

Parallel cholinergic circuit in oculomotor nucleus to control eye movements and REM sleep

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Parts of this Peer Review File have been redacted as indicated to remove third-party material.

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)

The manuscript by Jiang and co-workers investigated the role of ChAT neurons in oculomotor in regulation of eye-movement and REM sleep. The authors discovered the novel neural pathways (NP-OMN and OMN-vIPAG) gating REM sleep. The manuscript is well-written. However, several concerns need to be addressed before being accepted for publication.

- 1) 8 hours sleep deprivation and recovery sleep may efficiently NREM sleep-active neurons. These data are consistent with the Calca sleep-active neurons discovery in pIII region (Zhang et.al, 2019). In particular, REM sleep deprivation should be performed to determine the c-fos expression of OMN ChAT neurons.
- 2) Although cell ablation strategy was used to determine the necessity of OMN ChAT neurons for controlling EM and REM sleep, acute optogenetic inhibition would provide more information on brain state transition.
- 3) Antegrade tracing data in Fig.5a is not clear; it seems labeling of soma of vIPAG and other downstream neurons instead of axon terminals.
- 4) The conclusion of NP-OMN and OMN-vIPAG connections is only drawn from AAV-assisted tracing. To confirm these functional connections, either mono-synaptic Rabies tracing or optogenetics-assisted electrophysiological recordings are recommended.

Reviewer #2

(Remarks to the Author)

The authors use the term oculomotor nucleus (OMN) throughout the manuscript without mentioning the fact that there are three oculomotor nuclei and defining the specific roles of each of them. This should be specified in the introduction. It is also welcome since line 76 they show which type of oculomotor movements are generated by the 3rd nucleus specifically. There is a large literature on the role of OMN in eye movements and it would be of great help for them and the readers to define it. Strangely, the authors state in the line 51 in the Introduction that activation of OMN neurons occurs after sleep deprivation not rebound.

Interestingly, they found that 37% of the OMN neurons were cFos after a REM rebound despite their low phasic activity during REM displayed by calcium imaging. They use fiber photometry to monitor activity of OMN neurons. I wonder whether they control that they did not record also the activity of EW cholinergic neurons?

Line 108 they look at activity of the OMN neurons at transitions and state that it is also increased during REM sleep outside the EMs. Unfortunately fiber photometry is not a method sensitive enough to monitor such modifications.

The description line 112-119 of the fiber photometry recordings results is not clear at all and cannot lead to the conclusion stated that OMNChat neurons are more active during REM without EMs. For that, the authors would need to make electrophysiological unit recordings of the OMNChat neurons.

The authors then ablate the OMNChat neurons to look at the effect on REM sleep. Strangely, they don't directly determine

whether it abolishes the EMs both during wakefulness and REM sleep. This is really strange. I anticipate that the effect might not be that impressive since both the 4th and 6th cranial nuclei are known to be involved. It is crucial that the authors analyze whether EMs are impacted by their lesion.

It is even more important since they report an increase in REM sleep and a decrease in Wake after such ablation. What are their criteria to define the two states? Is it muscle and EEG? And in such case, are they sure of their distinction between the two?

When conversely they activate the OMNChat neurons they obtain an increase in EMs and a termination of REM sleep.

Line 155 what the authors mean with high sleep stress levels?

In summary, the authors found that OMNChat neurons induce EMs as previously described and I wonder whether their stimulation do not indirectly induces wakefulness.

Then the authors attempt to specifically eliminate the OMNChat projecting to the extra ocular muscle using retrograde tracing from them. They found that 33% of the neurons OMN neurons are retrogradely labeled and concluded that the remaining ones don't project to the extraocular muscles. They cannot state so since it completely depends on the efficacy of their retrograde tracer. In fact, no retrograde tracer is sensitive enough to label all neurons projecting to an injected area. This claim is supported by their demonstration that optogenetic stimulation of the remaining neurons still induces EMs although it was logically decreased.

Again after stimulation, the quantity of REM sleep is decreased at the expense of wakefulness. It might be also that stimulating a large number of OMN neurons might induce a long lasting contraction of the oculomotor muscles and therefore inhibit EMs and induces waking.

In support of such hypothesis, when the authors record individual OMN neurons they report that some are activated and some are inhibited at the onset of EMs.

Line 245-282, they made correlation analysis to distinguish two subpopulation of OMN neurons. I'm not convinced by the methodology used and their conclusions that two subpopulations of neurons exist. More simple analysis would be more appropriate here to distinguish potential subpopulations. What is in particular the correlation with the EMs of the two subpopulations? Is really only one of the subpopulations increasing its activity at REM-W transitions? In such case what is their activity during the EMs?

Then, the authors look at the afferents to the OMNChat neurons using retrograde tracing. Line 287 they write they are looking to their downstream targets but it is their afferents not their targets they are looking at?

Strangely line 295 they look at the projection of OMNChat neurons to the vIPAG and not the opposite they just demonstrated? The same for LC, they show that OMN neurons project there? It has never been described in the numerous previous papers that OMN cholinergic neurons project to the LC and the vIPAG. All this part is very confusing. The confusion is confirmed in the next experiment in which they found that some OMN neurons project to the extraocular muscles and some to the vIPAG (which is very close to the OMN by the way... and this could induce a methodological problem). A simpler way to look at the efferents of the OMN Chat neurons would have been to use anterograde tracing after their transfection with a GFP expressing Cre AAV.

In the next chapter, they used a retroAAV injection into the OMN to label the afferents to the cholinergic neurons. It is really confusing since they also talked of retrograde tracing using mWGA-mCherry virus injection in the OMN and also wrote that it is retrograde tracing and they indeed show labeled soma in some regions. WGA is indeed a retrograde tracer so all of this is extremely confusing.

In the last chapter, they confirm previous results showing that the papillo nucleus project to the OMN and induce EMs and REM to wake transition.

In summary, the authors reproduced previous results showing that the OMN neurons are activated during REM sleep to participate in the REM generation. They further state that some OMN neurons terminate REM sleep and induce W. As explained above I'm not sure that such conclusion is accurate since wake might be induced indirectly when stimulating EMs generating neurons of the OMN.

Reviewer #3

(Remarks to the Author)

In the current study, Jiang et al. found that the overall activity of oculomotor nucleus (OMN) cholinergic neurons (ChAT) are higher during eye movements (EMs) and REM sleep. Ablation of OMN ChAT neurons reduced amplitude of EMs and REM episode number while increased REM duration. Optogenetic activation of OMN ChAT neurons induced EMs and decreased REM sleep. Optogenetic activation of non-extraocular muscle projecting OMN ChAT neurons did not affect EMs but terminated REM sleep and increased wakefulness. Calcium imaging showed distinct populations of OMN ChAT neurons that are active during EMs and REM-to-wake transitions. vIPAG projecting OMN ChAT neurons regulated REM-to-wake transitions. The nucleus papilio (NP) innervated OMN ChAT neurons and regulates both EMs and REM-to-wake transitions. The circuit mechanism for the coordination of EMs and REM sleep is interesting, while the goal was not accomplished in the present study. Although two populations of ChAT neurons in the OMN were found to be responsible for EMs and REM sleep regulation respectively, how these two parallel circuits coordinate with each other was unexplored. There is also a lack of rigorousness in preparing the manuscript. These issues reduced my enthusiasm for the paper. Specific comments:

1. The example traces in Fig.1f showed no obvious EMs during REM, and EMs happened mostly during wakefulness, especially in wakefulness episodes right after REM. To better visualize the temporal dynamics, it would be good to show the traces of EMs-free episodes and EMs-enriched episodes separately for each brain state.
2. In Fig.1h -1k, how did the author normalize time?
3. For the long-term optogenetic experiment in Extended Data Fig.5g, the effects became smaller as stimulation progressed and became comparable to control by the end of the 1-h stimulation. The authors should provide some insight into this observation.
4. In Extended Data Fig.6b-c, the example images seemed not to fit with the quantification. CTB555 labeled cells in the

contralateral part of OMN seems to comprise much more than the author claimed of ChAT+ cells (11.50%). The authors need to provide more details on how they did the quantification. How many animals and how many sections in each animal were used for the quantification?

5. What is the effect of optogenetic inactivation of OMN ChAT neurons on EMs and brain states?

6. Line 213-214, the number 51.9%, 18.7%, 20.0% did not fit with the numbers presented in Fig. 3l, any explanation on that?

7. In Supplementary Fig. 3e, the data shown looks like that of when stimulation were presented during wakefulness rather than the stated NREM sleep, since the probability of wakefulness is 1 before stimulation. Please explain the discrepancy between the figure and the statement.

8. Does optogenetic activation of extraocular muscle-projecting OMN ChAT neurons affect brain states?

9. In Fig. 4, how many mice and how many sessions were used for the calcium imaging?

10. Line 293, citation 6 and 24 does not seem to be appropriate.

11. The description of Figure 5 is chaotic, which reduced the credibility of this study. For example, 1) line 286, it should be anterograde tracing rather than retrograde tracing, right? 2) line 287-288, the author used mWGA-mCherry virus, while in Fig. 5a, the author stated in the legend that shown are GFP-labeled cells (line 1090). What is the explanation for this confusion? 3) Line 296, the author stated that they used AAV-Ef1a-ChrimsonR-mCherry virus, while in Fig. 5b and the legend, it was AAV2/9-hEF1 α -DIO-hChR2(H134R)-GFP. Which one did the author exactly use?

12. Does activating vPAG-projecting OMN ChAT neurons affect EMs?

13. In Fig. 5, are the vPAG projecting and LC projecting OMN neurons the same or distinct populations? Other than vPAG and LC, did the author test the effects of activating other targets (e.g., VTA, MPB) on sleep?

14. The description and labeling of Extended Data Fig. 8 is also chaotic and does not make sense. For example, in panel a, the author injected hSyn-FLEX-ChrimsonR-mCherry virus into NP of ChAT-cre mice, while ChAT seems not to be expressed in NP. Same issue for panel f. In panel a, it should be GCaMP6s virus rather than ChR2 virus, correct?

15. What is the neuronal cell type in NP that would project to OMN? How does NP coordinate EMs and REM-to-wake transitions through OMN? Does the same NP neuron innervate both extraocular muscle-projecting and vPAG-projecting OMN neurons?

Minor:

Line 72, Extended Data Fig. 1a?

Line 134, Fig. 2j-k?

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

The authors have conducted comprehensive experiments to address my concerns. I thank the authors for their efforts; all of my concerns have been fully addressed.

Reviewer #2

(Remarks to the Author)

Overall the authors provide a large number of additional data to the original study strongly strengthening it. There are a number of typos in many places like in the Title of Fig. R1-2 in which it is written ablation and not optogenetic inhibition. Concerning the anterograde labeling using mWmC, they did control the projection to the vPAG using an AAV which is a good addition. They show and tested projections to LC, MBP and VTA but I'm not convinced from their photomicrograph that there are anterogradely labeled fibers in each of these nuclei. Compared to the fibers in the vPAG the staining is not looking like terminals and for the LC it seems outside the core body of the nucleus. In the VTA it does not look like fiber while in the MBP it seems there are no fibers.

Reviewer #3

(Remarks to the Author)

The authors have addressed my previous critiques, and the manuscript is much improved, while I have a few comments remaining:

1. The authors incorrectly interpreted the cited ref. 21, as according to ref 21 there is decreased rather than increased c-fos in OCM after REM sleep deprivation. Please make sure the appropriate citation and statement. (line 59)

2. Line 123, since EMs-free REM episodes had higher Ca²⁺ activity compared to that of EMs-enriched REM episodes, it might not be appropriate to conclude that "EMs contribute to elevated calcium signals in both REM and wake states", it was true for wake states but not for REM.

3. In Fig. 5f and supplementary Fig. 5d, the traces of spontaneous and the laser-evoked spike wave form look almost the same, could there be any mistakes in preparing these figures? The authors should double check their data or provide the source data of these figures.

4. Supplementary Fig. 14, panel a, should it be WT mice instead of ChAT-Cre mice?

5. Supplementary Fig. 15, in panel i and k, the diagrams on the left seemed to mismatch.

Version 2:

Reviewer comments:

Reviewer #2

(Remarks to the Author)

Thank you the authors did address all my requests.

Reviewer #3

(Remarks to the Author)

The authors have successfully addressed my concerns. I have no further comments.

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Response to Reviewers' Comments

Text in blue: Comments/questions from reviewers

Text in black: Response from the authors

Text in green: Text in the original submission

Text in red: Revisions made to the manuscript

Reviewer #1

General comments:

The manuscript by Jiang and co-workers investigated the role of ChAT neurons in oculomotor in regulation of eye-movement and REM sleep. The authors discovered the novel neural pathways (NP-OMN and OMN-vIPAG) gating REM sleep. The manuscript is well-written. However, several concerns need to be addressed before being accepted for publication.

Response:

We sincerely thank the reviewer for the positive evaluation of our work and for acknowledging the novelty of the neural pathways we identified in the regulation of REM sleep. We also appreciate your recognition of the overall quality of the manuscript. We are grateful for your thoughtful suggestions, which have significantly contributed to the refinement of our work.

In this revision, we have carefully addressed all of your concerns and thoughtful suggestions in a point-by-point manner. We **conducted new experiments** to clarify the functional connections between nIII^{ChAT} neurons and its upstream/downstream targets, namely NP and vIPAG. We also revised the manuscript accordingly to improve its clarity, scientific rigor, and presentation. As suggested by Reviewer #2, we have updated the revised manuscript by replacing all occurrences of the abbreviation “OMN” with “nIII” throughout the main text, figures, and the response to reviewers. **The major revisions are summarized as follows:**

- C-fos screening after REM sleep deprivation using closed-loop sleep interference

system was added as both new figures of **Supplementary Fig.2d-j** and corresponding descriptions in the **Results** section;

- The effects of optogenetic inhibition on eye movements (EMs) and sleep architecture have been added as both new figures of **Fig.2l-o** and corresponding descriptions in the **Results** section.
- Typos have been corrected and double-checked throughout the manuscript;
- Optogenetics-assisted *in vivo* electrophysiological recordings verifying the NP-NIII and NIII-vIPAG connections have been added as both new figures of **Fig.5e-h**, **Fig.6e-h** and corresponding descriptions in the **Results** section.

A summary of the revisions in response to the reviewers' comments can be found at the end of the rebuttal letter. We hope that you now find the revised manuscript suitable for publication.

Comment 1:

8 hours sleep deprivation and recovery sleep may efficiently NREM sleep-active neurons. These data are consistent with the Calca sleep-active neurons discovery in OMN region (Zhang et.al, 2019). In particular, REM sleep deprivation should be performed to determine the c-fos expression of OMN ChAT neurons.

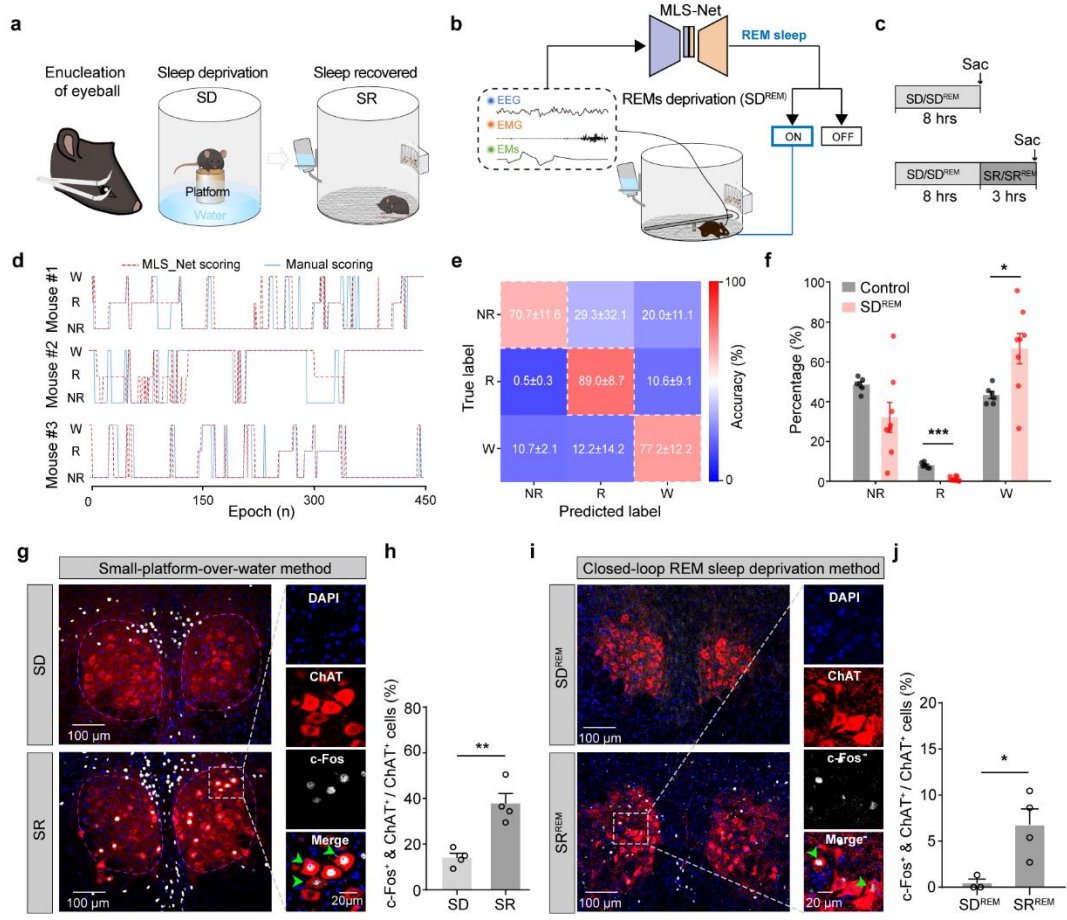
Response:

Thank you for your constructive and insightful comment. We agree with the reviewer that in order to accurately assess c-fos expression in OMN^{ChAT} (nIII^{ChAT} in the revised manuscript as suggested by reviewer #2) neurons, it is important to selectively deprive REM sleep (REMs).

To further improve the specificity of REMs deprivation, **we conducted new sleep deprivation experiments using a close-loop sleep interference system** based on our previously developed autonomous sleep classifier, MLS-Net (**Fig. R1-1b**)(Jiang et al. 2024). EEG/EMG/EMs signals were recorded in real-time and segmented into 4-second

epochs. These were then analyzed online by MLS-Net to determine vigilance states. Upon detection of a REMs epoch, a TTL signal was sent via Arduino to trigger a rotarod, thereby interrupting REMs with minimal disruption to other states. The SD^{REM} (REMs deprivation) and SR^{REM} (REMs recovery after deprivation) groups in this protocol followed the same 8-hour deprivation and 3-hour recovery paradigm, respectively (**Fig. R1-1c**). Following REMs deprivation, c-Fos immunostaining was performed.

We first evaluated the performance of the close-loop sleep interference system in detecting and classifying REM sleep episodes. The accuracy of online sleep staging by MLS-Net was validated by comparison with manual offline scoring. The confusion matrix revealed average classification agreement rates of 70.7% for NREMs, 89.0% for REMs, and 77.2% for wake between MLS-Net and manual scoring (**Fig. R1-1d-e**), demonstrating the reliability of the closed-loop system. During the 8-hour deprivation period, the proportion of REMs was reduced from 8.1% in the control group to 1.2% in the deprived group, accompanied by a significant increase in wakefulness (**Fig. R1-1f**). After 8 hrs sleep deprivation and following 3 hrs sleep recovery, there was a significant increase in c-fos expression in nIII^{ChAT} neurons in the SR group compared to the SD group in the new closed-loop sleep deprivation system (**Fig. R1-1i-j**).



(Newly added Supplementary Fig.2) Fig. R1-1. | The c-Fos expression in nIII^{ChAT} neurons after sleep deprivation. **a** The schematic of REM sleep deprivation (SD) and sleep recovered (SR) using small-platform-over-water method. **b** Schematic of REMs specific deprivation (SD^{REM}) and REMs specific rebound using a closed-loop sleep deprivation system. **c** The timeline of sleep deprivation and c-Fos screening. **d** The output hypnogram predicted by real-time autonomous sleep classifier compared with manual scoring. **e** The average confusion matrix comparing the true and predicted sleep labels for the input sleep-wake epochs on the MLS-Net model. **f** The percentage of different vigilance states for control and REM sleep deprivation mice using a closed-loop sleep deprivation system. N = 8 from SDREM group, n = 7 from control group. *p<0.05, ***p<0.001. Two-sided unpaired t-test. **g** Representative image of the c-Fos expression in the coronal sections of the nIII in the SD or SR mouse using small-platform-over-water method. Dashed box highlights the c-Fos (white) and ChAT (red) double-labeled neurons. **h** Percentages of c-Fos+/ChAT+ co-expressing neurons in the

nIII region. Each dot indicates data from one mouse ($n = 4$ mice). $**p < 0.01$. Two-sided unpaired t-test. **i** Representative image of the c-Fos expression in the coronal sections of the nIII in the SDREM or SRREM mouse using a closed-loop sleep deprivation system. Dashed box highlights the c-Fos (white) and ChAT (red) double-labeled neurons using small-platform-over-water method. **j** Percentages of c-Fos⁺/ChAT⁺ co-expressing neurons in the nIII region. Each dot indicates data from one mouse ($n = 4$ mice). $*p < 0.05$. Two-sided unpaired t-test.

The corresponding description have been added in the revised manuscript:

(Page 3 line 96-108) To investigate the activity of nIII^{ChAT} neurons during sleep, we conducted a c-Fos staining experiment after 8 hrs sleep deprivation (SD, using the small-platform-over-water method) or during the subsequent sleep recovered (SR) in binocularly enucleated mice (Supplementary Fig. 2a,c). We observed that the proportion of c-Fos⁺ChAT⁺ neurons among nIII^{ChAT} neurons was higher in the SR group (37.74%) compared with the SD group (14.01%) (Supplementary Fig. 2g-h). We also deprived the REMs of mouse specifically by employing a closed-loop sleep interference system (Supplementary Fig. 2b), which achieves a state-of-the-art performance in REMs classification (Supplementary Fig. 2d-e)²³. During the 8-hour deprivation period, the proportion of REMs was reduced from 8.1% in the control group to 1.2% in the deprived group, accompanied by a significant increase in wakefulness (Supplementary Fig. 2f). Subsequently, we observed a significantly increased c-Fos expression in the SR^{REM} group compared to the SD^{REM} group (Supplementary Fig. 2i-j). These evidences indicate that the activation of nIII^{ChAT} neurons was associated with sleep, especially REM episodes.

(Page 16 line 679-694) The mice were categorized into two groups: the sleep deprivation (SD) and the sleep rebound (SR) group. Small-platform-over-water method and a closed-loop sleep interference system were used. For sleep deprivation using small-platform-over-water method, mice were gently placed on a handcrafted small-round platform (diameter: 2.5 cm) situated within a water tank (1 cm under the platform)

at the start of the light period (7 am). For sleep deprivation using closed-loop sleep interference system, the mice were implanted with EEG and EMG electrode. The mice were habituate the polysomnographic recording chamber at least 2 days prior to sleep deprivation. On the third day, EEG, EMG, and EM signals were recorded in real time and segmented into 4 s epochs. The segmented signals were subsequently transmitted to MLS-Net, an autonomous online sleep classifier, for analysis and determination of vigilance states. Upon detection of a REMs epoch, a TTL signal was sent via Arduino to trigger a rotarod in the bottom of the chamber, thereby interrupting REMs. After 8 hrs of sleep deprivation, mice in the SR group were transferred to their home cages for sleep recovery with polygraphic recording concurrently. Once the mice had slept for at least 3 hrs, they were immediately anesthetized and sacrificed for further c-Fos immunostaining and histological procedures. In contrast, mice in the SD group were directly sacrificed for immunostaining without undergoing a recovery period.

Comment 2:

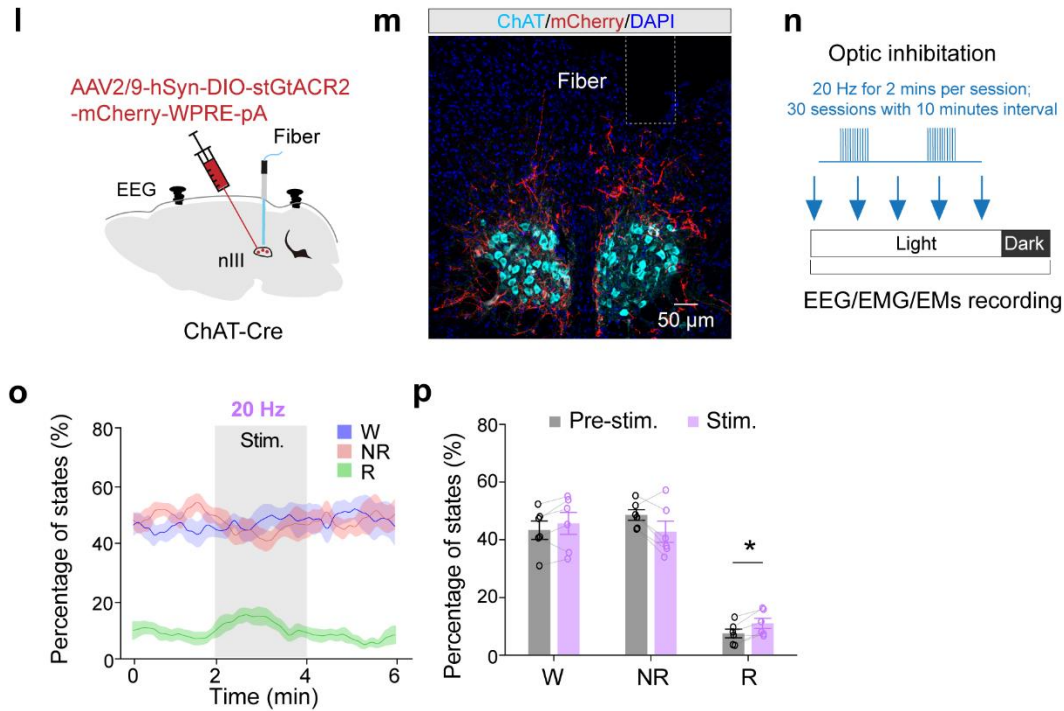
Although cell ablation strategy was used to determine the necessity of OMN ChAT neurons for controlling EM and REM sleep, acute optogenetic inhibition would provide more information on brain state transition.

Response:

Thanks for your constructive suggestion. To further clarify the role of nIII^{ChAT} neurons in regulating REM sleep, **we performed new experiments to optogenetically inhibit nIII^{ChAT} neurons.**

Specifically, we injected AAV2/9-hSyn-DIO-stGtACR2-mCherry-WPRE-pA (stGtACR2 being an inhibitory opsin) virus into the nIII of ChAT-Cre mice (**Fig. R1-2l-m**). During the light phase (12:00pm-18:00pm), a total of 30 photoinhibition sessions were conducted, each consisting of a 2-minute stimulation block (20 Hz, indicated by blue arrows), delivered at 10-minute intervals to selectively silence nIII^{ChAT} neurons (**Fig. R1-2n**). Optogenetic inhibition of nIII^{ChAT} neurons resulted in increased proportion of REMs (**Fig. R1-2o-p**).

These complementary results provide additional functional evidences to strengthen the conclusion that $nIII^{ChAT}$ activity is necessary for regulating REM sleep occurrence. We have added these results into the revised manuscript.



(Newly updated Fig. 2) Fig. R1-2 | Ablation of $nIII^{ChAT}$ attenuates REMs episodes and elongates REM duration. **I** Schematic depicting stGTACR-mediated inhibition of $nIII^{ChAT}$ neurons. **m** Confocal image of viral expression and the placement of optical fibers above the nIII. The dashed grey box indicates the trace of the optical fiber. **n** Two-minute blocks (blue arrows) of stimulations were delivered with intervals of 10 to 25 min throughout the light phase to photo-inhibit $nIII^{ChAT}$ neurons. **o** Percentage of wake, NREM, or REM state before, during, and after optogenetic inhibition in ChAT-Cre mice ($n=6$). The gray shielding indicates the time window of stimulation. The red, blue and green shielding indicates the 95% confidence intervals (CIs). **p** Quantification of the percentage of wake, NREM, or REM state before (pre-stim.), and during (stim.) optogenetic inhibition in ChAT-Cre mice ($n=6$). Each dot indicates individual mice. * $p<0.05$. Two-sided paired t-test. Error bars represent SEM across mice.

The corresponding description have been added in the revised manuscript:

(Page 5 line 188-206) To clarify the necessity of nIII^{ChAT} neurons in regulating REMs, beyond the cell ablation approach, we have performed acute and cell-type-specific optogenetic inhibition of nIII^{ChAT} neurons during polysomnographic recordings. We injected the inhibitory opsin (AAV2/9-hSyn-DIO-stGtACR2-mCherry-WPRE-pA) into the nIII of ChAT-Cre mice (Fig. 2l). Three weeks post-injection, stGtACR2 was stably co-expressed with the cholinergic marker ChAT (Fig. 2m). During the light phase (12:00pm-18:00pm), a total of 30 photoinhibition sessions were conducted, each consisting of a 2 min stimulation block (20 Hz, indicated by blue arrows), delivered at 10 min intervals to selectively silence nIII^{ChAT} neurons (Fig. 2n). We found that optogenetic inhibition of nIII^{ChAT} neurons induced a reliable EMs in nasal-to-dorsotemporal direction (Supplementary Fig. 6f-g). We further examined the dynamic changes in EEG, EMG, and EMs signal across different vigilance states (Supplementary Fig. 7). We found that the occurrence rate of EMs rapidly increased during the stimulation window across multiple trials (Supplementary Fig. 6h, Supplementary Fig. 7). The EEG power spectra and EMG of each brain state during inhibition were similar to those displayed during spontaneous sleep-wake cycle indicating that the optogenetic-evoked EMs did not interfere EEG/EMG signals, thus not affecting the accurate identification of sleep stages (Supplementary Fig. 7a-f). We then evaluate the sleep architecture and observed that optogenetic inhibition of nIII^{ChAT} neurons significantly increased the proportion of REMs (Fig. 2o-p). These results is consistent with the nIII^{ChAT} neuronal ablation experiment, indicating that silencing nIII^{ChAT} neurons could lead to the generation and maintenance of REMs.

Comment 3:

Antegrade tracing data in Fig.5a is not clear; it seems labeling of soma of vIPAG and other downstream neurons instead of axon terminals.

Response:

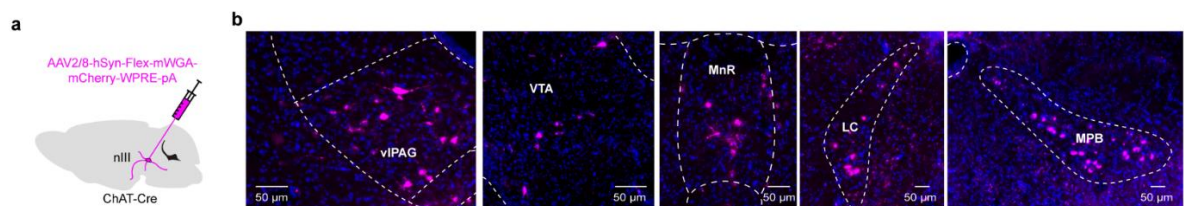
Thank you for pointing out this issue. We apologize for the confusion caused by

our misstatement in the previous manuscript. As the reviewer correctly pointed out, the anterograde trans-synaptic tracer we used labels the postsynaptic somata of downstream neurons, rather than the axon terminals of nIII^{ChAT} neurons. **We have carefully revised the relevant texts in the revised manuscript. Details can be seen below.**

AAV2/8-hSyn-Flex-mWGA-mCherry (mWmC) that we used in our manuscript was validated by Nicole Y. Tsai et al. (Tsai et al. 2022) through striatal injections, which led to strong labeling of neuronal somata in the substantia nigra pars reticulata (SNr) (Fig. R1-3), thereby supporting its efficiency in trans-synaptic labeling. Building upon this validation, we injected the mWmC virus into the nIII to identify direct postsynaptic targets of nIII^{ChAT} neurons. We observed distinct somatic mCherry signals in several downstream regions, including the ventrolateral periaqueductal gray (vlPAG), ventral tegmental area (VTA), median raphe nucleus (MnR), locus coeruleus (LC), and medial parabrachial nucleus (MPB) (Fig. R1-4).

[Figure Redacted]

(Fig. 4m in the cited article) **Fig. R1-3 | m** Anterograde trans-synaptic tracing from substantia nigra (SNr) via stereotaxic injections into the Striatum. From Nicole Y. Tsai et al 2022.



(Fig. 5) Fig. R1-4 | vIPAG-projecting nIII^{ChAT} population suppress REM sleep. a Schematic of anterograde trans-synaptic tracing of the nIII^{ChAT} neurons to label the downstream nuclei. **b** Example image showing mCherry-labelled somatic in several different downstream regions. vIPAG: the ventrolateral periaqueductal grey, VTA: ventral tegmental area, MnR: median raphe nucleus, LC: locus coeruleus, MPB: medial parabrachial nucleus. Scale bar, 50 μ m.

The corresponding description have been added in the revised manuscript:

(Page 9 line 369-379) To further investigate the neural circuit mechanisms by which the nIII regulates REMs, we conducted anterograde tracing to identify their downstream targets. AAV2/8-hSyn-Flex-mWGA-mCherry-WPRE-pA (mWmC) virus was a genetically encoded monosynaptic anterograde tracer developed by Nicole Y. Tsai et al. 29. The anterograde and trans-synaptic labeling capabilities of mWmC have been validated through striatal injections, which resulted in robust mCherry-labeled somas in the substantia nigra pars reticulata (SNr)²⁹. We then injected the mWmC virus into the nIII in ChAT-Cre mice to identify direct postsynaptic targets of nIII^{ChAT} neurons (Fig. 5a). We observed distinct somatic mCherry signals in several downstream regions, including the ventrolateral periaqueductal gray (vIPAG), ventral tegmental area (VTA), median raphe nucleus (MnR), locus coeruleus (LC), and medial parabrachial nucleus (MPB) (Fig. 5b).

Comment 4:

The conclusion of NP-OMN and OMN-vIPAG connections is only drawn from AAV-assisted tracing. To confirm these functional connections, either mono-synaptic Rabies tracing or optogenetics-assisted electrophysiological recordings are recommended.

Response:

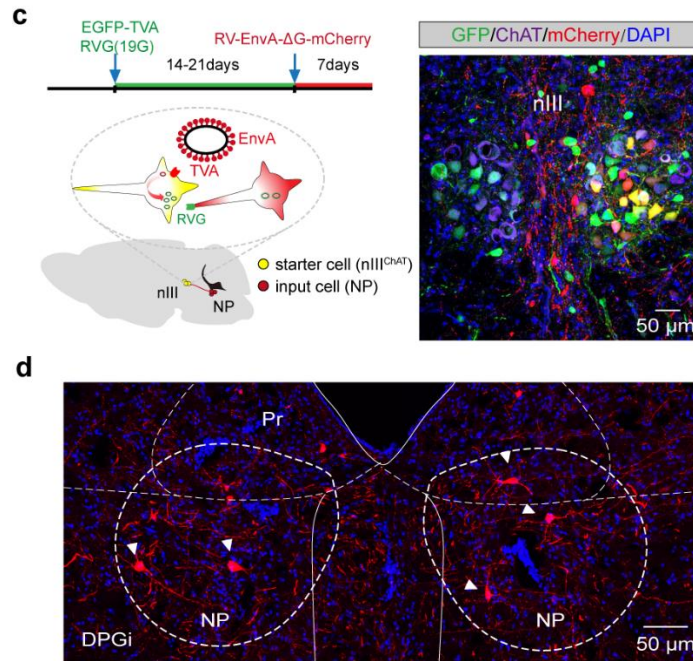
We thank the reviewer for this constructive suggestion. We performed new experiments to validate the functional connections of NP-nIII and nIII-vIPAG using both monosynaptic rabies tracing and optogenetics-assisted *in vivo*

electrophysiological recordings.

