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Microglial TNFα orchestrates protein phosphorylation in the cortex during sleep period and controls homeostatic sleep

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(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. Depending on transfer agreements, referee reports obtained elsewhere may or may not be included in this compilation. Referee reports are anonymous unless the Referee chooses to sign their reports.)

1st Editorial Decision **13th Jun 2022**

Dear Dr. Bessis,

Thank you for submitting your manuscript for consideration by the EMBO Journal. It has now been seen by three referees whose comments are shown below.

Should you be able to address these criticisms, we could consider a revised manuscript. It is EMBO Journal policy to allow a single round of revision and therefore, acceptance or rejection of the manuscript will depend on the completeness of your responses in this revised version. Although we have already discussed that you will provide some new mechanistic data in the resubmission, we always offer authors the chance to discuss the entire plan to address referee concerns. I am available in the coming weeks if you would like to discuss your plan via zoom or email.

Along with your revised manuscript, please include a detailed point-by-point response to the referees' comments. Please bear in mind that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process, please visit our website: https://www.embo.org/embo-press

We generally allow three months as standard revision time. As a matter of policy, competing manuscripts published during this period will not negatively impact on our assessment of the conceptual advance presented by your study. However, we request that you contact the editor as soon as possible upon publication of any related work, to discuss how to proceed. Should you foresee a problem in meeting this three-month deadline, please let us know in advance and we may be able to grant an extension. I have attached a guide for revisions for your convenience.

Thank you for the opportunity to consider your work for publication. I look forward to your revision.

Kind regards,
Kelly

Kelly M Anderson, PhD
Editor
The EMBO Journal
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Referee #1:

In the article titled "Microglial TNFα orchestrates brain phosphorylation during the sleep period and controls homeostatic sleep", by Pinto MJ et. al, addressed the role of microglial TNFa mediated phosphorylation and its impact on sleep homeostasis. Using phosphoproteome analysis of frontal cortex samples collected from control and micTNFa-KO mice they observed key differences in the phosphorylation status of many proteins predominantly in the sleep period (S) rather than in the wake period (W). Further, they identified significant changes in some key kinases involved in sleep homeostasis between these two samples especially during the sleep period and observed these differences in the synaptic proteins involved in sleep regulation. Finally, using SWA analysis by EEG, authors showed that micTNFa-KO mice have modestly impaired build-up of sleep need in response to prolonged wake which supports the role of microglial TNFa in homeostatic sleep regulation.

The manuscript feels like two-thirds of a story. First, is microglial TNFa important in sleep? Figure 5 says yes, but only modestly. (I'd like to know how other interventions compare - is this change similar to other manipulations?)

So what's the mechanism whereby TNFa knockout regulates sleep needs? Well, there are changes in phosphorylation (both up and down) in the sleep phase when TNFa is deleted from microglia. Why are there changes in phosphorylation? Probably because TNFa is a potent signaling molecule that is upstream of many pathways. TNFa is a very well-studied cytokine that regulates NF-kB, MAPK and apoptotic pathways, and that may correlate with some of the specific changes they see, but in the manuscript, none of these downstream pathways are robustly examined. Finally, after cataloging all the phosphorylation

changes, which, if any, of these phosphorylation changes or altered pathways in the sleep phase cause the modest change in sleep needs? This is not tested. So my overall assessment is that this is an interesting descriptive study of TNFa regulation of phosphorylation, but the study lacks substantial mechanistic insight.

It's odd to me that they start with the hypothesis that TNFα regulates sleep, but this is not tested until the last figure. Might it make more sense to start with the microglial TNFα knockout and see if there is indeed a significant difference in various parameters? And then they could move on to test the next hypothesis, microglia TNFα regulates sleep via its effect on protein phosphorylation networks? And then wrap up the story by testing if any of the TNFα regulated phosphorylation networks also control sleep?

Specific comments:
The fold changes and FDR cut-offs used are not very clear in the results and figure legends. It would be helpful to explicitly state these.

