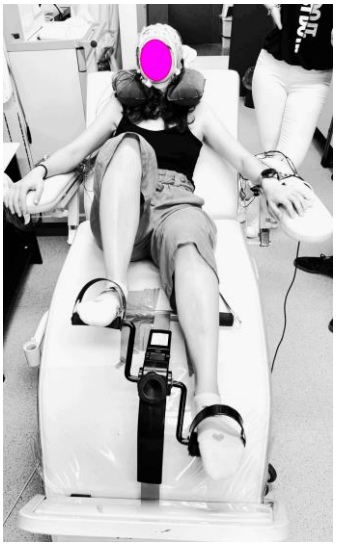
To address this valid methodological point raised by the reviewer, we performed an extensive control experiment using 12 volunteers in the TMS-EEG setup with stationary cycling, as shown in the picture below (not included in the manuscript). Here, the two conditions compared were rhythmic leg movements versus resting. We observed a significant decrease of the late component for both response amplitude and PLF (Figure S2C). Given this new result now included in the revised version of the manuscript, we can comfortably state that the late

component of the evoked responses elicited by cortical stimulation in both humans and mice is modulated by the state of the cortico-thalamic network.



TMS-EEG experimental setup for the stationary cycling motor task.

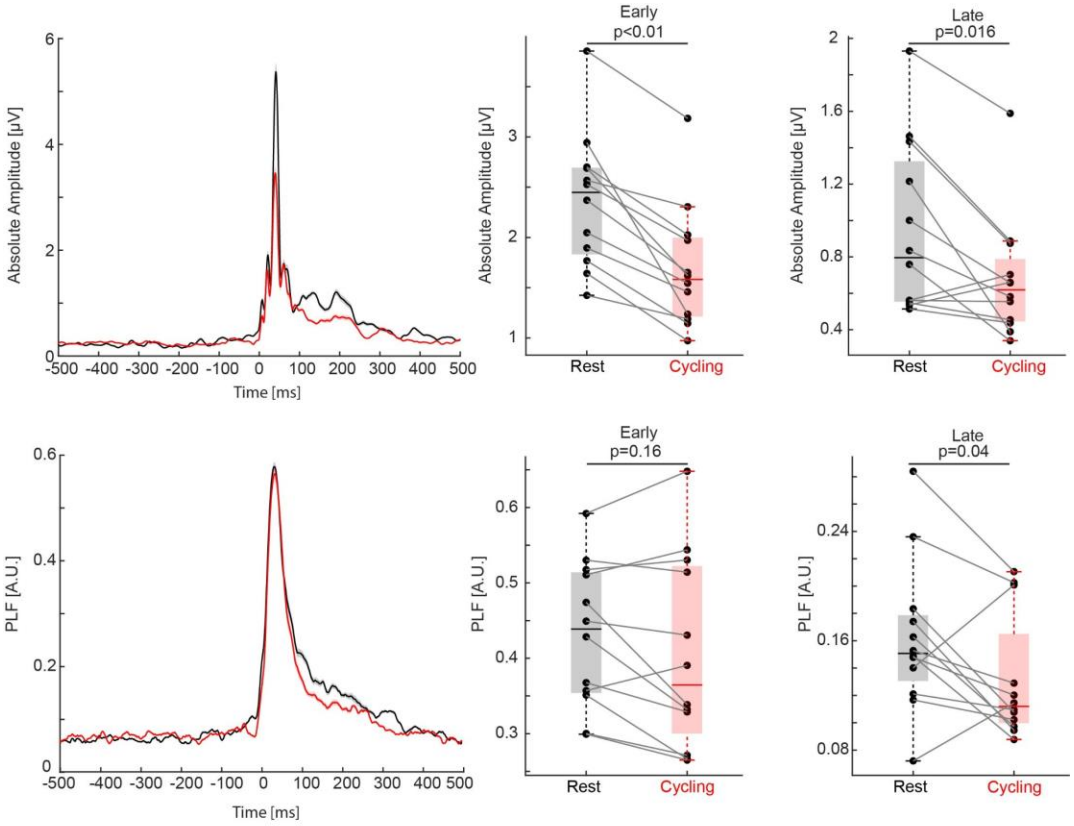


Figure S2C: ***Behavioral-state-dependent modulation of EEG responses evoked by magnetic stimulation of premotor cortex in humans during a stationary cycling motor task.*** *Modulation of the EEG responses to TMS delivered to the premotor cortex in humans at*

rest and during cycling. Top left to right: Grand-average (12 subjects) during rest (black) and cycling (red). Rectified amplitude average of the early (3-50 ms) and late component (150-250 ms; shaded gray) during rest and cycling for each subject (Wilcoxon signed rank test. Same subjects are connected by lines). Bottom left to right: PLF as a function of time from the stimulation onset and averaged PLF calculated over the early (3-50 ms) and late response time window (150-250 ms, shaded gray) at rest (black) and cycling (red).

We added the following text (line 129): "*In a set of 12 subjects, we also performed a control experiment involving rhythmic leg movements of the lower limbs. In this case the active condition was continuous, stationary cycling versus a resting condition of the legs. Similar to the upper limb task, we observed a significant state-related modulation in amplitude and PLF of the late component (amplitude: p=0.016; PLF: p=0.04, Figure S2C).*"

Human experiments: In the human experiments, if I understand well, the TMS pulses during the behavioral motor task (squeezing a ball) were randomly applied. This is in my view somehow problematic as it has been demonstrated in different studies that the application of TMS pulses and its evoke potential depends strongly on the stage of the motor act (e.g. preparatory phase, movement phase etc.) intrahemispherically (e.g., O'Shea et al. 2007 EJN, Heise et al. 2013 J Neuroscience), as well as interhemispherically (e.g. Murase et al. 2004 Ann Neurol; Liuzzi et al. 2019 Cerebral Cortex; Liuzzi et al. 2014 Neurology; Lazzari et al.2022 Nat Comm). Were TMS pulses only applied in specific windows of the movement process? How much did the task performance (strength of squeezing impact on these parameters? How well was the strength controlled? how did these windows compare to the stimulation windows in the running tasks?

To address this criticism, we performed additional control experiments on 12 subjects in the TMS-EEG setup where we recorded the electromyographic (EMG) signal of the *abductor pollicis brevis* muscle during the hand squeezing condition. From the EMG signal we were able to identify the active contraction corresponding to when the subject squeezed the ball as opposed to when the subject opened his hand (Figure S2G). We then sorted the TMS pulses based on when they occurred with respect to this signal, leading to a classification of the EEG-evoked responses based on the phase of the movement as contraction versus relaxation. We computed the same quantification of the amplitude of the late component which showed no significant differences between the two phases of the task (Figure S2G). This new result suggests that the modulation described in this study is not (or only weakly) dependent on the phase of the movement *per se*. Rather, it appears to depend on a more generalized activity state of the network, supporting our interpretation. As opposed to the studies mentioned by the reviewer, the task we asked the subject to perform is a continuous movement where the two phases of the movement are less than 1 second apart as shown by the raw EMG signal (Figure S2G); hence there is not a well-separated preparatory phase of the movement, but simply a continuous execution of it. Similarly, in the mice data, we discriminated between periods of running (or walking) versus periods of restfulness based on the speed of the wheel (sampled at 100 kHz). This signal does not provide the different phase of the movement; however, we can

change the threshold of the wheel speed used to classify running and resting trials as the reviewer suggested (see our response below).

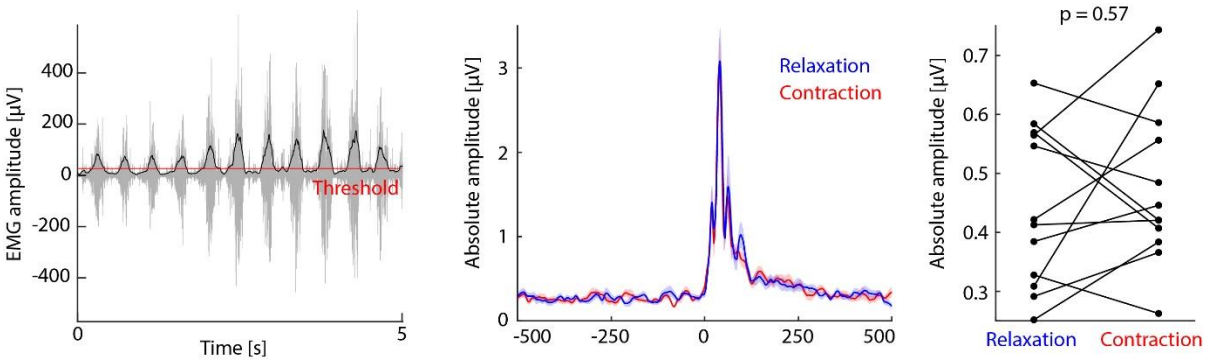


Figure S2G: TMS-EEG control experiments on 12 subjects showing the lack of a modulation based on the phase of the movement (Contraction vs Relaxation) for the hand squeezing motor task. From left to right: raw EMG signal of the first dorsal interosseus muscle during the hand squeezing condition showing the muscle tone during the two phases of the movement (hand closed VS hand open) and the threshold used to sort the trials based on the phases of the squeezing movement. EEG responses classified based on the two movement phases with the corresponding quantification of the late component amplitude showing no significant differences between the two movement phases (Wilcoxon signed rank tests, p>0.05).