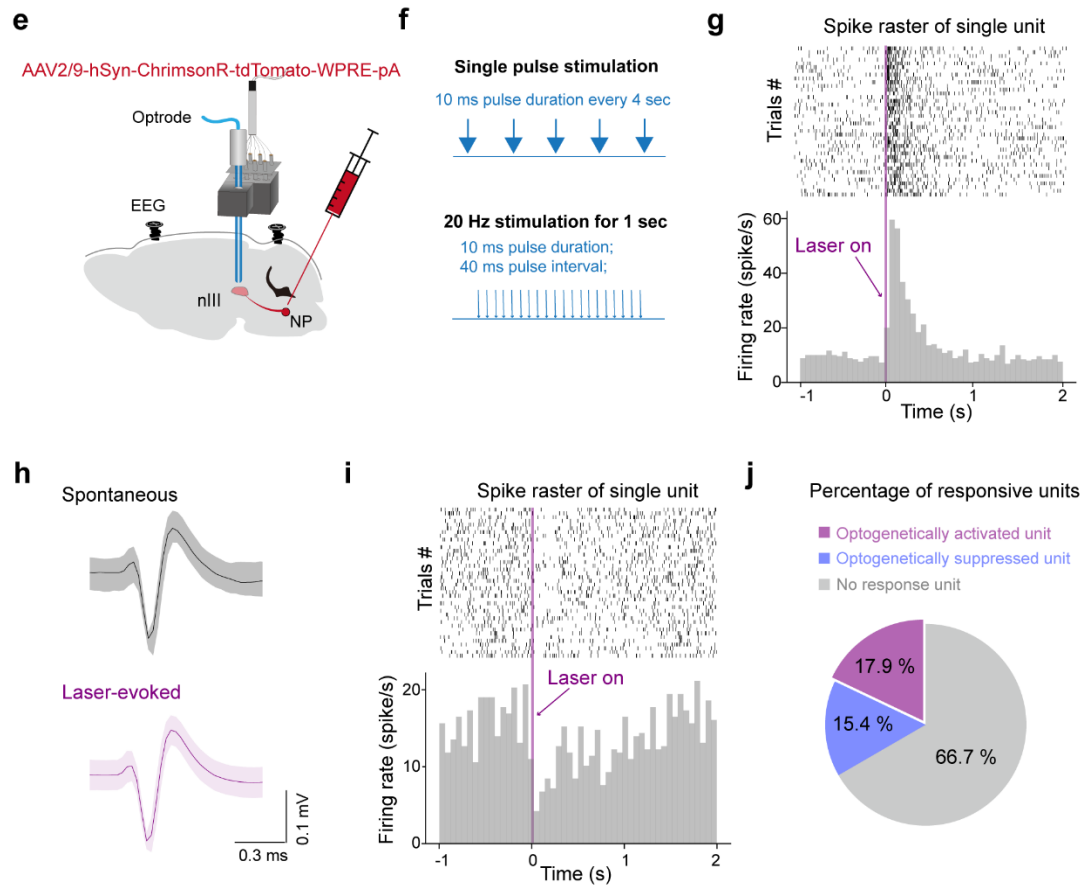
To confirm the NP-nIII connection, we injected a Cre-dependent monosynaptic retrograde pseudorabies virus into the nIII in ChAT-Cre mice (**Fig. R1-5c**). We found mCherry-positive somata in the nucleus papilio (NP) (**Fig. R1-5d**), indicating a direct monosynaptic input from NP neurons to nIII^{ChAT} neurons. To assess the functional connection between NP and nIII, we injected AAV2/9-hSyn-ChrimsonR-tdTomato-WPRE-pA (a red-shifted optogenetic activator) into the NP of C57 mice and implanted an optrode in the nIII for optogenetic-tagged single-unit recording *in vivo* (**Fig. R1-6e, R1-7a-b**). Both single-pulse stimulation (10 ms pulse every 5 minutes) and high-frequency trains of stimulation (20 Hz; 10 ms per pulse; 20 pulses per train) induced a pulse-dependent increase or decrease in firing rate of single-unit (**Fig. R1-6f-i, Fig. R1-7c**). Among 40 single units in nIII recorded from 3 mice, 17.9% exhibited laser-evoked excitatory responses (**Fig. R1-6j, R1-7d**), and 15.4% showed inhibitory responses (**Fig.s R1-6i-j, R1-7e**), demonstrating that NP neurons can modulate nIII activity through both excitatory and inhibitory synaptic inputs.

We also verified the nIII-vIPAG connection by injecting the AAV2/2Retro-hSyn-Cre-WPRE-pA into vIPAG of C57 mice. We then injected the Cre-dependent TVA and RVG into the nIII (**Fig. R1-8e**). Using this strategy, we can specifically label the vIPAG-projecting nIII^{ChAT} neurons, which is confirmed by GFP and mCherry double-labelled neurons expressing ChAT (**Fig. R1-8f**). To further confirm the functional connectivity of nIII-vIPAG projection, we conducted optogenetic-tagged electrophysiological recording by injecting AAV2/9-hEF1 α -DIO-hChR2(H134R)-EYFP virus into the nIII of ChAT-Cre mice and implanted an optrode in the vIPAG (**Fig. R1-9e**). Using the same optical stimulation protocol as in **Fig. R1-6f** (20 Hz; 10 ms per pulse; 20 pulses per train), we observed reliable optogenetically-evoked spiking activity in a subset of vIPAG neurons upon photostimulation of nIII^{ChAT} axons terminals within the vIPAG (**R1-9f-h**). Among the 61 units recorded from 3 mice, 32.8% exhibited optogenetically activated responses (**Fig. R1-9i, Fig. R1-10a**), and 9.8% showed optogenetically suppressed responses to nIII^{ChAT} axon activation (**Fig. R1-9i, Fig. R1-10b**).

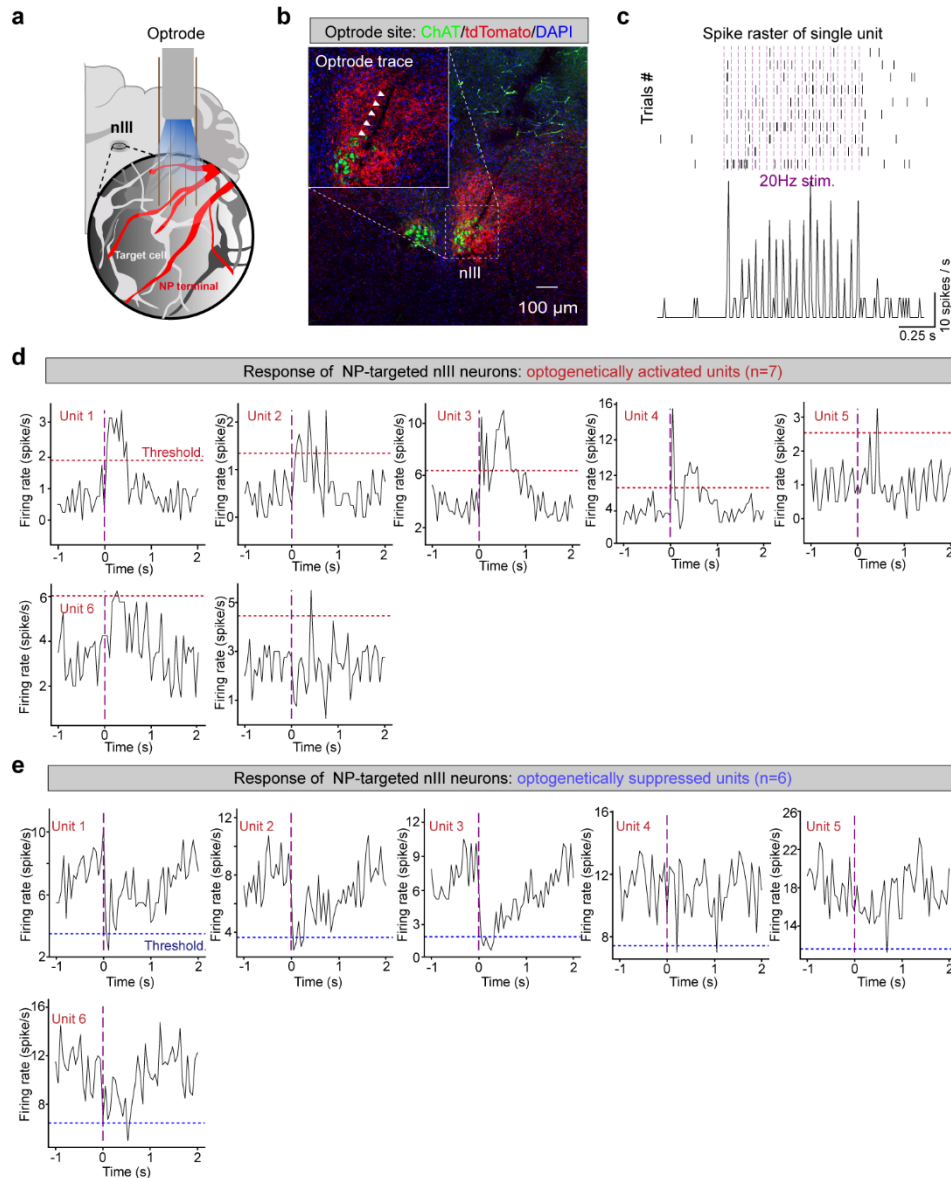
Together, these new results from viral tracing and optogenetic-assisted *in vivo* electrophysiology provide convergent evidence supporting a functional monosynaptic connection between NP-nIII^{ChAT} neurons and nIII^{ChAT}-vIPAG neurons. We have added these new results into the revised manuscript.



(Newly updated Fig.6) Fig. R1-5. | NP mediates nIII cholinergic activity to modulate EMs and REM sleep. **c** Left: Schematic diagram of injection of helper viruses, including recombinant adeno-associated virus (AAV) expressing tumor virus receptor A (TVA) with a green fluorescent protein (rAAV-EF1 α -DIO-EGFP-T2A-TVA) or expressing rabies glycoprotein(RG) (rAAV-EF1 α -DIO-oRVG(19G)) into the nIII in ChAT-Cre mouse, followed by injection of modified rabies virus (RV) expressing mCherry (RV-EnvA-G-mCherry). Right: confocal image showing a coronal section of the nIII 10 days post-RV injection. Starter nIII^{ChAT} neurons appear yellow due to co-expression of GFP and mCherry from helper and RV vectors, respectively. **d** The confocal image of NP neurons labeled by RV-mediated monosynaptic retrograde tracing from the nIII.

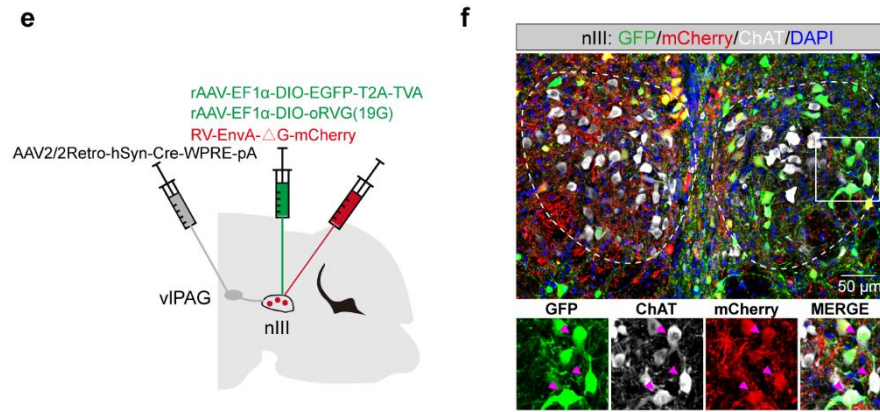


(Newly updated Fig.6) Fig. R1-6. | The functional connectivity of the NP–nIII circuit was confirmed by optogenetics-assisted electrophysiological recordings. e Schematic of viral injection in the NP and optrode implantation in the nIII. **f** The schematic of stimulus diagram. **g** Representative raster plot (upper) and stimulus time histogram (lower) for laser-evoked spikes of an example ChR2-tagged unit. Dashed line, laser onset time. Laser duration, 10 ms. Power density, 2 mW. **h** Averaged waveform of spontaneous (gray) and laser-evoked (purple) spike of an example unit. **i** Representative raster plot (upper) and stimulus time histogram (lower) for laser-inhibited spikes of an example ChR2-tagged unit. **j** Percentages of optogenetically activated, suppressed and no-response units among all recorded electrode in the nIII. Optogenetically activated and suppressed units were defined based on whether the post-stimulation peak firing rate exceeded a threshold of mean $\pm$ 3 standard deviations (SD) of the baseline activity.

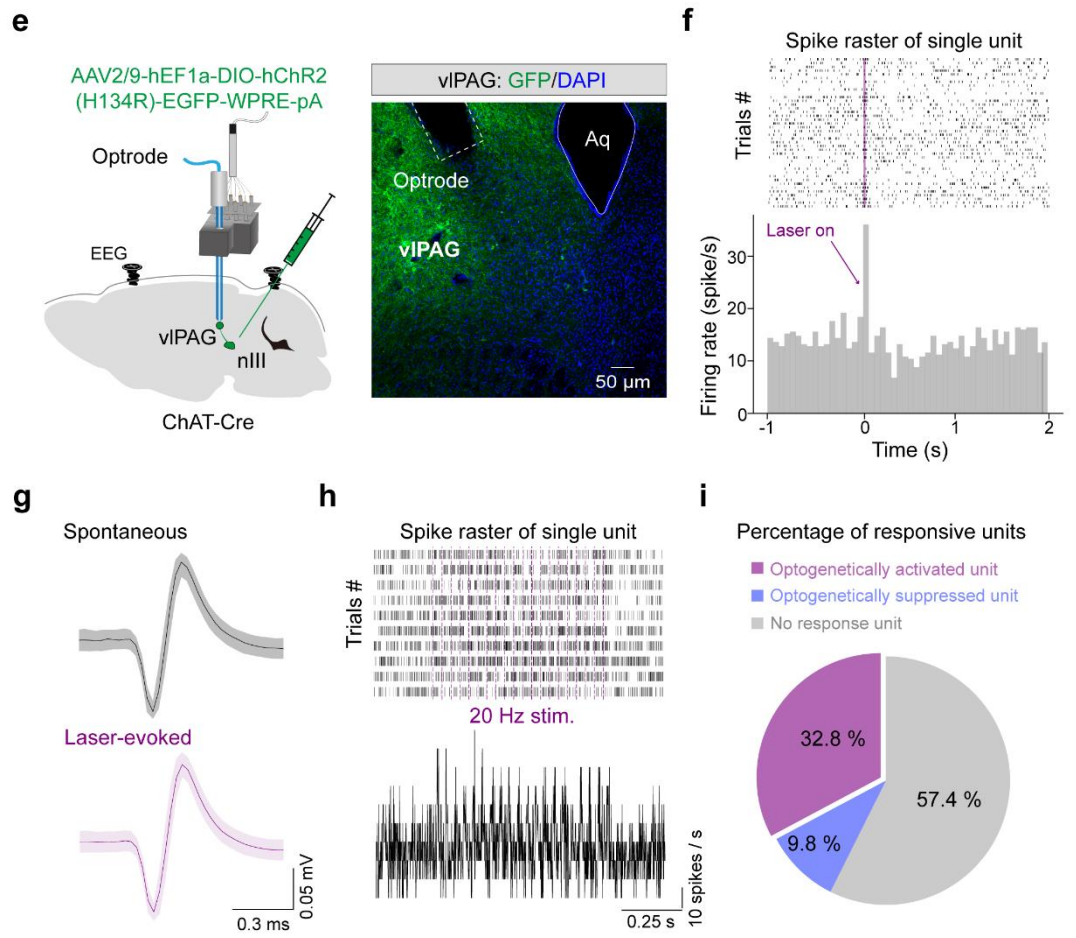


(Newly added Supplementary Fig. 22) Fig. R1-7 | Optogenetic-assisted electrophysiological recording of NP-targeted nIII neurons. **a** Schematic drawing of ChrimsonR-targeted nIII neurons using electrophysiology recording after co-injection of AAV2/9-hSyn-ChrimsonR-tdTomato (red) in the NP. **b** Confocal image showing the viral expression and fiber location within the nIII. The arrows in the magnified region show the trace of the electrode. **c** Representative ChrimsonR-targeted unit within the nIII showing pulse-dependent firing rate during continuous laser-pulse stimulation. Purple dashed lines indicate the laser pulse (20 Hz, 10 ms per pulse, lasting for 1 s). **d** Units activated by optogenetic activating the NP neuronal terminal within the nIII. N = 7 units from 3 mice. The optogenetically activated units were defined

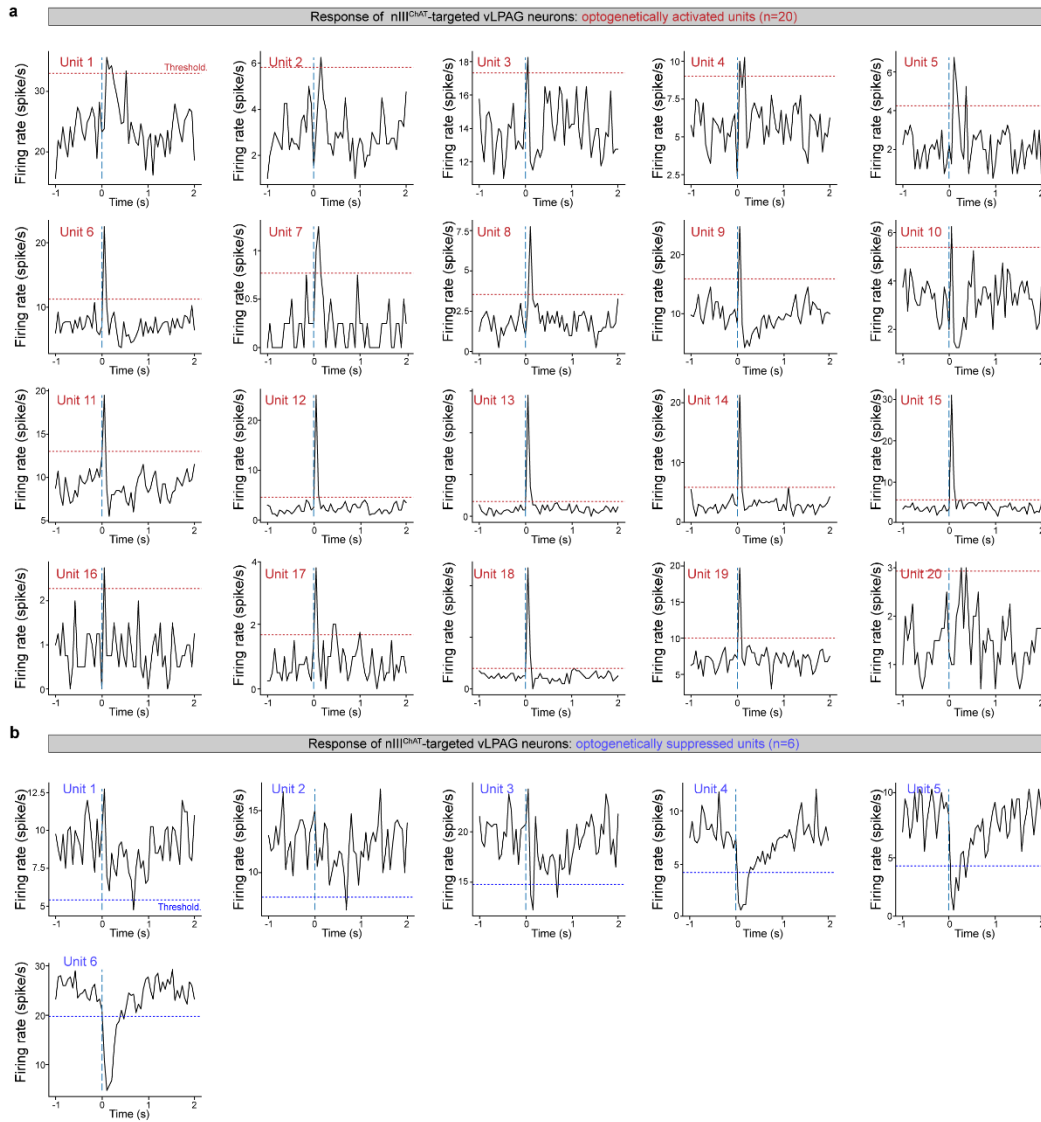
based on whether the post-stimulation peak firing rate exceeded a threshold of mean + 3 standard deviations (SD) of the baseline activity. Horizontal red dashed lines indicate response threshold. Vertical purple dashed lines indicate the onset of stimulus. Pulse duration, 10 ms. Pulse density, 2 mW. **e** Units suppressed by optogenetic activating the NP terminal within the nIII. N = 6 units from 3 mice. The optogenetically suppressed units were defined based on whether the post-stimulation peak firing rate below a threshold of mean - 3 standard deviations (SD) of the baseline activity. Horizontal blue dashed lines indicate response threshold. Vertical purple dashed lines indicate the onset of stimulus. Pulse duration, 10 ms. Pulse density, 2 mW.



(Newly updated Fig7) Fig. R1-8 | Anatomical connection between nIII and vlPAG revealed by retrograde rabies virus tracing. e Schematic diagram of strategy to label the vlPAG-projecting NP neuron. **f** Confocal image showing a coronal section of the nIII 10 days post-RV injection. The dashed square region in the nIII is magnified below. vlPAG-projecting nIII^{ChAT} neurons appear yellow due to co-expression of GFP and mCherry from helper and RV vectors, respectively.



(Newly updated Fig5) Fig. R1-9 | vIPAG-projecting nIII^{ChAT} population suppress REM sleep. **e** Left: Schematic of viral injection in the nIII and optrode implantation in the vIPAG. Right: representative image to show the locations of optrode in the vIPAG. **f** Raster plot (upper) and stimulus time histogram (lower) for laser-evoked spikes of an example ChR2-tagged unit. Dashed line, laser onset time. Laser duration, 10 ms. Power density, 2 mW. **g** Averaged waveform of spontaneous (gray) and laser-evoked (purple) spike of an example unit. **h** Representative ChR2-tagged unit showing pulse-dependent firing rate during continuous laser-pulse stimulation. Purple dashed lines indicate the laser pulse (20 Hz, 10 ms per pulse, lasting for 1 s). **i** Percentages of optogenetically activated, suppressed and no-response units among all recorded electrode in the vIPAG. Optogenetically activated and suppressed units were defined based on whether the post-stimulation peak firing rate exceeded a threshold of mean $\pm$ 3 standard deviations (SD) of the baseline activity.



(Newly added Supplementary Fig. 18) Fig. R1-10 | Laser-evoked response of nIII^{ChAT}-targeted vLPAG neurons. a Units activated by optogenetically activating the nIII^{ChAT} neuronal terminal within the vLPAG. N = 20 units from 3 mice. The optogenetically activated units were defined based on whether the post-stimulation peak firing rate exceeded a threshold of mean + 3 standard deviations (SD) of the baseline activity. Horizontal red dashed lines indicate response threshold. Vertical blue dashed lines indicate the onset of stimulus. Pulse duration, 10 ms. Pulse density, 2 mW. **b** Units suppressed by optogenetically activating the nIII^{ChAT} neuronal terminal within the vLPAG. n = 6 units from 3 mice. The optogenetically suppressed units were defined based on whether the post-stimulation peak firing rate below a threshold of mean - 3 standard deviations (SD) of the baseline activity. Horizontal blue dashed lines indicate

response threshold. Vertical blue dashed lines indicate the onset of stimulus. Pulse duration, 10 ms. Pulse density, 2 mW.

The corresponding description have been added in the revised manuscript:

(Page 11 line 441-465) We first validate this connection by injecting an anterograde trans-synaptic AAV1/2-hSyn-Cre-WPRE into the NP and CTB488 into EOMs of transgenic Ai14 mice, and found cholinergic neuronal soma intermingled with tdTomato-labeled soma in the ipsilateral nIII area (Fig. 6a-b, Supplementary Fig. 21a-b). There is also a subset of CTB488/tdTomato co-labeled nIII^{ChAT} neurons (Supplementary Fig. 21b-c), indicating that the NP neurons innervate both EOMs-projecting and non-EOMs-projecting nIII^{ChAT} neurons. Additionally, we employed a rabies virus (RV)-mediated trans-synaptic retrograde tracing approach, which has been shown to efficiently label monosynaptic inputs to selectively infected starter cells³². To specifically label nIII^{ChAT} neurons, we first injected two Cre-dependent helper viruses (rAAV-EF1 α -DIO-EGFP-T2A-TVA and rAAV-EF1 α -DIO-oRVG(19G)) into the nIII (Fig. 6c). After 2 weeks, glycoprotein (G) gene-deleted RVs expressing mCherry (RV-EnVA-G-mCherry) were injected into the same site. The starter neurons, which are shown in yellow, were characterized by the expression of both the helper virus and RV in cholinergic neurons (Fig. 6c). Immunofluorescence staining revealed robust mCherry-positive somata in the NP (Fig. 6d). These results provided direct anatomical evidence of a synaptic connection from the NP to nIII^{ChAT} neurons.

In addition to RV-mediated anatomical tracing, we also measured functional connections between NP and nIII^{ChAT} neurons using *in vivo* optogenetics-assisted electrophysiological recordings. We injected AAV2/9-hSyn-ChrimsonR-tdTomato-WPRE-pA into the NP and implanted an optrode in the nIII (Fig. 6e, Supplementary Fig. 22a-b). Optogenetic activation of NP terminals within the nIII induced a pulse-dependent increase or decrease in firing rate of single-unit (Fig. 6 f-g, Supplementary Fig. 22c). Among 40 well-identified nIII units recorded from 3 mice, 17.9% exhibited optogenetically activated responses, and 15.4% showed optogenetically suppressed

responses (Fig. 6h, Supplementary Fig. 22d-e), demonstrating that nIII received both excitatory and inhibitory synaptic inputs from the NP.

(Page 9 line 385-395) Previous studies suggest that vlPAG played a crucial role in gating REMs, while activating vlPAG GABAergic neurons in mice suppresses the initiation and maintenance of REMs^{12,20}. To further confirm the functional connectivity of nIII-vlPAG projection, we injected AAV2/9-hEF1 α -DIO-hChR2(H134R)-EYFP into the nIII of ChAT-Cre mice and implanted an optrode in the vlPAG to record downstream neuronal activity (Fig. 5e). We observed clear axonal projections from nIII^{ChAT} neurons to vlPAG along rostral-caudal axis in coronal sections (Supplementary Fig. 17a-b). Upon photostimulation of nIII^{ChAT} terminal within the vlPAG with blue laser pulses (480 nm), we observed reliable optogenetically evoked spiking in a single unit (Fig. 5f-g). Among the 61 well-isolated units recorded from 3 mice, 32.8% exhibited optogenetically activated responses, and 9.8% showed optogenetically suppressed responses to nIII^{ChAT} axon activation (Fig. 5h, Supplementary Fig. 18).

Reviewer #2

General response:

Thank you for your detailed and constructive comments.

In this revision, we have carefully addressed all of your concerns in a point-by-point manner. We performed new experiments to directly examine the role of nIII^{ChAT} neurons in regulating EMs and REM sleep. Importantly, the evoked EMs had no significant impact on the sleep-wake architecture. We also revised the manuscript to improve its clarity, scientific rigor, and presentation. **The major revisions are summarized as follows:**

- Additional analyses have been included in the **Results** section to confirm that both the neural recordings and optogenetic manipulations were localized to the oculomotor nucleus (nIII), and not confounded by potential signals from the neighboring Edinger-Westphal nucleus;
- Results from new experiments of *in vivo* electrophysiological recording in nIII^{ChAT} neurons across sleep-wake cycle were added as new figures of **Supplementary Fig. 4** and **Supplementary Fig. 5**, as well as corresponding descriptions in the **Results** section;
- Results from new experiments of chronic ablation of the nIII^{ChAT} neurons and their effect on the EMs during spontaneous sleep-wake cycle has been added as new figures of **Supplementary Fig. 6a-b** and as corresponding descriptions in the **Results** section;
- Results from new experiments of how optogenetically-evoked EMs impact EEG and EMG signals have been added to both as new figures of **Supplementary Fig. 7** and **Supplementary Fig. 10** and corresponding descriptions in the **Results** section;
- The definitions of sleep pressure and the criteria of sleep state classification have been clarified and added to both in the **Methods** and **Results** sections;
- Results from new experiments examining the effects of optogenetic activation of nIII^{ChAT} neurons projecting to the extraocular muscles (EOMs) and those

specifically projecting to the vlPAG on sleep architecture and EMs have been added as new figures of **Fig. 2l-p, Supplementary Fig. 14, Supplementary Fig. 17** and corresponding descriptions in the **Results** section;

- Results from new experiments of the anterograde tracing to label the terminal of nIII^{ChAT} neurons in the vlPAG has been added as new figures of **Supplementary Fig. 19a-c** and corresponding descriptions in the **Results** section;
- New analyses to cross-validate the functional specificity of EMs-active and R-W transition-active nIII^{ChAT} neurons have been added as new figure of **Fig. 4k** and corresponding descriptions in the **Results** section;
- Typos have been corrected and double-checked throughout the manuscript;

A summary of the revisions in response to all the reviewers' comments can be found at the end of the rebuttal letter. We hope that you find the revised manuscript suitable for publication.

Comment 1:

The authors use the term oculomotor nucleus throughout the manuscript without mentioning the fact that there are three oculomotor nuclei and defining the specific roles of each of them. This should be specified in the introduction. It is also welcome since line 76 they show which type of oculomotor movements are generated by the 3rd nucleus specifically. There is a large literature on the role of OMN in eye movements and it would be of great help for them and the readers to define it.

Response:

Thank you for your constructive and insightful comment. We agree with the reviewer that the roles of the other two oculomotor nuclei (i.e., the trochlear nucleus and the abducens nucleus) should be clearly defined in the **Introduction** to help readers better understand the anatomical and functional context of the oculomotor system. **To better differentiate the third oculomotor nucleus from other oculomotor-related**

nuclei, we replaced the abbreviation ‘OMN’ with ‘nIII’ to denote the oculomotor nucleus in the revised manuscript and added new descriptions of the three oculomotor nuclei in the Introduction of the revised manuscript. Details of the descriptions can be seen below.

Eye movements are coordinated by three nuclei, namely oculomotor nucleus (nIII), the trochlear nucleus and the abducens nucleus. The nIII is located in the rostral midbrain at the level of the superior colliculus, medial to the medial longitudinal fasciculus. The trochlear nucleus lies caudally in the midbrain, just ventral to the periaqueductal gray and dorsal to the medial longitudinal fasciculus. The abducens nucleus is situated in the caudal pons, ventral to the fourth ventricle and medial to the facial nerve fibers.

We also specified the modality of eye movements generated by the nIII, the trochlear nucleus and the abducens nucleus in the revised manuscript. Abducting EMs are mediated by motoneurons in the abducens nucleus, whereas the torsional rotation of the eye is controlled by the trochlear nucleus via its innervation of the superior oblique muscle (Escudero and Marquez-Ruiz 2008, Fritzsche 2024). nIII neurons innervate the majority of extraocular muscles. Although several studies have emphasized the nIII’s role in mediating eye elevation and adduction, others have proposed its involvement in vertical and torsional components of oculomotor function (Aladdin et al. 2022, Fritzsche 2024). In our study, optogenetic activation and inhibition of nIII^{ChAT} neurons respectively drive eye movements from the temporal side toward the nasal side, and from the nasal side toward the dorsotemporal direction.

The corresponding revisions in the revised manuscript:

(Page 1-2 line 37-48) REM sleep (REMs) is an “active” phase of sleep hallmarked by prominent EMs (EMs)¹. During REMs, postural motor neurons are inhibited and muscles are relaxed, with the exception of EOMs². As a prominent pool of cholinergic neurons within the midbrain^{3,4}, the oculomotor nucleus (nIII) has been suggested to modulate oculomotility, not only during wakefulness⁵ but also during REMs although

there are some discrepancies^{6,7}. In alert animals, spontaneous EMs consist of voluntary and involuntary saccades that keep and move the eyes to a visual target, enabling visual acquisition^{8,9}. These modality of EMs are coordinated by nIII and another nuclei, trochlear nucleus, and the abducent nucleus. Specifically, abducting and adducting EMs are mediated by motoneurons in the abducens nucleus and nIII, respectively, whereas the torsional rotation of the eye is controlled by the trochlear nucleus via its innervation of the superior oblique muscle^{9,10}. With regard to EMs in sleep, Herrera et al. provided evidence that the activation of the nIII is responsible for the occurrence of EMs during REMs⁷, spawning new data on the behavior of this visuomotor system during sleep.

Comment 2:

Strangely, the authors state in the line 51 in the Introduction that activation of OMN neurons occurs after sleep deprivation not rebound.

Response:

We thank the reviewer for this constructive comment. We apologize for the mistake in the original statement on line 51 of the Introduction, and we have revised the text accordingly in the revised manuscript. The sentence “We first confirmed the activation of nIII neurons occurs after sleep deprivation” has been revised to “We first confirmed the **elevated c-Fos expression** of nIII^{ChAT} neurons during **recovery sleep after** deprivation in mice.” in the revised manuscript.

The corresponding revisions in the revised manuscript:

(Page 2 line 64-65) **We first confirmed the elevated c-Fos expression of nIII^{ChAT} neurons during recovery sleep after deprivation in mice.**

Comment 3:

Interestingly, they found that 37% of the OMN neurons were cFos after a REM rebound despite their low phasic activity during REM displayed by calcium imaging. They use fiber photometry to monitor activity of OMN neurons. I wonder whether they control

that they did not record also the activity of EW cholinergic neurons?

Response:

We thank the reviewer for this insightful comment. We have also added new analyses to confirm that the recorded and activated neurons are located in the nIII, rather than the Edinger-Westphal nucleus (EW). Details can be seen below.

Anatomically, the EW nucleus is located rostral to the nIII in coronal slices. The EW nucleus comprises two major neuronal populations, namely preganglionic parasympathetic neurons (EWpg) and centrally projecting neurons (EWcp). EWpg, although cholinergic, constitute a small population that is cytoarchitecturally distant from the nIII (**Fig. R2-1, Fig. R2-2**). In contrast, the EWcp is located in close proximity to the nIII and consists of non-cholinergic neurons (**Fig. R2-1, Fig. R2-3**) (Yi et al. 2023). Both Tamás Kozicz et al. and Tingting Yi et al. demonstrated that cholinergic neurons in the EW nucleus and those in the nIII do not overlap in the coronal plane (Kozicz et al. 2011, Yi et al. 2023). This spatial separation, together with the limited lateral collection volume below the tip of the optical fiber (typically restricted to a radius of ~200–400 μm below the fiber tip) (Gradinaru et al. 2009, Yizhar et al. 2011), minimizes the possibility of signal contamination from the EW nucleus during fiber photometry recordings targeting the nIII.

In our study, we employed a Cre-loxP strategy in ChAT-Cre mice, thereby excluding the non-cholinergic EWcp neurons from our recordings. Moreover, when we implanted the optical fiber above the nIII for optogenetic stimulation, we did not observe laser-evoked pupillary constriction but EM responses (**Fig. R2-4e-f**), indicating that EMcp neurons were not involved in these experiments.

Together, these anatomical, genetic, and functional evidences strongly indicate that our fiber photometry signals reflect the activity of nIII^{ChAT} neurons, not EW^{ChAT} neurons. We have added the above analysis into the Results and Discussion of the revised manuscript.

[Figure Redacted]

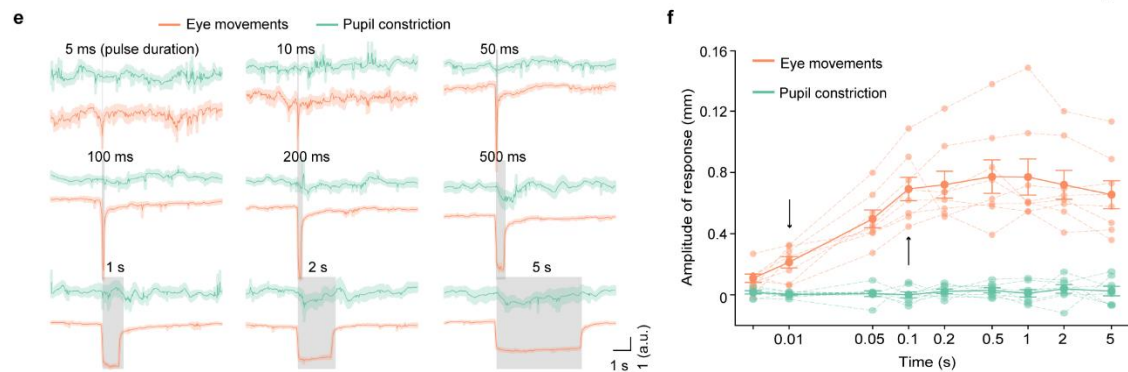
(Fig. 5 in the cited article) Fig. R2-1 | Confocal images from rostral to caudal sections illustrate the spatial distribution of cholinergic neurons. (ChAT⁺, red) within the III and EW. From Tamás Kozicz et al 2010.