The data in figure 1 lead to the conclusion, "...that microglial TNFα massively dampens variations in phosphorylation." It would be helpful to the reader if this sentence was a topic sentence, and then the data interpreted instead of presented value-free. This leads to fig 2, which would more appropriately be titled "Microglial TNFα acts during the sleep period to suppress changes in the cortical phosphoproteome."

p. 4 :" demonstrating that modulation of these phosphorylation events by microglial TNFα occurs specifically in the light phase irrespective of sleep." Do they mean, these events are probably circadian-influenced rather than sleep-influenced?

"The peptides threshold was decreased to 1 peptide across 3 biological replicates of a group to consider a phosphosite in the downstream analysis." Does this mean, the same peptide had to be found in 3 of 5 biological replicates?

Fig 1B: it took me a while to figure out why there were three columns instead of two. Could this information be presented more clearly, or could the figure legend explain it more?

So Fig 1B and 1C show to my eye that TNFα knockout in microglia leads to a 7-fold INCREASE in S/W phosphorylation events. Thus, TNFα signaling suppresses changes....

I don't understand this point - could you re-state it differently? "Strikingly, most of this modulation depends on microglial TNFα since 70.7% of these phosphosites showed no more significant changes between S and W in micTNFα-KO."

The changes in kinase phosphorylation is probably really interesting but I can't readily find the data. The sentence "We also found that phosphorylation sites known to control the activity of kinases are regulated by microglial TNFα during the sleep period, such as Thr286 on CaMKIIα, Tyr205/Tyr185 on MAPK3/1 and Ser21 on GSK3α (table S2)." I need help from the authors to find this data and see the quantitation that supports this sentence. Table S2 has 8 tabs, and is 583 kb in size. When I checked GSKa I see the result is less clearcut than the text "Q2NL51-Phospho:S21.S41" So is it S21, or S41? This should be checked with the commercially available very good anti-phosphoepitope antibodies.

Fig 3E and F are not very compelling. First, what are the sites they are probing? What was the prediction from the MS data? Should the p-value (which is borderline) be corrected for multiple testing, as I expect they tested more than these antibodies before deciding which data to use? I understand that the method casts a wide net and the magnitude of the effect is diluted by other sites that might not change. But the authors should tell me this, and use it as an indirect indicator supporting the MS data.

In the methods, I got lost here: "For analysis of the amplitude of change, the log2 value of the fold change of unique phosphosites was imputed to 5 and -5 (according to the group in which the phosphosite was unique)." Is this imputation sort an extrapolation of missing data? Why 5 and -5? Please explain and provide some reference or justification for this step.

Minor comments:
Methods: tree AttractSPE disk should be three?
There is always a space between numerals and units, e.g. 65 mph, not 65mph. likewise, 2 ug, not 2ug.

Standard writing: The first line of a paragraph should be indented, unless there is extra space between paragraphs. Microsoft Normal format screws this up.

Plppr4 is not a phosphoprotein phosphatase. It is a lipid phosphatase.

Suggestion: "Taken together, these data show that during the sleep period, microglial TNFα modulates the cortical phosphoproteome in a manner dependent on the dark/light cycle and on sleep-intrinsic mechanisms.
Might be made more clear by saying "Taken together, these data show that during the sleep period, microglial TNFα modulates the cortical phosphoproteome both in a manner dependent on the dark/light cycle and in addition, on sleep-intrinsic mechanisms."

Referee #2:

The manuscript presents a detailed analysis of the impact of TNFalpha knockout (KO) in microglia on the frontal cortex phosphoproteome in mice. This is done in considering the distribution of wakefulness and sleep as well as the light/dark cycle. It also reports a sleep phenotype, in particular regarding NREMS SWA dynamics, in the same mouse model. The manuscript is well written and carefully organized. It presents a comprehensive literature review, and citations of key relevant papers is certainly a strength in addition to the integration of existing datasets with the current findings. The study design is adequately controlling for the light phase versus the effect of sleep itself; thus, featuring interesting observations of light-driven (or circadian-driven) versus sleep-driven changes in phosphoproteome after micTNFalpha-KO. The findings are globally adequately interpreted, show originality, and complement the existing literature on the topic by uniting TNFalpha-suggested and microglia-suggested roles in wake-sleep regulation.