We added the following text (line 116): "*This modulation was only observed when comparing the active and resting state. Indeed, when we sorted the trials based on the phase of the squeezing movement by comparing the responses evoked by the stimulation occurring during the closure of the hand versus its opening, as monitored with electromyography (EMG), we observed no significant differences in the magnitude and PLF of the late EEG component in 12 subjects (Figure S2G).*"

Intensities of Stimulation Human rodents (page 6): Could the authors comment on the applied intensities in humans and rodents. How comparable are there? Subthreshold, suprathreshold, adjustment to motor threshold in humans, in the animal model are the intensities of stimulation also adjusted to a motor threshold?

The stimulation intensities across stimulation modalities (TMS and ES) were standardized in both species based on motor threshold by setting the highest current intensity below that threshold. Specifically, for the human experiments, we stimulated each site at the highest stimulation intensity that did not evoke any motor twitches both at rest and during movement, within a maximum intensity. For the human TMS experiments, we also had the additional criterion of evoking a response of amplitude above 6μV without scalp contractions according to previously described guidelines (Casarotto, Journal of Neuroscience Methods, 2022).
For the ES human experiments, the maximum intensity was limited to 5 mA due to safety clinical limitations. For the ES mice experiments, the stimulation intensity was limited to 100 μA due to IACUC protocol limitations. Notice that even when smaller current intensities were used

for the mice experiments, the behavioral-state-dependent modulation was still observable (Figure S4). These details are included in the methods section.

Rodent exps: Rest vs running was defined by a threshold of wheel speed greater or smaller 0.1 cm/sec. Is this an arbitrary definition? Is this an established definition? How do the results change if the threshold would be 1.5cm/sec or 0.5 cm/sec?

As the reviewer pointed out, we used a threshold of 0.1 cm/s calculated over a 1 second window centered at the stimulation onset as an arbitrary and conservative threshold to separate resting from running trials. Mice usually move at higher speeds (Lemieux, Frontiers Neurosci, 2016) on average, thus the conservative choice allowed us to account for the noise of the experimental setup. As suggested by the reviewer, we studied how the modulation rest vs. movement changes as a function of the chosen threshold. We observed a significant decrease of the magnitude of late component at the EEG, LFP and CSD level from resting to running when we set the threshold to 0.5 cm/s, 1 cm/s, 1.5 cm/s and 5 cm/s, although the p-value progressively increased to values closer to 0.05 for the threshold of 5 cm/s, as expected (Figure below). This result makes our observation statistically more robust; and we thank the reviewer for the comment. We added in the manuscript the following at line 171: "*Note that the same EEG, LFP, and CSD modulations were also observed when using speed thresholds up to 5 cm/s.*"

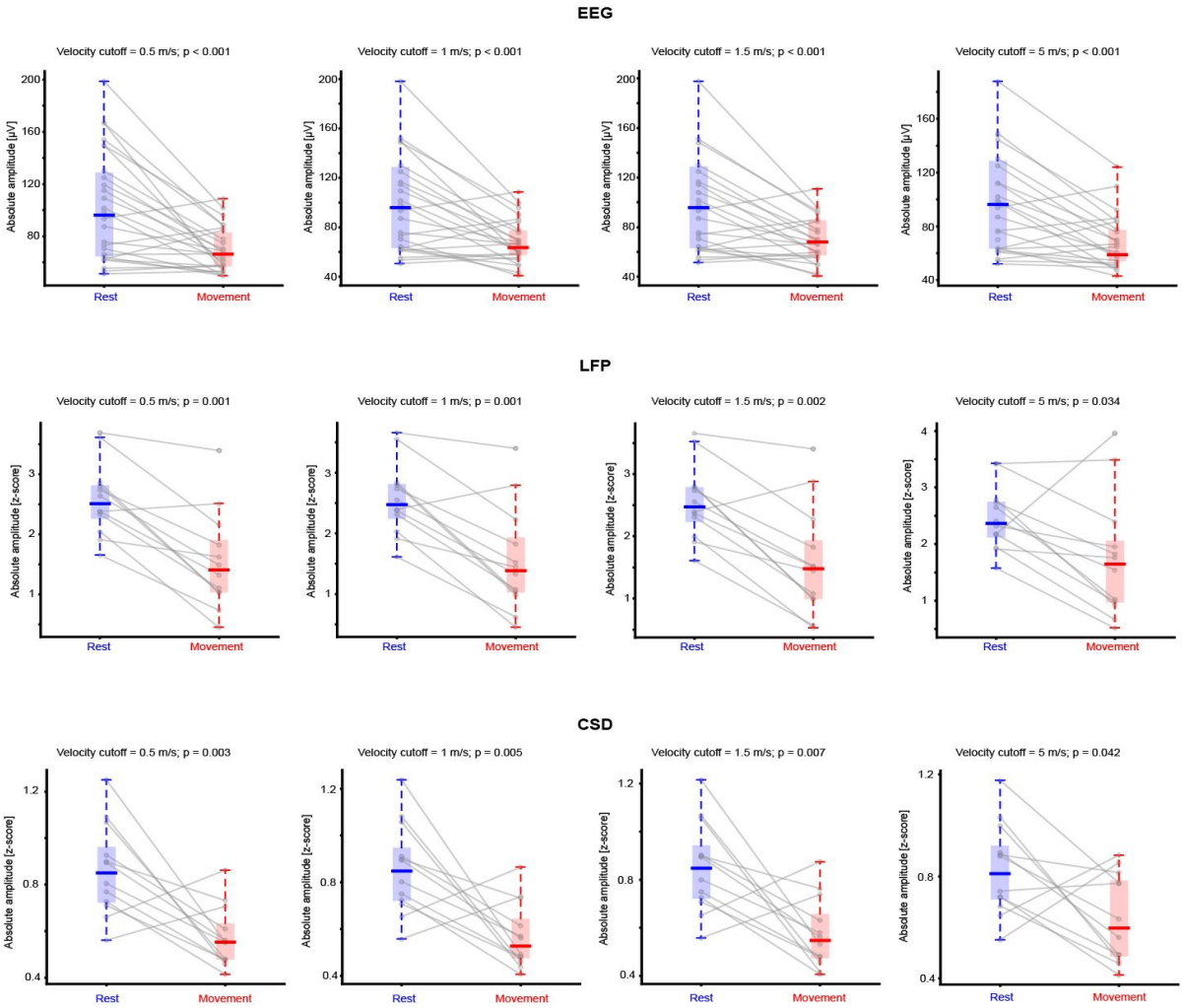


Figure (not included in the manuscript): Quantification of the magnitude of the late component at the EEG, LFP and CSD level as function of speed threshold used to define resting and running trials (from left to right: threshold= 0.5 cm/s, 1 cm/s, 1.5 cm/s, 5 cm/s).

Control-Exp. represented in Fig2Sc: This experiment is done in n=4 subjects. In my view this does not provide reliable evidence in one or the other direction, as can be seen already in the individual data heterogeneity. As the sample of the study is already rather small with n=12 subjects, the control exps should also be done in n=12 subjects, ideally the same ones.

We addressed this point by adding more subjects to the control experiment for a total of n=12 subjects. Figure S2D is replaced by the figure below, showing the lack of a modulation for early and late components during active and passive movement when the stimulation is delivered to posterior cortical areas in 12 subjects.