[Figure Redacted]

(Fig. 7 in the cited article) Fig. R2-2 | The 3-D organization of EWpg and EWcp. From Tamás Kozicz et al 2010.

[Figure Redacted]

(Fig. 3 in the cited article) Fig. R2-3 | The identification of cell types in the EW (Dashed line marked area) using IF and fluorescence in situ hybridization. From Tingting Yi et al 2023.



(New updated Supplementary Fig.8) Fig. R2-4 | Effect of activating nIII^{ChAT} neurons on the oculomotor and pupil constriction. e Amplitudes of ipsilateral EMs (orange) and pupil constriction (cyan) induced by optical stimuli of varying durations (5 ms to 5 s) in ChAT-Cre mice. The gray shading area indicates the time window for laser stimulus. Orange and cyan shading indicates 95% confidence intervals (n = 8 mice). f Amplitude of laser-evoked EMs and pupil constriction induced by optical stimuli of varying durations (5 ms to 5 s). Error bars represent SEM across mice in each states (n = 8 mice).

The corresponding revisions in the revised manuscript:

(Page 5 line 210-224) **Given that the fundamental role of the nIII is oculomotor control,**

we then examine the dual function of nIII by unilateral injection of AAV2/9-hEF1 α -DIO-hChR2(H134R)-EYFP into the nIII (Supplementary Fig. 8a-b). Three weeks after viral expression, optogenetic activation was performed using 480 nm laser illumination, and the eyeball position of head-fixed mice was recorded with an infrared camera (Supplementary Fig. 8c). We observed a reliable ipsilateral EMs in temporal-to-nasal direction but had no effect on the pupil constriction (Supplementary Fig. 8d-f). These results confirm that we selectively activated the nIII without affecting the adjacent Edinger-Westphal (EW) nucleus. The amplitude of EMs increased as the pulse durations increased from 5 ms to 100 ms, and reached a plateau as the durations increased further (Supplementary Fig. 8e-f). When the duration of the optogenetic pulse exceeded 500 ms, it might induce a prolonged contraction of the oculomotor muscles, which was subsequently followed by sustained ocular immobility (Supplementary Fig. 8e). Moreover, time-locked EMs induced by low-frequency laser pulses (3.3 Hz, 100 ms duration) were robust, while 20 Hz optical stimulation induced very smaller amplitude of EMs (Supplementary Fig. 8g-h, Supplementary Video1 and 2).

(Page 12-13 line 521-535) The nIII is located in close proximity to the Edinger-Westphal (EW) nucleus, which also contains cholinergic neurons. The cholinergic population in the EW represents preganglionic parasympathetic neurons (EWpg), which are primarily confined to the rostral part of the EW and are cytoarchitecturally distant from the nIII⁴⁴. Previous studies by Tingting Yi et al. and Tamás Kozicz et al. demonstrated that cholinergic neurons in the EW and those in the nIII do not overlap in the coronal plane^{18,44}. This anatomical separation, combined with the limited lateral spread of signal collection in fiber photometry recordings (typically within a radius of ~200–400 μ m below the fiber tip)^{45,46}, minimizes the possibility of signal contamination from the EW when targeting the nIII. Although the EW also contains non-cholinergic, centrally projecting neurons (EWcp) that have been implicated in the regulation of NREM sleep¹⁸, our use of a Cre-loxP strategy in ChAT-Cre mice ensured that these non-cholinergic EWcp neurons were excluded from our recordings. Furthermore, when we implanted the optical fiber above the nIII for optogenetic

stimulation, we observed laser-evoked eye movement responses but not pupillary constriction (Supplementary Fig. 8), further indicating that EW neurons were not involved in these experiments.

Comment 4:

Line 108 they look at activity of the OMN neurons at transitions and state that it is also increased during REM sleep outside the EMs. Unfortunately fiber photometry is not a method sensitive enough to monitor such modifications. The description line 112-119 of the fiber photometry recordings results is not clear at all and cannot lead to the conclusion stated that OMN^{Chat} neurons are more active during REM without EMs. For that, the authors would need to make electrophysiological unit recordings of the OMN Chat neurons.

Response:

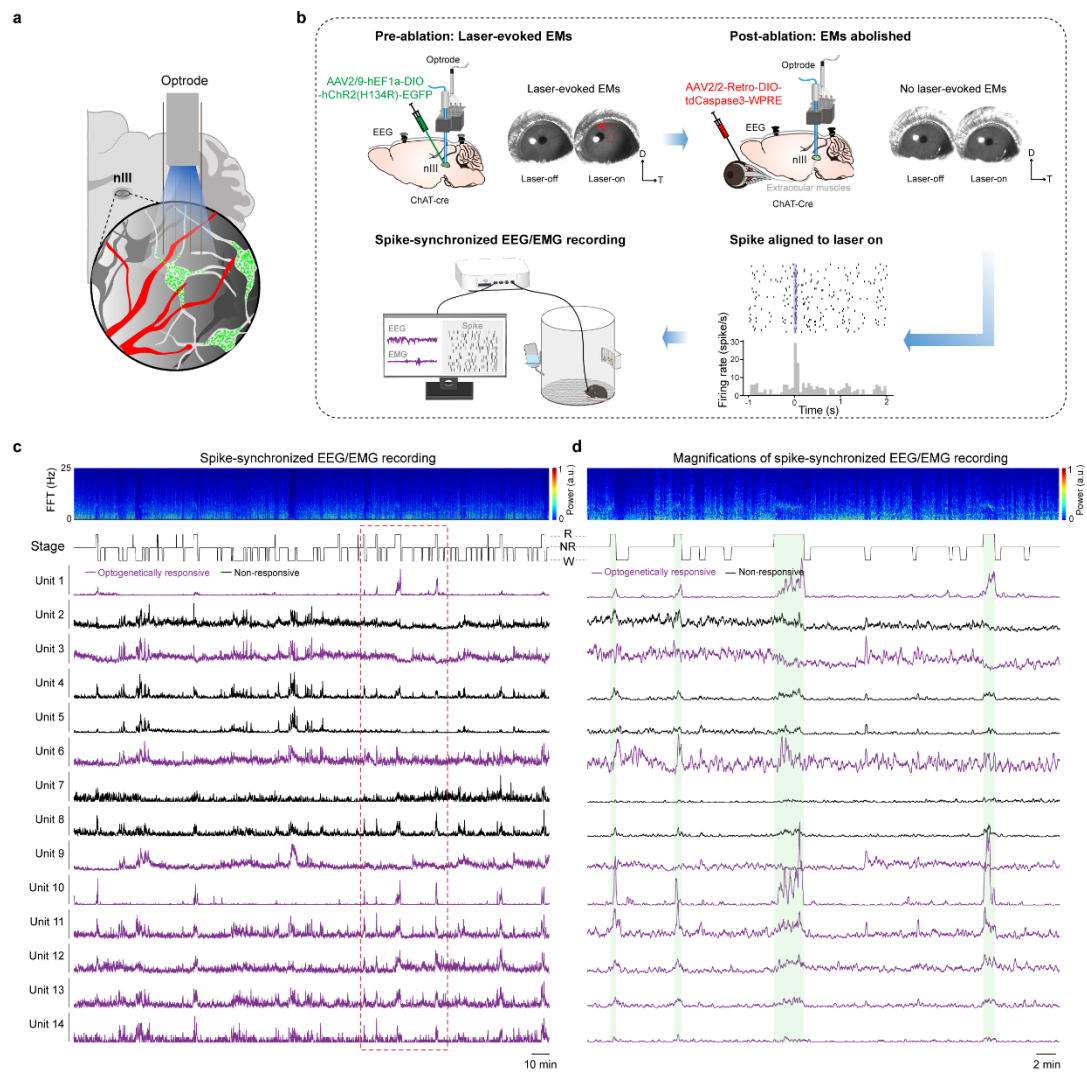
Thank you for your constructive and insightful suggestions. **We performed new electrophysiology experiments to validate our conclusions and revised the corresponding statements in the manuscript.**

To record neuronal activity from non-extraocular muscle-projecting nIII^{ChAT} neurons, we first injected AAV2/9-hEF1 α -DIO-hChR2(H134R)-EYFP into the nIII of ChAT-Cre mice and implanted optrode at nIII. Each optrode comprises 20 channels in total, including 16 nickel-titanium alloy wire electrodes (20 μ m internal diameter; impedance 250-900 k Ω) for spike recording arranged around an optical fiber, along with 4 additional silver wire electrodes for EEG and EMG recordings. Using optrode, we were able to identify ChR2-expressing neurons based on laser-evoked firing and EMs while simultaneously recording EEG/EMG signals (**Fig. R2-5a-b**). Subsequently, we injected retrograde DIO-tdCaspase3-WPRE into the extraocular muscles to selectively ablate nIII^{ChAT} neurons that project to these muscles (**Fig. R2-5b**). This ablation abolished laser-evoked EMs (**Fig. R2-6a-b**), confirming that the recorded neurons no longer innervated extraocular targets.

We performed single-unit optrode recordings in combination with EEG/EMG

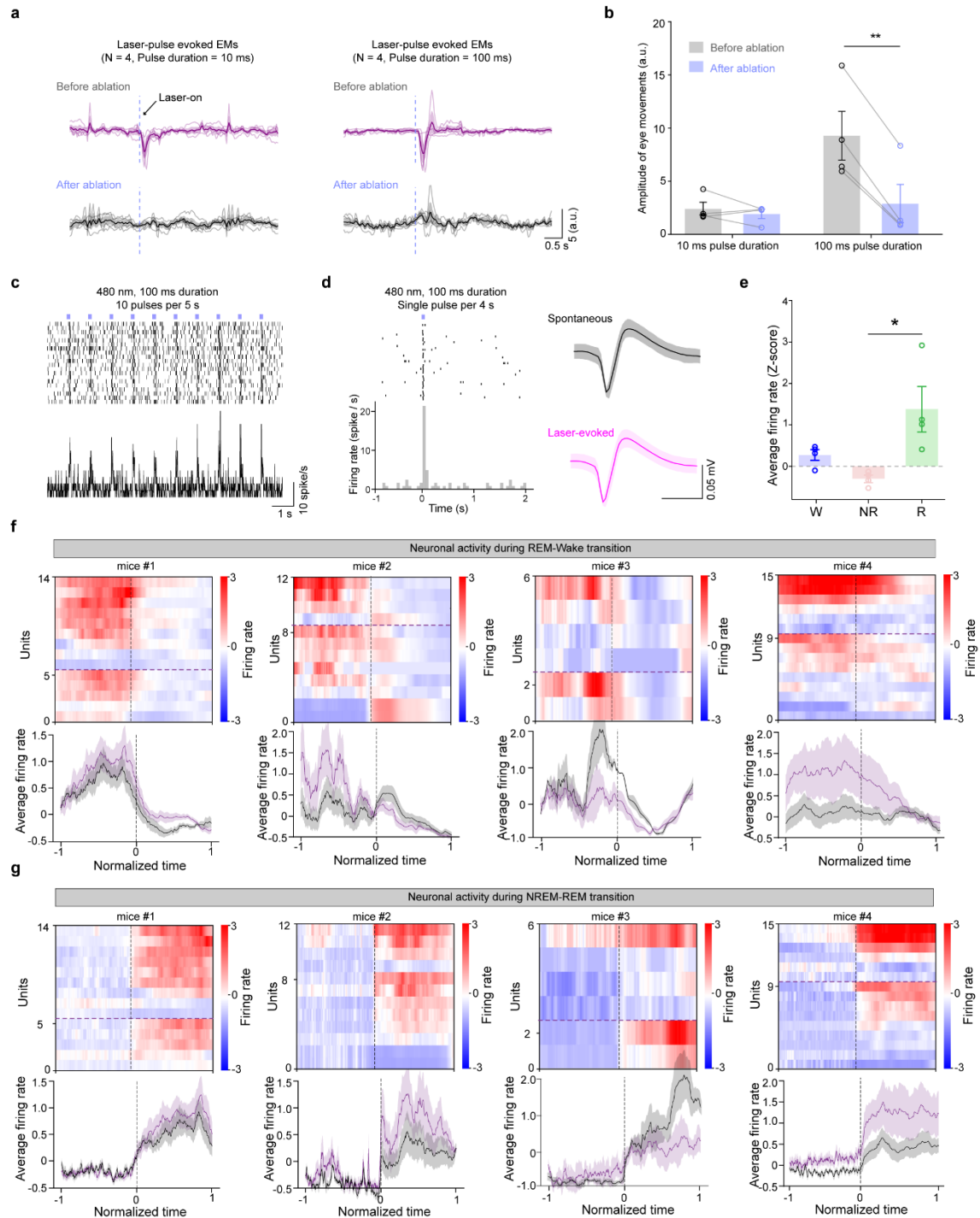
during natural sleep. The purple traces in **Fig. R2-5c-d** represent the spontaneous activity of optogenetically responsive $nIII^{ChAT}$ neurons across the sleep-wake cycle. We observed that the non-extraocular muscles-projecting $nIII^{ChAT}$ neurons exhibited higher firing rates during REM sleep compared to NREMs and wakefulness (**Fig. R2-6e**). Interestingly, the firing rates of $nIII^{ChAT}$ neurons increased gradually over the course of each REM sleep episode before the transition to wakefulness or after the transition from NREM sleep (**Fig. R2-6f-g**). The consistency of this activity pattern across recorded units supports the conclusion that $nIII^{ChAT}$ neurons are indeed active during REM sleep, even in the absence of EMs.

We have added these new results in the Result section and have included a detailed description in the corresponding sections of Method in revised manuscript.



(New added Supplementary Fig.4) Fig. R2-5 | Optogenetics-assisted electrophysiological recording the activity nIII^{ChAT} neurons across sleep-wake cycle. **a** Schematic drawing of ChR2-tagged nIII^{ChAT} neurons (green) using electrophysiology recording after co-injection of AAV2/2-Retro-DIO-tdCaspase3 (red) in the EOMs and AAV2/9-hEF1α-DIO-hChR2-EGFP (green) in the nIII. **b** The schematic of the strategy to recording the activity of non-EOMs-projecting nIII^{ChAT} neurons. The AAV2/9-hEF1α-DIO-hChR2-EGFP was injected into the nIII followed by the optrode implantation. Once the laser-evoked EMs were recorded, the AAV2/2-Retro-DIO-tdCaspase3 was injected into the EOMs to ablate the EOMs-projecting nIII^{ChAT} neurons and the evoked-EMs were disappeared. The spike and EEG/EMG were concurrently recorded to monitoring the activity of non-EOMs-projecting nIII^{ChAT}

neurons across sleep-wake cycle. **c** Representative firing rates recorded from multiple units across sleep-wake episodes. The purple trace indicates a unit that exhibits pulse-dependent responses during optogenetic stimulation. **d** Enlarged view of the dashed time window shown in panel **c**.



(New added Supplementary Fig.5) Fig. R2-6 | Fire rates of the non-EOMs-projecting nIII^{ChAT} neurons during REM transition. a Average traces of laser-

evoked EMs before (upper) and after (lower) ablation of EOM-projecting nIII^{ChAT} neurons. Left: 10 ms pulse duration at 2 mW power density; Right: 100 ms pulse duration at 2 mW power density. Each dot indicates data from one mouse (n = 4 mice).

a Quantification of Laser-evoked EMs amplitude before and after cellular ablation. ****p** = 0.003 and $t_5 = 3.071$. Two-sided paired t-test. **c** Representative ChR2-tagged unit showing pulse-dependent firing rate across multiple laser-pulse stimulation. The purple boxes indicate the laser pulse (10 ms per pulse). **d** Left: raster plot (upper) and stimulus time histogram (50 bins, lower) for laser-evoked spikes of an example ChR2-tagged unit. The purple boxes indicate the laser pulse. Laser duration, 10 ms. Power density, 2 mW. Right: averaged waveform of spontaneous (gray) and laser-evoked (purple) spike of an example unit. **e** Quantification of the average fire rate of non-EOMs-projecting nIII^{ChAT} neurons cross different vigilance state episodes. Each dot represents one mouse (n=4 mice). One-way ANOVA followed by Bonferroni's post hoc analysis between within states. Data are presented as mean $\pm$ SEM. ***p** < 0.05. **f** Upper: the heatmap show the fire rate of non-EOMs-projecting nIII^{ChAT} neurons during REM-to-wake (R-W) transition from 4 mice. The units above the horizontal dashed line are that exhibits pulse-dependent responses during optogenetic stimulation. The vertical dashed line indicates the time point of R-W transition. Lower: the average fire rate of selected units (purple, units above the horizontal dashed line in the heatmap) and non-selected units (gray) during R-W transition. The shielding indicates the 95% confidence intervals (CIs). **g** Upper: the heatmap show the fire rate of non-EOMs-projecting nIII^{ChAT} neurons during NREM-to-REM (NR-R) transition from 4 mice. The units above the horizontal dashed line are that exhibits pulse-dependent responses during optogenetic stimulation. The vertical dashed line indicates the time point of NR-R transition. Lower: the average fire rate of selected units (purple, units above the horizontal dashed line in the heatmap) and non-selected units (gray) during NR-R transition. The shielding indicates the 95% confidence intervals (CIs).

The corresponding revisions in the revised manuscript:

(Page 4 line 143-163) To further corroborate these finding, we performed *in vivo* electrophysiological recordings of non-EOMs-projecting nIII^{ChAT} neurons (Supplementary Fig. 4a). we first injected AAV2/9-hEF1 α -DIO-hChR2(H134R)-EYFP into the nIII of ChAT-Cre mice and implanted optrode at nIII. Each optrode comprises 20 channels in total, including 16 nickel-titanium alloy wire electrodes for spike recording arranged around an optical fiber, along with 4 additional silver wire electrodes for EEG and EMG recordings. Using optrode, we were able to identify ChR2-expressing nIII^{ChAT} neurons based on laser-evoked firing and EMs while simultaneously recording EEG/EMG signals (Supplementary Fig. 4b). Subsequently, we injected retrograde DIO-tdCaspase3-WPRE into the EOMs to selectively ablate nIII^{ChAT} neurons that project to EOMs. This ablation almost completely abolished laser-evoked EMs (Supplementary Fig. 5a-b), confirming that the recorded neurons barely innervated EOMs.

After 3 weeks recovery, single units exhibiting reliable laser-evoked spiking from ChR2-tagged, putative non-EOMs-projecting nIII^{ChAT} neurons (Supplementary Fig. 5c-d). We then performed long-term spike recordings combined with EEG and EMG monitoring across the sleep-wake cycles (Supplementary Fig. 4b). Supplementary Fig. 4c-d shows the activity of non-EOMs-projecting nIII^{ChAT} neurons across the sleep-wake cycle. In line with the fiber photometry findings (Fig. 1g), nIII^{ChAT} neurons exhibited the highest firing rate during REMs. Their firing rate increased gradually over the course of each REMs episode before the transition to wake or after the transition from NREMs (Supplementary Fig. 5f-g). The consistency of this activity pattern across recorded units supports the notion that nIII^{ChAT} neurons are indeed active during REMs, even in the absence of EMs.

Comment 5:

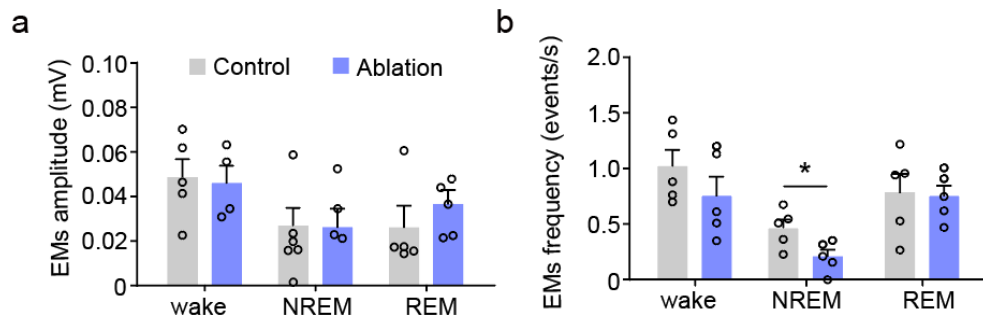
The authors then ablate the OMNChat neurons to look at the effect on REM sleep. Strangely, they don't directly determine whether it abolishes the EMs both during wakefulness and REM sleep. This is really strange. I anticipate that the effect might not

be that impressive since both the 4th and 6th cranial nuclei are known to be involved. It is crucial that the authors analyze whether EMs are impacted by their lesion.

Response:

Thank you for your insightful comment. As the reviewer has correctly pointed out, the nIII is not the sole nucleus involved in oculomotor control, with the trochlear (4th) and abducens (6th) nuclei also playing important roles.

We conducted new analysis to evaluate the change of EMs across sleep-wake cycles after nIII^{ChAT} neuronal ablation. The ablation of nIII^{ChAT} neurons did not lead to a significant change in either the amplitude or frequency of EMs during wakefulness and REM sleep (Fig. R2-7a-b). We have added these new results in the Result section in revised manuscript.



(New added Supplementary Fig.6) Fig. R2-7 | Effects on EMs and sleep architecture after silencing of nIII^{ChAT} neurons. a-b Effects of bilateral ablation of the nIII on overall amplitude (a) and frequency (b) of EMs cross vigilance states (n=5 for control and n=4 for ablation group). *p<0.05 for EMs frequency during NREM sleep. Two-sided unpaired t-test.

The corresponding revisions in the revised manuscript:

(Page 5 line 181-187) The ablation of nIII^{ChAT} neurons had no significant effect on the duration and number of wake or NREMs episodes (Supplementary Fig. 6d-e). We also did not observe a significant change in the overall amplitude and frequency of EMs during wake and REMs (Supplementary Fig. 6b-c). The frequency of EMs during NREMs was significantly decreased (Supplementary Fig. 6c). These findings indicate that ablation of nIII^{ChAT} neurons likely facilitates the maintenance of REMs and

consequently impairs the physiological transition from REMs to wake.

Comment 6:

It is even more important since they report an increase in REM sleep and a decrease in Wake after such ablation. What are their criteria to define the two states? Is it muscle and EEG? And in such case, are they sure of their distinction between the two? When conversely they activate the OMNchat neurons they obtain an increase in EMs and a termination of REM sleep.

Response:

Thank you for your constructive comments. We first clarified our criteria to define REM and Wake states. We also conducted new analysis to investigate whether activation of nIII Chat neurons will affect the definition of sleep status. Details can be seen below.

In mammalian models, sleep states are identified using electroencephalogram (EEG) recordings of cortical activity in combination with electromyogram (EMG) recordings of skeletal muscle tone (Liu and Dan 2019). In our study, sleep-wake states were identified according to the previous reported criteria (Liu and Dan 2019, Jiang et al. 2024). We defined wakefulness as desynchronized, low-amplitude, and high-frequency EEG rhythms with elevated EMG activity. NREM sleep was defined as high-amplitude, and low frequency (0.5-4 Hz) EEG activity with lower tonic EMG tone, whereas REM sleep was defined as containing prominent theta (6-9 Hz) oscillations on EEG and minimal EMG activity.

In our study, EEG signals were collected from two stainless-steel screws implanted over the frontal (AP +1 mm, ML +1.5 mm) and parietal (AP -2 mm, ML -1.5 mm) cortices. For nIII^{ChAT}-ablated mice, the EEG power spectra across different behavioral states (Wake, NREM, REM) were comparable to those of control mice during the same states (**Fig. R2-8a**). The characteristic spectral peaks of EEG for each state were clearly preserved following the nIII^{ChAT} ablation (**Fig. R2-8a**).

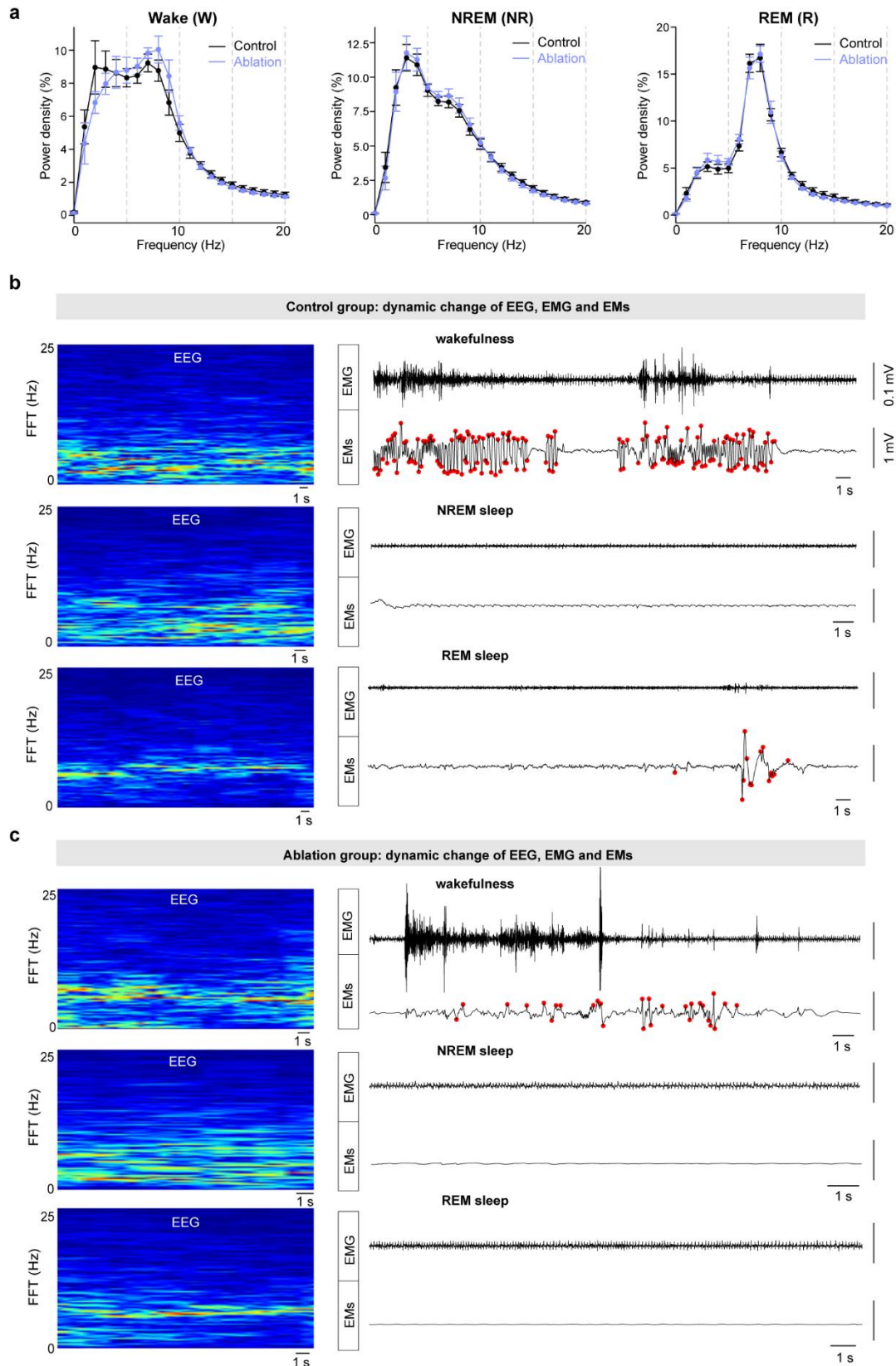
EMG signals in our study were recorded from two electrodes inserted into the neck

muscles. These signals primarily reflect **locomotor activity and postural muscle tone of animal**. As shown in the top panel of **Fig. R2-8b-c**, wake episodes are characterized by intense muscle activity, whereas both NREM and REM sleep episodes were accompanied by profound muscle atonia and minimal EMG signal. The ablation of $nIII^{ChAT}$ neurons shows no detectable change in the EMG waveform during wake, NREM and REM sleep compared to those in control mice.

We also conducted new analysis to inspect whether EEG and EMG signals were affected by optogenetically-induced EMs. As shown in **Fig. R2-9**, we visualized the temporal dynamics of EEG, EMG, and EMs during the pre-stimulation (pre-stim), pre-stimulation-to-stimulation (pre-stim.>>stim.), and stimulation (stim.) periods. The EEG and EMG signals exhibited typical features of wakefulness, NREM, and REM sleep, as above-mentioned. Upon optogenetic stimulation, pulse-dependent EMs were reliably induced, as indicated by the blue dots on the EMs trace. Importantly, the laser-evoked EMs had no observable effect on the patterns of EEG and EMG across all vigilance states (wakefulness, NREM, and REM) (**Fig. R2-9a, c, e**, data from the optogenetic activation of $nIII^{ChAT}$ neurons). Notably, the induced EMs did not trigger arousal, indicated by the absence of EMG elevation. Furthermore, during optogenetic activation of $nIII^{ChAT}$ neurons, the characteristic spectral peaks for each state were preserved, indicating that modulation of $nIII^{ChAT}$ activity did not perturb the overall EEG architecture (**Fig. R2-9b, d, f**).

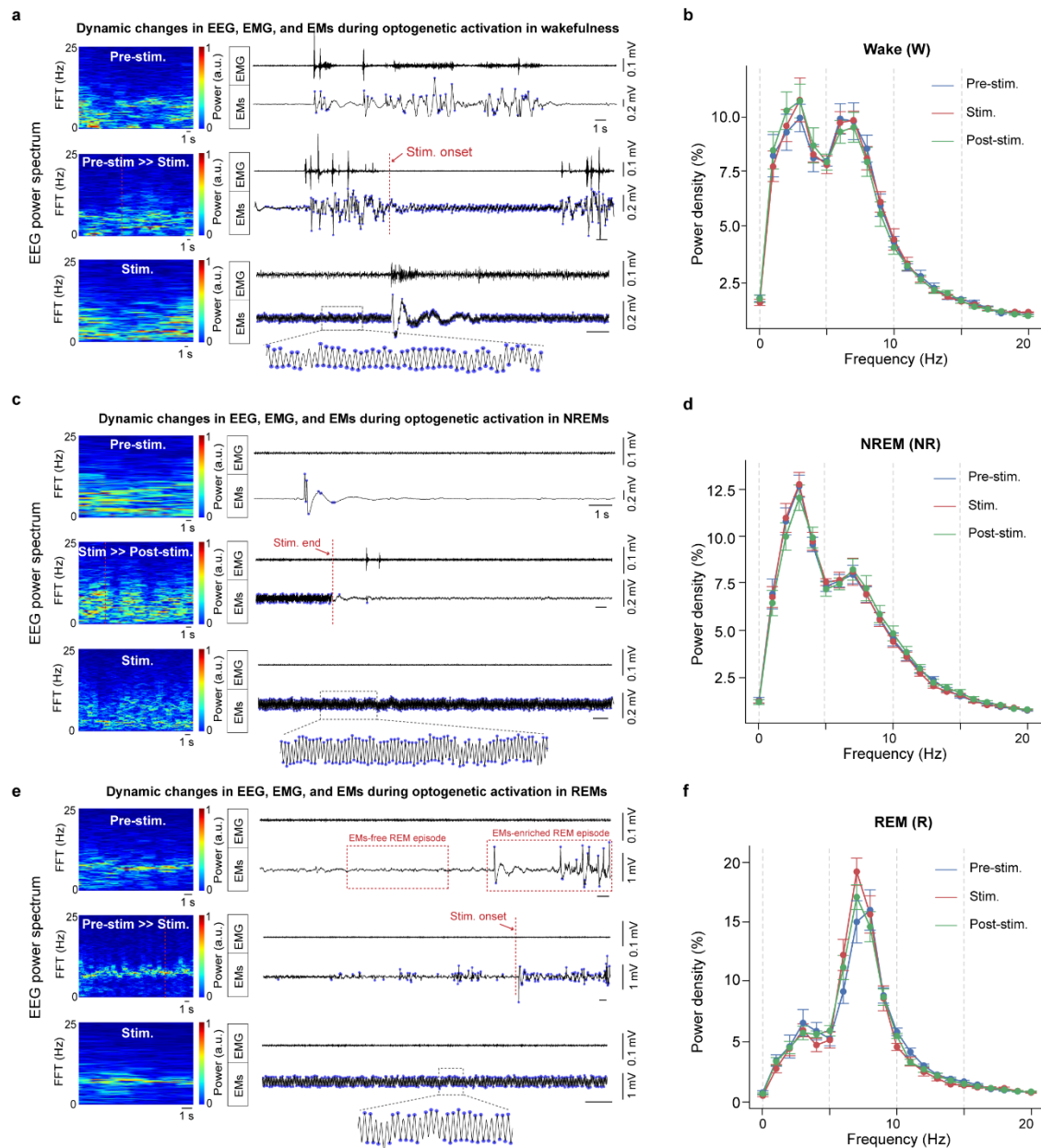
Taken together, these results demonstrate that both spontaneous and optogenetically evoked EMs do not interfere with EEG or EMG recordings, hence do not compromise the reliability of sleep-wake state classification in our study.

We have added these results in the Result section and have included a detailed description in the corresponding sections of Method in revised manuscript.



(New added Supplementary Fig. 6) Fig. R2-8 | EEG, EMG and EMs during wake, NREMs and REMs in $nIII^{ChAT}$ -ablated mice. a EEG power density in wake, NREM sleep, and REM sleep for control ($n = 6$) and $nIII^{ChAT}$ neuronal ablation group ($n = 7$).

Two-way ANOVA revealed no significant differences. **b** Dynamic change in EEG power spectrum (left), EEG and EMs (right) during wake, NREMs and REMs episodes in normal mice. The wake episodes is enriched with periodic EMs, and high amplitude of EMG. The NREM episodes are characterized by absence of EMs and low amplitude of EMG. The REM episodes are characterized by tonic or phasic EMs and low amplitude of EMG. The red dots in the EMs trace indicate the EMs event. **c** Dynamic change in EEG power spectrum (left), EEG and EMs (right) during wake, NREMs and REMs episodes in nIII^{ChAT}-ablated mice. The red dots in the EMs trace indicate the EMs event.



(New added Supplementary Fig. 10) Fig. R2-9 | Dynamic change of EMs, EEG and EMG during optogenetic activation. **a** Dynamic change in EEG power spectrum (left), EEG and EMs (right) during optogenetic activation of nIII^{ChAT} neurons in wake episodes. The wake episodes is enriched with periodic EMs, and high amplitude of EMG. The optogenetic activation evoked sustained high-frequency EMs. The blue dots in the EMs trace indicate the automatically detected EMs event. **b** Quantification of EEG power density during optogenetic activation of nIII^{ChAT} neurons in wakefulness (n = 6). Two-way ANOVA revealed no significant differences. **c** Dynamic change in EEG power spectrum (left), EEG and EMs (right) during optogenetic inhibition in NREM

sleep. The NREM episodes are characterized by a paucity of EMs and low amplitude of EMG. The optogenetic activation evoked sustained high-frequency EMs. The blue dots in the EMs trace indicate the automatically detected EMs event. **d** Quantification of EEG power density during optogenetic activation of $nIII^{ChAT}$ neurons in NREM sleep ($n = 6$). Two-way ANOVA revealed no significant differences. **e** Dynamic change in EEG power spectrum (left), EEG and EMs (right) during optogenetic activation in REM sleep. The REM episodes are characterized by tonic or phasic EMs and low amplitude of EMG. The optogenetic activation evoked sustained high-frequency EMs. The dashed red box indicate the EMs-free or EMs-enriched REM episodes. The blue dots in the EMs trace indicate the automatically detected EMs event. **f** EEG power density during optogenetic activation of $nIII^{ChAT}$ neurons in REM sleep ($n = 6$). Two-way ANOVA revealed no significant differences.