Nevertheless, improvements of the overall work could be made by incorporating missing crucial information in the Results, clarifying multiple methodological aspects, performing more rigorous and in-depth analysis of the EEG, and adding nuance to some interpretations. The following specific comments could help authors in this way:

Major comments

1. Abstract (and elsewhere regarding 'time spent asleep' tracking 'sleep need'); it is written that 'time we spend asleep is adjusted to previous time spent awake, and therefore believed to be under tight homeostatic control'. This sentence should be revised given that sleep intensity (much more than time spent asleep) is tightly linked to previous wake/sleep duration as later adequately indicated by authors. Additionally, the abstract could point out to the exact kinases found to be more closely involved according to their findings (e.g., MAPK, CDK, PKC).

2. The introduction is missing the required information about the justification for the brain region investigated as well as information about findings in a given brain region. TNFalpha-derived from microglia should be expected to be influencing phosphoproteome in a sleep-relevant manner in specific brain regions. At the beginning of the Results, a justification can be found but this should be moved to the Introduction. Also, it seems important to expand the discussion in this regard (end of page 10) to address the likelihood of brain region-specific effects of microglia-derived TNFalpha.

3. Some required methodological details need to be described in the Results section (which precedes the Materials and Methods). For instance, the very beginning of the Results should describe the animal model used (i.e., later life KO of microglial TNFalpha using tamoxifen in CX3CR1-CREert2 and TNF-f/f crossed mice). In addition, at the top of page 6, authors should specify from which brain region organotypic slices (even need to be indicated in the Methods on page 17 where only coronal slices are indicated) were prepared for the TNFalpha neutralization experiment and how the neutralization antibody was validated. These experimental details are required in the main text to understand the relevance of the conducted experiments.

4. The data in Fig. 3E are not convincing in showing a genotype effect for MAPK/CDK substrates in S, and blots do not appear to match the quantification. It is required that the bands quantified as indicative of substrates be shown/indicated. Also, the apparent use of vinculin versus tubulin for data normalization does not seem to be reflected in quantifications of the right side of Fig. 3E.

5. Middle of page 7; the authors write 'at the onset of the light (sleep) period, which is the time of highest accumulation of sleep need,..' whereas the highest level is reached during the dark phase, as can clearly been seen from the data in Fig. 5. Please revise the sentence.

6. The use of 10-sec epochs for the identification of wake/sleep states is not adequate for interrogating wake/sleep variables in the mouse because states in the mouse change more rapidly (in comparison to humans). The use of shorter epoch length (e.g., 4-sec) is required to reliably determine the fine sleep-wake architecture (see Mang and Franken, 2012, reference #98 cited in the manuscript), and especially report on number of state bouts and their duration such as in Table S4.

7. The temporal resolution of the SWA time course is very poor (i.e., 2 time points during the baseline dark and 4 time points during the baseline light), and some differences could have been missed at other times with elevated homeostatic sleep need (e.g., end of the dark phase). For instance, the citation of the authors in support of the use of equal intervals (i.e., Mang and Franken, 2012, #98) is using 18 intervals (12 during the light and 6 during the dark), which should be implemented by the authors to ensure relevant times are not missed.

8. Moreover, a full spectral composition of states is important to incorporate to evaluate additional differences that could be

associated with sleep homeostasis (e.g., wake theta/gamma activity, PMID 28630298). In this sense, a time course analysis of frequency bands other than SWA would be relevant to include, and also to further supplement the previous work conducted by the authors (https://www.biorxiv.org/content/10.1101/2022.02.21.481254v1.full). Importantly, the previous findings of delta peak activity shift and SW amplitude/duration changes in the same mouse model should be adequately discussed.

9. Nonsignificant interactions found in ANOVA should not be decomposed by post hoc or planned comparisons. The authors should thus avoid comparisons of Sham and SD for CTL in NREMS in Fig. 5B and NREMS bouts mean duration in Table S4 (and remove any reference to change between Sham and SD for these parameters in CTL).