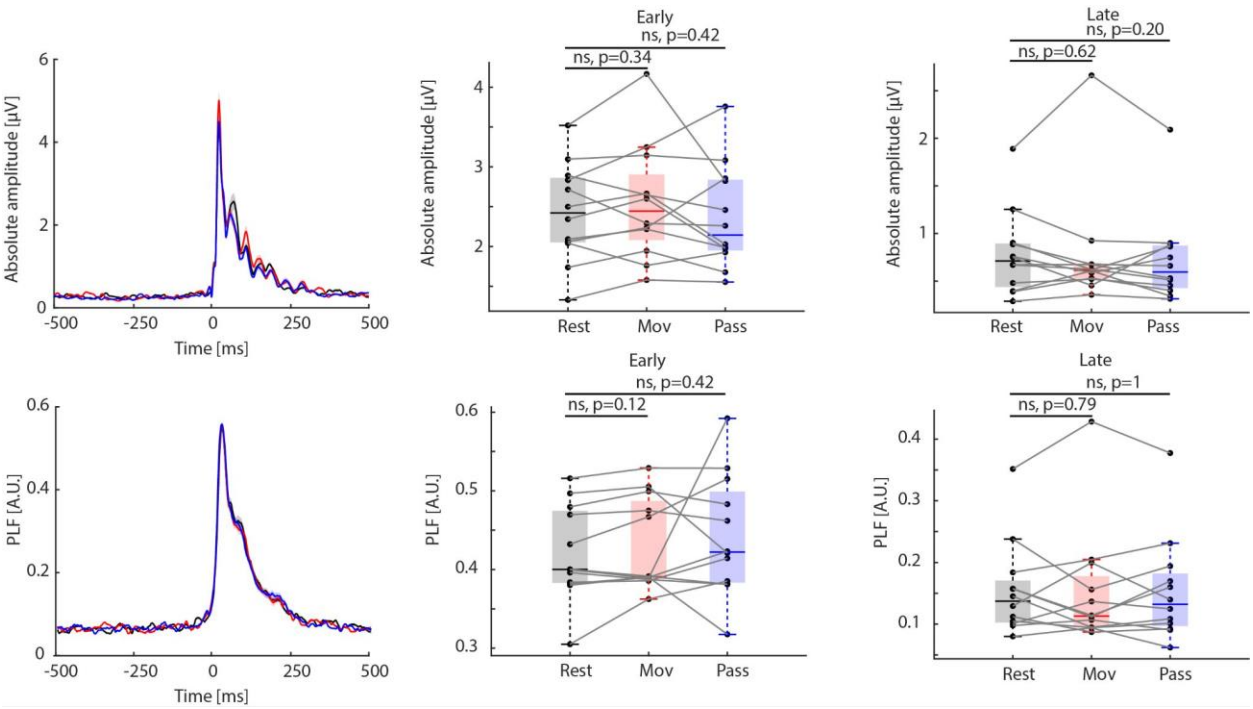


Figure S2D: Lack of modulation of EEG responses to TMS delivered to occipital cortical areas in humans at rest (black), and during active (red) and passive (blue) movement in 12 subjects. From left to right. Schematics of the setup with a TMS coil stimulating the occipital area. Evoked EEG rectified amplitude over time during rest, active and passive movement. EEG rectified amplitude average calculated over the early response time window (3-50 ms, shaded gray) for the three conditions (Wilcoxon signed rank tests; rest-movement: p=0.34, ns; rest-passive: p=0.42, ns). EEG rectified amplitude average calculated over the late response time window (150-250 ms, shaded gray) for the three conditions (Wilcoxon signed rank tests; rest-movement: p=0.62, ns; rest-passive: p=0.20, ns). Evoked EEG phase locking factor (PLF) over time for the three conditions. EEG PLF calculated over the early (3-50 ms, shaded gray) and late (150-250 ms, shaded gray) response time windows for each subject (Wilcoxon signed rank tests; early and late responses rest-movement: p=0.12, ns; p=0.79, ns; early and late responses rest-passive: p=0.42, ns; p=1, ns).

Minor:

- Ref 35 is missing

We added the reference, thank you for noticing it.

- Sometimes data for analyses is z-scored sometimes not, can the authors provide rational for it.

In this study we included data acquired via different modalities in humans and mice. The results are shown in a format consistent with the literature relative to each modality. Specifically the studies based on intracranial EEG (iEEG) usually report the z-scored signals because of the

variability of the raw signals due to the large differences in the contact impedance which leads to large differences in the iEEG voltages (even of orders of magnitude within the same subject; see for example Trebaul et al., Neuroimage, 2018, Russo, Neuroimage, 2021; Keller, J Neurosci, 2014; Silverstein, Neuroimage, 2020; Levison, J Neurosci Methods, 2024). To be consistent with the practice in the human literature, we reported the LFP and CSD mouse signals in the same way (z-scored). On the other hand, in the human EEG literature, the signals are usually reported without z-scoring them (Schomer, Donald L., and Fernando H. Lopes da Silva (eds), Niedermeyer's Electroencephalography: Basic Principles, Clinical Applications, and Related Fields, New York, 2017; online edn, Oxford Academic), this is because the impedance of the EEG contacts are generally very similar across contacts, hence the signal variability across contacts is relatively small. We refer to these studies in the methods of the revised manuscript.

Reviewer #2 (Remarks to the Author):

This is an exemplary study demonstrating similar features of cortically evoked activity in mice and humans using a variety of methods and modelling. Since the two species display similar features and the mechanisms of cortico-thalamic influence can be studied in detail in mice the authors may draw clinically relevant inferences on the human condition.
I have the following advice to improve the presentation:
Major:
1) The impact of corticothalamic afferents on thalamic networks has been investigated in detail in the past decades (E.g. Kalil and Chase 1970 J. Neurophysiol; Deschenes and Hu, 1990 EJN or Olsen et al., 2012 Nature). More specifically, the early and late component of the response to cortical stimulation together with the long-lasting inhibition and its state dependency have also been described using in vivo intracellular recordings and modelling (For a review see e.g Destexhe 2000 J. Physiol Paris). The direct impact of cortex on RTN has also been studied (Fuentealba et al., 2004 EJN). The authors have to make it clear which of these early data have been confirmed by their novel approach and which must be revised.

We thank the reviewer whose deep command of the literature allowed us to expand and improve the discussion of our results.

At line **426** we added: "*Our results are in line with previous findings showing that stimulation of the cortico-thalamic pathway triggers a prominent depolarization followed by a long-lasting hyperpolarization frequently ending with a burst discharge of thalamic relay cells (Deschênes and Hu 1989, Roy et al., 1984). In some cases, the hyperpolarization remained even after lesioning the reticular thalamic complex (Deschênes and Hu 1989), suggesting that sources other than the TRN can induce IPSP. Although both studies (Deschênes and Hu 1989, Roy et al., 1984) attributed the remaining IPSPs to other thalamic interneurons, an earlier experiment that inactivated visual cortex via cooling led to a decrease of baseline firing rate and changes of response features to visual stimuli in the directly connected units of the lateral geniculate thalamic nucleus (Kalil and Chase 1970 J. Neurophysiol). This demonstrated that the lack of*

cortical inputs can suppress thalamic activity, as shown by our simulated (Figure 4) and optogenetic data (Figure 2, S8). This mechanism coexists with the IPSPs followed by a rebound burst of TC neurons induced by TRN (Destexhe 2000) and shaped by neuromodulators associated with different states of arousal.

Crucially, one important difference between our study and these early studies is that they were conducted on anesthetized cats, which could affect the baseline membrane potential and the time course of the responses compared to wakefulness. However, the time course of the off-period here described is in line with a reduction of cortical excitatory PSPs (EPSPs) and a reduction of firing probability of TC neurons evoked by a thalamic electrical pulse when a conditioning paired pulse was delivered to the cortex of anesthetized cats up to 100 ms before it (Fuentealba et al., 2004 EJN)."

2) The exact nuclear localization of thalamic recording electrodes has to be described in detail and should be demonstrated in a figure. The impact of cortex on thalamic activity depends hugely on nuclear identity and ranges from negligible to very strong (see e.g Diamond et al., 1992 JCN but also Guo et al., 2017 Nature). The authors implicate cortical disfacilitation during the long thalamic inhibition before the rebound response. If this interpretation is correct the activity in first and higher order nuclei (see Sherman and Guillery's work) is expected to differ during the inhibitory phase of the cortically induced activity.

We thank the reviewer for catching this missed information. The location of the probes is verified based on the histology and an imaging process we described at length in Claar et al 2023. In the revised version of the manuscript, we added a section in the methods describing the whole procedure (see line 1032 and 1094: **Ex vivo imaging and localization of electrodes**), including an additional figure showing the exact location of each Neuropixels probe targeting the thalamic nuclei across all the recordings included in this study (Fig S11A). We also performed an analysis showing the evoked responses following ES by separating the different thalamic nuclei and did not see a difference across nuclei (Figure S11A).

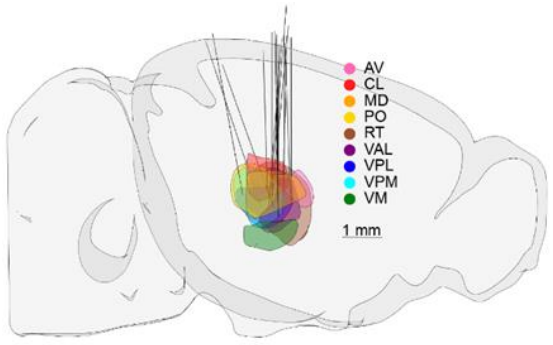


Figure S11A (left): sagittal projection of the reconstruction of an average mouse brain (grey), SM-TH thalamic nuclei (color coded), and the spatial location of the Neuropixels 1.0 probes targeting thalamic nuclei across mice (black lines, N=15).