The corresponding revisions in the revised manuscript:

(Page 4-5 line 174-181) The hourly amounts of REMs in ChAT-Cre mice were markedly increased at the expense of wake during both the light and dark phases following the ablation of $nIII^{ChAT}$ neurons (Fig. 2g-h). The EEG power spectra of each brain state (wake, NREMs and REMs) following ablation-induced increase of REMs were similar to those displayed during spontaneous sleep- wake (Supplementary Fig. 6a). Detailed analysis revealed that the substantial increase in total REMs time observed in $nIII^{ChAT}$ -ablated mice was due to an augmentation not in the number but in the duration of REMs episodes (Fig. 2i-j). As a consequence of less fragmentation of REMs in $nIII^{ChAT}$ -ablated mice, the number of R-W transitions was decreased (Fig. 2k). (Page 6 line 225-240) To elucidate the role of $nIII^{ChAT}$ neurons in R-W transition, acute optogenetic activation were delivered for 2 min per trial during polysomnographic recordings, with stimulation starting at least 20 s after the onset of stable NREMs or REMs. A total of 10-15 blocks were conducted, with a minimum interval of 10 minutes between blocks (Supplementary Fig. 9a). Optogenetic stimulations were conducted between 12:00-17:00, a period when mice exhibit high sleep stress and increased

propensity to fall asleep²⁶. We also examined the dynamic changes in EEG, EMG, and EMs across different vigilance states (Supplementary Fig. 10). We found that optogenetic activation reliably induced EMs regardless of the current vigilance state, with rapidly increased EMs rate during the stimulation window (Supplementary Fig. 9b-c). The EEG power spectra, EMG of each brain state during optogenetic activation were similar to those displayed during spontaneous sleep-wake cycles indicating that the evoked EMs did not interfere with EEG/EMG signals, thus not affecting the accurate identification of sleep stages (Supplementary Fig. 10a-f). We found that activating nIII^{ChAT} neurons awakened the mice, producing EEG and EMG waveforms characteristic of wake (Supplementary Fig. 9d-e), accompanied by a significant reduction in the theta wave oscillation during stimulation (which is typically dominant during REMs) (Supplementary Fig. 9f).

(Page 19 line 817-825) All sleep-wake scoring was semi-automatic based on spectral signatures of EEG and EMG waveforms in 4 s epochs. Sleep-wake states were classified according to previously reported criteria^{23,24}. Sleep-wake state classification is manually performed based on visual and spectral signatures of EEG and EMG signals. States were assigned across consecutive non-overlapping 4 s windows. We defined wakefulness as desynchronized, low-amplitude, and high-frequency EEG rhythms with elevated EMG activity. NREM sleep was defined as high-amplitude, and low frequency (0.5-4 Hz) EEG activity with lower tonic EMG tone, whereas REM sleep was defined as containing prominent theta (6-9 Hz) oscillations on EEG and minimal EMG activity.

Comment 7:

Line 155 what the authors mean with high sleep stress levels?

Response:

Thank you for your valuable comment. We agree with the reviewer that the word “sleep stress” is confusing and needs revision.

In mammals, prolonged wakefulness leads to an increased propensity to fall asleep (Silverman et al. 2025, Tatsuki et al. 2016). **We used ‘increased propensity to fall**

asleep’ instead of ‘sleep stress’ to describe in the revised manuscript.

The corresponding revisions in the revised manuscript:

(Page 6 line 227-230) A total of 10-15 blocks were conducted, with a minimum interval of 10 minutes between blocks (Supplementary Fig. 9a). Optogenetic stimulations were conducted between 12:00-17:00, a period when mice exhibit high sleep stress and increased propensity to fall asleep²⁶.

Comment 8:

In summary, the authors found that OMNChat neurons induce EMs as previously described and I wonder whether their stimulation do not indirectly induces wakefulness.

Response:

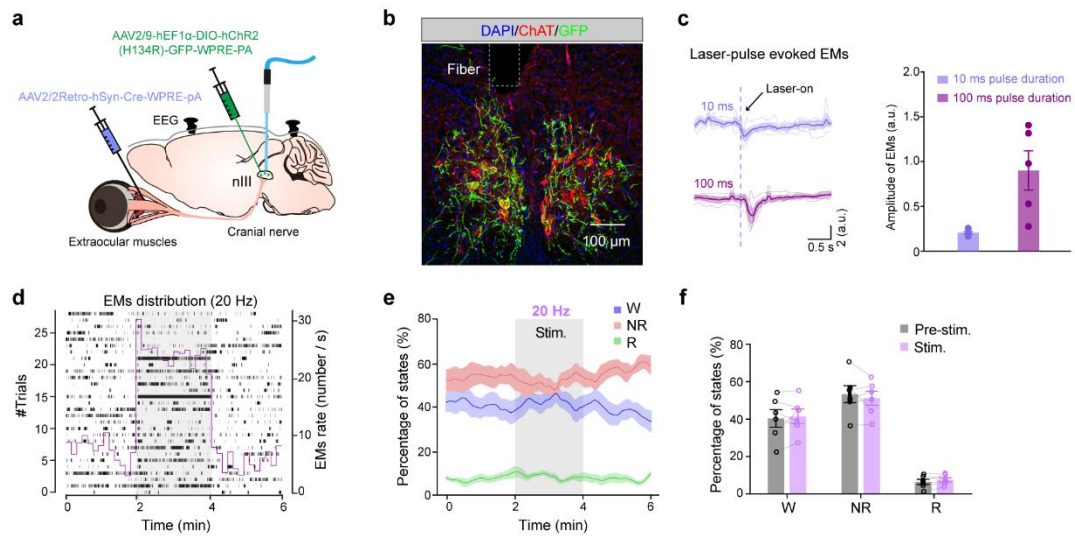
Thank you for your insightful comment. We agree that it is important to distinguish whether activation of nIII^{ChAT} neurons modulates REM-to-wake transitions directly or indirectly through induced EMs. **We first conducted new experiments to demonstrate that activating extraocular-muscle-projecting nIII^{ChAT} neurons does not induce wakefulness. We then activated non-extraocular-muscle-projecting nIII^{ChAT} neurons (to prevent the induction of EM) to examine their role in wakefulness induction.** Details are shown below.

To examine whether nIII-induced-EMs causes wakefulness, we first conducted new experiments to inject retroAAV-hSyn-Cre-WPRE into the extraocular muscles of C57BL/6 mice and subsequently injected AAV2/9-hEF1 α -DIO-ChR2(H134R)-GFP-WPRE into the nIII to selectively express ChR2 in nIII^{ChAT} neurons projecting to the extraocular muscles (**Fig. R2-10a-b**). Optogenetic activation of these neurons induced robust EMs (**Fig. R2-10c-d**), yet did not significantly alter the proportion of time spent in different sleep states (**Fig. R2-10e-f**). These results support the conclusion that activation of nIII^{ChAT} neurons directly drives REM-to-wake transitions, rather than exerting an indirect influence via evoked EMs.

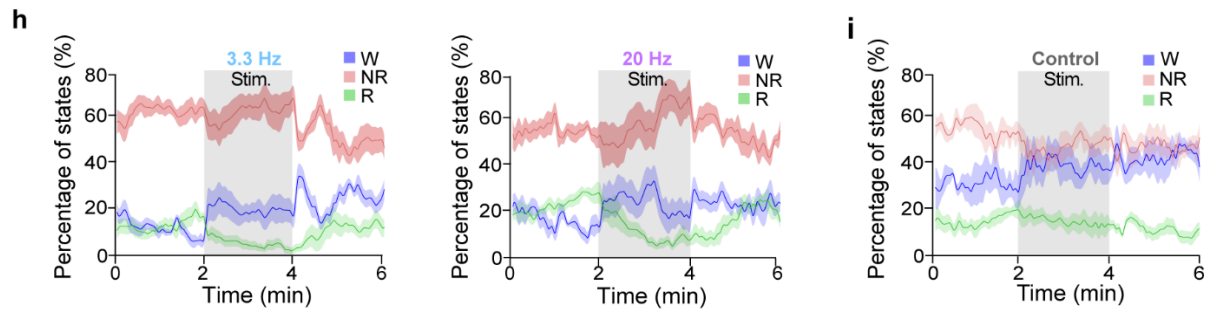
To further elaborate on the importance of the reviewer’s comment, our conclusion

of nIII^{ChAT} neurons being REM sleep terminating is based on the experiments of optogenetically activating non-extraocular-muscle-projecting nIII^{ChAT} neurons, excluding the possibility of inducing EMs (**Fig. R2-11h-i**).

We have added these new results in the Result section and included a detailed description in the corresponding sections of Method in the revised manuscript.



(New added Supplementary Fig.14) Fig. R2-10 | Effect of optogenetic activation of EOMs-projecting nIII^{ChAT} neurons on sleep. **a** Schematic of viral injection and fiber implantation. **b** The confocal image of viral expression and location of fiber in the nIII. **c** Left: EMs trace induced by optogenetic activation of EOMs-projecting nIII^{ChAT} neurons. Right: quantification of laser-evoked EM amplitudes under 10 ms and 100 ms pulse durations. Each dot represents an individual mouse. **d** Raster plot of EMs events during optogenetic activation of EOM-projecting nIII^{ChAT} neurons. Gray shading indicates the time window of optogenetic stimulation. **e-f** Percentage (e) and quantification (f) of wake, NREM, or REM states before, during, and after optogenetic activating the EOMs-projecting nIII^{ChAT} neurons. The gray shielding indicates the time window of stimulation with 20 Hz. Gray shading indicates the time window of optogenetic stimulation. The red, blue and green shielding indicates the 95% confidence intervals (CIs).



(New updated Fig.3) Fig. R2-11 | Change of sleep architecture following optogenetic activating the non-EOMs-projecting nIII^{ChAT} neurons. **h** Percentage of wake, NREM, or REM state before, during, and after optogenetic activating non-EOMs-projecting nIII^{ChAT} neurons. Left: 3.3 Hz at 480 nm, n=8. Right: 20 Hz at 480 nm, n=8. The gray shielding indicates the time window of stimulation. The red, blue and green shielding indicates the 95% confidence intervals (CIs). **i** Percentage of wake, NREM, or REM states before, during, and after 590nm optogenetic stimulation (control, n=8 mice). The gray shielding indicates laser stimulation window. The red, blue and green shielding indicates 95% confidence intervals (CIs).

The corresponding revisions in the revised manuscript:

(Page 7-8 line 296-309) When the optical stimulation was specifically delivered during the REMs period, the probability of REMs decreased drastically from 55.6% to 19.7% upon 3.3 Hz optogenetic stimulus or to 16.3% by 20 Hz optogenetic stimulus (Fig. 3j-k). However, no discernible alteration was noted when the optogenetic stimulus were presented during wakefulness or NREMs (Supplementary Fig. 12e-h). Consistently, the latency from the onset of light stimulus to the R-W transition was significantly shortened upon optogenetic stimulations (Fig.3m-o).

To further dissociate EMs control from REMs modulation, we injected retroAAV-hSyn-Cre-WPRE-pA into the EOMs of C57 mice and subsequently injected AAV2/9-hEF1 α -DIO-ChR2(H134R)-GFP-WPRE into the nIII to selectively express ChR2 in nIII^{ChAT} neurons projecting to the EOMs (Supplementary Fig. 14a-b). Optogenetic activation of these neurons induced robust EMs (Supplementary Fig. 14c-d), yet did not significantly alter the proportion of time spent in different sleep states

(Supplementary Fig. 14e-f). These results support the conclusion that activation of nIII^{ChAT} neurons directly drives R-W transitions, rather than exerting an indirect influence via evoked EMs.

Comment 9:

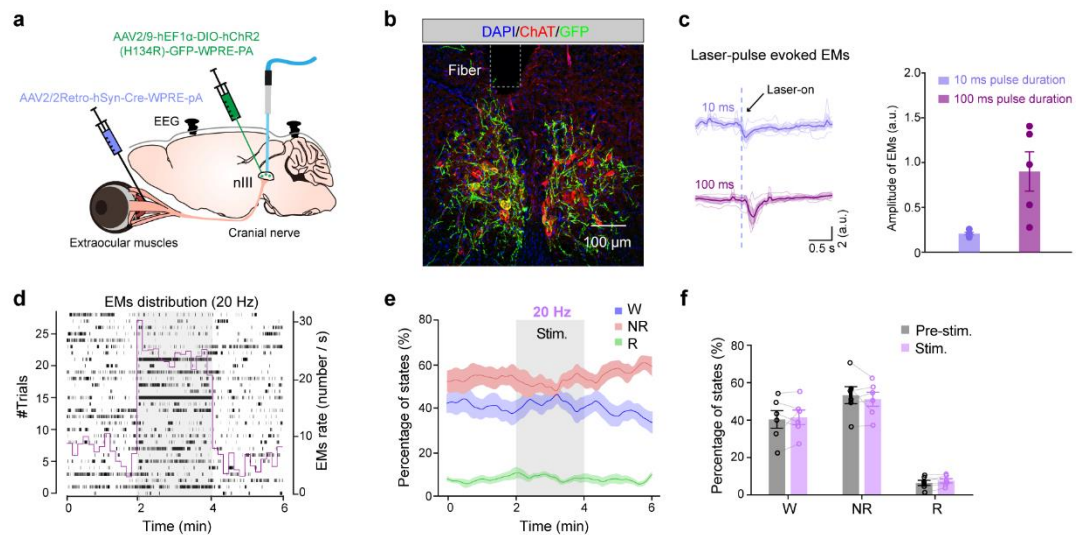
Then the authors attempt to specifically eliminate the OMNchat projecting to the extraocular muscle using retrograde tracing from them. They found that 33% of the OMN neurons are retrogradely labeled and concluded that the remaining ones don't project to the extraocular muscles. They cannot state so since it completely depends on the efficacy of their retrograde tracer. In fact, no retrograde tracer is sensitive enough to label all neurons projecting to an injected area. This claim is supported by their demonstration that optogenetic stimulation of the remaining neurons still induces EMs although it was logically decreased.

Response:

We thank the reviewer for raising this important point. We fully agree with the reviewer that retrograde tracing methods may not achieve 100% labeling efficiency. **We have revised the manuscript to clearly state the possibility that remaining neurons may still induce EMs.**

As can be seen in **Reply to Reviewer 2 Comment 8 (Fig. R2-12)**, to make our conclusions on the roles of nIII^{ChAT} neurons more solid given that optogenetic stimulation of remaining nIII neurons may still induce EMs, we conducted new experiments to provide evidences that activating extraocular-muscle-projecting nIII^{ChAT} neurons did not change sleep status. Results from this experiment further supports the notion that non-extraocular-muscle-projecting nIII^{ChAT} neurons promotes REM-to-wake transition.

We have clarified these limitations in the discussion section of revised manuscript.



(New added Supplementary Fig.14) Fig. R2-12 | Effect of optogenetic activation of EOMs-projecting nIII^{ChAT} neurons on sleep. **a** Schematic of viral injection and fiber implantation. **b** The confocal image of viral expression and location of fiber in the nIII. **c** Left: EMs trace induced by optogenetic activation of EOMs-projecting nIII^{ChAT} neurons. Right: quantification of laser-evoked EM amplitudes under 10 ms and 100 ms pulse durations. Each dot represents an individual mouse. **d** Raster plot of EMs events during optogenetic activation of EOM-projecting nIII^{ChAT} neurons. Gray shading indicates the time window of optogenetic stimulation. **e-f** Percentage (**e**) and quantification (**f**) of wake, NREM, or REM states before, during, and after optogenetic activating the EOMs-projecting nIII^{ChAT} neurons. The gray shielding indicates the time window of stimulation with 20 Hz. Gray shading indicates the time window of optogenetic stimulation. The red, blue and green shielding indicates the 95% confidence intervals (CIs).

The corresponding revisions in the revised manuscript:

(Page 7 line 274-285) To this end, we first investigated the projections of nIII^{ChAT} neurons to the EOMs, finding that these projections comprised 33.60% of ipsilateral and 11.50% of contralateral nIII^{ChAT} neurons, based on data from 6 mice, with 3 representative sections analyzed per animal (Supplementary Fig. 11a-c). We eliminated these EOMs-projecting nIII^{ChAT} neurons by injecting AAV2/2-Retro-DIO-tdCaspase3-

WPRES into EOMs in ChAT-Cre mice. Subsequently, AAV-hEF1 α -DIO-hChR2-GFP was injected into the nIII followed by optic fiber implantation (Fig. 3a). Despite anatomical labeling and caspase expression in EOMs-projecting nIII^{ChAT} neurons may not be complete, the genetic ablation resulted in a 39% reduction in the number of nIII^{ChAT} neurons (Fig. 3b,c). The amplitude of EMs elicited by optogenetic stimulation of nIII^{ChAT} neurons was dramatically decreased (Fig. 3d and Supplementary Fig. 12a, Supplementary Video 3 and 4).

(Page 13 line 556-559) Our findings laid out a circuit substrate in nIII, connecting to previously identified switching circuitry for REMs⁴⁸. Given current methodological limitations, it remains challenging to determine whether these two pathways share the same neuronal substrate, warranting further exploration.

Comment 10:

Again after stimulation, the quantity of REM sleep is decreased at the expense of wakefulness. It might be also that stimulating a large number of OMN neurons might induce a long lasting contraction of the oculomotor muscles and therefore inhibit EMs and induces waking. In support of such hypothesis, when the authors record individual OMN neurons they report that some are activated and some are inhibited at the onset of EMs.

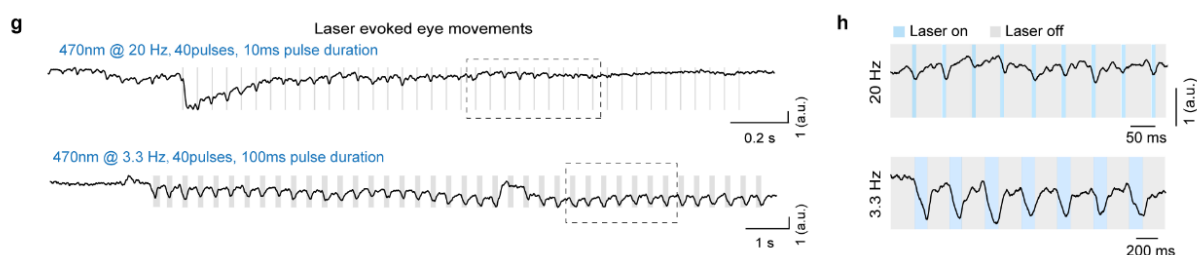
Response:

We thank for the reviewer's insightful comments. **We have conducted new experiments to elucidate the effect of optogenetic stimulation on the muscle contraction.**

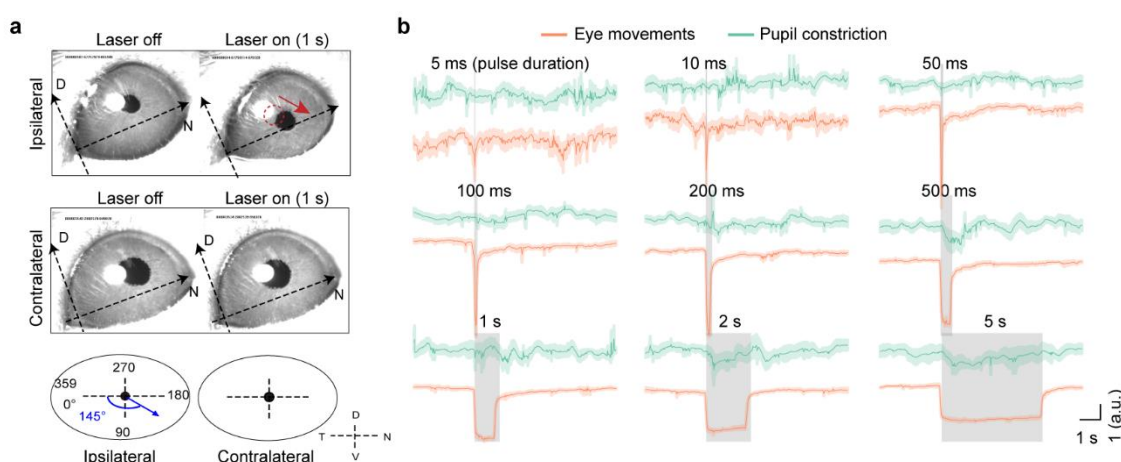
Under the optogenetic stimulation conditions that induced a reduction in REM sleep (20 Hz, 10 ms pulse duration and 40 ms pulse interval lasting for 2 min, 2 mW; 3.3 Hz, 100 ms pulse duration and 200 ms pulse interval lasting for 2 min, 2 mW), rapid eye movements were elicited, without producing sustained muscle contraction (**Fig. R2-13**). As the reviewer correctly noted, we found that when the duration of a single pulse exceeded 500 ms, the pulse induced a prolonged contraction of the oculomotor

muscles, followed by sustained ocular immobility (**Fig. R2-14**).

We also conducted new experiments and confirmed that laser-induced rapid eye movements themselves did not alter the overall sleep-wake state (**see more details in Reply to Reviewer 2 Comment 6, Comment 8 and Comment 9**). Together, these findings reinforce the conclusion that the observed sleep phenotype is not simply a consequence of residual EMs or muscle contraction, but rather reflects a dedicated neural mechanism within nIII^{ChAT} neurons for modulating REM sleep.



(New updated Supplementary Fig.8) Fig. R2-13 | Effect of 3.3 Hz and 20 Hz optogenetic activation of nIII^{ChAT} neurons on the oculomotor. g Traces of EMs induced by optogenetic activation of nIII^{ChAT} neurons using 20 Hz stimulation (10 ms pulse width) and 3.3 Hz stimulation (100 ms pulse width). **h** Enlarged view of the dashed time window shown in panel g. The gray shielding indicates laser-off and the blue shielding indicates laser-on.



(New updated Supplementary Fig.8) Fig. R2-14 | Effect of single-pulse optogenetic activation of nIII^{ChAT} neurons on the oculomotor and pupil constriction. d Upper: the position of ipsilateral or contralateral eyeball before (laser off) and after (laser on)

single-pulse (1 s) optogenetic activation of the nIII^{ChAT} neurons. Lower: schematic diagram of ipsilateral or contralateral EMs traces induced by optogenetic activating the nIII^{ChAT} neurons. Blue area indicates the stimulation time window. D: dorsal, V: ventral, T: temporal, N: nasal. Laser duration, 1 s; 2 mW. **b** Dynamic change of ipsilateral EMs (orange) and pupil constriction (cyan) induced by optogenetic activation with varying durations (5 ms to 5 s) in ChAT-Cre mice. The gray shading area indicates the time window for laser stimulus. Orange and cyan shading indicates 95% confidence intervals (n = 8 mice).

The corresponding revisions in the revised manuscript:

(Page 5-6 line 214-224) We observed a reliable ipsilateral EMs in temporal-to-nasal direction but had no effect on the pupil constriction (Supplementary Fig. 8d-f). These results confirm that we selectively activated the nIII without affecting the adjacent Edinger-Westphal (EW) nucleus. The amplitude of EMs increased as the pulse durations increased from 5 ms to 100 ms, and reached a plateau as the durations increased further (Supplementary Fig. 8e-f). When the duration of the optogenetic pulse exceeded 500 ms, it might induce a prolonged contraction of the oculomotor muscles, which was subsequently followed by sustained ocular immobility (Supplementary Fig. 8e). Moreover, time-locked EMs induced by low-frequency laser pulses (3.3 Hz, 100 ms duration) were robust, while 20 Hz optical stimulation induced very smaller amplitude of EMs (Supplementary Fig. 8g-h, Supplementary Video1 and 2).

Comment 11:

Line 245-282, they made correlation analysis to distinguish two subpopulation of OMN neurons. I'm not convinced by the methodology used and their conclusions that two subpopulations of neurons exist. More simple analysis would be more appropriate here to distinguish potential subpopulations. What is in particular the correlation with the EMs of the two subpopulations? Is really only one of the subpopulations increasing its

activity at REM-W transitions? In such case what is their activity during the EMs?

Response:

Thank you for the reviewer's constructive comments. To address these concerns, we conducted new analysis and answered the reviewer's questions in detail below.

1. What is in particular the correlation with the EMs of the two subpopulations? Is really only one of the subpopulations increasing its activity at REM-W transitions? In such case what is their activity during the EMs?

In our previous manuscript, we classified 88 nIII^{ChAT} neurons recorded from five mice into two distinct subpopulations: EMs active neurons (25/88, magenta) and R-W active neurons (28/88, green). Within these groups, there are 14 EMs specific neurons, 17 R-W specific neurons and 11 overlapping neurons (**Fig. R2-15a**). The classification process is illustrated in **Fig. R2-15a**. Namely, neurons were clustered separately using two criteria: activity correlation with eye movements or R-W transition. R-W specific neurons exhibited increased activity preceding the termination of REM sleep (**Fig. R2-15b,f**), but showed no significant activation during EMs (**Fig. R2-15c**). Conversely, the EMs specific neurons didn't display significant activation during R-W transitions (**Fig. R2-15g**).

2. New approach for calcium imaging analysis to classify nIII^{ChAT} neurons

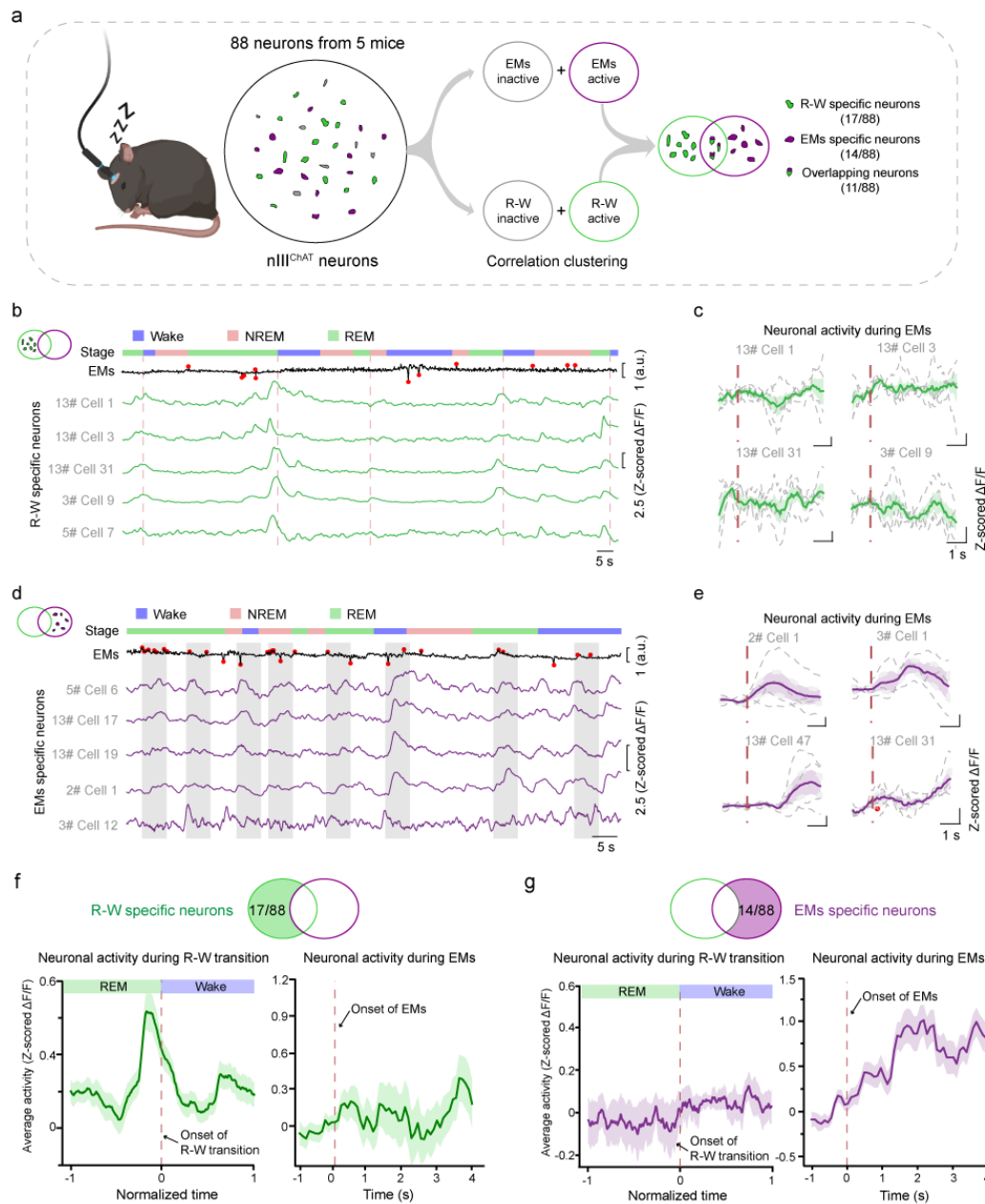
Following the reviewer's suggestion of utilizing a simpler analysis method, we utilized a threshold-based approach, which is commonly used to identify neurons associated with specific states or events in sleep studies (Izawa et al. 2019, Tseng et al. 2022). EMs active neurons (38/88) were defined as those whose peak calcium signal within the 4-s window following EMs onset exceeded 2.5 standard deviations above baseline (corresponding to the 99% confidence interval) (**Fig. R2-16a, R2-17a**). R-W active neurons (36/88) were defined as those whose peak calcium signal within the state transition window (16 s before to 16 s after the transition point) exceeded 2.5 standard deviations above baseline (**Fig. R2-16e, R2-17a**). A small subset of neurons (8/88) was active in both EMs and R-W transitions. Comparison with the correlation-based clustering results revealed a 67.9% concordance for R-W active neurons-(**Fig. R17b**).

These results, obtained through two independent analytical approaches, consistently support the existence of two functionally distinct populations of nIII^{ChAT} neurons.

3. Why did we choose the correlation-based clustering method?

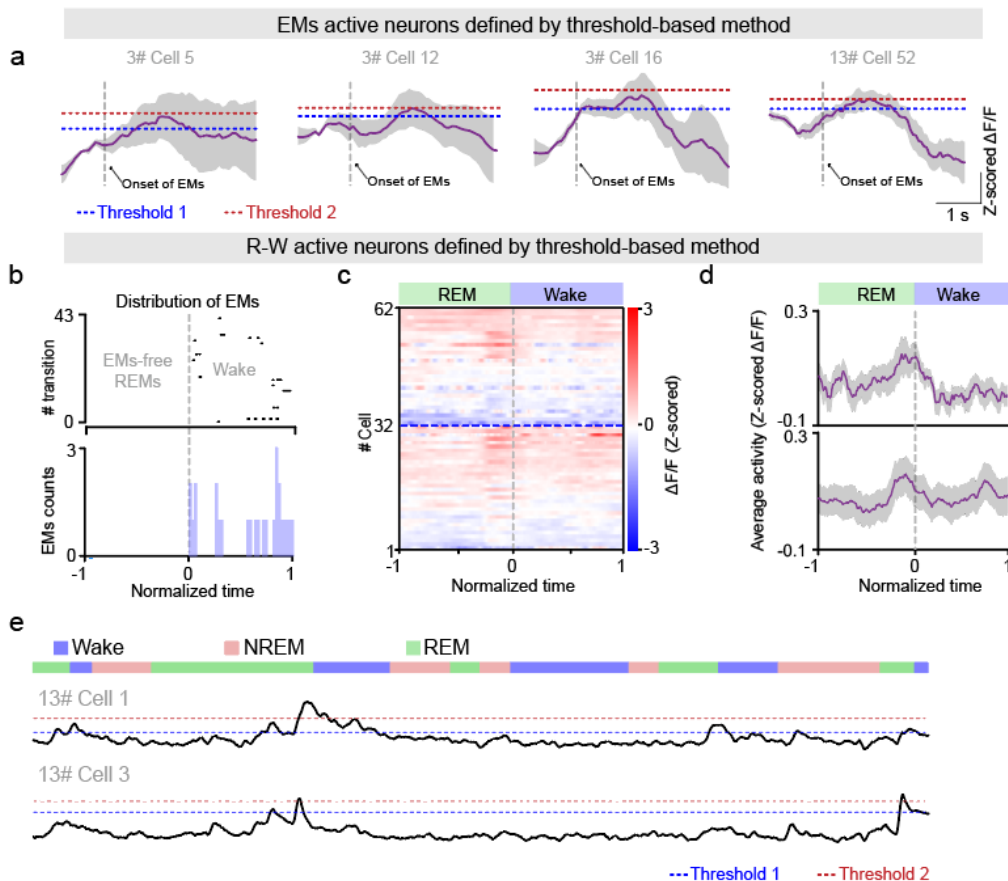
The correlation analysis followed by hierarchical clustering employed in our study is a well-established approach for identifying functionally coherent neuronal subpopulations (Oesch et al. 2020, Perez-Ortega et al. 2021, Yang et al. 2023). One potential drawback in the thresholding method is its sensitivity to the choice of threshold criteria. For example, the neurons shown in **Fig. R2-16a** were classified as EMs-active when the threshold was set at mean + 2.5 SD (threshold 1); however, the same neurons would be classified as EMs-inactive if the threshold were increased to mean + 3 SD (threshold 2). A similar limitation applies to the definition of R-W transition-active neurons (**Fig. R2-16e**).

We have incorporated these new results in the Result section and have included a detailed description in the corresponding sections of Method in revised manuscript.



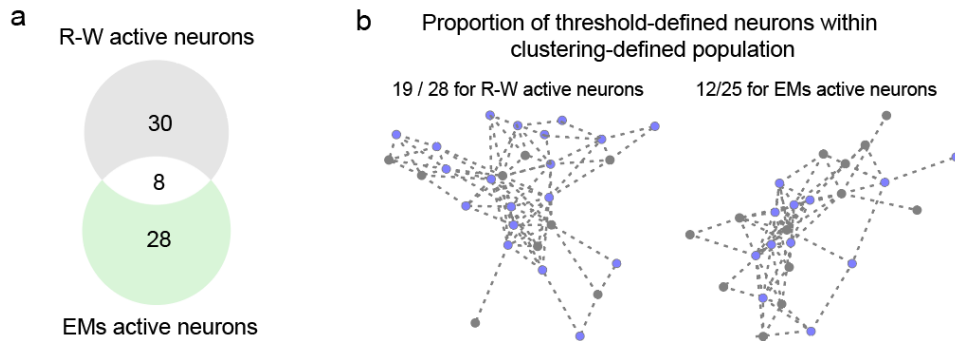
(New updated Fig.4 and supplementary Fig.16) Fig. R2-15 | Two subpopulations of nIII^{ChAT} neurons defined by a correlation-based approach. **a** The schematic of process to clustering the EMs active and R-W active neurons. **b** $\Delta F/F$ trace of R-W active nIII^{ChAT} neurons across sleep-wake states, recorded concurrently with EMs signals. The red dots in the EMs trace indicate the events of eye movements. **c** The average calcium fluorescence of four representative R-W active neurons in response to EMs. The dashed line indicates the onset of EMs. Shading area indicates 95% confidence intervals (CIs). **d** $\Delta F/F$ trace of EMs active nIII^{ChAT} neurons across sleep-

wake states, recorded concurrently with EMs signals. The red dots in the EMs trace indicate the events of eye movements. **e** The average calcium fluorescence of four representative EMs active neurons in response to EMs. The dashed line indicates the onset of EMs. Shading area indicates 95% confidence intervals (CIs). **f** The average calcium signal of R-W active neurons during R-W transition (left) and their corresponding activity in response to the EMs during REM sleep (right). Each REM and wake episode was temporally normalized. The dashed line indicates the time point of transition or onset of EMs. The shaded area indicates the 95% confidence intervals (CIs). **g** The average calcium signal of EMs active neurons during R-W transition (left) and in response to EMs (right). The dashed line indicates the onset of EMs or time point of transition. The shaded area indicates the 95% confidence intervals (CIs).



(New analysis) Fig. R2-16 | Two subpopulations of nIII^{ChAT} neurons defined by a correlation-based approach. **a** The average calcium signal of four representative EMs-active neurons in response to occurrence of eye movements. The vertical dashed

lines indicate the onset of eye movements. The horizontal dashed line indicates the amplitude cutoff threshold. Horizontal dashed lines indicate the thresholds: Threshold 1 corresponds to mean + 2.5 SD, and Threshold 2 corresponds to mean + 3 SD. Shading area indicates 95% confidence intervals (CIs). **b** The raster plot (upper) and peri-event time histogram (PESH, lower) of EMs during R-W transition episodes. **c** The activity heatmap for 30 R-W transition-active neurons aligned to R-W transition episodes. Cells from 60-88 were the identified R-W transition-active neurons. All cells are sorted by the average of activities. **d** The average activity of R-W transition-active neurons during R-W transition. Time 0 represents the transition time point in R-W transition. **e** Calcium traces of representative R-W transition-active neurons identified using a threshold-based approach. The same neurons were also defined as R-W transition active neurons using correlation-based method. Horizontal dashed lines indicate the thresholds: Threshold 1 corresponds to mean + 2.5 SD, and Threshold 2 corresponds to mean + 3 SD.



(New analysis) Fig. R2-17 | The proportion of neurons identified by threshold-based analysis that overlapped with the same neuronal subtype classified by correlation-based clustering analysis. a The venn diagram number of EMs-active neurons and REMs active. **b** The proportion of neurons identified by threshold-based analysis that overlapped with the same neuronal subtype classified by clustering analysis. The purple dots indicate the neurons defined by threshold-based approach, the gray dots indicate the neurons defined by the correlation clustering.