10. Please discuss the divergence between the large effects on the phosphoproteome under baseline conditions and the small effects on baseline sleep (e.g., page 10 highlights effect only under SD conditions for SWA whereas phosphorylated sites are importantly changed under baseline conditions and validated by SD)

11. Please discuss the potential for age-related effects of the KO. Given that tamoxifen was provided at adulthood (and young adulthood for some EEG-recorded mice), a developmental compensation should be avoided. Nevertheless, microglia activity/reactivity was shown to be impacted by age, and the choice of different ages (2 to 5 months) for experiments should be discussed.

12. Methods, page 13; the middle paragraph indicates that the experiment were conducted 5-6 weeks after tamoxifen treatment while the last paragraph indicates 2 weeks. This apparent discrepancy should be clarified and corrected if necessary.

13. Methods, page 15; please add relevant/applicable citations for the sentences 'The phosphosite localization accuracy was estimated by using the PtmRS node in PD, in PhosphoRS mode only.' and ' To estimate the significance of the change in protein abundance, a linear model (adjusted on peptides and biological replicates) was performed, and p-values were adjusted using the Benjamini-Hochberg FDR procedure.'. Please also define PD in the first of the two sentences.

14. Methods, page 19; the authors need to define on which signal was the EEG activity interrogated. Was only the frontal EEG electrode used or was the signal used a bipolar signal between the frontal and cerebellar sites.

Minor comments

15. Stars in Fig. 1E are not easily visible and a change in position is recommended (e.g., above datapoints instead of near average).

16. Results description; changing wording to be more precise/appropriate; the word 'regulated' mostly represents an interpretation and the correct description would rather be 'changed'/'modified'/'altered' in micTNFalpha-KO (top of page 4).

17. Top of page 6; please define 'activation loop of MARKs' (what is the loop in terms of components/cascades, and the relevance).

18. Fig. 3C; please define the grey datapoints (similar to what has been done for red, blue and dark green dots). Grey color seems to be defined in Fig. 3D but no grey identification is used in this panel. Please revise accordingly. Also, in 3D, it could be interesting to show the behavior for wake of the same kinases represented for sleep (instead of showing an empty wake panel).

19. Given the role of microglia in shaping sex-dependent brain maturation and behavior, discussing the sole use of males in the current study could be of relevance.

Referee #3:

This is an interesting study that attempts to address a gap in knowledge about how microglia, specifically, microglia produced TNF-a, regulate sleep-wake behavior in the rodent. While there is a strong scientific premise for this study, there are weaknesses in the manuscript that should be addressed. There are claims made that are not supported by literature in the field. There are missing details, the ARRIVE guidelines are not followed, and the statistics are incomplete.

Major concerns:

The Introduction is too brief. There is not enough background information for the reader to be introduced to key topic necessary to understand the study. This should be significantly expanded.

It is not accurate to say TNF-a is mostly, if not exclusively produced by microglia. This concept is overstated throughout the manuscript. There are several publications, more recent than the manuscripts cited in the Introduction, that confirm that other

glia cells (e.g., astrocytes) and neurons make cytokines. TNF-R1 and TNF-R2 have been documented for decades on neurons, astrocytes, and microglia throughout the CNS.

The Results section should only contain results from experiments, per the journal instructions. Currently, each Results section has introductory information, study design details, and interpretation of data. The Results section should be strictly a presentation of data and results from the analyses. All interpretation of results should be in the Discussion.

There are no study limitations listed in the Discussion. This is a relatively low-powered study (n=5/group) which is a weakness. There is no justification for not including female mice. There is no mention of limitations with methods.

It is unclear how many mice are used in each experiment. There is no number of mice listed for the entire study. There are inconsistencies with animal details. In "Animals and housing" the mice are males 4-5 months old. In "Electrode Implantation" mice are listed as 2-4 months of age, and no sex is specified. What mice are used for what experiment? A study design figure would be helpful that included animal numbers and what outcome measures were derived from what mice.

That statistics are incomplete. All F and p values should be included in the main document (not supplemental material). These values are pertinent for the reader to make inferences about the data.