Line 1032: “*Up to three Neuropixels 1.0 probes were inserted targeting MO, SS, VIS, and thalamic nuclei. We restricted our analysis of thalamic nuclei to those that had strong projections to and/or from motor and SS areas (Guo et al., 2017; Harris et al., 2019): anteroventral, central lateral, mediodorsal, posterior, RT, ventral anterior-lateral, ventral posterolateral, ventral posteromedial, and ventral medial. We refer to these collectively as the SM-TH, disregarding the distinction between first and higher order thalamic regions.”*

Line 1094: ***Ex vivo imaging and localization of electrodes***

The day of the experiment before insertion, all Neuropixels probes and the stimulating electrode were coated with a fluorescent dye (Vybrant DiI/DiO/DiD, ThermoFisher Scientific, Waltham, MA, USA) by repeatedly immersing them in a well filled with the dye and removing each probe slowly allowing the dye to dry on the surface. To localize the probes, we followed the procedure we extensively described in Claar et al, 2023[21]. Briefly, after extracting the brains, we sliced them into 100 µm coronal sections using a vibratome (Leica VT1000S) and imaged with a fluorescent microscope (Olympus VS110/120) at 10x magnification, or whole brains were imaged using serial two-photon tomography[118,119]. Images of the 100 µm coronal sections were aligned to the Allen Institute Common Coordinate Framework (CCFv3) following the process detailed by McBride et al., 2023[120] and images from serial two-photon tomography were aligned to the CCFv3 following the process detailed by Oh et al., 2014[118]. Fluorescent tracks corresponding to the location of the Neuropixels probes, and the stimulation electrode were manually identified in the aligned images. For each Neuropixels probe the locations of major structural boundaries along the track was manually aligned with the physiology data[38,121].

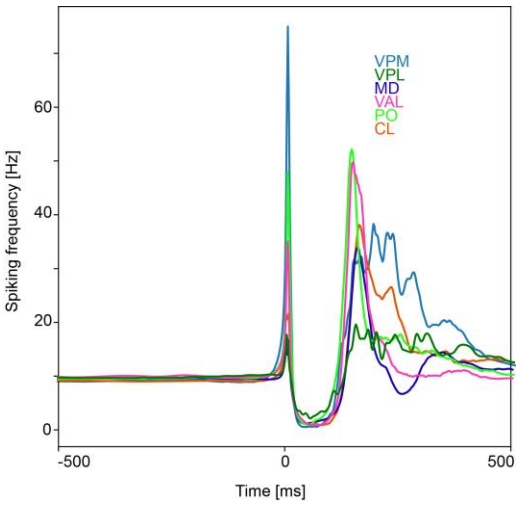


Figure S11A (right): ES evoked firing rate over time for each sampled thalamic nucleus (color coded; AV, RT, and VM excluded because of a small number of recorded units) showing the consistent occurrence of the triphasic pattern across nuclei.

3) The impact and significance of the study could be greatly enhanced by the inclusion of spontaneous activity that evokes a pattern of response similar to cortical stimulation. The most plausible would be e.g. interictal activity in epileptic patients with intracranial electrodes.

As the reviewer mentioned, the electrically evoked responses resemble spontaneous activity during interictal activity in epileptic patients as shown here below. However, we excluded from the study patients with a seizure onset zone in the premotor areas to prevent the engagement of pathological circuits and the associated risk to trigger a seizure through electrical stimulation. In the updated version of the manuscript, we explicitly mention this resemblance in the discussion at line 443, suggesting that a similar mechanism can potentially be at the origin of these waveforms. If the reviewer believes it is important, we could include the figure below as a supplementary figure, however we think it may be a point of confusion for the reader given that any data showing epileptic activity was intentionally excluded from the analyzed dataset.

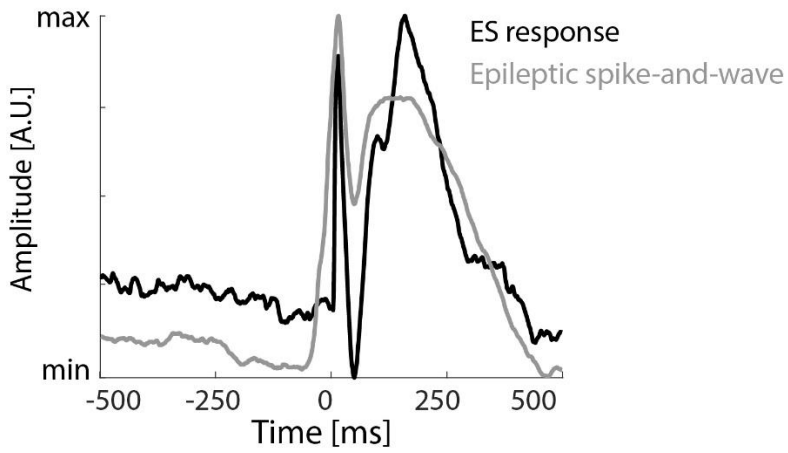


Figure (not included in the manuscript): normalized iEEG traces showing a representative averaged response to ES from one contact (black) overlapped to a representative interictal-spikes average signal (gray).

At line 443: "*Interestingly, the electrically evoked responses elicited by cortical stimulation resemble the interictal spikes spontaneously occurring in epileptic patients. From a mechanistic perspective, both electrical stimulation and epileptic spikes are associated with the abrupt recruitment of a local ensemble of neurons and thus may engage similar mechanisms, suggesting that the cortico-thalamo-cortical loop may contribute to the generation of this pathological activity*".

4) The cortically induced responses seem to show large intertrial variability even in the same behavioral conditions. The authors may characterize this variability and could provide a hypothesis for its source using the mouse data.

Figure S13 shows how evoked response frequency, response onset variability and amplitude of the EEG evoked responses are correlated. These are, in turn, correlated with the baseline firing rate. The analysis is performed at the single trial level, hence the intertrial variability is due to

the variability of the thalamic and cortical excitability captured by baseline firing rate. This is modulated by behavioral state as we showed in Figure 3 but also by arousal state which can also vary within the same behavioral state.

5) The early response component in the thalamus may reflect both ortodromic as well as antidromic activation. Since these two options result in different recruitment of RTN (cortical vs thalamic) it should be clarified. Using peristimulus time histograms, the variability of the response latency and possibly collision tests in case of individual neurons orto- vs. antidromic activation of thalamic neurons could be differentiated.

To address this very valid point, we performed two quantifications whose statistical results are now included in the manuscript at line 376 (Figure below). We calculated the variability trial-by-trial of the first spike latency after stimulation onset in MOs and SM-TH. MOs shows an average variability of 1 ms while SM-TH showed an average variability of 6 ms, significantly larger than the cortical neurons ($p < 0.001$ Wilcoxon Rank Sum test). We also quantified and compared the latency of the first spike in SM-TH and TRN which are both peaking on average at 11 ms after stimulation onset and they are not significantly different. These analyses together suggest that the early thalamic response is due to an orthodromic activation from the cortex.

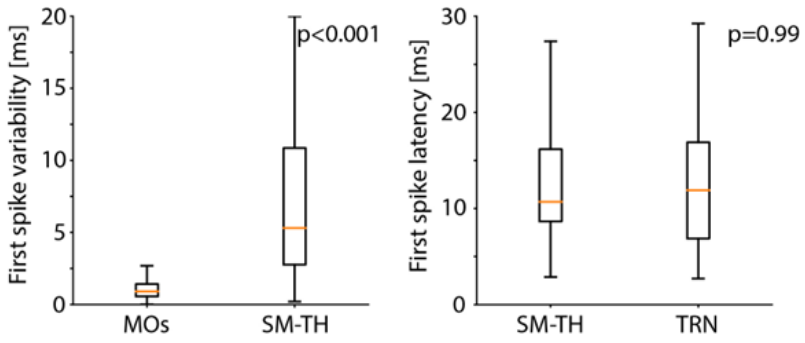


Figure (not included in the manuscript): Quantification of the variability of the first spike latency after stimulation onset in the early response window [0 - 50 ms] for neurons in MOs and in SM-TH ($p < 0.001$ Wilcoxon Rank Sum test) (left). Quantification of the latency of the first spike from stimulation onset for SM-TH neurons and TRN neurons which are not significantly different ($p=0.99$ Wilcoxon Rank Sum test) (right).

Line 376: "*This generates an initial excitation in cortex and in the thalamus mainly via orthodromic activation from the cortex as suggested by the significantly larger variability of the first spike latency in the SM-TH compared to MOs (Wilcoxon test; $p<0.001$) and the lack of significant differences between SM-TH nuclei and TRN in the latency of the first spike (Wilcoxon test; $p=0.99$).*"

6) Were low intensity stimulations used in humans as well? If yes, could the omission of the second component be demonstrated in case of low intensity stimulations?

Due to time constraints in the human experiments, we only administered one stimulation intensity, so, unfortunately, we do not have the data to experimentally address whether lower TMS intensity evoked the late response. However, a simulation of TMS-induced neural activation found that even at low intensities, TMS activates L5 pyramidal neurons (Aberra et al., Brain Stimulation, 2020) likely including cortico-thalamic neurons, hence we would expect to evoke the late component even at lower TMS intensities. In the Discussion at line 466, we specified: "*This result confirms that the late components evoked by confounds-controlled TMS are likely generated by a direct cortico-thalamic activation – even for low TMS intensities (Abarra et al, 2020) - that engages similar mechanism as the one elicited by invasive ES in humans and mice (Figures 1, 5)*."