The corresponding revisions in the revised manuscript:

(Page 9 line 352-359) For the R-W specific neurons, we observed elevated neuronal activity at the EMs-free R-W transition (Fig. 4j left, Supplementary Fig. 15i, j). However, no responses were detected when aligned with the occurrence of EMs (Fig. 4j right, Supplementary Fig. 15h). In contrast, EMs specific neurons exhibited notably elevated GCaMP6s signals during EMs, but not during R-W transition (Fig. 4k, Supplementary Fig. 15k-l). We also examined the activity of R-W specific neurons across three other types of vigilance state transitions and found these neurons did not alter their activity during transitions from W-NR, NR-R and NR-W (Supplementary Fig. 16a-c).

Comment 12:

Then, the authors look at the afferents to the OMN^{Chat} neurons using retrograde tracing. Line 287 they write they are looking to their downstream targets but it is their afferents not their targets they are looking at?

Response:

We thank the reviewer for pointing out this error in our description. We apologize for the confusion: the term “retrograde” in the sentence “To further investigate whether non-extraocular muscle-projecting nIII^{ChAT} neurons were part of the sleep network to regulate REM sleep, we conducted **retrograde** tracing to identify their downstream targets” should be “**anterograde**”. We have changed the word in the revised manuscript accordingly.

The corresponding revisions in the revised manuscript:

(Page 9 line 369-370) To further investigate the neural circuit mechanisms by which the nIII regulates REMs, we conducted **anterograde** tracing to identify their downstream targets.

Comment 13:

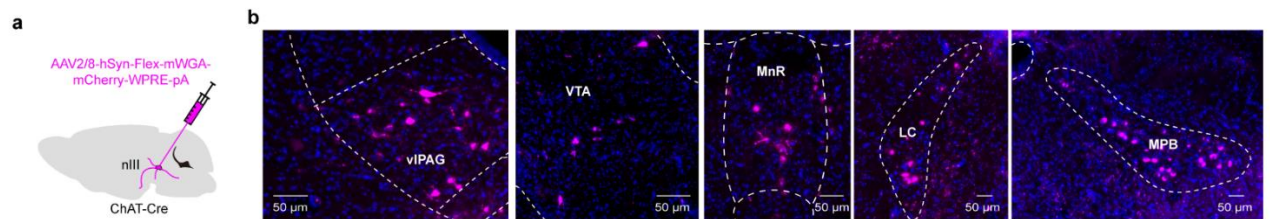
Strangely line 295 they look at the projection of OMNchat neurons to the vlPAG and not the opposite they just demonstrated? The same for LC, they show that OMN neurons project there? It has never been described in the numerous previous papers that OMN cholinergic neurons project to the LC and the vlPAG. All this part is very confusing. The confusion is confirmed in the next experiment in which they found that some OMN neurons project to the extraocular muscles and some to the vlPAG (which is very close to the OMN by the way... and this could induce a methodological problem). A simpler way to look at the efferents of the OMN Chat neurons would have been to use anterograde tracing after their transfection with a GFP expressing Cre AAV.

Response:

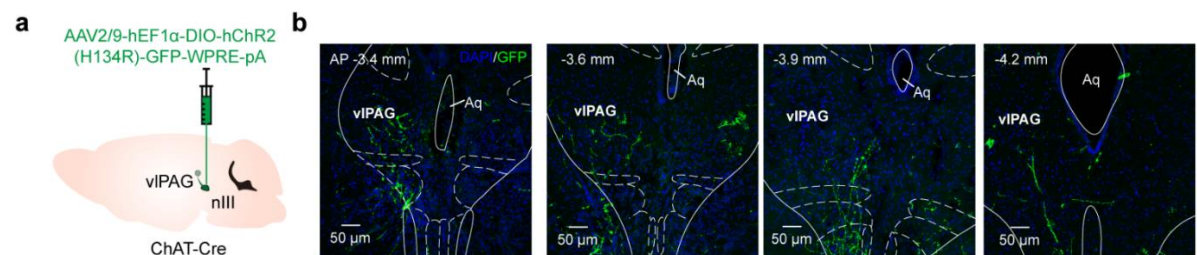
Thank you for your insightful and constructive comments. We first apologize for the confusion caused by the wrong description in this section. We performed **trans-synaptic anterograde** tracing experiment using genetically engineered WGA in the initial manuscript (**Fig. R2-18**), which was mistakenly written as “**retrograde**” in the original manuscript. We made the correction in the revised manuscript.

We fully agree with the reviewer that these results should be interpreted with caution, particularly given the anatomical proximity of the nIII and vlPAG (nIII: AP – 4.0 mm, ML 0.1 mm, DV 3.3 mm; vlPAG: AP –3.5 mm, ML 0.5 mm, DV 2.5 mm). As the reviewer suggested, we conducted new anterograde tracing experiments by injecting AAV2/9-hEF1 α -DIO-hChR2-(H134R)-GFP-WPRE-pA into the nIII of ChAT-Cre mice (**Fig. R2-19a**). As shown in **Fig. R2-19b**, we observed clear axonal projections from nIII^{ChAT} neurons to vlPAG along rostral-caudal axis in coronal sections, which further confirming the mWGA results.

We have revised the manuscript accordingly to clarify the anatomical tracing strategy and to temper the interpretation of these novel projections.



(Newly updated Fig. 5) Fig. R2-18 | The downstream targets of nIII^{ChAT} neurons were mapped using anterograde trans-synaptic tracing. a Schematic of anterograde trans-synaptic tracing of the nIII^{ChAT} neurons to label the downstream nuclei. **b** Example image showing mCherry-labelled somatic in several different downstream regions. vlPAG: the ventrolateral periaqueductal grey, VTA: ventral tegmental area, MnR: median raphe nucleus, LC: locus coeruleus, MPB: medial parabrachial nucleus. Scale bar, 50 μm.



(Newly updated Supplementary Fig.17) Fig. R2-19 | Axon terminals of nIII^{ChAT} neurons within the vlPAG. a The schematic of virus injection in ChAT-Cre mice. **b** The distribution of nIII^{ChAT} neuronal terminal within the vlPAG along the rostral-caudal axis.

The corresponding revisions in the revised manuscript:

(Page 9 line 369-370) To further investigate the neural circuit mechanisms by which the nIII regulates REMs, we conducted anterograde tracing to identify their downstream targets.

(Page 9 line 385-395) Previous studies suggest that vlPAG played a crucial role in gating REMs, while activating vlPAG GABAergic neurons in mice suppresses the initiation and maintenance of REMs^{12,20}. To further confirm the functional connectivity of nIII-vlPAG projection, we injected AAV2/9-hEF1α-DIO-hChR2(H134R)-EYFP into the nIII of ChAT-Cre mice and implanted an optrode in the

vIPAG to record downstream neuronal activity (Fig. 5e). We observed clear axonal projections from nIII^{ChAT} neurons to vIPAG along rostral-caudal axis in coronal sections (Supplementary Fig. 17a-b). Upon photostimulation of nIII^{ChAT} terminal within the vIPAG with blue laser pulses (480 nm), we observed reliable optogenetically evoked spiking in a single unit (Fig. 5f-g). Among the 61 well-isolated units recorded from 3 mice, 32.8% exhibited optogenetically activated responses, and 9.8% showed optogenetically suppressed responses to nIII^{ChAT} axon activation (Fig. 5h, Supplementary Fig. 18).

Comment 14:

In the next chapter, they used a retroAAV injection into the OMN to label the afferents to the cholinergic neurons. It is really confusing since they also talked of retrograde tracing using mWGA-mCherry virus injection in the OMN and also wrote that it is retrograde tracing and they indeed show labeled soma in some regions. WGA is indeed a retrograde tracer so all of this is extremely confusing.

Response:

Thank you for your constructive suggestion. We apologize for the confusion caused by the mistake in using the word “retrograde” (which should be “anterograde”, **also see the response to Comment 12**). **We have revised the text to better explain the functionality of the anterograde transsynaptic tracer mWGA-mCherry. Please see details below.**

WGA is capable of both anterograde and retrograde transport depending on how it is delivered (as a protein or via gene expression) and used (with or without viral vectors) (Crossland 1985). To investigate the downstream targets of nIII^{ChAT} neurons, we employed a genetically encoded anterograde transsynaptic tracer, AAV2/8-hSyn-DIO-mWGA-mCherry (mWmC). This tracer, developed by Nicole Y. Tsai et al. (Tsai et al. 2022), is specifically designed to cross a single synapse and label postsynaptic neurons at the soma level. The anterograde and trans-synaptic labeling capabilities of mWmC have been previously validated by injections into the striatum, leading to labeled somas

in the substantia nigra pars reticulata (SNr), as reported by the developer (**Fig. R2-20**).

[Figure Redacted]

(Fig. 4m in the cited article) Fig. R2-20 | m Anterograde trans-synaptic tracing from substantia nigra (SNr) via stereotaxic injections into the Striatum. From Nicole Y. Tsai et al 2022.

The corresponding revisions in the revised manuscript:

(Page 9 line 369-376) To further investigate the neural circuit mechanisms by which the nIII regulates REMs, we conducted anterograde tracing to identify their downstream targets. AAV2/8-hSyn-Flex-mWGA-mCherry-WPRE-pA (mWmC) virus was a genetically encoded monosynaptic anterograde tracer developed by Nicole Y. Tsai et al. 29. The anterograde and trans-synaptic labeling capabilities of mWmC have been validated through striatal injections, which resulted in robust mCherry-labeled somas in the substantia nigra pars reticulata (SNr)²⁹. We then injected the mWmC virus into the nIII in ChAT-Cre mice to identify direct postsynaptic targets of nIII^{ChAT} neurons (Fig. 5a).

Comment 15:

In the last chapter, they confirm previous results showing that the papillo nucleus project to the OMN and induce EMs and REM to wake transition. In summary, the authors reproduced previous results showing that the OMN neurons are activated during REM sleep to participate in the REM generation. They further state that some

OMN neurons terminate REM sleep and induce W. As explained above I'm not sure that such conclusion is accurate since wake might be induced indirectly when stimulating EMs generating neurons of the OMN.

Response

Thank you for your constructive and insightful comments. To better address the reviewer's concerns, we have divided our response into two parts and reply the comments one by one.

Comment 15-1: "In the last chapter, they confirm previous results showing that the papillo nucleus project to the OMN and induce EMs and REM to wake transition. In summary, the authors reproduced previous results showing that the OMN neurons are activated during REM sleep to participate in the REM generation."

Reply to comments 15-1:

As the reviewer correctly pointed out, the study by C. Gutierrez Herrera et al. (Gutierrez Herrera et al. 2019) demonstrated the anatomical projection from NP to nIII using viral tracing and showed that optogenetic activation of NP terminals within nIII during REM sleep induces EMs. In this study, we found that optogenetic activation of the NP-nIII^{ChAT} projection promotes REM sleep. To elucidate the circuit and cellular mechanisms underlying the contribution of NP-nIII pathway to ocular control and REM sleep regulation, we conducted new *in vivo* electrophysiology, retrograde labeling, as well as RNAscope experiments.

We first performed *in-vivo* electrophysiological recordings from nIII neurons while optogenetically activating NP neurons (**Fig. R2-21e**). We found that 17.9% of nIII neurons were activated and 15.4% were suppressed in their firing rates by optogenetic activation of NP terminals within the nIII (**Fig. R2-21f-j**). To determine the cell-type specificity of NP-nIII projection, we employed both monosynaptic retrograde rabies tracing and RNA scope to label NP neurons that project to either vPAG-projecting or extraocular-muscle-projecting nIII neurons (**Fig. R2-22**). *Vglut2*-expressing neurons in the NP preferentially project to nIII^{ChAT} neurons that project to extraocular muscles (**Fig. R2-22d**), while *Vgat*-expressing NP neurons project to nIII^{ChAT} neurons that innervate

the vIPAG, thus regulating REM sleep (Fig. R2-22h).

Together, these findings indicate that two parallel NP-nIII circuits independently regulate distinct physiological functions: the NP^{Vgat}-nIII-vIPAG pathway governs REM-to-wake transitions, while the NP^{Vglut2}-nIII-extraocular muscles pathway controls EMs. We have added these new results into the revised manuscript.

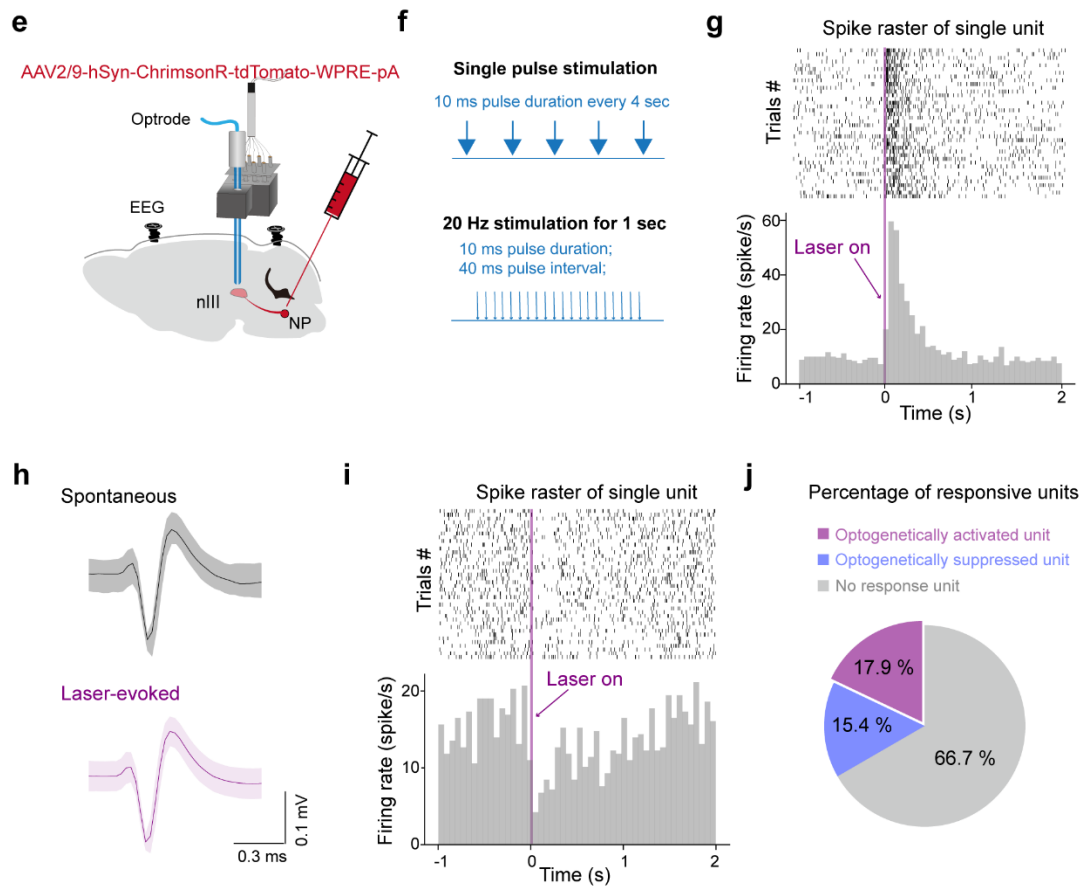
Comment 15-2: “They further state that some OMN neurons terminate REM sleep and induce W. As explained above I’m not sure that such conclusion is accurate since wake might be induced indirectly when stimulating EMs generating neurons of the OMN.”

Reply to comments 15-2:

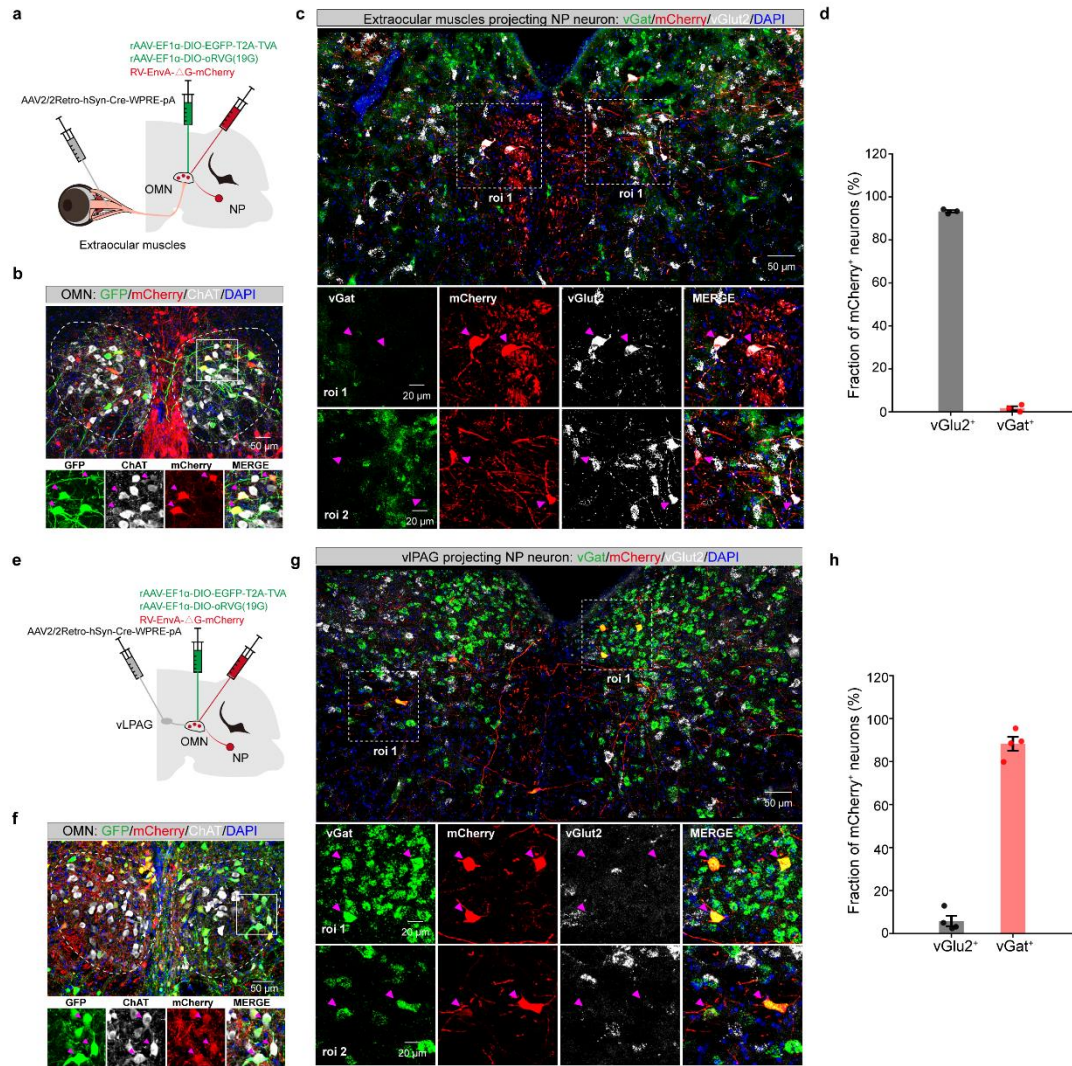
We agree with the reviewer that it is important and essential to clarify whether the observed REM-to-wake transitions are directly mediated by nIII^{ChAT} neuronal activity or indirectly induced through evoked eye movements (EMs). To address this concern, we conducted new experiments and revised our manuscript accordingly. Details can be seen below.

We first conducted new experiments to express ChR2 in nIII^{ChAT} neurons projecting to the extraocular muscles by injecting retrograde AAV into the muscles and Cre-dependent AAV into the nIII. Optogenetic activation of these neurons robustly evoked EMs without altering the overall proportion of REM, NREM, or wake (see details in Reply to Reviewer #2 Comment 8 and Comment 10). To further rule out the possibility that EMs affect sleep architecture or scoring, we conducted new analysis to verify that neither spontaneous EMs nor optogenetically-induced EMs changed the EEG or EMG signals used for sleep-wake determination (see details in Response to Comment 6). Together, these results support the conclusion that nIII^{ChAT} neurons’ role in REM-to-wake transitions is likely not a secondary consequence of EM induction.

We have added these new results to the revised manuscript and have included a detailed discussion of their potential implications in the corresponding sections of the Results and Discussion.



(Newly updated Fig.6) Fig. R2-21. | The functional connectivity of the NP–nIII circuit was confirmed by optogenetics-assisted electrophysiological recordings. e Schematic of viral injection in the NP and optrode implantation in the nIII. **f** The schematic of stimulus diagram. **g** Representative raster plot (upper) and stimulus time histogram (lower) for laser-evoked spikes of an example ChR2-tagged unit. Dashed line, laser onset time. Laser duration, 10 ms. Power density, 2 mW. **h** Averaged waveform of spontaneous (gray) and laser-evoked (purple) spike of an example unit. **i** Representative raster plot (upper) and stimulus time histogram (lower) for laser-inhibited spikes of an example ChR2-tagged unit. **j** Percentages of increased, decreased and no-significant response units among all recorded electrode in the nIII. Increased and decreased units were defined based on whether the post-stimulation peak firing rate exceeded a threshold of mean $\pm$ 3 standard deviations (SD) of the baseline activity.



(Newly added Fig.7) Fig. R2-22. | nIII^{ChAT} innervated NP neurons express mutually exclusive marker genes. Schematic diagram of strategy to label the EOMs-projecting NP neuron. After 3 weeks of AAV2/2-retro-hSyn-Cre expression in the extraocular muscles (EOMs), two helper (rAAV-EF1α-DIO-EGFP-T2A-TVA and rAAV-EF1α-DIO-oRVG(19G)) were injected into the nIII in C57 mice, followed by injection of modified rabies virus (RV) expressing mCherry (RV-EnvA-G-mCherry). **b** Confocal image showing a coronal section of the nIII 10 days post-RV injection. The dashed square region in the nIII is magnified below. EOMs-projecting nIII^{ChAT} neurons appear yellow due to co-expression of GFP and mCherry from helper and RV vectors, respectively. **c** Co-expression of mCherry-labeled EOMs-projecting NP neurons with the glutamatergic marker *Vglut2* mRNA. **d** Percentages of mCherry-labelled neurons

in the NP region that express *Vglut2* mRNA or *Vgat* mRNA. Each dot indicates data from one mouse (n = 3 mice). **e** Schematic diagram of strategy to label the vIPAG-projecting NP neuron. **f** Confocal image showing a coronal section of the nIII 10 days post-RV injection. The dashed square region in the nIII is magnified below. vIPAG-projecting nIII^{ChAT} neurons appear yellow due to co-expression of GFP and mCherry from helper and RV vectors, respectively. **g** Co-expression of mCherry-labeled vIPAGs-projecting NP neurons with the GABAergic marker *Vgat* mRNA. **h** Percentages of mCherry-labelled neurons in the NP region that express *Vglut2* mRNA or *Vgat* mRNA. Each dot indicates data from one mouse (n = 4 mice).

The corresponding revisions in the revised manuscript:

(Page 11-12 line 478-502) Lodged at the dorsomedial boundary of the dorsal paragigantocellular nucleus (DPGi), the NP comprises a heterogeneous population of neurons, including inhibitory GABAergic neurons, excitatory glutamatergic neurons, and a substantial number of neuropeptidergic neurons³³. To further identify the neuron types upstream of nIII^{ChAT} neurons contributing to motor behavior and sleep regulation, we employed a rabies virus (RV)-mediated trans-synaptic retrograde tracing approach. To specifically label NP neurons projecting to EOMs-innervating nIII neurons and to vIPAG-projecting nIII neurons, we first injected AAV2/2-Retro-hSyn-Cre-WPRE-pA into either the extraocular muscles or the vIPAG of C57 mice (Fig. 7a, Fig. 7e). Two Cre-dependent helper viruses rAAV-EF1 α -DIO-EGFP-T2A-TVA and rAAV-EF1 α -DIO-oRVG(19G) were unilaterally injected into the nIII (Fig. 7a, Fig. 7e). After 2 weeks, RV-EnVA- Δ G-mCherry was injected into the nIII, as previously described in Fig. 6c. Using this strategy, the RVs can only infect TVA-expressing cells and required RG expression to spread retrogradely into presynaptic cells. The starter neurons, which are shown in yellow, were limited to the nIII region and characterized by the expression of both the helper virus and RV in cholinergic neurons (Fig. 7b, Fig. 7f). We mapped the afferent neurons in the NP by imaging 3 brain slices from each of four mice. Immunofluorescence and RNAscope assays shows that 93.3% of the

monosynaptic inputs from NP to EOMs-projecting nIII^{ChAT} neurons expressed the glutamatergic marker *Vglut2* mRNA, while scarcely expressing the GABAergic marker *Vgat* mRNA (Fig. 7c-d). On the other hand, 88.3% of NP neurons innervating vIPAG-projecting nIII^{ChAT} neurons expressed the GABAergic marker *Vgat*, but not the glutamatergic marker *Vglut2* mRNA (Fig. 7g-h). No co-expression of *Vgat* and *Vglut2* was observed in NP neurons (Fig. 7d-h). We also examined neurons expressing ChAT and Cart; none of these neurons exhibited detectable co-expression with mCherry (Supplementary Fig. 23). This finding suggests that the functional heterogeneity of nIII^{ChAT} neurons most likely arises from differences in the gene profiles of upstream NP neurons.

(Page 11 line 457-465) In addition to RV-mediated anatomical tracing, we also measured functional connections between NP and nIII^{ChAT} neurons using *in vivo* optogenetics-assisted electrophysiological recordings. We injected AAV2/9-hSyn-ChrimsonR-tdTomato-WPRE-pA into the NP and implanted an optrode in the nIII (Fig. 6e, Supplementary Fig. 22a-b). Optogenetic activation of NP terminals within the nIII induced either an increase or decrease in the firing rate of single units upon photostimulation (Fig. 6 f-g, Supplementary Fig. 22c). Among 40 well-identified nIII units recorded from 3 mice, 17.9% exhibited optogenetically activated responses, and 15.4% showed optogenetically suppressed responses (Fig. 6h, Supplementary Fig. 22d-e), demonstrating that nIII received both excitatory and inhibitory synaptic inputs from the NP.

Reviewer #3

General comments:

In the current study, Jiang et al. found that the overall activity of oculomotor nucleus (OMN) cholinergic neurons (ChAT) are higher during eye movements (EMs) and REM sleep. Ablation of OMN^{ChAT} neurons reduced amplitude of EMs and REM episode number while increased REM duration. Optogenetic activation of OMN ChAT neurons induced EMs and decreased REM sleep. Optogenetic activation of non-extraocular

muscle projecting OMN^{ChAT} neurons did not affect EMS but terminated REM sleep and increased wakefulness. Calcium imaging showed distinct populations of OMN^{ChAT} neurons that are active during EMS and REM-to-wake transitions. vIPAG projecting OMN^{ChAT} neurons regulated REM-to-wake transitions. The nucleus papilio (NP) innervated OMN^{ChAT} neurons and regulates both EMS and REM-to-wake transitions. The circuit mechanism for the coordination of EMS and REM sleep is interesting, while the goal was not accomplished in the present study. Although two populations of ChAT neurons in the OMN were found to be responsible for EMS and REM sleep regulation respectively, how these two parallel circuits coordinate with each other was unexplored. There is also a lack of rigorousness in preparing the manuscript. These issues reduced my enthusiasm for the paper. Specific comments:

Response:

We sincerely thank the reviewer for the thoughtful and constructive comments. We are encouraged that the reviewer found “the circuit mechanism for the coordination of eye movements (EMS) and REM sleep to be interesting”.

In response to the reviewer’s concerns, we have **conducted several new experiments to provide more detailed evidences about the two parallel circuits**. We also corrected imprecise statements, improved figure captions, and reorganized parts of the **Results and Methods** sections to enhance readability and scientific rigor.

As requested by Reviewer #2, we have updated the revised manuscript by replacing all occurrences of the abbreviation “oculomotor nucleus (OMN)” with “nIII” throughout the main text, figures, and the response to reviewers. All changes have been highlighted in the revised manuscript. **A summary of the revisions in response to the reviewers’ comments can be found at the end of the rebuttal letter.** We hope that you find all your concerns addressed in the revised manuscript.

The main responses to Reviewer #3’s comments are summarized as follows:

- New *in vivo* electrophysiological recording of nIII^{ChAT} neuronal activity under

long-term optogenetic activation was added as both new figures of **Supplementary Fig. 11** and corresponding descriptions in the **Results** section;

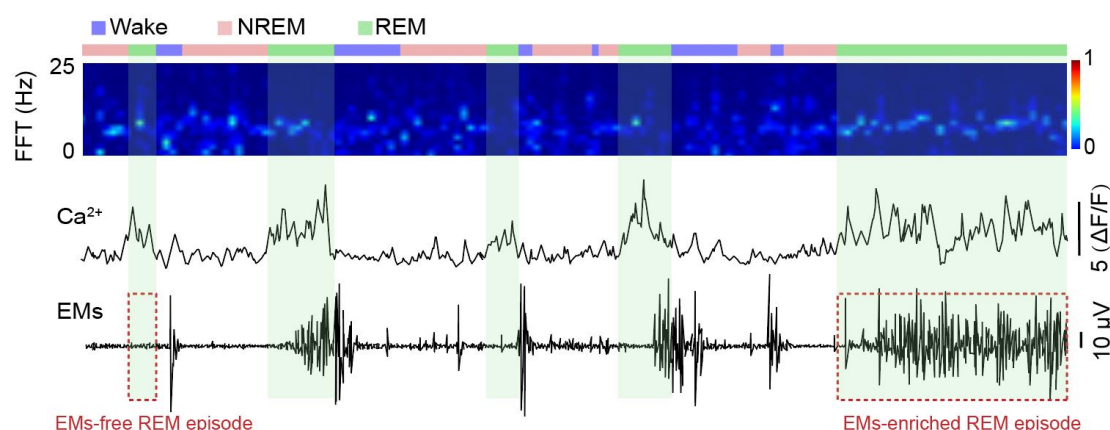
- New figures about the effects of optogenetic inhibition on EMs and sleep architecture were added as both new figures of **Fig. 2l-p** and corresponding descriptions in the **Results** section;
- New figures about the effect of optogenetic activation of extraocular muscle (EOMs)-projecting nIII^{ChAT} neurons on sleep-wake transition have been added as both new figures of **Supplementary Fig. 14** and corresponding descriptions in the **Results** section.
- New figures about the effect of optogenetic activation of vIPAG-projecting nIII^{ChAT} neurons on EMs have been added as both new figures of **Supplementary Fig. 17** and corresponding descriptions in the **Results** section;
- New figures about the effects of optogenetic activation of nIII^{ChAT} neuron projections to the MPB and VTA on sleep–wake transitions have been added as both new figures of **Supplementary Fig. 19** and corresponding descriptions in the **Results** section;
- New figures about the neuron type of NP-nIII-EOMs and NP-nIII-vIPAG have been added as both new figures of **Fig. 7** and corresponding descriptions in the **Results** section;
- The citations were updated in the **Text** and **Reference** section;
- The number of mice and detailed analytical methods have been added to the **Methods** section.;
- Typos have been corrected and double-checked throughout the manuscript;

Comment 1:

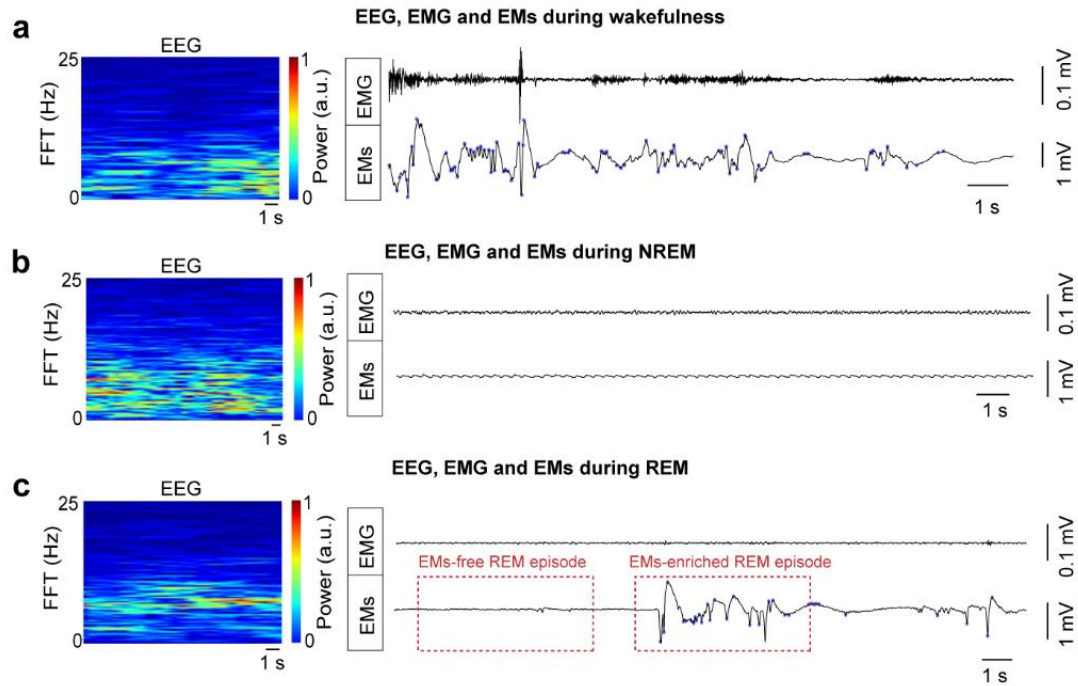
The example traces in Fig. 1f showed no obvious EMs during REM, and EMs happened mostly during wakefulness, especially in wakefulness episodes right after REM. To better visualize the temporal dynamics, it would be good to show the traces of EMs-free episodes and EMs-enriched episodes separately for each brain state.

Response:

Thank you for pointing out this issue. We apologize that the resolution of the original figure was low, making it difficult to clearly see the EMs during REM sleep. In the revised manuscript, we provided higher-resolution traces that clearly illustrate both EMs-free and EMs-enriched episodes across different sleep-wake states (**Fig. R3-1, Fig. R3-2**). The dashed red box indicated the EMs-free or EMs-enriched REM episodes.



(New added Supplementary Fig. 3) Fig. R3-1 | Activity of nIII^{ChAT} neurons across sleep-wake cycles in synchrony with EM recordings. Representative recording of relative EEG power, EMG, EMs traces, and fluorescence traces of nIII^{ChAT} neurons across spontaneous sleep-wake cycles. Green-shaded areas represent REMs episodes, while red dashed boxes highlight EMs-free and EMs-enriched REM episodes.



(New added Supplementary Fig. 7) Fig. R3-2 | Dynamic change of EMs, EEG and EMG across sleep-wake cycle. a Dynamic change in EEG power spectrum (left), EEG and EMs (right) during wake episodes. The wake episodes is enriched with periodic EMs, and high amplitude of EMG. The blue dots in the EMs trace indicate the automatically detected EMs event. **b** Dynamic change in EEG power spectrum (left), EEG and EMs (right) during NREM sleep. The NREM episodes are characterized by a paucity of EMs and low amplitude of EMG. **c** Dynamic change in EEG power spectrum (left), EEG and EMs (right) during REM sleep. The dashed red box indicate the EMs-free or EMs-enriched REM episodes. The REM episodes are characterized by tonic or phasic EMs and low amplitude of EMG. The optogenetic activation evoked sustained high-frequency EMs. The blue dots in the EMs trace indicate the automatically detected EMs event.

The corresponding revisions in the revised manuscript:

(Page 3 line 115-124) Taking the average calcium signal of the entire recording as the baseline, the overall activities of $nIII^{ChAT}$ neurons was highest during REMs compared to wake and NREMs (Fig. 1g). Moreover, we categorized REMs episodes into EMs-

enriched and EMs-free episodes and found that EMs-enriched REM episodes exhibited slightly lower activity than EMs-free REM episodes (Fig. 1g, Supplementary Fig. 3a). The calcium signals of nIII^{ChAT} neurons during wake were smaller than those during REMs episodes and showed more negative values in EMs-free wake episodes compared to EMs-enriched wake episodes (Fig. 1g). These observations suggest that the nIII^{ChAT} neurons are hyperactive during REMs, and that EMs contribute to elevated calcium signals in both REM and wake states.