The ARRIVE guidelines for reporting animal research have not been followed, as requested by scientific journals. Were any investigators scoring sleep blinded to the experimental conditions? How were animals randomized in the sleep disruption studies? What were the exclusion criteria for each experiment? Based on the degrees of freedom listed in the supplementary table, there were analyses where mice were excluded. Were these outliers? What outlier test was used?

Most of the text on the figures is not legible. At 300% zoom they can barely be seen. Figures should be updated and size of text when printed on a journal page should be considered.

Minor concerns:

There are grammatical errors throughout, and the manuscript should be read by all authors and edited for grammar and typographical errors.

TNF-a abbreviation is not defined in first paragraph of the Introduction.

ZT is not defined in the Results.

S and W are not explicitly defined in the Results section.
There are inconsistencies with using S-W cycle and sleep-wake cycle.

The term "sleep period" is misleading. There is a light and dark period. During the light period the mice sleep more but there is 20-40% of this period that the mice are not sleeping.

In the Results, figure parts are referenced out of order.

Reference in order, or re-order the figures.

Figure 1 you cannot see the asterisks on panel E.

To facilitate the identification of the changes, we have now numbered the lines of the manuscript.

Please note that due to new additions to the supplementary material, the numbering of the supplementary figures has changed. The same holds true for the tables.

Referee 1:

1. The manuscript feels like two-thirds of a story. First, is microglial TNFa important in sleep? Figure 5 says yes, but only modestly. (I'd like to know how other interventions compare - is this change similar to other manipulations?)

As noted by this referee, Figure 5 shows that microglial TNFα is important in sleep.

After SD, SWA after SD decreases from 219 to 192 from CTL to microglial TNFα KO mice (representing a 12% decrease). SWA after SD has been measured in comparable interventions. For instance:

-Ingiosi et al. 2020 Curr Biol (PMID: 32976809) show that (Fig 6c) reduced intracellular calcium in astrocytes induces a “modest” decrease of NREM delta power one hour after SD (from about 170 to 150; a 12% decrease)

-Halassa et al. 2009 Neuron (PMID: 19186164) show (Fig 2c) that reduced exocytosis in astrocytes leads to a decrease in “normalize delta power” two hours after SD (from about 200 to 160; a 20% decrease)

We therefore believe that the effect of microglial TNFα on sleep is in the expected range. We also would like to emphasize the fact that figure 5B shows that sleep rebound after sleep deprivation is completely blunted when microglial TNFα is not expressed.

We thus believe that the effect of microglia TNFα is biologically significant.

2. So what's the mechanism whereby TNFa knockout regulates sleep needs? Well, there are changes in phosphorylation (both up and down) in the sleep phase when TNFa is deleted from microglia. Why are there changes in phosphorylation? Probably because TNFa is a potent signaling molecule that is upstream of many pathways. TNFa is a very well-studied cytokine that regulates NF-kB, MAPK and apoptotic pathways, and that may correlate with some of the specific changes they see, but in the manuscript, none of these downstream pathways are robustly examined. Finally, after cataloging all the phosphorylation changes, which, if any, of these phosphorylation changes or altered pathways in the sleep phase cause the modest change in sleep needs? This is not tested. So my overall assessment is that this is an interesting descriptive study of TNFa regulation of phosphorylation, but the study lacks substantial mechanistic insight.

As suggested, we assessed activation of NF-kB and apoptotic pathways by western blot using specific markers. The results were now included in this version of the manuscript in figure S4 and suggest no overt changes in the activation of NF-kB pathway between controls and micTNF-KOs and reveal no induction of apoptosis in any of the tested groups (main text lines 147-152, sup material lines 39-50). These results are consistent with our MS data as a Reactome Pathway Analysis to microglial TNFα substrates (both at a proteomic and phosphoproteomic level) does not show enrichment of apoptosis and/or NF-κB-related pathways (fig 4, S2 and data not shown).