7) The experimental data in mice clearly demonstrate a major role of the evoked RTN inhibition in the pause-rebound response and less support for the cortical disfacilitation hypothesis (there is very little thalamic rebound after cortical inhibition alone, the timing of thalamic but not cortical inhibition alters cortical rebound). These data are supported by the earlier results as well. In contrast the modelling data describe that RTN inhibition is dispensable and place more emphasis on cortical disfaciliation. Discussion is quite ambiguous on this matter. Please clarify exactly what supports the role of cortical vs thalamic inhibition in timing the rebound and examine the discrepancies between experimental and in silico results.

We thank the reviewer for this comment which gave us the opportunity to expand the analysis on the in-silico data now shown in Figure 4I in the revised manuscript. In this subplot, we quantified the amount of thalamic hyperpolarization (color coded) following ES by varying the connectivity strength of GABA-A interneurons in cortex (x-axis) and in TRN (y-axis) independently. The result of this analysis shows that both cortical and thalamic GABAergic neurons have a role in inducing the thalamic off-period (Figure 4I). For the in-vivo data, we added a quantification in Figure S8C showing that optogenetic activation of MOs GABAergic neurons or TRN induce a significant decrease of firing rate in both cortex and thalamus (see also response to the next point raised by the reviewer). We think that both simulated data and in-vivo data are therefore supporting the role of both cortical and thalamic interneurons in generating the thalamic off-period.

We clarified this point in the main manuscript at line 303: "*We independently varied the cortical and thalamic GABA-A connectivity strength, observing stimulus-induced hyperpolarization effects in TC neurons (Figure 4I). This suggests that both cortical and thalamic GABAergic neurons contribute to the transient hyperpolarization of the thalamic TC neurons, triggering the thalamic rebound. Importantly, our optogenetic experiments confirmed that the activation of cortical or thalamic GABAergic units alone is sufficient to induce conjoint cortical and thalamic off-periods (Figs. 2H, S8C).*"

At line 406: "*The magnitude of the thalamic hyperpolarization is indeed affected by the strength of GABA-A connectivity in both cortex and thalamus (Figure 4I), further supporting the*

hypothesis that GABAergic neurons in both cortex and thalamus concurrently contribute to generate the thalamic pause-rebound pattern."

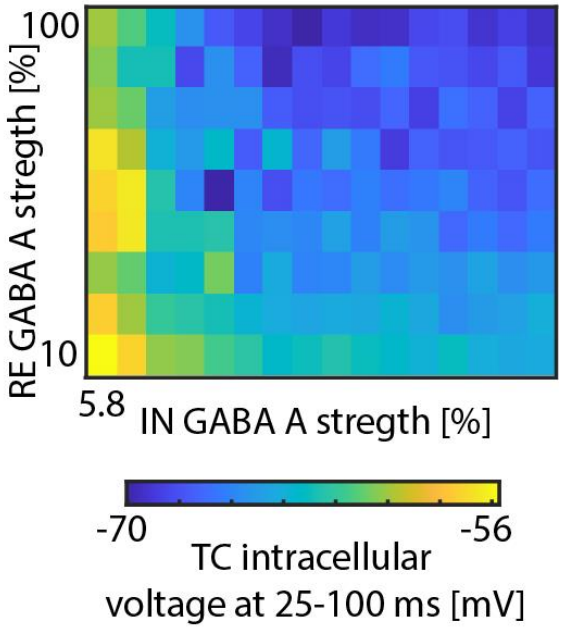


Figure 4I: I) Stimulus-induced hyperpolarization as function of cortical (IN) and thalamic (TRN) GABA-A connectivity strength. The plot shows the average intracellular voltage during the off-period (25-100 ms) in 50 centrally-located TC neurons across different GABA-A connectivity strength in the IN->PY (x-axis) and TRN->TC (y-axis) connections. Both connections independently contribute to generating stronger stimulus-induced hyperpolarization in TC cells.

8) Along the same argument the difference between the unit response to optogenetic inhibition of MO vs SM-Th (Fig S8C) should be better quantified.

We quantified the suppression induced by the optogenetic inhibition as the reviewer suggested (Figure S8C). This quantification confirmed our qualitative observation, showing that optogenetically inhibiting SM-TH or MOs caused a significant suppression of activity not only to the area targeted by the optogenetic manipulation but also to the area directly connected to it.

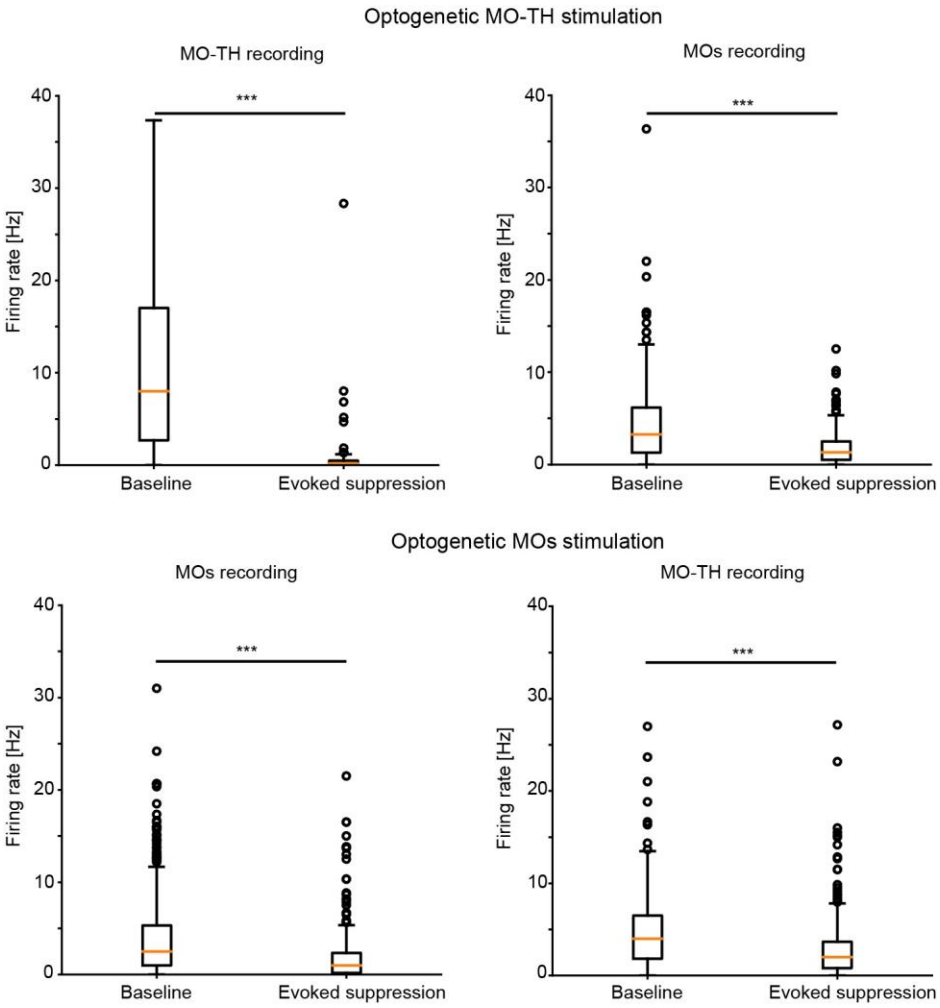


Figure S8C: Suppression of firing rate induced by stimulation alone of GABAergic neurons in cortex and thalamus. Left: average firing rate of SM-TH units (129 units, left) and MOs units (156 units, right) evoked by optogenetic activation of thalamic GABAergic neurons strongly decreases firing rate of SM-TH and MOs units with respect to baseline (0-50 ms from light onset; Wilcoxon signed rank tests; both p<0.001). The thalamic suppression is followed by a large rebound excitation. Right: average firing rate of SM-TH units (417 units, left) and MOs units (440 units, right) evoked by optogenetic activation of MOs GABAergic neurons showing a strong decrease in firing rate for both SM-TH and MOs units with respect to baseline (0-50 ms from light onset; Wilcoxon signed rank tests; both p<0.001).

9) It is unclear what is exactly shown in FigS2C and the corresponding rodent Fig S6 what is the rationale and exactly what these experiments want to demonstrate. Were all the recordings made in MO or also in the stimulated cortices as well? What EEG refers to in Fig S6a? Both the rectified amplitude and the PLF show very different shape in case of the posterior cortical stimulations (FigS2C) and the rebound response differs also from MO in Fig S6B as well. The early and late components are unclear in these examples. Why we expect a rebound in MO if

posterior cortices are stimulated, when rebound is generated by topographically organized TC cells, which project back to posterior cortex? Does the pause-rebound response have cortical region specificity. I.e. if parietal cortex is the stimulation and recording site as well do we have the same two components as in MO? What is exactly meant by “control” cortices? Are posterior cortices are not involved in movements? Movement is thought to involve and modulate all cortical areas.