Comment 2:

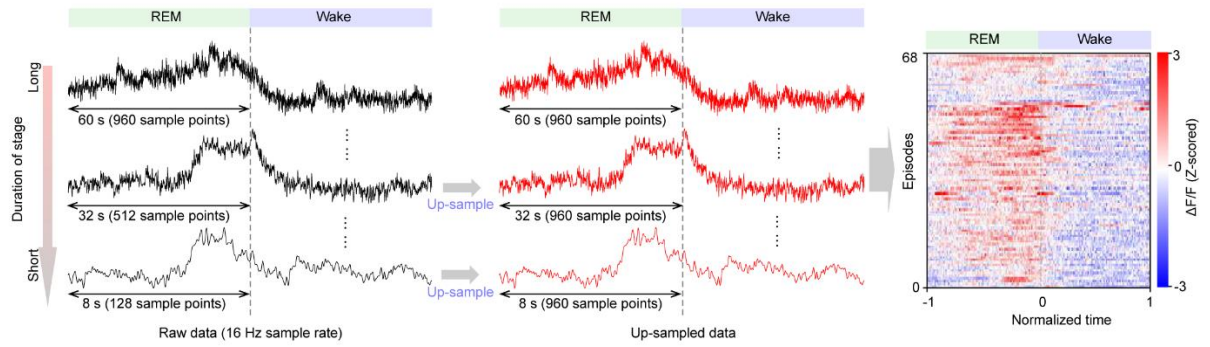
In Fig.1h -1k, how did the author normalize time?

Response:

Thank you for your constructive comment. We have expanded the description of the time normalization procedure in the **Methods** section to improve clarity and ensure better understanding. Please see details below.

We conducted time normalization following the previously reported approach (Hong et al. 2023, Weber et al. 2018). Specifically, if the duration of a target episode exceeded 60 s, we extracted $\Delta F/F$ signals within a 60 s window before and after the transition point. For episodes shorter than 60 s, signals were extracted using the actual episode duration both preceding and following the transition. The extracted traces were then interpolated to generate 60 s time-normalized activity profiles. Finally, all trials were temporally aligned to the transition point and visualized as a heatmap (**Fig. R3-3**).

We have added corresponding descriptions to the Method of the revised manuscript.



(New updated Supplementary Fig. 3) Fig. R3-3 | Procedures for temporal normalization. Episodes of different durations were upsampled to adjust the number of sampling points, thereby generating time-normalized traces of equal length.

The corresponding revisions in the revised manuscript:

(Page 3 line 125-131) Given that REMs is typically followed by wakefulness in a physiological context^{12,25}, here we further examined the neuronal activity during REM-to-wake (R-W), NREM-to-REM (NR-R), NREM-to-wake (NR-W) and wake-to-NREM (W-NR) transition. To account for variability in episode duration, the time axis was normalized to enable direct comparisons of neuronal activity across transitions (Supplementary Fig. 3d). We found that nIII^{ChAT} neurons displayed increased activity preceding R-W transitions, which declined upon entry into wakefulness (Fig. 1h).

(Page 20 line 864-875) Due to the variable duration of each state episode, we performed time normalization as previous reported^{12,72} and then averaged the $\Delta F/F$ under the same type of state transition across trials (Fig. 1 and 4). Specifically, if the duration of a target episode exceeded 60 s, we extracted $\Delta F/F$ signals within a 60 s window before and after the transition point. For episodes shorter than 60 s, signals were extracted using the actual episode duration both preceding and following the transition. The extracted traces were then interpolated to generate 60 s time-normalized activity profiles. Finally, all trials were temporally aligned to the transition point and visualized as a heatmap. To analyze the relationship between the duration of REMs preceding its termination, the average calcium signal, and the probability of REMs transition episodes, the Z-scored $\Delta F/F$ values of the last epoch at the NREM-to-REM transition or the first epoch of the

R-W transition were averaged across all extracted trials from all mice.

Comment 3:

For the long-term optogenetic experiment in Extended Data Fig.5g, the effects became smaller as stimulation progressed and became comparable to control by the end of the 1-h stimulation. The authors should provide some insight into this observation.

Response:

Thank you for your insightful comment. To address the attenuation of effects observed during the prolonged 1-hour optogenetic stimulation (20 Hz, 10 ms pulse duration, 40 ms inter-pulse interval; applied in 1-s on/1-s off cycles) in **Supplementary Fig. 9m** (Extended Data Fig. 5g in the original manuscript), we performed new experiments to investigate the underlying mechanism.

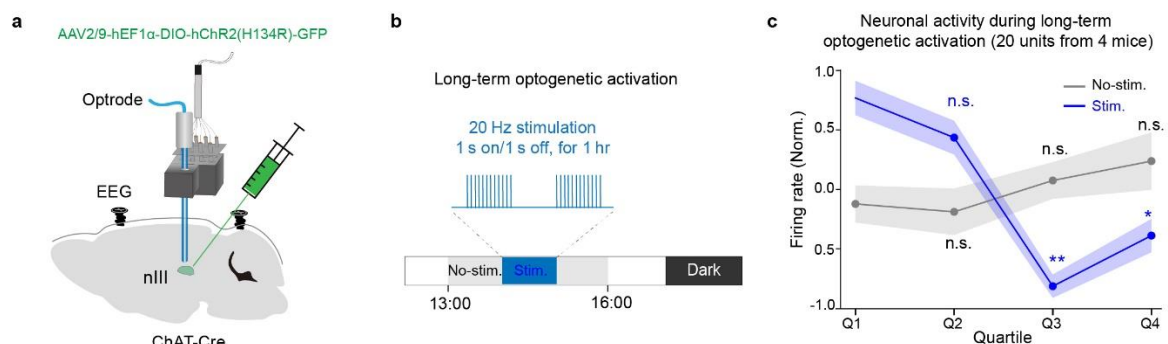
Microbial opsins such as ChR2 undergo desensitization during sustained or repetitive illumination, resulting in reduced neuronal responses over time. Consistent with this, Mattis et al. (Mattis et al. 2011) demonstrated a decline in spike fidelity in ChR2-expressing neurons during 10 s pulse trains (**Fig. R3-4**).

To test the temporal fidelity of ChR2-tagged nIII^{ChAT} neurons in our system, we conducted new *in vivo* optogenetics-assisted electrophysiological recordings of nIII^{ChAT} neuronal activity by delivering continuous 1 hr optical stimulation (20 Hz, 10 ms pulse width, 40 ms interval). As shown in **Fig. R3-5**, evoked spike counts progressively declined with stimulation time, particularly after 30 minutes, while the spontaneous firing activity in the control group (no-stim., no light was delivered) remained stable. This supports the notion that the diminishing effects on REM sleep reduction towards the end of 1-hr stimulation period likely reflect a progressive reduction in neuronal firing rates.

We have added these new results in the Result section and have included a detailed description in the corresponding sections of Method in revised manuscript.

[Figure Redacted]

(Fig. 2b in the cited article) Fig. R3-4 | Temporal stationarity of depolarizing tools to 20 Hz optogenetic stimulation. Vertical and horizontal scale bars represent 40 mV and 1 s, respectively. From Joanna Mattis et al., Nature Methods, 2012.



(Newly added Supplementary Fig.11) Fig. R3-5 | Temporal dynamics of nIII^{ChAT} neuronal firing during prolonged optogenetic stimulation. **a** Schematic of viral injection and fiber implantation. **b** Schematic of prolonged optogenetic activation protocol. Optogenetic stimulation was delivered at 20 Hz (10 ms pulse width), with each stimulation train lasting 1 sec followed by a 1 sec interval. This 1-s on/1-s off cycle was repeated continuously from 14:00 to 15:00 at light phase. **c** Temporal stationarity of ChR2-tagged nIII^{ChAT} neuron firing during 1 hr optogenetic stimulation (20 Hz, 10 ms pulse width, 2 mW; n = 20 units from 4 mice). The firing rates during stimulation were divided into four quartiles, and the average firing rate was calculated for each quartile. Spontaneous firing activity recorded 1 hr prior to stimulation served as the baseline control (No-stim.). Data are presented as mean ± SEM. Asterisks indicate

statistical significance compared to the first quartile: * $p < 0.05$, ** $p < 0.01$. Two-sided paired t-test.

The corresponding revisions in the revised manuscript:

(Page 6-7 line 255-269) To evaluate whether the REMs-suppressing effect of optogenetic activation of $nIII^{ChAT}$ neurons could be influenced by homeostatic sleep pressure, we also performed long-term (1 hr) optogenetic experiment while simultaneously recording the electrophysiological activity of ChR2-tagged $nIII^{ChAT}$ neurons using an optrode (Supplementary Fig. 11a-b). Prolonged activation also led to a significant decrease in the probability of REMs and an increase in the probability of wake compared to the control condition, although this effect progressively diminished toward the end of the long-term stimulation (Supplementary Fig. 9m). Similarly, the firing rate of single-unit also shows a gradual decline with stimulation time, while the spontaneous firing activity in the control group (no-stim.) remained stable (Supplementary Fig. 11c). That means that the progressively reduced effect by the end of the long-term stimulation may owing to the fatigue and desensitization of ChR2-tagged neurons²⁸. Additionally, the amount of time mice spent in REMs significantly decreased, accompanied with an increase in the duration of wake episodes, compared to the control group (Supplementary Fig. 9n). Taken together, these results provide evidences that activating $nIII^{ChAT}$ neurons can resulted in the REMs exit and promote the R-W transition.

(Page 23 line 997-1002) To analyze $nIII^{ChAT}$ neuron firing rates across sleep-wake transitions, we applied time normalization and neuronal activity visualization procedures analogous to those used in the fiber photometry analysis. To assess the temporal fidelity of ChR2-tagged $nIII^{ChAT}$ neurons during prolonged optogenetic stimulation, we applied continuous optical stimulation for 1 h. The stimulation period was divided into four 15 min quartiles, and neuronal firing rates were quantified by averaging within each quartile.

Comment 4:

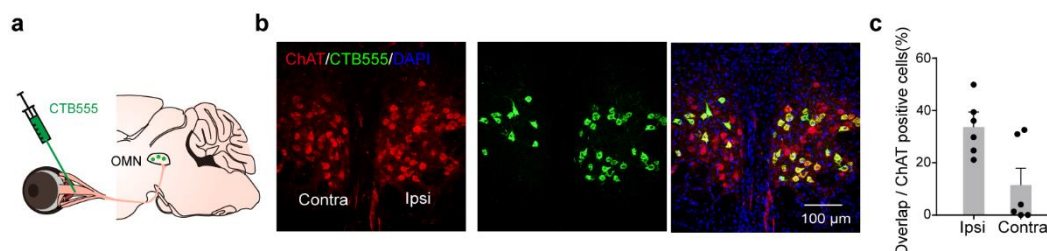
In Extended Data Fig.6b-c, the example images seemed not to fit with the quantification. CTB555 labeled cells in the contralateral part of OMN seems to comprise much more than the author claimed of ChAT⁺ cells (11.50%). The authors need to provide more details on how they did the quantification. How many animals and how many sections in each animal were used for the quantification?

Response:

We thank the reviewer for pointing out the mistake. We sincerely apologize for this mistake. In Extended Data Fig. 6b, the ‘contra’ and ‘ipsi’ labels in the example images were inadvertently misplaced and have been corrected in the revised figure (Fig. R3-6).

For the quantification, we analyzed 6 animals, with 3-4 representative sections per animal encompassing the rostral-caudal extent of the nIII. In each section, we quantified the number of CTB555-positive neurons, ChAT-positive neurons, and the double-labeled neurons on both the ipsilateral and contralateral sides. The percentage of double-labeled neurons was calculated for each animal and then averaged across animals to obtain the reported values.

We have included these details in the revised figure legend and methods section in the revised manuscript.



(Newly updated Supplementary Fig.12) Fig. R3-6 | Proportion of EOMs-projecting neurons in the bilateral nIII regions. a Schematic of unilaterally injecting CTB555 into EOMs of C57 mice. **b** The confocal image of CTB555 (green) labelled ChAT⁺ (red) neurons in ipsilateral and contralateral nIII region. **c** Percentage of ChAT⁺

and CTB555-labeled neurons in ipsilateral and contralateral nIII region (Ipsi = 33.60±5.37%, contra= 11.50±6.65%).

The corresponding description have been added in the revised manuscript:

(Page 7 line 274-277) To this end, we first examined the projections of nIII^{ChAT} neurons to the extraocular muscles, finding that that 33.60% of ipsilateral and 11.50% of contralateral nIII^{ChAT} neurons were labeled, based on data from 6 mice, with 3 representative sections analyzed per animal (Extended Data Fig.12a-c).

(Page 23-24 line 1005-1023) All images for cell counting were acquired using a confocal microscope (Olympus FV3000, Tokyo, Japan) equipped with a 20× objective lens. Z-stack images were obtained with a step size of 1.5 μm across 7–8 layers, resulting in a total thickness of approximately 25 μm. The acquired Z-stacks were processed using maximum intensity projection and saved in TIFF format. Cell quantification was performed using the counting analysis tool in Adobe Photoshop (Adobe Systems Incorporated, San Jose, CA).

For immunostaining, three consecutive 30-μm-thick coronal sections were used per mouse; for FISH experiments, 20-μm-thick sections were used. For cellular ablation analysis, the proportion of ChAT-positive neurons co-labeled with NeuN was manually quantified in four mice. To assess projection patterns of nIII^{ChAT} neurons to the extraocular muscles (EOMs), we counted CTB555⁺ neurons, ChAT⁺ neurons, and their double-labeled counterparts on both ipsilateral and contralateral sides relative to the injection site in six mice. For quantification of projection ratios from nIII^{ChAT} neurons to the vlPAG and LC, we calculated the percentage of double-labeled neurons (GFP and mCherry co-expressing) relative to either GFP⁺ or mCherry⁺ populations from three mice. To determine neurotransmitter identity, the proportion of *Vglut2*⁺ or *Vgat*⁺ cells among mCherry-labeled neurons was quantified. This was performed in three mice for NP-EOMs projections and in four mice for NP-vlPAG projections. Values were averaged across animals to obtain group-level estimates. All quantification was performed by experimenters who were blinded to experimental conditions and

treatment groups.

Comment 5:

What is the effect of optogenetic inactivation of OMN^{ChAT} neurons on EMs and brain states?

Response:

Thanks for your constructive suggestions. Following the reviewer's suggestion, we performed new experiments of optogenetic inhibition on nIII^{ChAT} neurons.

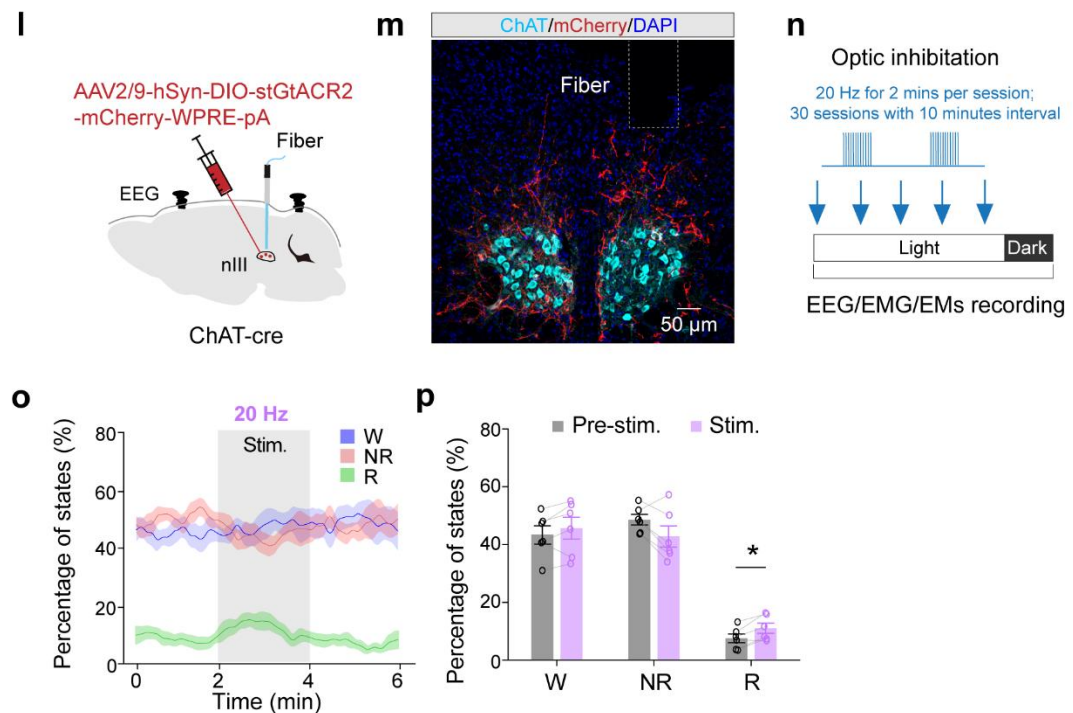
We injected AAV2/9-hSyn-DIO-stGtACR2-mCherry-WPRE-pA, a Cre-dependent inhibitory opsin, into the nIII of ChAT-Cre mice to selectively inhibit nIII^{ChAT} neurons (**Fig. R3-7l-m**). Three weeks after the injection, we applied optical stimulation using the same parameters as in the optogenetic activation experiments (20 Hz for 2 min, 10 ms pulse width, 2mW) (**Fig. R3-7n**). Optogenetic inhibition of nIII^{ChAT} neurons significantly increased the proportion of REM sleep (**Fig. R3-7o-p**), accompanied by a reduction in NREM sleep. This effect was consistent with that observed during optogenetic activation of nIII^{ChAT} neurons, which suppressed REM and increased wakefulness.

We also found that single-pulse optogenetic inhibition of nIII^{ChAT} neurons induced transient EMs (**Fig. R3-8e-f**), and high-frequency optogenetic inhibition of nIII^{ChAT} neurons triggered EMs time-locked to each stimulation pulse (**Fig. R3-8g, Fig. R3-9**). While activation of nIII^{ChAT} neurons led to temporally-to-nasal EMs on the ipsilateral side (see more detail in **Reply to Reviewer #2 Comment 10**), inhibition caused nasal-to-dorsotemporally EMs in ipsilateral eye (**Fig. R3-8**).

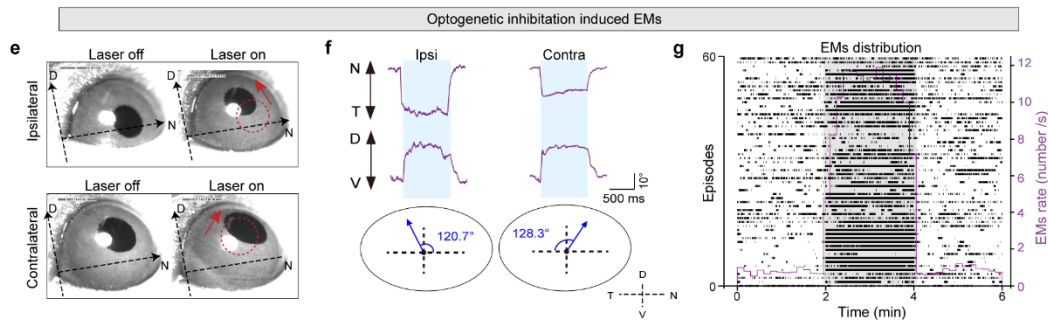
We would like to also bring to the reviewer's attention that in **Reply to Reviewer #2 Comment 8 as well as Reply to Reviewer #3 Comment 8**, we demonstrated that optogenetically-induced EMs has no effect on the brain states.

Together, these new findings demonstrate that optogenetic suppression of nIII^{ChAT} neurons induce EMs and promote the REM sleep. **We have incorporated these new results in the Result section and have included a detailed description in the**

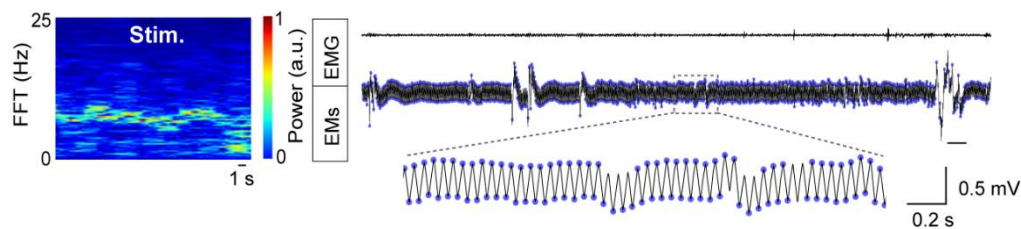
corresponding sections of Method in revised manuscript.



(New updated Fig.2) Fig. R3-7 | Non-EOMs-projecting nIII^{ChAT} neurons modulate R-W transition. **l** Schematic depicting stGtACR-mediated inhibition of nIII^{ChAT} neurons. **m** Confocal image of viral expression and the placement of optical fibers above the nIII. The dashed grey box indicates the trace of the optical fiber. **n** Two-minute blocks (blue arrows) of stimulations were delivered with intervals of 10 to 25 min throughout the light phase to photo-inhibit nIII^{ChAT} neurons. **o** Percentage (left) and quantification (right) of wake, NREM, or REM states before, during, and after optogenetic inhibition in ChAT-Cre mice. Left: 3.3 Hz at 470 nm, n=8. Right: 20 Hz at 470 nm, n=8. The gray shielding indicates the time window of stimulation. The red, blue and green shielding indicates the 95% confidence intervals (CIs). Each dot indicates individual mice.



(New updated Supplementary Fig.6) Fig. R3-8 | Effects on EMs and sleep architecture after silencing of nIII^{ChAT} neurons. **e** The position of ipsilateral or contralateral eyeball before (laser off) and after (laser on) optogenetic inhibition. Laser duration, 1 s. Power density, 2 mW. **f** Upper: example of ipsilateral or contralateral EMs traces induced by optogenetic inhibiting the nIII^{ChAT} neurons. Lower: schematic diagram of EMs directions during optogenetic activation of inhibition (n = 3 mice). Blue area indicates the stimulation time window. D: dorsal, V: ventral, T: temporal, N: nasal. **g** An example raster plot of EMs distribution across multiple optogenetic trials. The shaded area indicates the time window of optogenetic stimulation.



(New updated Supplementary Fig.7) Fig. R3-9 | Dynamic change of EMs, EEG and EMG during optogenetic inhibition. Dynamic change in EEG power spectrum (left), EEG and EMs (right) during optogenetic inhibition in sleep. The optogenetic inhibition evoked sustained high-frequency EMs. The blue dots in the EMs trace indicate the automatically detected EMs event.

The corresponding description have been added in the revised manuscript:

(Page 5 line 188-206) To clarify the necessity of nIII^{ChAT} neurons in regulating REMs, beyond the cell ablation approach, we have performed acute and cell-type-specific

optogenetic inhibition of nIII^{ChAT} neurons during polysomnographic recordings. We expressed AAV2/9-hSyn-DIO-stGtACR2-mCherry-WPRE-pA in the nIII^{ChAT} neurons in ChAT-Cre mice (Fig. 2l-m). Three weeks post-injection, optogenetic inhibition of nIII^{ChAT} neurons was performed during the light phase (12:00–18:00) in 30 sessions, each comprising a 2-min, 20-Hz stimulation block (indicated by blue arrows) delivered at 10-min intervals (Fig. 2n). We found that optogenetic inhibition of nIII^{ChAT} neurons induced a reliable EMs in nasal-to-dorsotemporal direction (Supplementary Fig. 6f-g). We further examined the dynamic changes in EEG, EMG, and EMs signal across different vigilance states (Supplementary Fig. 7). We found that the occurrence rate of EMs rapidly increased during the stimulation window across multiple trials (Supplementary Fig. 6h, Supplementary Fig. 7). The EEG power spectra and EMG of each brain state during inhibition were similar to those displayed during spontaneous sleep-wake cycle indicating that the optogenetic-evoked EMs did not interfere EEG/EMG signals, thus not affecting the accurate identification of sleep stages (Supplementary Fig. 7a-f). We then evaluate the sleep architecture and observed that optogenetic inhibition of nIII^{ChAT} neurons significantly increased the proportion of REMs (Fig. 2o-p). These results is consistent with the nIII^{ChAT} neuronal ablation experiment, indicating that silencing nIII^{ChAT} neurons could lead to the generation and maintenance of REMs.

(Page 15 line 627-639) The following recombinant adeno-associated viral vectors (AAVs) were packaged and purchased from Shanghai Taiertu Biological Technology Co., Ltd.: AAV2/9-hEF1 α -DIO-hChR2(H134R)-EYFP, AAV2/2-Retro-hSyn-hChR2(H134R)-EYFP, AAV2/2Retro-Plus-hEF1 α -DIO-hChR2(H134R)-mCherry-WPRE-pA, AAV2/9-hSyn-DIO-stGtACR2-mCherry-WPRE-pA, AAV2/9-hSyn-mCherry-WPRE, AAV2/9-CAG-DIO-taCaspase3-WPRE, AAV2/9-hSyn-FLEX-GCaMP6s-WPRE, pAAV-EF1 α -DIO-GTACR1-GFP, AAV1/2-hSyn-cre-tdTomato, AAV2/8-hSyn-Flex-mWGA-mCherry-WPRE-pA, AAV2/9-hSyn-Flex-ChrimsonR-tdTomato-WPRE-pA, AAV2/9-hSyn-ChrimsonR-tdTomato-WPRE-pA, and

AAV2/2Retro-hSyn-Cre-WPRE-pA. The retrograde tracer rabies virus V-EnVA-G-mCherry, together with the helper viruses rAAV-EF1 α -DIO-EGFP-T2A-TVA and rAAV-EF1 α -DIO-oRVG(Δ G), were purchased from Brain Case (Shenzhen) Biotechnology Co., Ltd. The titer was estimated to be $\sim 10^{12}$ to $\sim 10^{13}$ genome copies ml^{-1} and diluted to $\sim 10^{12}$ with 5% glycerin and 95% PBS prior to administration.

(Page 22 line 942-945) For optogenetic inhibition of nIII^{ChAT} neurons, a 590 nm laser at a frequency of 20 Hz with a power of 2 mW was applied for 2 min per trial with intervals of 15 min during 1:00 pm-6:00 pm at light phase. Each mouse underwent a total of 30 trials within a 24 hrs period.

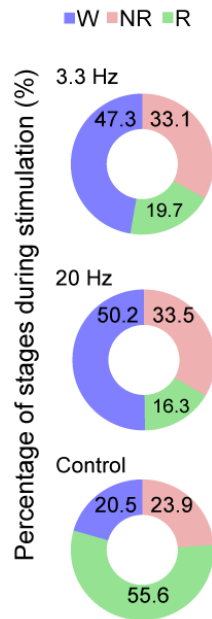
Comment 6:

Line 213-214, the number 51.9%, 18.7%, 20.0% did not fit with the numbers presented in Fig.3l, any explanation on that?

Response:

Thank you for pointing out this mistake. We sincerely apologize for the typographical error in the original manuscript. **We have now corrected the corresponding values to ensure consistency with Fig. R3-10** (Fig.3l in the original manuscript). Details can be seen below.

When optogenetic stimulation was selectively delivered during REM sleep, the probability of REM sleep markedly decreased from 55.6% to 19.7% with 3.3 Hz stimulation for 2 minutes, and to 16.3% with 20 Hz stimulation for 2 minutes. These corrected values in text have been updated in the revised manuscript accordingly.



(New updated Fig.3) Fig. R3-10 | Non-EOMs-projecting nIII^{ChAT} neurons modulate R-W transition. k Pie charts showing changes in brain states during laser stimulation when specifically activating the nIII^{ChAT} neurons during REM sleep.

The corresponding description have been added in the revised manuscript:

(Page 7 line 296-298) When the optical stimulation was specifically delivered during the REM sleep period, the probability of REM sleep decreased drastically from 55.6% to 19.7% upon 3.3 Hz optogenetic stimulus for 2 min or to 16.3% by 20 Hz optogenetic stimulus for 2 min.

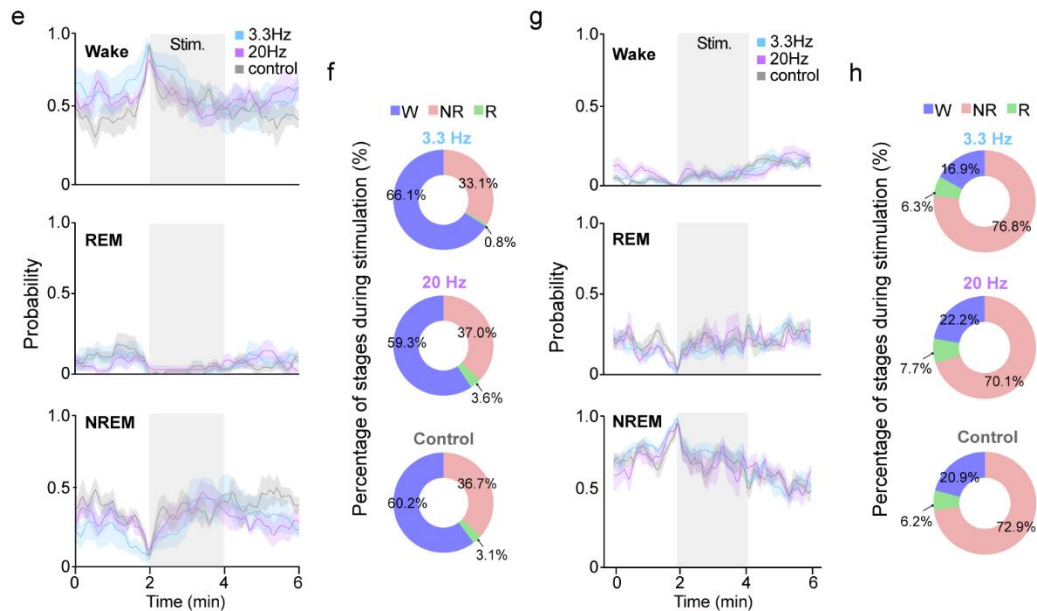
Comment 7:

In Supplementary Fig.3e, the data shown looks like that of when stimulation were presented during wakefulness rather than the stated NREM sleep, since the probability of wakefulness is 1 before stimulation. Please explain the discrepancy between the figure and the statement.

Response:

Thank you for pointing out the mistake. We apologize for the typographical error in Supplementary Fig.3e in the original manuscript. As the reviewer correctly pointed out, the data we mistakenly presented in the original manuscript were result of

optogenetic activation during wakefulness. In the revised manuscript, we provided data on the alterations in sleep-wake architecture when stimulation was applied during both wakefulness (**Fig. R3-11e-f**) and NREM sleep (**Fig. R3-11g-h**).



(New updated Supplementary Fig.13) Fig. R3-11 | Change of EMs and sleep architecture following optogenetic activating the non-EOMs-projecting nIII^{ChAT} neurons. **e** The probabilities of different vigilance states in ChAT-Cre mice when optogenetic activation during wake in Chr2-tagged nIII^{ChAT} neurons in ChAT-Cre mice. The gray shielding indicate the optogenetic stimulation coverage time window. **f** Pie charts showing changes in brain states during laser stimulation when specifically activating the nIII^{ChAT} neurons during wake. **g** Probabilities of different sleep state in ChAT-Cre mice when specifically activating the nIII^{ChAT} neurons during NREMs in Chr2-tagged ChAT-Cre mice. The gray shielding indicates the time window of stimulation. **h** Pie charts showing changes in brain states during laser stimulation when specifically activating the nIII^{ChAT} neurons during NREMs.

The corresponding description have been added in the revised manuscript:

(Page 7 line 296-301) When the optical stimulation was specifically delivered during the REMs period, the probability of REMs decreased drastically from 55.6% to 19.7%

upon 3.3 Hz optogenetic stimulus or to 16.3% by 20 Hz optogenetic stimulus (Fig. 3j-k). However, no discernible alteration was noted when the optogenetic stimulus were presented during wake or NREMs (Supplementary Fig. 12e-h). Consistently, the latency from the onset of light stimulus to the R-W transition was significantly shortened upon optogenetic stimulations (Fig. 3m-o).

Comment 8:

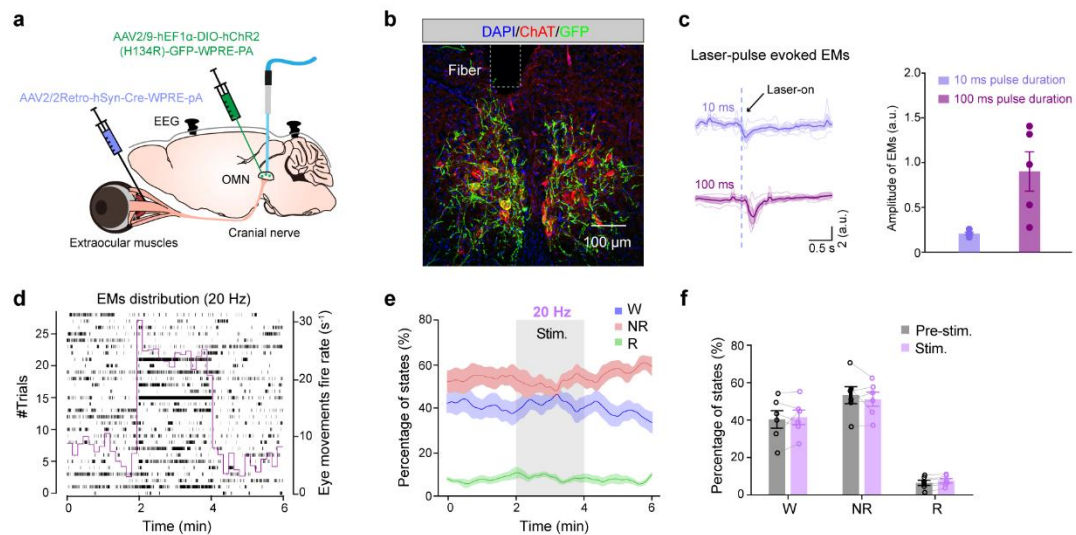
Does optogenetic activation of extraocular muscle-projecting OMN^{ChAT} neurons affect brain states?

Response:

Thank you for your insightful comment. Following the reviewer's suggestion, we conducted new experiments to specifically assess whether optogenetic activation of nIII^{ChAT} neurons that project to the extraocular muscles influences brain states.

To achieve specific expression of ChR2 in nIII^{ChAT} neurons innervating extraocular muscles, we injected retroAAV-hSyn-Cre-WPRE into the extraocular muscles of C57BL/6 mice, followed by AAV2/9-hEF1 α -DIO-ChR2(H134R)-GFP-WPRE injection into the nIII (Fig. R3-12a-b). Upon optogenetic stimulation, we observed robust and consistent EMs (Fig. R3-12c-d). Importantly, activation of these neurons did not lead to significant changes in the proportion of time spent in REM sleep, NREM sleep, or wakefulness (Fig. R3-12e-f). These results provide strong evidence that activation of extraocular muscle-projecting nIII^{ChAT} neurons did not affect brain states.

We have incorporated these new results in the Result section and have included a detailed description in the corresponding sections of Method in revised manuscript.



(New added Supplementary Fig.14) Fig. R3-12 | Effect of optogenetic activation of EOMs-projecting nIII^{ChAT} neurons on sleep. **a** Schematic of viral injection and fiber implantation. **b** The confocal image of viral expression and location of fiber in the nIII. **c** Left: EMs trace induced by optogenetic activation of EOMs-projecting nIII^{ChAT} neurons. Right: quantification of laser-evoked EM amplitudes under 10 ms and 100 ms pulse durations. Each dot represents an individual mouse. **d** Raster plot of EMs events during optogenetic activation of EOM-projecting nIII^{ChAT} neurons. Gray shading indicates the time window of optogenetic stimulation. **e-f** Percentage (**e**) and quantification (**f**) of wake, NREM, or REM states before, during, and after optogenetic activating the EOMs-projecting nIII^{ChAT} neurons. The gray shielding indicates the time window of stimulation with 20 Hz. Gray shading indicates the time window of optogenetic stimulation. The red, blue and green shielding indicates the 95% confidence intervals (CIs).

The corresponding description have been added in the revised manuscript:

(Page 7-8 line 302-30) To further dissociate EMs control from REMs modulation, we injected retroAAV-hSyn-Cre-WPRE-pA into the EOMs of C57 mice and subsequently injected AAV2/9-hEF1α-DIO-ChR2(H134R)-GFP-WPRE into the nIII to selectively express ChR2 in nIII^{ChAT} neurons projecting to the EOMs (Supplementary Fig. 14a-b). Optogenetic activation of these neurons induced robust EMs (Supplementary Fig. 14c-d), yet did not significantly alter the proportion of time spent in different sleep states

(Supplementary Fig. 14e-f). These results support the conclusion that activation of nIII^{ChAT} neurons directly drives R-W transitions, rather than exerting an indirect influence via evoked EMs.