We thank the reviewer for raising this point which gave us the opportunity to clarify the rationale of this control experiment. These control experiments in both humans and mice aimed to show that the modulation is specific to the cortico-thalamic network activated by the task. When the stimulated area is a motor cortical area in both mouse and human activated by an active or passive task, we observed a modulation of the late component compared to a resting condition. This is also confirmed in the extra control experiments we performed in humans where the task involved the lower limbs (cycling), to make it more comparable with the mouse task (Figure S2C). However, when the stimulation is targeting a cortical area outside of the cortico-thalamic circuit directly activated by the task, we did not observe any modulation of the late EEG component as defined here peaking within 150 ms and 250 ms from stimulation onset (Figure S2D). As the reviewer pointed out the waveform of the electrically evoked responses elicited by the stimulation of posterior areas are different from the waveform of the responses elicited by the stimulation of premotor areas, confirming findings previously described (Rosanova et al., J of Neuroscience, 2009). Therefore, we were not expecting to see similar waveforms, but we wanted to quantify the modulation at the EEG level to verify the network specificity of the described effects. We agree with the reviewer that including the LFP and CSD of MOs responses to the stimulation of posterior areas in mice (referred to as “control cortices”) is confusing and we removed figures S6B and S6C in the revised manuscript as they don’t add meaningful information, and they don’t show the pause-rebound pattern as expected. We do think though that Figure S6A for the mouse data and the updated Figure S2D for the human data, now showing 12 subjects as requested by reviewer 1,are important controls showing the lack of global movement-related modulation when the stimulation targeted cortices outside the cortico-thalamic network involved in the task.

10) It would be important to state whether increasing stimulation intensity can turn LF cells to HF cells or these are two distinct cell types recruited by different intensities.

Figure S12 does show indeed that LF thalamic units progressively turn to HF thalamic units when higher stimulation intensities are applied. This is an important observation which confirms that these neurons are functionally the same neurons, but their response rebound pattern is dependent on the amount of synaptic input from the cortex (see also Figure S14 from the simulated data). Accordingly, we modified the text to state it clearly.

Line 455: “*In-vivo data further supported this prediction, showing that the intensity of the electrical stimulus affected not only the overall number of responsive units, but also the relative size of the two subsets, such that higher current intensities showed a progressive shift from LF to HF response patterns (Figure S12).”*

11) It would be nice to directly show that synchrony between LF cells, as opposed to HF cells decrease during movements e.g. by analyzing cross correlograms.

Following the reviewer's insightful suggestion, we computed the cross correlograms of HF and LF neurons at rest and during movement (now included in the manuscript as Figure S13D and in the text at line 258). This analysis confirms the response onset variability (ROV) analysis, showing that LF neurons are significantly more synchronous at rest (black) compared to the active state (red), while the synchrony of HF neurons is unchanged between conditions.

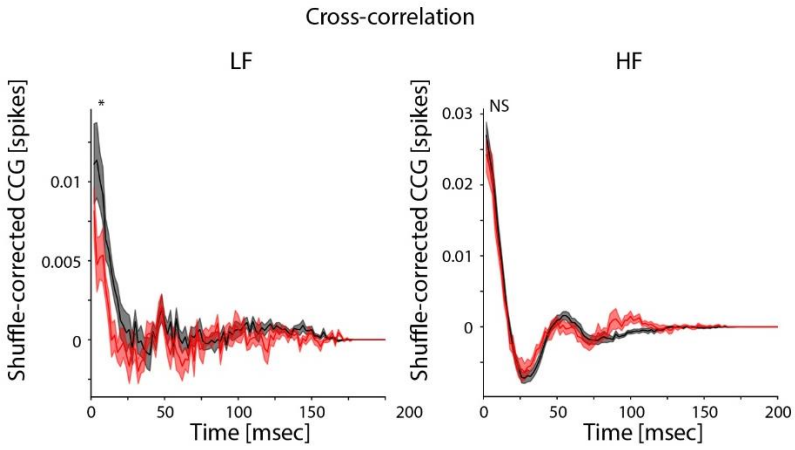


Figure S13D: Cross-correlation of HF and LF SM-TH units across rest and active state (black and red traces, respectively). LF, but not HF, units exhibit higher cross-correlation values during rest compared to active state (10 ms window, Wilcoxon signed rank test; LF: p=0.013; HF=0.57).

Minors

a) The author write „As opposed to the late component, the early component of the EP (3-50 ms from stimulus onset) showed a reduction in amplitude, but not in phase locking…….. Figure S2A)".

b) Both the early and the late components show a reduction in amplitude thus „As opposed to…" is disturbing here.

Line 125 now reads: "*). In the premotor TMS, the early component of the EP (3-50 ms from stimulus onset) showed a reduction in amplitude, but not in phase locking* [...]"

c) In Suppl Fig 1C please indicate the cut-off value for the low vs high signal-to-noise ratio and plot the same results showing the used, high signal-to-noise data points. In this dataset no correlation is expected.

In Fig S1C the cut-off value is represented in the plot by the horizontal dashed line and we now report the exact value of 1.5 in the legend. On the right side of the figure we performed the analysis only on the high signal-to-noise data points. As the reviewer correctly stated we do not

expect to have a correlation between SNR and PLF, hence we removed the linear regression line from the plot.

d) In Fig S10, there is large difference between the two subjects in thalamic responses while cortical and RTN responses are similar. How this interindividual variability can be explained.
Figure S10 aims to show the responses from two subjects where we successfully recorded the reticular nucleus which showed the evoked activity elicited by the stimulation. Although we had other animals where we successfully recorded reticular neurons, they did not show evoked activity induced by the stimulation. This is because it is possible that the targeted neurons in those cases were not directly connected with the cortical neurons activated by the stimulation. In the same way we also observed for some subjects none or few SM-TH units were responsive to the stimulation. Hence the variability noticed by the reviewer can be explained in terms of connectivity profile between the stimulated neurons and the recorded ones. Despite this variability, for all our analyses only the units whose evoked activity exceeded 7 standard deviations from baseline (see Methods) were considered, thus the non-responding units were excluded.

e) Since the reference of Sinkkonen et al (1995) is not so well known and the phase locking factor (PLF) is not described, as such, in this reference but PLF is a highly important measure here please describe it in a bit more detail, how it is calculated and what it is sensitive for etc.
Following line 1304, we write: "*The phase locking factor (PLF) for each condition was computed as described by Sinkkonen et al[37]. In brief, the time-resolved phase of each recording site was obtained from the Hilbert transform of each single-trial signal divided by its absolute value and averaged across trials. As such, PLF captures the phase consistency across trials in an amplitude-independent and time-resolved manner. Then, single-channel PLF was computed by averaging the real part of the phase-vector in the window of interest (early response: 3-50 ms; late response: 150-250 ms).*"

f) Please indicate significance with stars above the lines in the figures rather than in the text, it is simply faster to read.

We changed how we indicate significance as suggested by the reviewer.

g) Abbreviation for the reticular thalamic nucleus (RTN vs TRN) seems to be inconsistent throughout the ms.

We corrected the inconsistency.

h) Please indicate in what state (move or rest) the optogenetic inferences have been delivered.
The optogenetic manipulation was applied independent of the state of the animals hence the results include data from both states. At line 199 we added "[..] *independent of the behavioral state of the animal*"

i) In Suppl F10A clearly label Ctx and TRN.

Done!

j) Please provide a reference for the similarity of Ih and It between primates and rodents.
Cavaretta et al., The Cerebral Cortex and Thalamus, 2023

Reviewer #2 (Remarks on code availability):

N/A

Reviewer #3 (Remarks to the Author):

In this paper, Russo and colleagues explore the mechanisms underlying an early and late component of the cortical population response to stimulation in humans and mice. For both species and independently of the stimulation method, they find both an early and late component of the cortical population response, with the late component being modulated by behavioral state. Recordings from thalamus and biophysical modelling reveal that the late component originates from rebound spiking mediated by thalamic hyperpolarization, likely through activation of both cortical and thalamic (TRN) inhibition.

While the results are not surprising to researchers working on thalamocortical circuits, they constitute important interpretative guidelines for clinical results involving cortical stimulation and are therefore a significant contribution to the field. I only have a few suggestions for further enhancing the clarity of the manuscript.

MAJOR:
- Please add page and line numbers to facilitate the communication about the manuscript.

We apologize for this oversight; the revised manuscript now contains the line numbers.

- The merged manuscript file does not contain the figure captions for the supplementary figures (they only appear in the separate Supplemental information file). This made more difficult than usual it to assess the supporting information.

We apologize for the inconvenience, which is beyond our control, given that the merge file has been automatically generated by the journal platform. We are going to try to submit the revised material in another way.

- I would like to ask the authors to be less specific about the interpretation of the active condition. In the abstract, they refer to the manipulation by “behavioral state”; later on, they frequently refer to the condition as “movement” condition. In my view, active state / active condition describes the condition more appropriately. To understand whether movement was

indeed required, one would need to do control experiments, in which the human subjects or animals would be aroused or in an activated behavioral state but without movement. The passive movement condition could also induce higher arousal than rest, so the results obtained in this control would not contradict the sentence “The modulation observed during passive movement suggests that peripheral inputs are sufficient to change the state of the thalamus.” Given what we know about modulations of thalamic activity, the described mechanism would not require movement, but would also work with state-dependent modulation of thalamus. Hence, I would suggest to consistently use the term active condition / state.

We indeed expect to observe a similar modulation (rest VS active) for other cortico-thalamic circuits not exclusively for the motor circuit considered here, although a different and specific task targeting the activation of that circuit would need to be employed. Hence our results, as the reviewer noted, could be generalized to other cortico-thalamic networks. Accordingly, and as suggested by the reviewer, we refer to the “movement” state as “active” throughout the revised manuscript in the text and in all figures. In the introduction we also specify at line 85: “*Here we show that, despite the differences between species (humans versus mice) and between magnetic and electrical pulses, the late responses evoked by cortical stimulation are remarkably similar and likewise modulated by the state of the targeted cortico-thalamic network, defined here as either active or resting state*.”

I am not convinced that the interpretation “progressively delayed both thalamic and cortical units’ responses” is correct. An alternative interpretation that is fully in line with the authors’ conclusion could be that the strong activation of TRN will strongly hyperpolarize the thalamus, which will then respond with rebound activity at the offset. This would not be a “response” to the cortical ES, but a mechanism that would override the native response to ES and elicit its own rebound through strong hyperpolarization and rebound excitation. I would therefore suggest avoiding the term “delay the responses”.

As the reviewer states the optogenetic stimulation could indeed overwrite the response of the electrical stimulation by suppressing the thalamus and preventing it from depolarizing with a rebound. However, the final outcome is a delayed rebound given that the thalamus eventually rebounds but at a later time as also shown by the raw signals in Figure 2. To acknowledge the fact that the optogenetic stimulation could indeed overwrite the rebound of the ES response we specify in the revised manuscript at line 414: “*A possible explanation is that optogenetic TRN activation keeps thalamic relay cells hyperpolarized such that the h-current is not sufficient to depolarize the membrane, thus overwriting the response to ES and then generating an independent rebound burst (low-threshold Ca^{2+} spike) at a later time.* ”

At line 420: “.. *hence overwriting and generating a delayed rebound in cortex and thalamus*”. We also modified the language in the result section.

I have a few questions regarding the placement of electrodes: For the iEEG experiments, in which layer were the two adjacent contacts used for iEEG stimulation? How did the response pattern look like at more distant electrodes?

The iEEG contacts have a diameter of 2 mm with a gap between contacts of 1.5 mm. Since the thickness of the human cortex ranges between 1 mm and 4.5 mm (Fischl, PNAS, 2000), the electrical stimulation delivered between two adjacent contacts is most likely engaging all the cortical layers, preventing us from accurately defining the target of ES in these human experiments. Moreover, the standard algorithm used to determine the location of the iEEG contacts has an accuracy of the order of millimeters (Narizzano, BMC Bioinformatics, 2017), also limiting the estimation of their localization.

The intracerebral response at more distant electrodes resulted in smaller EPs (Figure below) compared to the EPs of the electrodes close to the stimulation site (Figure 1B). The small early component together with a preserved, even though reduced, late component in distant contacts is coherent with previous works (Veit et al., PNAS, 2021).

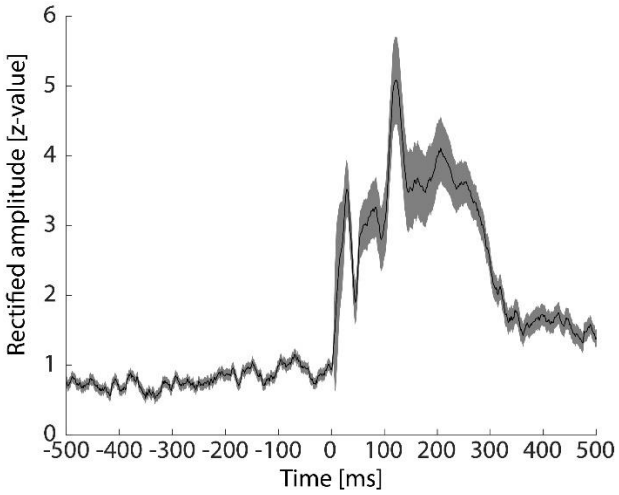


Figure (not included in the manuscript): iEEG EP of distant electrodes from the stimulated site (>3 cm) at rest.

For the experiments in mice, please give a few more details on how the units were assigned to thalamic nuclei and TRN. Please also give the stereotaxic coordinates for the TRN fibre implant.

Please see our response to point 2 of reviewer 2. The location of the probes is verified based on the histology and an imaging process we described at length in Claar et al 2023 and we added in the revised version of the manuscript (see line 1032 and 1094: **Ex vivo imaging and localization of electrodes**). We also generated a figure showing the exact location of the Neuropixels probes targeting the thalamic nuclei across all the recordings included in this study (Figure S11A).

The fiber was implanted at this coordinate: AP -1.7, ML -2.0, DV –3.25. We added this information in line 1020 of the revised manuscript.

For the comparisons of latency across SM-TH and MO units, does the difference also hold when matching the firing rates in the two structures? Maybe this is already answered in Figure

S7B, but given that these are simultaneous recordings in cortex and thalamus during the mouse experiments, could the authors please do the comparisons of latencies on a session-by-session basis?

The baseline firing rate of SM-TH and MOs are different as shown in figures 3A and S9A, thus finding trials where the two are matching is particularly difficult. The comparison on a session-by-session basis has been shown in Figure S7A and Figure 5 of Claar et al 2023. Figure S7B as the reviewer mentioned is also showing the latencies of simultaneously recorded SM-TH and MO units but at the single trial level for all sessions.
At line 189 we added: "*see also Figure 5 Claar et al. 2023*".

I cannot follow the argument: "…evoked firing rate in MOs and SM-TH units was not modulated by movement, unlike EEG, LFP, and CSD responses. Therefore, MOs and SM-TH firing rate changes did not explain the movement-related modulation of the EPs (Figures, 3A, S9, S10 p=0.03, rest lower than movement)". Why is p=0.03 not considered significant? Why is it not taken into account that the baseline firing rate is enhanced during movement (Figure 3A)? If this was the case, wouldn't the modulation be even further enhanced? I also do not understand the subsequent argument: in Figure 3E, it seems like there is a reduction in the HF response frequency with movement without a concomitant decrease in HF ROV; at the same time there is an increase in LF ROV, but no change in response frequency. I therefore cannot fully follow why ROV should underlie the changes in response frequency.

We acknowledge that the writing is not clear. p=0.03 is indeed considered significant, but in this case the active condition is larger than the rest condition, hence it goes in the opposite direction compared to the modulation of the EPs at the EEG, LFP and CSD level. To make it clear we rephrased as follows in the manuscript at line 225: "*Upon further investigating rebound activity of thalamic units, we found that the evoked firing rate in MOs and SM-TH units was not similarly modulated by the subject's state. This stands in contrast to EEG, LFP, and CSD responses. We indeed observed an increase in the rebound firing rate during the active state as opposed to the decrease of the late component described above (Figures, 3A, S9, S10; p=0.03, rest lower than active state). Therefore, MOs and SM-TH firing rate changes in the rebound response window did not explain the state-related modulation of the EPs.*"

The state of the subject modulates the baseline firing rate, which in turn modulates the synchronicity of the LF units (quantified by ROV which is the inverse of synchronicity given that it quantifies the variability of the first spike latency across units) and the burstiness (quantified as response frequency of the rebound spikes) of the HF thalamic units. Given that LF and HF units are two different response patterns, rather than different neuronal populations, of the rebound response it is reasonable that they are characterized by two different features modulated by the state of the animal. These two features are ROV and rebound firing rate which both correlate with baseline firing rate (Figure 3). Line 254 now reads: "*By correlating baseline firing rate, ROV, response frequency and late EP component amplitude at the EEG level, we found that the active state almost doubled the thalamic baseline firing rate (from 6.78±4.4 Hz at rest to 12.48±4.6 Hz during active state; p<0.001), correlating with a decreased synchronicity as*

quantified by the increased onset variability of LF units (from 35.7±11.8 ms at rest to 40.7±8.2 ms during active state; p<0.001; a cross-correlational analysis confirmed the synchronicity modulation as function of state, Figure S13D) and to a smaller response frequency of HF units (from 309±126 Hz at rest to 241±116 Hz during active state; p<0.001)."