(Page 17 line 725-730) To selectively activate the non-EOMs-projecting nIII^{ChAT} neurons, we injected retroAAV-hSyn-Cre-WPRE into the extraocular muscles of C57BL/6 mice, followed by AAV2/9-hEF1 α -DIO-ChR2(H134R)-GFP-WPRE injection into the nIII. To activate the EOMs-projecting neurons, we injected retroAAV-hSyn-Cre-WPRE-pA into the EOMs of C57 mice and subsequently injected AAV2/9-hEF1 α -DIO-ChR2(H134R)-GFP-WPRE into the nIII to selectively express ChR2 in EOMs-projecting nIII^{ChAT} neurons.

Comment 9:

In Fig.4, how many mice and how many sessions were used for the calcium imaging?

Response:

Thank you for your constructive comment. For single-cell calcium imaging, 5 mice were used, with each mouse recorded across 2 independent sessions. We have added this information to the revised manuscript, both in the main **text** and **method** for clarity.

The corresponding description have been added in the Result and Method section of revised manuscript:

(Page 8 line 316-323) The expression of GCaMP6s in nIII^{ChAT} neurons was shown by immunostaining 4 weeks post-injection (Fig. 4c). We recorded the activity profiles of nIII^{ChAT} neurons for 2 hours per session from 5 mice, at least two sessions per mouse. Each session encompassed multiple episodes of wake, NREMs, and REMs (Fig.4d, Supplementary Video5). We observed prominent calcium signals in spatially separated individual nIII^{ChAT} neurons during EMs in REMs or wake (cell 15, 18, 31 in Fig.4e,f and Supplementary Fig. 14a,b), as well as during R-W transition (cell 3, 4 in Fig.4e,f and Supplementary Fig. 14e,f). There were also some neurons exhibited non-specific responses across these episodes (Fig. 4f).

(Page 21 line 884-890) While minor changes in field of view were inevitable due to long-term imaging sessions, all data were analyzed independently to explicitly align cell signals across imaging sessions. Images were collected at a rate of 11 Hz with an LED power of 1 mW/mm² and a gain of 6. A total of 5 mice were recorded, each with at least two imaging sessions. Each imaging session lasted for 1-2 hrs, and the calcium movies from all of the acquired sessions were motion corrected using default settings in the inscopix data processing software. Single-cell activity traces were obtained using CNMF-E algorithm

Comment 10:

10.Line 293, citation 6 and 24 does not seem to be appropriate.

Response:

Thank you for the point out this mistake. We have replaced citations 6 and 24 with appropriate references (**Chen et al. 2022, Weber et al. 2018**) in the revised manuscript.

Comment 11:

The description of Figure 5 is chaotic, which reduced the credibility of this study. For example, line 286, it should be anterograde tracing rather than retrograde tracing, right? line 287-288, the author used mWGA-mCherry virus, while in Fig.5a, the author stated in the legend that shown are GFP-labeled cells (line 1090). What is the explanation for this confusion? Line 296, the author stated that they used AAV-Ef1a-ChrimsonR-mCherry virus, while in Fig. 5b and the legend, it was AAV2/9-hEF1 α -DIO-hChR2(H134R)-GFP. Which one did the author exactly use?

Response:

We thank the reviewer for pointing out these mistakes in our original manuscript. We sincerely apologize for the typographical error in the descriptions of Figure 5. Detailed corrections are provided as follows:

In line 286, the word “retrograde” should be “**anterograde**”.

In lines 287-288, “GFP-labeled cells” should be “**mCherry-labeled cells.**”

In line 296, “AAV-Ef1a-ChrimsonR-mCherry” should be “**AAV2/9-hEF1 α -DIO-hChR2(H134R)-GFP**”.

We have corrected these mistakes in the revised manuscript.

The corresponding description have been added in the revised manuscript:

(Page 9 line 369-376) To further investigate the neural circuit mechanisms by which the nIII regulates REMs, we conducted anterograde tracing to identify their downstream targets. AAV2/8-hSyn-Flex-mWGA-mCherry-WPRE-pA (mWmC) virus was a genetically encoded monosynaptic anterograde tracer developed by Nicole Y. Tsai et al.²⁹. The anterograde and trans-synaptic labeling capabilities of mWmC have been validated through striatal injections, which resulted in robust mCherry-labeled somas in the substantia nigra pars reticulata (SNr)²⁹. We then injected the mWmC virus into the nIII in ChAT-Cre mice to identify direct postsynaptic targets of nIII^{ChAT} neurons (Fig. 5a).

(Page 10 line 396-399) To examine whether vlPAG-projecting nIII^{ChAT} neurons regulates REMs through nIII-vlPAG circuit, we stereotactically injected AAV2/9-hEF1 α -DIO-hChR2(H134R)-GFP into the nIII of ChAT-Cre mice and implanted an optic fiber into the vlPAG, along with an EEG/EMG/EMs polysomnography recording device (Fig. 5i).

(Page 39 line 1433-1435) **Fig. 5 | vlPAG-projecting nIII^{ChAT} population suppress REMs.** **a** Schematic of anterograde tracing of the nIII^{ChAT} neurons to label the downstream nuclei. **b** Example image showing mCherry-labelled somatic in several different downstream regions.

Comment 12:

Does activating vlPAG-projecting OMN^{ChAT} neurons affect EMs?

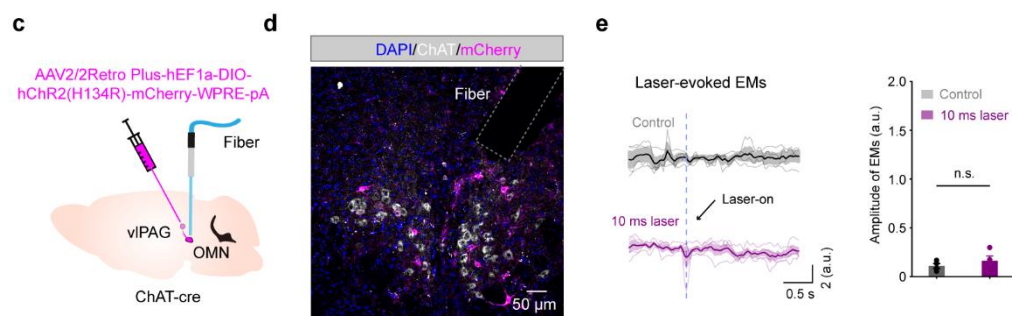
Response:

Thank you for the reviewer’s constructive comment. Taking the reviewer’s suggestion, we conducted new experiments of selectively activating the vlPAG-

projecting nIII^{ChAT} neurons.

We injected AAV2/9Retro-Plus-hEF1a-DIO-hChR2(H134R)-mCherry-WPRE-pA into the vIPAG of ChAT-Cre mice, and implanted an optical fiber above the nIII (**Fig. R3-13c-d**). Optogenetic activation at 480 nm (40 pulses, 10 ms pulse width, 4 s inter-pulse interval, 2 mW) did not elicit significant EMs compared to the control group (**Fig. R3-13e**).

We have incorporated these new results in the Result section and have included a detailed description in the corresponding sections of Method in revised manuscript.



(New added Supplementary Fig.17) Fig. R3-13 | Effect of optogenetic activating vIPAG-projecting nIII^{ChAT} neurons on the EMs. **a** The schematic of virus injection in ChAT-Cre mice. **b** The distribution of nIII^{ChAT} neuronal terminal within the vIPAG along the rostral–caudal axis. **c** The schematic of virus injection and fiber implantation in the nIII. **d** Representative confocal image demonstrating overlap between ChR2 and ChAT expression in vIPAG-projecting nIII neurons. **e** Left: EMs trace induced by optogenetic activation of vIPAG-projecting nIII^{ChAT} neurons. Control group consisted of the same mice subjected to 590 nm light stimulation (40 pulses, 10 ms pulse width, 4 s inter-pulse interval, 2 mW). Right: quantification of laser-evoked EMs amplitudes under 10 ms pulse durations compared to control group. Each dot represents an individual mouse. Two-sided paired t-test; n.s. indicates no significant difference

The corresponding description have been added in the revised manuscript:

(Page 10 line 403-410) We next sought to examine the effect of activation of vIPAG-

projecting $nIII^{ChAT}$ neurons on EMs. RetroAAV2/2-Plus-hEF1a-DIO-hChR2-mCherry-WPRE were injected into the vlPAG of ChAT-Cre mice, followed by implanted an optical fiber above the nIII (Supplementary Fig. 17c-d). Optogenetic stimulation at 480 nm was then applied in head-fixed mice. Laser-induced EMs were recorded by camera. As shown in Supplementary Fig. 17e, optogenetic activation of vlPAG-projecting $nIII^{ChAT}$ neurons did not elicit significant EMs compared to the control group. These anatomical and functional experiments provide evidence that vlPAG-projecting $nIII^{ChAT}$ neurons specifically regulate REMs rather than EMs.

Comment 13:

In Fig.5, are the vlPAG projecting and LC projecting OMN neurons the same or distinct populations? Other than vlPAG and LC, did the author test the effects of activating other targets (e.g., VTA, MPB) on sleep?

Response:

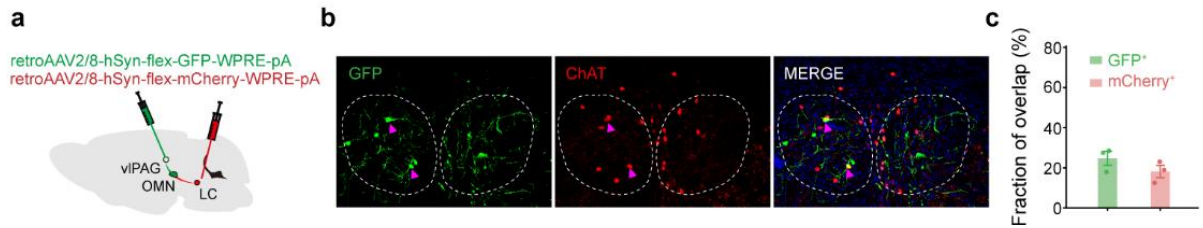
We thank the reviewer for the constructive comments. To address these questions, we conducted new experiments to investigate whether vlPAG projecting and LC projecting nIII neurons are the same populations and to evaluate the effects of activating VTA and MPB on sleep.

We first injected Cre-dependent retrograde viruses (retroAAV2/8-hSyn-FLEX-GFP-WPRE-pA and retroAAV2/8-hSyn-FLEX-mCherry-WPRE-Pa) into the vlPAG and LC, respectively, in ChAT-Cre mice (**Fig. R3-14a**). We then examined the co-localization of GFP (vlPAG-projecting nIII neurons) and mCherry (LC-projecting nIII neurons) signals in the nIII nucleus. As shown in **Fig. R3-14b-c**, the majority of labeled neurons exhibited non-overlapping GFP or mCherry expression. However, a small population of GFP⁺ and mCherry⁺ double-labeled cells was observed, accounting for 24.6% of vlPAG-projecting $nIII^{ChAT}$ neurons and 18.2% of LC-projecting $nIII^{ChAT}$ neurons, indicating that while there is some overlap between vlPAG projecting and LC projecting populations.

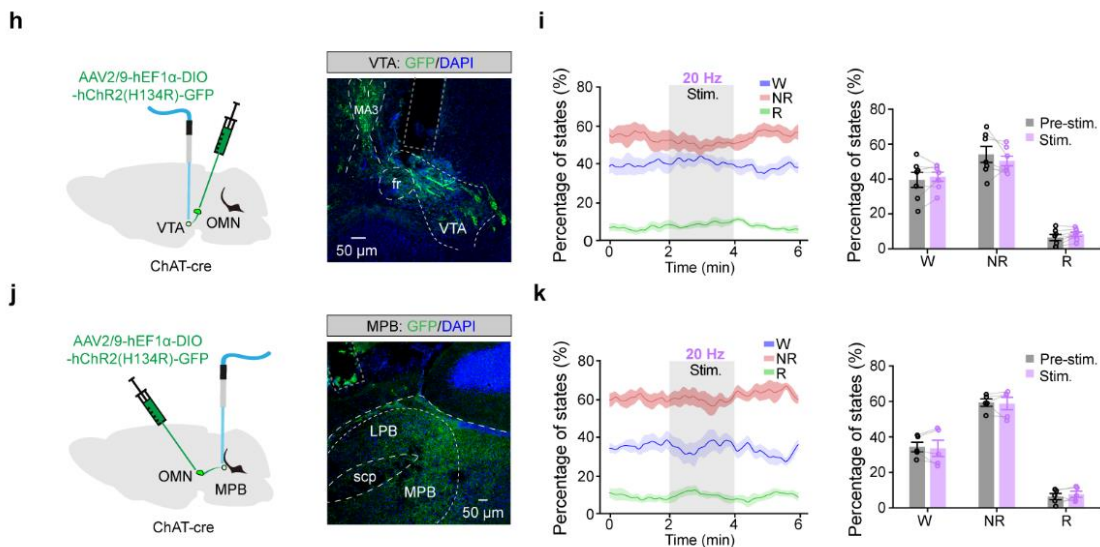
To further examine whether $nIII^{ChAT}$ projections to VTA and MPB are involved in

sleep regulation, we injected Cre-dependent ChR2-expressing viruses into the nIII of ChAT-Cre mice and performed optogenetic activation in the VTA and MPB in two separate groups of mice (**Fig. R3-15h-k**). Optogenetically stimulating these pathways did not result in significant changes to sleep architecture, suggesting that the nIII^{ChAT}-VTA and nIII^{ChAT}-MPB circuits are not likely to be directly involved in sleep regulation.

We have incorporated these new results in the Result section and have included a detailed description in the corresponding sections of Method into the revised manuscript.



(New added Supplementary Fig.19) Fig. R3-14 | Spatial distribution of nIII^{ChAT} neurons projecting to the vlPAG and LC. a The schematic of viral injection in ChAT-Cre mice. **b** Confocal image shows the vlPAG-projecting (GFP) and LC-projecting (mCherry) nIII^{ChAT} neuron. The arrows show the GFP and mCherry double labelling neurons. **c** Quantification of the proportion of double-labeled neurons among nIII^{ChAT} projections to both the LC and vlPAG.



(New added Supplementary Fig.19) Fig. R3-15 | The effect of optogenetic

activating the LC, VTA and MPB on sleep architecture. h The schematic of viral injection and fiber implantation in the VTA (left), the confocal image showing the nIII^{ChAT} neuronal terminal and the fiber placement within the VTA. The dashed box indicates the placement of fiber. **i** Percentage (left) and quantification (right) of wake, NREM, or REM states before, during, and after optogenetic activating the nIII^{ChAT} neurons terminal in the VTA. **j** The schematic of viral injection and fiber implantation in the MPB (left), the confocal image showing the nIII^{ChAT} neuronal terminal and the fiber placement within the MPB. The dashed box indicates the placement of fiber. **k** Percentage (left) and quantification (right) of wake, NREM, or REM states before, during, and after optogenetic activating the nIII^{ChAT} neurons terminal in the MPB.

The corresponding description have been added in the revised manuscript:

(Page 10 line 311-428) Additionally, LC located in the pons is also highly related to arousal and activating the LC noradrenergic neurons effectively induces the transition of mice from sleep to wakefulness^{30,31}. To determine whether the nIII neurons projecting to the vlPAG and LC constitute the same or distinct populations, we injected Cre-dependent retrograde viruses (retroAAV2/8-hSyn-Flex-GFP-WPRE-pA and retroAAV2/8-hSyn-Flex-mCherry-WPRE-pA) into the vlPAG and LC, respectively, in ChAT-Cre mice (Supplementary Fig. 19a). Co-localization analysis of GFP and mCherry signals in the nIII region revealed that a small subset of nIII^{ChAT} neurons (24.6% of GFP⁺ cells and 18.2% of mCherry⁺ cells) were double-labeled (Supplementary Fig. 19b-c), indicating partial overlap between the two projection populations.

To investigate the functional role of the nIII-LC pathway in sleep regulation, we implanted an optical fiber above the LC in ChR2-tagged ChAT-Cre mice and optogenetically stimulated LC-projecting nIII^{ChAT} axons (Supplementary Fig. 19d,e). In contrast to the REMs-suppressing effect observed with nIII-vlPAG pathway activation, activation of nIII-LC projections resulted in a slight increase in REMs probability (Supplementary Fig. 19f,g). Using the same approach, we also performed optogenetic activation of nIII^{ChAT} projections to the VTA and MPB (Supplementary Fig.

19h,j). However, activation of these pathways did not produce significant changes in sleep architecture, suggesting that the nIII^{ChAT}-VTA and nIII^{ChAT}-MPB circuits are not likely to be directly involved in sleep regulation(Supplementary Fig. 19i,k).

(Page 22 line 947-949) To activate nIII^{ChAT} neuron terminals within the VTA or MPB, 480 nm light pulses were delivered at 20 Hz. Each stimulation train lasted 2 min, and a total of 30 trains were applied with 15-min intervals.

Comment 14:

The description and labeling of Extended Data Fig.8 is also chaotic and does not make sense. For example, in panel a, the author injected hSyn-FLEX-ChrimsonR-mCherry virus into NP of ChAT-cre mice, while ChAT seems not to be expressed in NP. Same issue for panel f. In panel a, it should be GCaMP6s virus rather than ChR2 virus, correct?

Response:

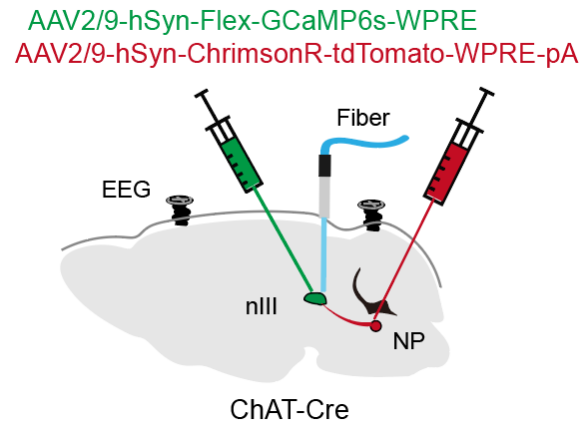
Thank you for the reviewer's constructive comments and pointing out these mistakes. We sincerely apologize for the sloppiness in writing up the descriptions and labels. **We have corrected these mistakes in the revised manuscript and carefully reviewed the entire text and figure legends to ensure accuracy in all viral descriptions and labeling.**

In panel a: "AAV2/9-hSyn-Flex-ChR2(H134R)-WPRE" should be "**AAV2/9-hSyn-Flex-GCaMP6s-WPRE**". "hSyn-FLEX-ChrimsonR-mCherry" should be "**AAV2/9-hSyn-ChrimsonR-tdTomato-WPRE-pA**". This experiments was performed in ChAT-Cre mice (**Fig. R3-16**).

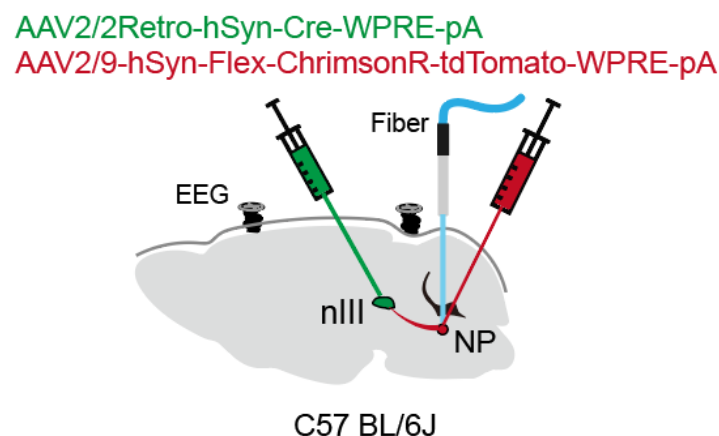
In panel f: "hSyn-FLEX-ChrimsonR-mCherry" should be "**AAV2/9-hSyn-Flex-ChrimsonR-tdTomato-WPRE-pA**". We first injected AAV2/2retro-hSyn-Cre-WPRE-pA into the nIII of C57BL/6 mice, followed by injection of AAV2/9-hSyn-Flex-ChrimsonR-tdTomato-WPRE-pA into the NP (**Fig. R3-17**).

We have corrected these mistakes in the revised manuscript. **Extended Data Fig. 8** has been reorganized into **Fig. 6** and **Supplementary Fig. 21** in revised manuscript. We have carefully reviewed both Fig. 6 and Supplementary Fig. 21 and confirmed that

no additional errors are present.



(New updated Supplementary Fig.21) Fig. R3-16 | Monitoring nIII^{ChAT} neuronal activity using calcium imaging. The schematic of the viral injection and fiber implantation.



(New updated Fig. 6) Fig. R3-17 | NP mediates nIII^{ChAT} neuronal activity to modulate EMs and REM sleep. The schematic of the viral injection and fiber implantation.

The corresponding description have been added in the revised manuscript:

(Page 11 line 468-472) As part of the dorsal paragigantocellular nucleus (DPGi), we wonder whether NP participates in nIII-mediated sleep regulation. To investigate this possibility, we injected AAV2/2Retro-hSyn-Cre-WPRE into the nIII and AAV2/9-Flex-

ChrimsonR-tdTomato-WPRE into the NP of C57 mice (Fig. 6i-j). Upon optogenetic activation of nIII-projecting NP neurons, we observed robust temporal-to-nasal EMs (Fig. 6k-l).

(Page 32 line 490-496 in Supplementary Information) **d** Schematic of virus injection and optical fibers implantation. AAV2/9-hSyn-ChrimsonR-tdTomato-WPRE-pA was injected into the NP and AAV2/9-hSyn-Flex-GCaMP6s-WPRE was injected into the nIII of ChAT-Cre mice. **e** Left: the heatmap of calcium signals from nIII^{ChAT} neurons in response to optical activating NP axon collateral within the nIII. Right: the averaged calcium signal across sessions. The blue dashed line indicates the onset of stimulation. Pulse duration, 1 sec, 5mW. The shaded area indicates the 95% confidence intervals (CIs).

Comment 15:

What is the neuronal cell type in NP that would project to OMN? How does NP coordinate EMs and REM-to-wake transitions through OMN? Does the same NP neuron innervate both extraocular muscle-projecting and vIPAG-projecting OMN neurons?

Response:

We thank the reviewer for the thoughtful comments. To address these important questions, we performed new experiments using monosynaptic rabies tracing and *in vivo* electrophysiology and address the questions one by one below:

1. What is the neuronal cell type in NP that would project to OMN? Does the same NP neuron innervate both extraocular muscle-projecting and vIPAG-projecting OMN neurons?

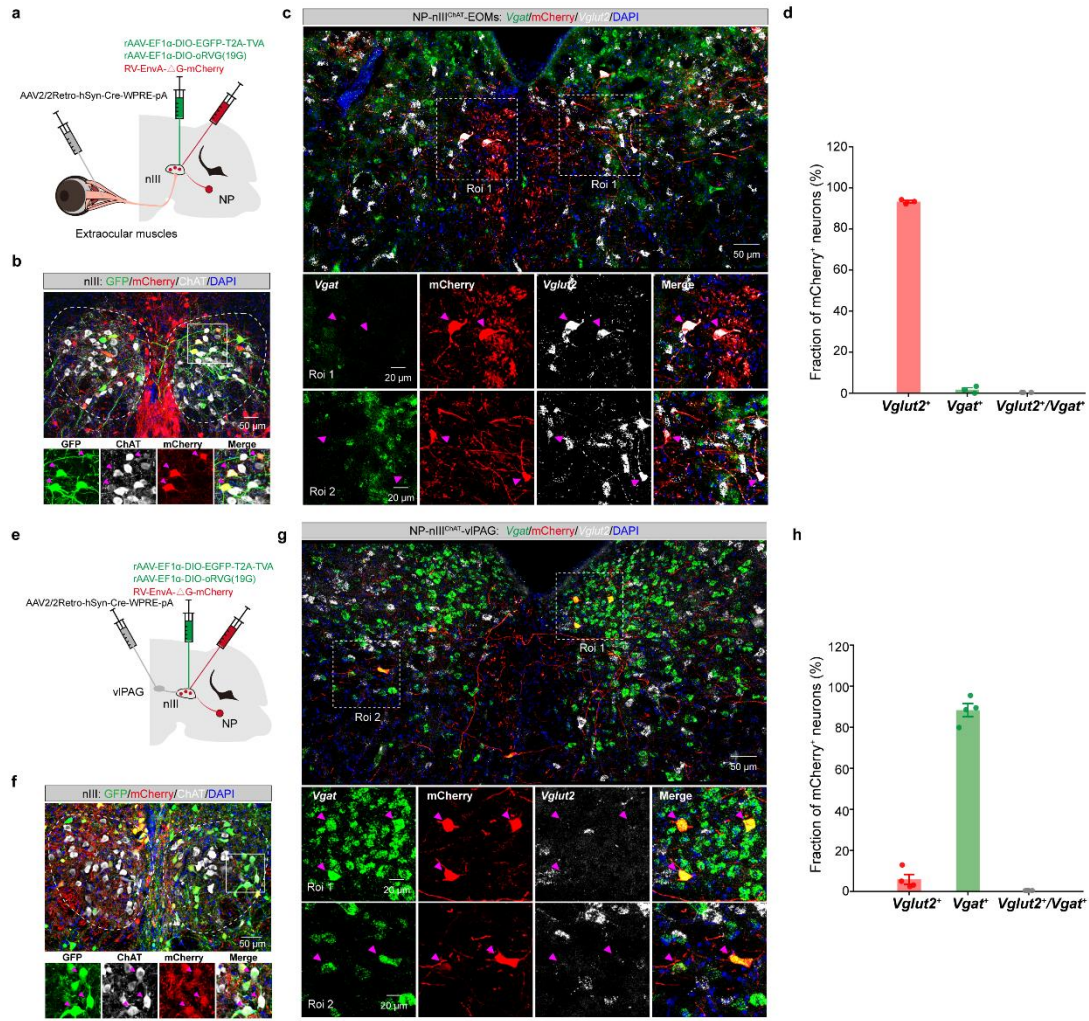
We have inspected the neuronal type in NP that project to nIII^{ChAT} neurons using a rabies virus (RV)-mediated trans-synaptic retrograde tracing approach. The RV virus was selectively expressed in extraocular muscle-projecting nIII^{ChAT} neurons or vIPAG-projecting nIII^{ChAT} neurons, respectively. Immunofluorescence and RNAscope assays

showed that 93.3% of NP neurons that innervate extraocular-muscle-projecting nIII^{ChAT} neurons expressed the glutamatergic marker *Vglut2*, but not the GABAergic marker *Vgat* (**Fig. R3-18c-d**). On the other hand, 88.3% of NP neurons innervating vIPAG-projecting nIII^{ChAT} neurons expressed the GABAergic marker *Vgat*, but not the glutamatergic marker *Vglut2* (**Fig. R3-18g-h**). No co-expression of *Vgat* and *Vglut2* was observed in NP neurons (**Fig. R3-18d-h**). We also examined neurons expressing ChAT and Cart: none of these neurons showed detectable expression (**Fig. R3-19**). In summary, glutamatergic NP neurons project to extraocular muscle-projecting nIII neurons, whereas GABAergic NP neurons project to vIPAG-projecting nIII neurons. **The same NP neurons is unlikely to innervate both extraocular muscle-projecting and vIPAG-projecting nIII neurons.**

2. How does NP coordinate EMs and REM-to-wake transitions through nIII?

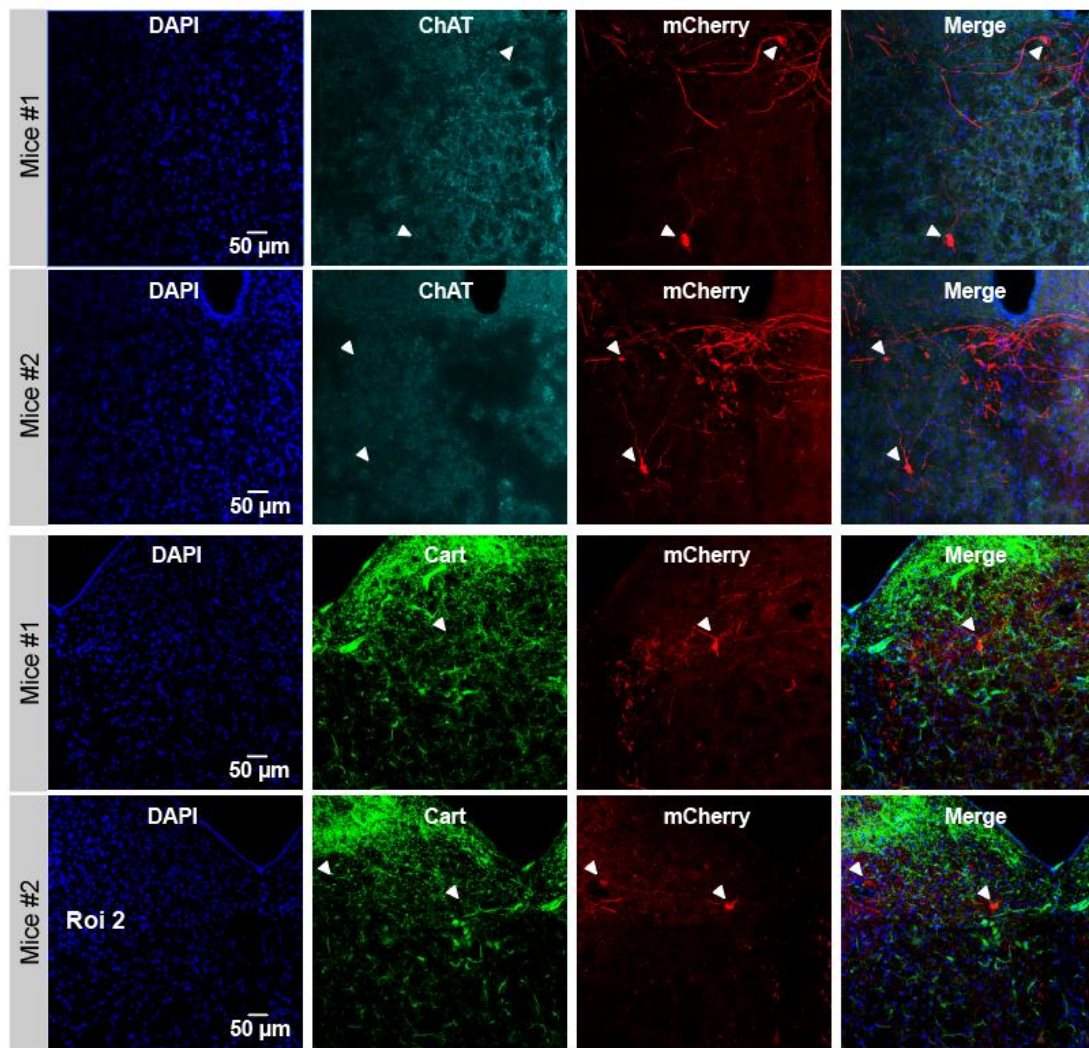
We found that optogenetic activation of NP terminals within the nIII nucleus resulted in 17.9% of nIII neurons being activated and 15.4% being suppressed in downstream neurons (**Fig. R3-20, see more details in Reply to Reviewer #1 Comment 4**). Combining the results from anatomical tracing and electrophysiology result, we hypothesize that NP^{*Vgat*}-nIII-vIPAG pathway mediates REM-to-wake transition, whereas NP^{*Vglut2*}-nIII-extraocular muscles pathway regulates EMs.

We have added these new results to the revised manuscript and have included a detailed discussion of their potential implications in the corresponding sections of the Results and Discussion.



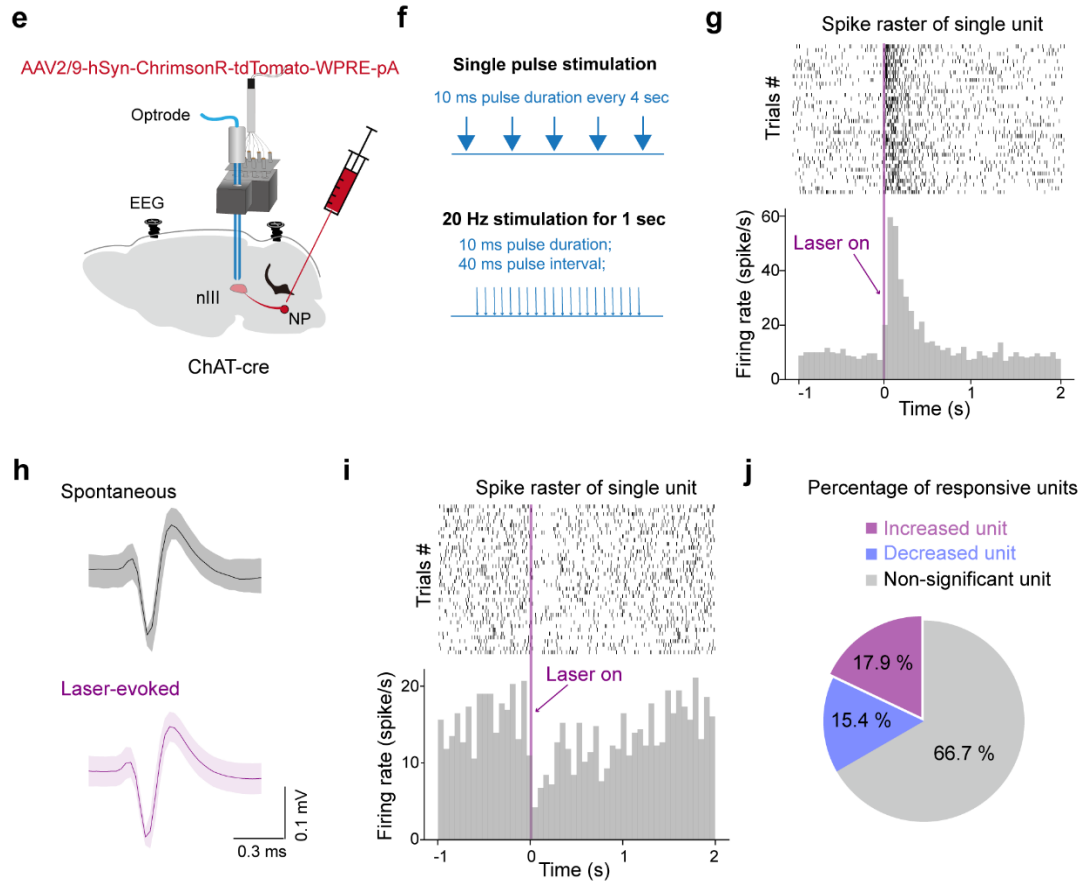
(Newly added Fig. 7) Fig. R3-18. | nIII^{ChAT} innervated NP neurons express mutually exclusive marker genes. **a** Schematic diagram of strategy to label the EOMs-projecting NP neuron. After 3 weeks of AAV2/2-retro-hSyn-Cre expression in the extraocular muscles (EOMs), two helper (rAAV-EF1 α -DIO-EGFP-T2A-TVA and rAAV-EF1 α -DIO-oRVG(19G)) were injected into the nIII in C57 mice, followed by injection of modified rabies virus (RV) expressing mCherry (RV-EnVA-G-mCherry). **b** Confocal image showing a coronal section of the nIII 10 days post-RV injection. The dashed square region in the nIII is magnified below. EOMs-projecting nIII^{ChAT} neurons appear yellow due to co-expression of GFP and mCherry from helper and RV vectors, respectively. **c** Co-expression of mCherry-labeled EOMs-projecting NP neurons with the glutamatergic marker *Vglut2* mRNA. **d** Percentages of mCherry-labelled neurons in the NP region that express *Vglut2* mRNA, *Vgat* mRNA and *Vglut2/Vgat* overlap.

Each dot indicates data from one mouse (3 slices from each mouse, n = 3 mice). **e** Schematic diagram of strategy to label the vIPAG-projecting NP neuron. **f** Confocal image showing a coronal section of the nIII 10 days post-RV injection. The dashed square region in the nIII is magnified below. vIPAG-projecting nIII^{ChAT} neurons appear yellow due to co-expression of GFP and mCherry from helper and RV vectors, respectively. **g** Co-expression of mCherry-labeled vIPAGs-projecting NP neurons with the GABAergic marker *Vgat* mRNA. **h** Percentages of mCherry-labelled neurons in the NP region that express *Vglut2* mRNA, *Vgat* mRNA and *Vglut2/Vgat* overlap. Each dot indicates data from one mouse (3 slices from each mouse, n = 4 mice).



(Newly added supplementary Fig. S23) Fig. R3-19. | Identification of NP neuronal subtypes by Immunostaining. Representative confocal image of NP in coronal section.

ChAT, choline acetyltransferase. mCherry, fluorescent reporter carried by the rabies virus (RV). Cart, cocaine-and-amphetamine-regulated transcript. The white arrows indicate the mCherry positive neurons. Scale bar, 50 μ m.



(Newly added Fig. 6) Fig. R2-20. | NP mediates nIII cholinergic activity to modulate EMs and REM sleep. **e** Schematic of viral injection in the NP and optrode implantation in the nIII. **f** The schematic of stimulus diagram. **g** Representative raster plot (upper) and stimulus time histogram (lower) for laser-evoked spikes of an example ChR2-tagged unit. Dashed line, laser onset time. Laser duration, 10 ms. Power density, 2 mW. **h** Averaged waveform of spontaneous (gray) and laser-evoked (purple) spike of an example unit. **i** Representative raster plot (upper) and stimulus time histogram (lower) for laser-inhibited spikes of an example ChR2-tagged unit. **j** Percentages of increased, decreased and no-significant response units among all recorded electrode in the nIII. Increased and decreased units were defined based on whether the post-stimulation peak

firing rate exceeded a threshold of mean $\pm$ 3 standard deviations (SD) of the baseline activity.

The corresponding description have been added in the revised manuscript:

(Page 11 line 457-465) In addition to RV-mediated anatomical tracing, we also measured functional connections between NP and nIII^{ChAT} neurons using *in vivo* optogenetics-assisted electrophysiological recordings. We injected AAV2/9-hSyn-ChrimsonR-tdTomato-WPRE-pA into the NP and implanted an optrode in the nIII (Fig. 6e, Supplementary Fig. 22a-b). Optogenetic activation of NP terminals within the nIII induced either an increase or decrease in the firing rate of single units upon photostimulation (Fig. 6 f-g, Supplementary Fig. 22c). Among 40 well-identified nIII units recorded from 3 mice, 17.9% exhibited optogenetically activated responses, and 15.4% showed optogenetically suppressed responses (Fig. 6h, Supplementary Fig. 22d-e), demonstrating that nIII received both excitatory and inhibitory synaptic inputs from the NP.

(Page 11-12 line 478-502) Lodged at the dorsomedial boundary of the dorsal paragigantocellular nucleus (DPGi), the NP comprises a heterogeneous population of neurons, including inhibitory GABAergic neurons, excitatory glutamatergic neurons, and a substantial number of neuropeptidergic neurons³³. To further identify the neuron types upstream of nIII^{ChAT} neurons contributing to motor behavior and sleep regulation, we employed a rabies virus (RV)-mediated trans-synaptic retrograde tracing approach. To specifically label NP neurons projecting to EOMs-innervating nIII neurons and to vlPAG-projecting nIII neurons, we first injected AAV2/2-Retro-hSyn-Cre-WPRE-pA into either the extraocular muscles or the vlPAG of C57 mice (Fig. 7a, Fig. 7e). Two Cre-dependent helper viruses rAAV-EF1 α -DIO-EGFP-T2A-TVA and rAAV-EF1 α -DIO-oRVG(19G) were unilaterally injected into the nIII (Fig. 7a, Fig. 7e). After 2 weeks, RV-EnVA- Δ G-mCherry was injected into the nIII, as previously described in Fig. 6c, Using this strategy, the RVs can only infected TVA-expressing cells and

required RG expression to spread retrogradely into presynaptic cells. The starter neurons, which are shown in yellow, were limited to the nIII region and characterized by the expression of both the helper virus and RV in cholinergic neurons (Fig. 7b, Fig. 7f). We mapped the afferent neurons in the NP by imaging 3 brain slices from each of four mice. Immunofluorescence and RNAscope assays shows that 93.3% of the monosynaptic inputs from NP to EOMs-projecting nIII^{ChAT} neurons expressed the glutamatergic marker *Vglut2* mRNA, while scarcely expressing the GABAergic marker *Vgat* mRNA (Fig. 7c-d). On the other hand, 88.3% of NP neurons innervating vIPAG-projecting nIII^{ChAT} neurons expressed the GABAergic marker *Vgat*, but not the glutamatergic marker *Vglut2* mRNA (Fig. 7g-h). No co-expression of *Vgat* and *Vglut2* was observed in NP neurons (Fig. 7d-h). We also examined neurons expressing ChAT and Cart; none of these neurons exhibited detectable co-expression with mCherry (Supplementary Fig. 23). This finding suggests that the functional heterogeneity of nIII^{ChAT} neurons most likely arises from differences in the gene profiles of upstream NP neurons.

Comment 16:

Line 72, Extended Data Fig. 1a? Line 134, Fig. 2j-k?

Thank you for the reviewer's constructive comments. We sincerely apologize for such mistakes. As the reviewer correctly pointed out, the citation in line 72 should be Extended Data Fig. 1a (Supplementary Fig. 1a in the revised manuscript), and the figure reference in line 134 should be **Fig. 2k**.

We have corrected these mistakes in the revised manuscript and carefully reviewed the entire text and figure legends to ensure accuracy in all viral descriptions and labeling.

The corresponding description have been added in the revised manuscript:

(Page 2-3 line 87-89) Subsequently, we recorded calcium signals of nIII^{ChAT} neurons during EMs induced by the optokinetic reflex (OKR) test (Supplementary Fig. 1a).

(Page 5 line 180-181) As a consequence of less fragmentation of REMs in nIII^{ChAT}-ablated mice, the number of R-W transitions was decreased (Fig. 2k).

Based on the Reviewers' comments, we have performed the following revisions in the revised Main Text:

1. Page 1-2, we have revised the Abstract, and Introduction to make it more concise and understandable; We also added relevant literature and clearly stated the main findings to enhance clarity and context;
2. Page 3, we have added citations about the function of nIII^{ChAT} neurons on oculomotor control, revised the description of the nIII^{ChAT} neuronal activity pattern during EMs in different direction; added new result of REM-sleep-selective deprivation;
3. Page 4, we have added the data for electrophysiological recording non-EOMs-projecting nIII^{ChAT} neurons activity across sleep-wake cycle; we updated the result to elucidated the effect of nIII^{ChAT} neuronal ablation on the EMs during spontaneous sleep-wake cycles;
4. Page 5, we have supplemented the effect of optogenetic activation of nIII^{ChAT} neurons on the sleep architecture and analysed the effect of optogenetic activation of nIII^{ChAT} neurons on the modality of Ems; We also discussed that nucleus-specific optogenetic activation did not affect the adjacent Edinger–Westphal (EW) nucleus;
5. Page 6, we have clarified the definition of sleep pressure and supplemented new result to explain the progressive decline in the efficacy of optogenetic stimulation;
6. Page 7, we have added new result of sleep change after specific activating the nIII^{ChAT} neurons during NREM sleep or wakefulness; we also added new result to demonstrate the effect of optogenetic activation the EOMs-projecting nIII^{ChAT} neurons on the sleep states ;
7. Page 7-8, we have added more details regarding the mice number and recording session number in micro-endoscopy imaging experiments;

8. Page 9, we have reorganized the presentation of the results, corrected typographical errors, added information on viral efficacy, and further validated the functional projection from nIII to vlPAG through in vivo electrophysiological recordings;
9. Page 9-10, we have added new result to demonstrate the effect of optogenetic activation the vlPAG-projecting nIII^{ChAT} neurons on EMs and added new experiments to demonstrate the overlap proportion of vlPAG-projecting and LC-projecting nIII^{ChAT} neurons;
10. Page 10-11, we have reorganized the presentation of the results, corrected typographical errors, added information on viral efficacy, and further validated the functional projection from NP to nIII through in vivo electrophysiological recordings;
11. Page 11-12, we have added new experiments to illustrate the neuron type within the NP which send projection to the nIII^{ChAT} neurons;
12. Page 12, we have updated the discussion about the oculomotor control function of nIII^{ChAT} neurons;
13. Page 13, we have discussed the methodological limitation, the role of nIII^{ChAT} in sleep-wake regulating system and updated the main findings in this work;
14. **Page 15-24, Materials and Methods Section**, we have added detailed information regarding the viral tools used, including dosage and injection parameters. Additionally, we included the experimental protocol for REM sleep-specific deprivation, the results of in situ hybridization experiments, and a description of the time normalization procedure. Furthermore, we have supplemented the Methods section with details on optrode implantation and in vivo electrophysiological signal analysis, as well as procedures for cell counting, data quantification, and statistical analysis.
15. **Page 25-29, Reference Section**, we have added new literature reference and revised the inappropriate reference;

16. **Page 30-43, Figure Section**, main figures section, we have updated the Fig.1-5 and added Fig.6 and Fig.7, and revised or added the description in the figure caption.

Based on the Reviewers' comments, we have performed the following revisions in the revised Supplementary Materials:

1. Page 1, we have updated the Supplementary Figure;
2. Page 3, we have added the c-Fos screening result after REM-sleep-selective deprivation;
3. Page 6-8, we have added newly **Supplementary Fig. 4** and **Supplementary Fig. 5** to recording the activity of nIII^{ChAT} neurons across sleep-wake cycle using in vivo electrophysiology approach and updated the captions;;
4. Page 9, we have added the EMs amplitude and frequency after nIII^{ChAT} neuronal ablation and the EMs pattern evoked by optogenetic inhibition in **Supplementary Fig. 6** and updated the captions;
5. Page 10-11, we added the dynamic change of EEG, EMG and EMs signals during wake, NREM and REM sleep under spontaneous condition or optogenetic activation condition in **Supplementary Fig. 7** and updated the captions;
6. Page 12-13, we have updated **Supplementary Fig. 8** to include the result of pupil constriction during optogenetic activation and updated the captions;
7. Page 16, we have newly added the result of ChR2-tagged nIII^{ChAT} neurons during long-term (1 hr) optogenetic activation in **Supplementary Fig. 10** and updated the captions;
8. Page 17, we have revised the figure legend in **Supplementary Fig. 11**;
9. Page 18, we have revised the figure legend and updated the result in **Supplementary Fig. 12**;
10. Page 19, we have added new result to illustrate the effect of optogenetic activation the EOMs-projection nIII^{ChAT} neurons on sleep in **Supplementary Fig. 13** and updated the captions;

11. Page 20-22, we has updated the figure numbering for **Supplementary Fig. 14-15**.
12. Page 23, we have added new result to verify the nIII-vIPAG connection and to assess the effect of optogenetic activation of vIPAG-projecting nIII^{ChAT} neurons on the EMs in **Supplementary Fig. 16**, and updated the captions;
13. Page 24, we have added the laser-evoked response of nIII^{ChAT}-targeted vIPAG neurons in **Supplementary Fig. 17**;
14. Page 25-26, we have added the result to evaluate the effect of optogenetic the nIII^{ChAT}-targed LC, VTA and MPB on sleep architecture in **Figure S18**;
15. Page 27-28, we updated the figure numbering for **Supplementary Fig. 19-20**.
16. Page 29, we have added new result to verify the NP-nIII connection in **Supplementary Fig. 21** and updated the captions;

Reference:

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Response to Reviewers' Comments

Text in blue: Comments/questions from reviewers

Text in black: Response from the authors

Text in green: Text in the original submission

Text in red: Revisions made to the manuscript

Reviewer #1 (Remarks to the Author):

The authors have conducted comprehensive experiments to address my concerns. I thank the authors for their efforts; all of my concerns have been fully addressed.

Response:

We are grateful for your recognition of our work and are happy to hear that the additional experiments and revisions have fully addressed your concerns.

Reviewer #2 (Remarks to the Author):

Overall the authors provide a large number of additional data to the original study strongly strengthening it.

Response:

We sincerely thank the reviewer for recognizing that the additional data have substantially strengthened this study.

In response to the remaining comments, we **have performed additional experiments, corrected imprecise statements, and carefully re-examined all figures and legends** to further improve clarity, accuracy, and overall scientific rigor. All changes have been highlighted in the revised manuscript. **A summary of the revisions in response to the reviewers' comments can be found at the end of the rebuttal letter.** We hope that you find all your concerns addressed in the revised manuscript.

Comment 1. There are a number of typos in many places like in the Title of Fig. R1-2 in which it is written ablation and not optogenetic inhibition.

Response:

We sincerely thank the reviewer for the additional constructive comment and for carefully identifying the typos.

As the reviewer correctly pointed out, the title of Fig. R1-2 incorrectly stated “ablation” instead of “optogenetic inhibition.” in **Reply letter**. In the original Fig. 2, we used both cellular ablation and optogenetic inhibition (Fig. R1-2) to examine the function of nIII^{ChAT} neurons. We have revised the corresponding figure Title in the manuscript (Fig. 2) to: **“Loss of nIII^{ChAT} function** reduces REMs frequency and prolongs REMs duration.” Furthermore, we have thoroughly proofread the entire manuscript and all figure titles/legends to eliminate similar typos and ensure consistency and accuracy throughout.

The corresponding description have been added in the revised manuscript:

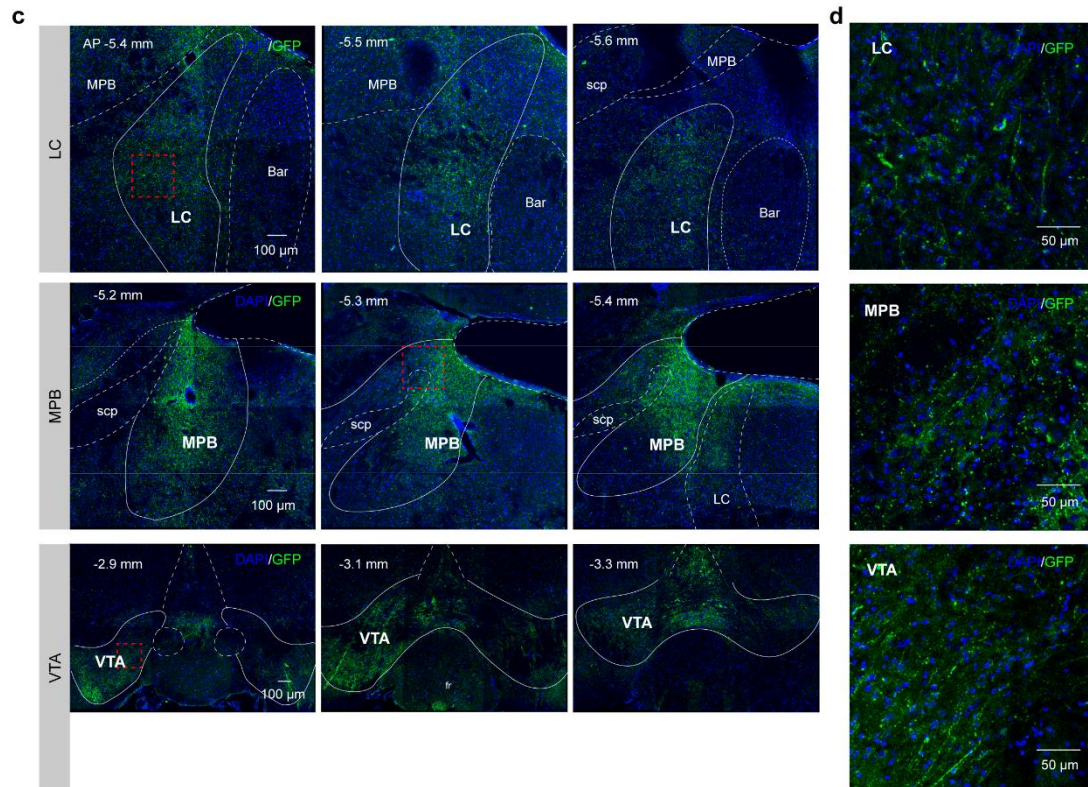
(Page 35 line 1313) **Loss of nIII^{ChAT} function attenuates REMs episodes and elongates REMs duration.**

Comment 2. Concerning the anterograde labeling using mWmC, they did control the projection to the vlPAG using an AAV which is a good addition. They show and tested projections to LC, MPB and VTA but I'm not convinced from their photomicrograph that there are anterogradely labeled fibers in each of these nuclei. Compared to the fibers in the vlPAG the staining is not looking like terminals and for the LC it seems outside the core body of the nucleus. In the VTA it does not look like fiber while in the MPB it seems there are no fibers.

Response:

We sincerely thank the reviewer for this additional constructive comment. We apologize that the relatively low resolution of the immunohistochemical images acquired using the Olympus VS120 microscope in the previous version made it difficult to clearly appreciate the labeled projections. To address this concern, **we performed additional experiments and acquired higher-resolution confocal images using an Olympus FV3000 microscope.** These new images allow clearer visualization of the labeled fibers within the LC, MPB, and VTA, and have been included in the revised manuscript.

Specifically, we performed additional anterograde tracing experiments by injecting AAV2/9-hEF1 α -DIO-hChR2(H134R)-GFP-WPRE-pA into the nIII of ChAT-Cre mice. As shown in **Fig. R2-1**, we observed dense GFP-labeled projections from nIII^{ChAT} neurons to the LC, MPB, and VTA along the rostral-caudal axis in coronal sections. The labeled fibers exhibited clear axonal morphology with terminal-like structures within the boundaries of these nuclei.



(Newly added Supplementary Fig.17) Fig. R2-1 | Axon terminals of nIII^{ChAT} neurons within the LC, MPB and VTA. **c** The distribution of nIII^{ChAT} neuronal terminal within the LC, MPB and VTA along the rostral-caudal axis. **d** Higher-magnification images of the LC, MPB, and VTA regions indicated by the red dashed boxes in (c).

The corresponding description have been added in the revised manuscript:

(Page 10 line 377-380) **By injecting AAV2/9-hEF1 α -DIO-hChR2(H134R)-GFP-WPRE-pA into the nIII of ChAT-Cre mice, we observed clear axonal projections within vIPAG, LC, MPB and LC along the rostral-caudal axis in coronal sections (Supplementary Fig. 17).**

Reviewer #3 (Remarks to the Author):

The authors have addressed my previous critiques, and the manuscript is much improved, while I have a few comments remaining:

Response:

We are sincerely thankful for the reviewer's thoughtful and constructive comment, which help us improve the manuscript a lot.

In response to the remaining comments, we have **refined the figure captions, corrected imprecise statements, and carefully rechecked all figures and legends** to enhance clarity, accuracy, and overall scientific rigor. All changes have been highlighted in the revised manuscript. **A summary of the revisions in response to the reviewers' comments can be found at the end of the rebuttal letter.** We hope that you find all your concerns addressed in the revised manuscript.

Comment 1. The authors incorrectly interpreted the cited ref. 21, as according to ref 21 there is decreased rather than increased c-fos in OCM after REM sleep deprivation. Please make sure the appropriate citation and statement. (line 59)

Response:

Thanks for reviewer's constructive suggestion. We have carefully re-examined ref. 21 (**Fig. R3-2**) and revised our statement accordingly in the revised manuscript.

The sentence in line 59 has been revised in the Introduction section to: **"Cholinergic neurons within the nIII displayed progressively augmented activity with increasing REM sleep deprivation duration, as evidenced by a marked increase in c-Fos expression in nIII^{ChAT} neurons (Ref 21, Santos et al., 2017)."**

[Figure Redacted]

(Fig. 6 in the cited 21) Fig. R3-1 | Oculomotor nucleus ChAT activation after REMSD and REB. From Patrícia Dos Santos et al 2017(Santos et al., 2017). REMSD, REM sleep deprivation, REB, REM sleep rebound.

The corresponding description have been added in the revised manuscript:

(Page 2 line 59-61) Cholinergic neurons within the nIII displayed progressively augmented activity with increasing REM sleep deprivation duration, as evidenced by a marked increase in c-Fos expression in nIII^{ChAT} neurons^[21].

Reference:

Santos, P.D., Targa, A.D.S., Nosedá, A.C.D., Rodrigues, L.S., Fagotti, J., and Lima, M.M.S. (2017). Cholinergic Oculomotor Nucleus Activity Is Induced by REM Sleep Deprivation Negatively Impacting on Cognition. *Mol Neurobiol* 54, 5721-5729.

Comment 2. Line 123, since EMs-free REM episodes had higher Ca²⁺ activity compared to that of EMs-enriched REM episodes, it might not be appropriate to conclude that “EMs contribute to elevated calcium signals in both REM and wake states”, it was true for wake states but not for REM.

Response:

We thank the reviewer for this important and insightful comment. We agree that the original statement “These observations suggest that the nIII^{ChAT} neurons are hyperactive during REMs, and that EMs contribute to elevated calcium signals in both REM and wake states.” was not sufficiently precise.

We have rephrased this sentence as: **“These observations suggest the existence of a subpopulation of nIII^{ChAT} neurons that are hyperactive during REMs episodes without EMs.”** in the revised manuscript.

The corresponding description have been added in the revised manuscript:

(Page 4 line 122-123) **These observations suggest the existence of a subpopulation of nIII^{ChAT} neurons that are hyperactive during REMs episodes without EMs.**

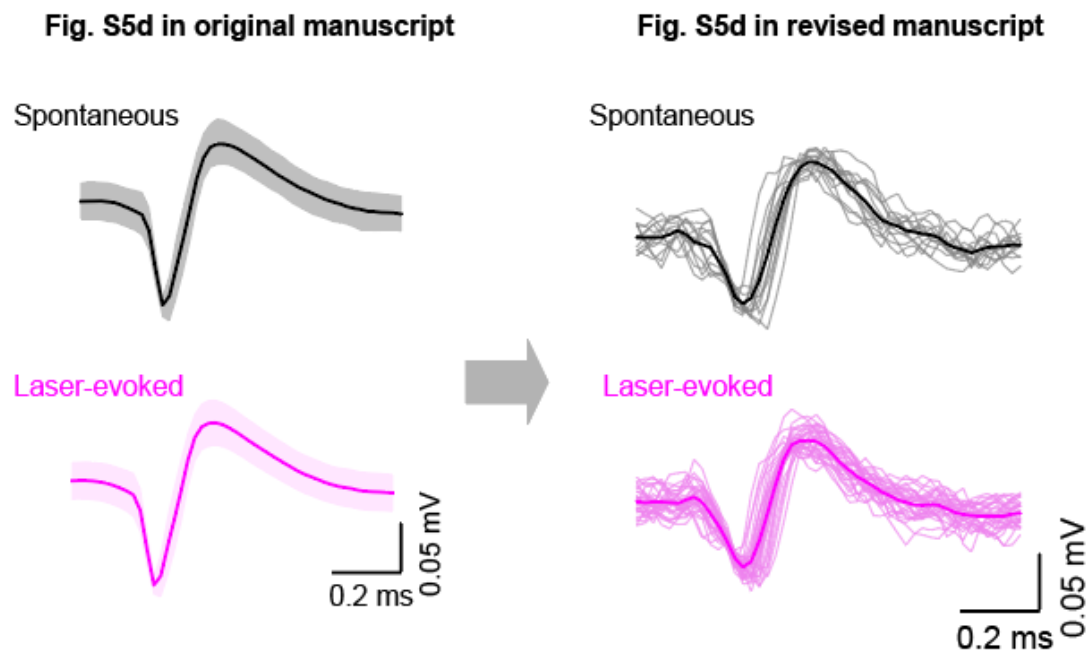
Comment 3. In Fig. 5f and supplementary Fig. 5d, the traces of spontaneous and the laser-evoked spike wave form look almost the same, could there be any mistakes in preparing these figures? The authors should double check their data or provide the source data of these figures.

Response:

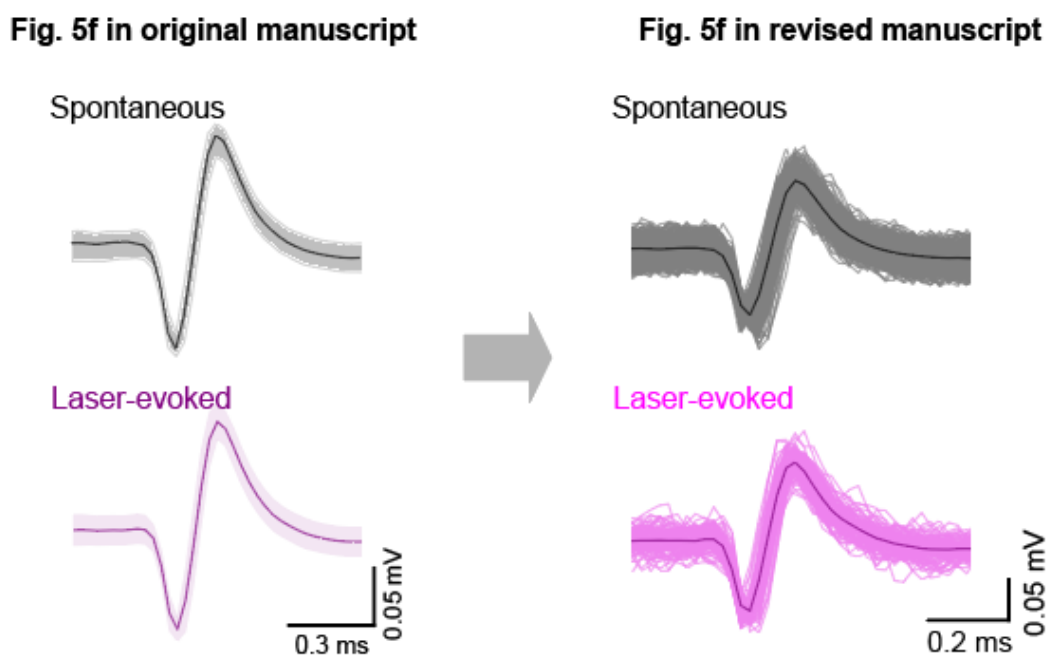
We thank the reviewer for this careful comment. We have re-examined the raw data corresponding to Fig. 5f and Supplementary Fig. 5d. The two datasets shown in Fig. 5f are correct; Supplementary Fig. 5d was inadvertently misplaced in the original manuscript. **We have revised and adjusted the presentation of these data in the revised manuscript, as shown in Fig. R3-2 (original Supplementary Fig. 5d) and Fig. R3-3 (original Fig. 5f).** We have also adjusted Fig. 6f to ensure consistency and accuracy (**Fig. R3-4**). The corresponding source data have now been provided in <https://doi.org/10.6084/m9.figshare.27684144>.

The observation that the laser-evoked spike waveform closely resembles the spontaneous spike waveform is expected. This similarity indicates that optogenetic stimulation effectively triggered physiological action potentials in the recorded neuron, rather than generating aberrant or artifactual events. Similar observations have been widely reported in previous optogenetic studies using Channelrhodopsin-2, where light-evoked spikes exhibit waveform characteristics comparable to spontaneous spikes (**Fig.**

R3-5) (Liang et al., 2021; Weber et al., 2015).



(Newly updated Supplementary Fig. 5d) Fig. R3-2 | Spontaneous and light-evoked spikes of nIII^{ChAT} neurons. Representative traces showing spontaneous (gray) and light-evoked (magenta) action potentials recorded from nIII^{ChAT} neurons. Each thin trace represents an individual spike, and the bold trace indicates the averaged waveform.



(Newly updated Fig. 5f) Fig. R3-3 | Spontaneous and light-evoked spikes of vIPAG

neurons. Representative traces showing spontaneous (gray) and light-evoked (magenta) action potentials recorded from vIPAG neurons. Each thin trace represents an individual spike, and the bold trace indicates the averaged waveform.

Fig. 6f in original manuscript

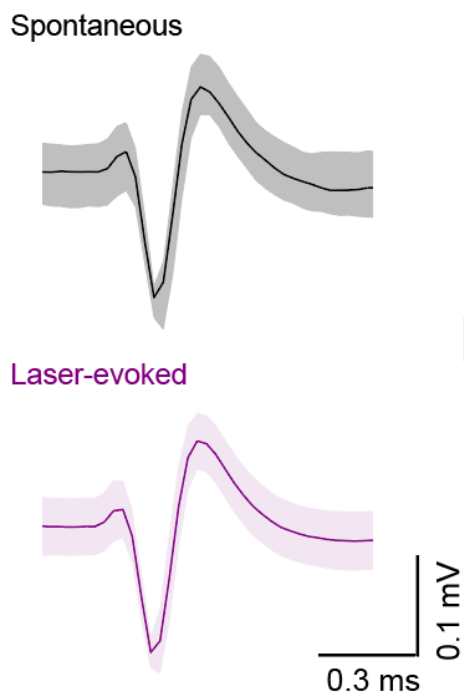
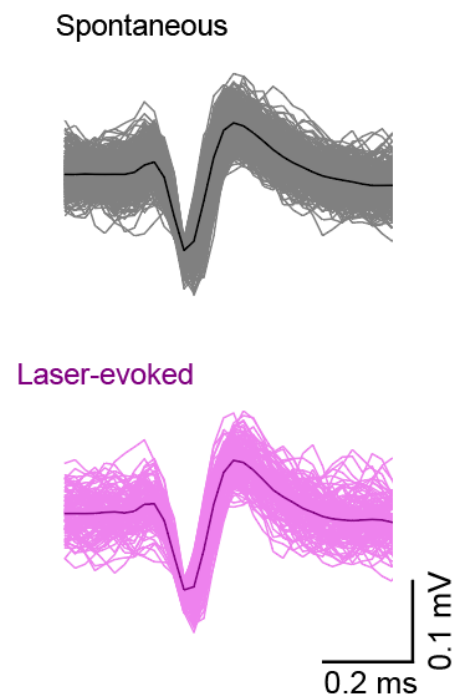


Fig. 6f in revised manuscript



(Newly updated Fig. 6f) Fig. R3-4 | Spontaneous and light-evoked spikes of NP-nIII^{ChAT} neurons. Representative traces showing spontaneous (gray) and light-evoked (magenta) action potentials recorded from nIII^{ChAT} neurons. Each thin trace represents an individual spike, and the bold trace indicates the averaged waveform.

[Figure Redacted]

(Figures in the reference) Fig. R3-5 | Spontaneous and laser-evoked spike. a Comparison between laser evoked (blue) and averaged spontaneous (red) spike waveforms from same unit. From Figure 3b in (Weber et al., 2015). b Averaged waveform of laser-evoked (blue) and spontaneous (gray) spike of an example neuron. From Figure 1H in (Liang et al., 2021).

Reference:

Liang, Y., Shi, W., Xiang, A., Hu, D., Wang, L., and Zhang, L. (2021). The NAergic locus coeruleus-ventrolateral preoptic area neural circuit mediates rapid arousal from sleep. *Curr Biol* 31, 3729-3742 e3725.

Weber, F., Chung, S., Beier, K.T., Xu, M., Luo, L., and Dan, Y. (2015). Control of REM sleep by ventral medulla GABAergic neurons. *Nature* 526, 435-438.

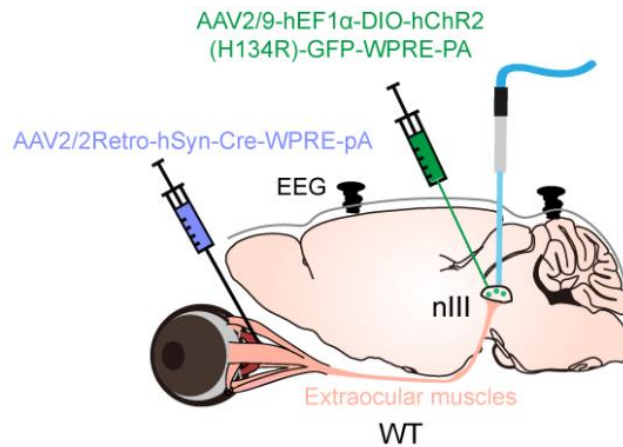
Comment 4. Supplementary Fig.14, panel a, should it be WT mice instead of ChAT-Cre mice?

Response:

We sincerely apologize for such mistake. As the reviewer correctly pointed out, the WT mice instead of ChAT-Cre mice were used in this experiment.

We have corrected these mistakes in the Fig. R3-6 and carefully reviewed the entire text and figure legends to ensure accuracy in all viral descriptions and

labeling in the revised manuscript.



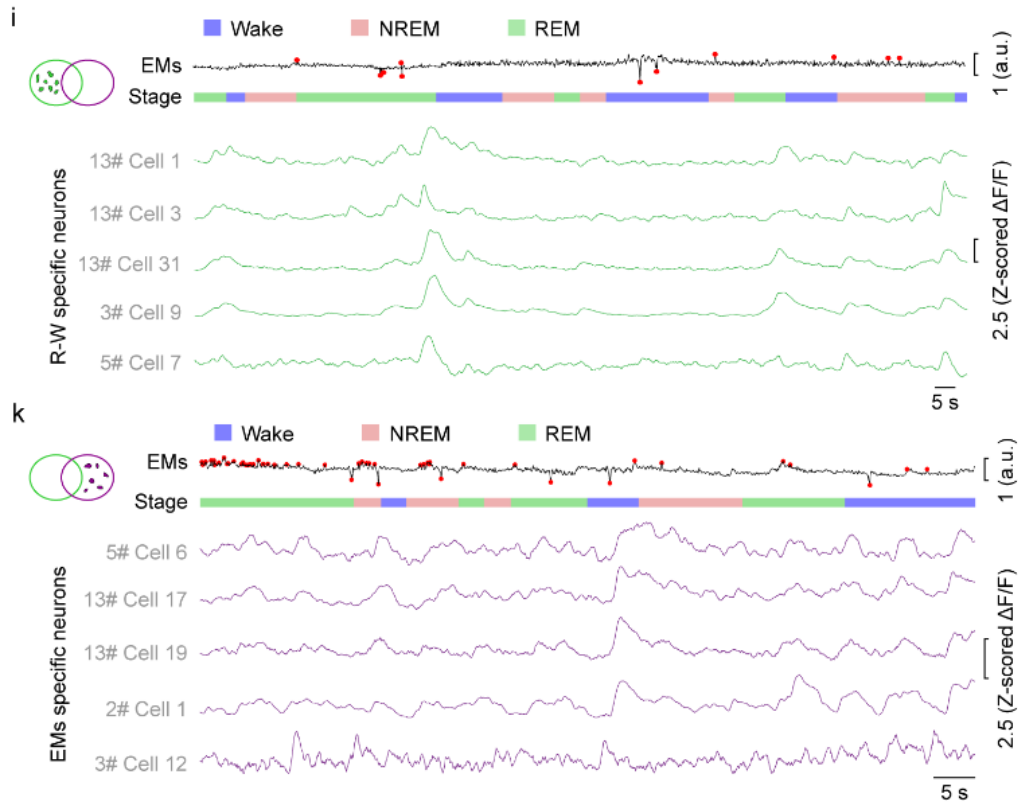
(Newly updated Supplementary Fig.14a) Fig. R3-6 | Schematic of viral injection strategy in WT mice to specifically label extraocular muscle-projecting nIII neurons.

Comment 5. Supplementary Fig. 15, in panel i and k, the diagrams on the left seemed to mismatch.

Response:

We sincerely apologize for this mistake. As the reviewer correctly pointed out, the schematic diagrams shown on the top-left corner of panel i and panel k in Supplementary Fig. 15 were mismatched.

We have now corrected the diagrams to ensure they accurately correspond to the respective experimental conditions in each panel (**Fig. R3-7**). We have carefully rechecked all figures and legends throughout the manuscript to ensure consistency and accuracy in the presentation.



(Newly updated Supplementary Fig. 15) Fig. R3-7 | Differential activity patterns of nIIIChAT neurons during EMs and R-W transition. i $\Delta F/F$ trace of R-W active nIII^{ChAT} neurons across the sleep-wake cycle. **k** $\Delta F/F$ trace of EMs-active nIII^{ChAT} neurons across the sleep-wake cycle, recorded concurrently with EMs signals (top). The red dots in the EMs trace indicate the events of EMs.

Based on the Reviewers' comments, we have performed the following revisions in the revised Manuscript:

1. Page 2, we have revised the statement in Introduction to enhance clarity and context;
2. Page 4, we have revised the description of the nIII^{ChAT} neuronal activity pattern during EMs in different states;
3. Page 10, we updated the result to clarify and elaborate on the direct projections from the nIII to the LC, MPB, and VTA;
4. Page 26, we have provided Source Data for Fig. 5f and supplementary Fig. 5d along with Source Data for other figures in <https://doi.org/10.6084/m9.figshare.27684144>.
5. Page 30-43, Figure Section, main figures section, We have updated Fig. 5, Supplementary Fig. 5, and Fig. 14, added Fig. 17, and revised or expanded the corresponding figure legends accordingly.