Regarding the introduction of the two thalamic response modes, I do not understand why the authors chose to first say "we identified two identical thalamic cell-types" before then saying that the "two subsets reflected two different response patterns to the cortical stimulation rather than two different cell types". I would suggest to rephrase the first statement to avoid sending the reader on the wrong track. For non-expert readers, I would suggest to also add a note about connectivity here - according to the authors own models, this is the reason why the two different "response patterns" to cortical stimulation arise.

We thank the reviewer for this comment.
At line 239 we rephrased as follow: "[…] *identified two distinct types of rebound activity likely generated by the same thalamic cell-types given the similarities across several features of their spike waveforms (Figure S11C)* "
At line 244: *"[..], suggesting that HF units may be more effectively activated by the electrically stimulated cortical neurons, in line with the simulated data (see section below)*".

I would suggest to move the figure panels with thalamic optogenetic stimulation alone and cortical stimulation alone to the main manuscript. This would give a chance to give some more space to the relative role of both sources of thalamic hyperpolarization.

We implemented this suggestion.

MINOR:
- 50 ms later,between -> blank missing

Corrected, Thanks!

- "These factors were modulated by their baseline firing rate" -> unclear, please rewrite. In particular, it is difficult to understand what "THEIR" refers to

At line 232 we rephrased as: "*These factors were modulated by the baseline firing rate of SM-TH units*".

- "response patterns to the cortical stimulation rather than two different cell types" -> please say even more clearly that this is expected given a topographic connectivity and is not related to intrinsic factors that might still determine "response patterns"

At line 249 we rephrased: "*response patterns to the cortical stimulation due to their different connectivity profile.*"

- “(Elbow method, Calinski- Harabasz score, and Davies-Bouldin score; metrics.calinski_harabasz_
score and metrics.davies_bouldin_score functions from sklearn - Python)” too much detail in the results, consider replacing by (see Methods) if wanted

We implemented the suggestion.

- “leading to an increased onset variability of LF units” - I don’t think ‘leading’ is appropriate, given that these are correlational analyses

At line 257 we corrected as: “*correlating with an increased onset variability of LF units*”

- “Our results show that the animal’s state (Figures 1, 3) and the intensity of the ES (Figure S4) modulate the late EP component via their effects on thalamic bursting frequency and synchronicity, rather than cortical and thalamic evoked firing rate (Figure 3, S9).” Is it possible to make such a strong statement? Isn’t the thalamic evoked firing rate directly related to depolarization and thus to bursting frequency?

At line 344 we corrected as: “*Our results show that the subject’s state (Figures 1, 3) and the intensity of the ES (Figure S4) modulate the late EP component via their effects on thalamic bursting frequency and synchronicity.*”

- Some typos in y-axis labels of Figure S8C

Corrected!

- Maybe I missed it, but I could not find the list of thalamic nuclei in the methods and how contacts were assigned to individual nuclei. Could the authors please provide a more explicit reference to the position within the article (“sensorimotor-related thalamic nuclei (SM-TH; see full list of thalamic nuclei in the Methods)”)

Please see our response to the comment the reviewer made earlier and also to point 2 of reviewer 2. We added a new section in the methods and a new figure to address this important point.

Reviewer #3 (Remarks on code availability):

I have not tried to install and run the code, but I browsed the repository. It seems like the readme file will provide enough details for installation, but it contains only limited instructions which files are associated with which analyses. In addition, I would urge the authors to provide a cleaned-up version of their code. For instance, folder names like "IreneScripts", "Anecdotic_Opto", should be renamed to more professional names. To reproduce the in silico results, the code for the thalamo-cortical model should also be made public (apologies if I couldn't identify the relevant parts of the code from browsing the folder structure).

We modified the folder structure and instructions as recommended by the reviewer. The repository for the thalamo-cortical model is at https://github.com/bazhlab-ucsd/sleep-stage-transition/tree/stim and the related analysis can be found at: https://doi.org/10.6084/m9.figshare.25773501.v1. We added these links in page 1 of the README.docx file of the shared code. We also added the links to the shared code in the main manuscript.

Reviewer #1 (Remarks to the Author):

Congratulations to the authors. They have addressed all my comments in an exemplary manner. I have no further remarks.

Reviewer #1 (Remarks on code availability):

na
Thank you very much!

Reviewer #2 (Remarks to the Author):

Congratulations for the authors they have thoroughly addressed all my questions and comments. I have no more question. Maybe one more suggestion.
Concerning the response to my point 3. I understand why the authors hesitate to include this nice figure to the present manuscript. However, the mechanisms of spike and wave discharges is still heavily debated, and the role of thalamus is usually underestimated. The present data provide compelling evidence for the role of thalamus in the wave component. Indeed, it may not be wise to bury the SWD data shown as a supplementary figure of this paper, but I strongly recommend the authors to consider a separate brief study on the similarities between evoked activity and spontaneous SWDs in humans.

We thank the reviewer for the positive comments and insightful suggestion. We agree that a dedicated study comparing SWD and activity evoked by cortical stimulation in human would provide a key advancement for identifying thalamic involvement in SWD. This investigation will be the focus of a future study in which we will compare SWD and evoked responses originating from the same cortical site and, thus, more likely to engage the same cortico-thalamo-cortical circuits.

Reviewer #3 (Remarks to the Author):

In this revised study, the authors investigated the circuit and network mechanisms underlying the late component evoked through cortical stimulation. They show that this late component depends on the thalamus and is affected by behavioral state. Through experiments and modelling the authors show that both thalamic intrinsic currents and

cortical and thalamic GABAergic neurons are involved in the late responses to cortical stimulation in both mice and humans.

I still have some issues with the clarity of the text, partly overlapping with my previous concerns.
More importantly, as mentioned in my previous review: The results are not surprising at all to researchers working on cortico-thalamo-cortical circuits; they seem to, however, constitute important interpretative guidelines for clinical results involving cortical stimulation. The editors would have to decide if the advance here is large enough to warrant publication in Nature Communications.

MINOR:

- l. 214: consider rephrasing (on the other hand is not preceded by on the one hand, and it is unclear what exactly is contrasted to what on the other hand; however)

Line 216: The statement now reads: “As opposed to thalamic inhibition, optogenetic stimulation of GABAergic neurons in MOs minimally affected the rebound response”
- l. 226: similarly to what?

Line 226 modified as: “Upon further investigating rebound activity of cortical and thalamic units, we found that the evoked firing rate in MOs and SM-TH units was not modulated by the subject’s state in the same way as the EEG, LFP, and CSD responses”
- l. 227: consider moving the reference to Fig. 3 after “active state” to avoid confusion

Done. Thank you for the suggestion.
- l. 227: is rebound rate here the same as late component? Yes, it is. To make is clearer we modified as: “late response time window”.
- l. 230-234: give a bit of intuition why this analysis was pursued

At Line 232 we added: “The EPs modulation was observed even at the level of the CSD showing a current sink between layers 2/3 and 5 (Figures 1E, S4C) suggesting thalamo-cortical input[39,40]. Hence, we further analyzed the thalamic rebound responses, and we found that the rectified amplitude of the EP late component was correlated to the synchronicity and bursting frequency of thalamic units (see below for more details, Figure S13).”
- l. 236: how can it be known that these are relay neurons as compared to local inhibitory neurons etc.

Line 239 now reads: “putative thalamic relay units (Figure 3B, C) (see Methods for units’ type classification)”.
- l. 250: can the authors please at this point already explain a bit more what is entailed

by “connectivity profile” here. I assume it could be how the thalamic neurons are connected to the specific site of cortical stimulation, assuming some topography between the cortex and the thalamus (as later illustrated for the model). Or do the authors mean that there is a specific class that is more strongly connected in general to cortex, as potentially implied in l. 244 (“class of more effectively modulated neurons”)

As the reviewer mentioned, we believe that the thalamic neurons connect to specific cortical neurons following topographic mapping between the cortex and the thalamus as shown by the model. To make it more clear at line 251 we state: “Furthermore, the strength of the electrical stimulus (i.e., current intensity) affected not only the overall number of responsive units, but also the relative size of the two subsets, such that higher stimulation intensities revealed progressively more HF units than LF units (Figure S12), corroborating the idea that these two subsets reflected two different response patterns to the cortical stimulation due to their different connectivity profile. Specifically, HF units may be more directly connected to the stimulated site, hence they receive more synaptic inputs from the stimulated cortical neurons (see next section for details).”

- l. 439: I would suggest to phrase this and the previous paragraph more carefully; the corticothalamic pathways in other systems has been widely investigated, also in awake animals

We rephrased the two statements at line 442 as followed: “This study demonstrated that the lack of cortical inputs can suppress thalamic activity[85], as also confirmed by our simulated (Figure 4) and optogenetic data (Figure 2, S8). This mechanism coexists with the IPSPs followed by a rebound burst of TC neurons induced by TRN[86] and shaped by neuromodulators associated to different states of arousal. Importantly the state of the subject, i.e. anesthetized versus awake, could affect the time course of the responses[21].”

Reviewer #3 (Remarks on code availability):

The code repo is revised according to my suggestions