We further assessed phosphorylation of MAPKs, specifically the phosphorylation sites on MAPK3 and MAPK1 identified by our MS data as being upregulated in micTNF-KOs at ZT6 (sleep period in the old version of the manuscript). Consistently, we now show a trend for an increase of MAPK phosphorylation in micTNF-KOs specifically at ZT6 (sleep period) (data now included in fig 3D and description in the text lines 174-182). This is in agreement with the reported increase in the phosphorylation of MAPK/CDK substrates and further supports MAPK pathway as a target of microglial TNFα in the light period.

To gain mechanistic insight into how microglial TNFα regulates sleep need, we now identified the targets of microglial TNFα phosphomodulation at the end of the sleep deprivation protocol (SD6 condition), a moment of maximal accumulation of sleep need. This new set of phosphoproteomic data was added to figure 5 (panels D-F) and to tables S2, S4 and S5. As discussed in the new version of the manuscript (lines 279-293), a total of 994 proteins had their phosphorylation regulated by microglial TNFα at high sleep need conditions, representing potential candidates acting downstream TNFα in the regulation of sleep need. These include many proteins whose phosphorylation has been linked to sleep control (i.e. SNIPPs and KSPs) and many proteins that have been causally linked to sleep regulation in the literature, including sleep homeostasis (fig

5F). This information was added to table S5 in which the above-mentioned candidates of interest are highlighted.

We agree with the reviewer that the next relevant step would be to identify which of these targets, and/or which specific phosphorylation change, acts downstream TNFα to control sleep homeostasis. However, as several candidates of interest stand out, we believe that the ideal approach would involve a sleep recording-based screening on several mutated/KO mice that is unfortunately not technically feasible in the frame of this study.

We further assessed by western blot TNFα-dependent changes at sleep deprivation conditions in the activation of NF-kB and apoptotic pathways (fig S4); and to the kinases identified as targets in the light period (i.e. GSK3, MAPKs, MARKs, PKCs) (fig S9). Once more, our data does not reveal an obvious candidate, suggesting a different landscape of phosphorylation cascades acting downstream microglial TNF during undisturbed sleep and when sleep homeostasis is perturbed.

3. It's odd to me that they start with the hypothesis that TNFα regulates sleep, but this is not tested until the last figure. Might it make more sense to start with the microglial TNFα knockout and see if there is indeed a significant difference in various parameters? And then they could move on to test the next hypothesis, microglia TNFα regulates sleep via its effect on protein phosphorylation networks? And then wrap up the story by testing if any of the TNFα regulated phosphorylation networks also control sleep?

We thank the reviewer for this proposition of a more accurate organization of our manuscript. We think that the feeling of an “odd organization” came from the fact that we did not start by clearly presenting in the introduction the known role of TNFα in sleep.

TNFα has indeed been identified as a sleep factor for many years and we are thus not testing this hypothesis. However, the cellular source or the molecular mechanisms underlying its effect on sleep have remained completely unknown and we now intend to provide insight into these mechanisms. We have now emphasized the role of TNFα as a sleep factor in a new paragraph of the introduction (lines 53-63). We hope that this will better support the organization of the manuscript.

4. The fold changes and FDR cut-offs used are not very clear in the results and figure legends. It would be helpful to explicitly state these.

As indicated in the material & methods, an FDR of 1% was set at the peptide level for the whole study (line 524). Proteins and phosphosites were considered significantly different in a pairwise comparison when the adjusted p-value was ≤ 0.05. Unique phosphosites on one condition of a given pairwise comparison were also considered significantly different. As suggested, we have now clearly indicated this in all figure legends. Reasoning that even small significant changes are biologically relevant, no cut-off on the fold change was applied in this study.

5. The data in figure 1 lead to the conclusion, "...that microglial TNFα massively dampens variations in phosphorylation." It would be helpful to the reader if this sentence was a topic sentence, and then the data interpreted instead of presented value-free. This leads to fig 2, which would more appropriately be titled "Microglial TNFα acts during the sleep period to suppress changes in the cortical phosphoproteome."

Please find the suggested changes in this new version (line 99).

6. p. 4 :" demonstrating that modulation of these phosphorylation events by microglial TNFα occurs specifically in the light phase irrespective of sleep." Do they mean, these events are probably circadian-influenced rather than sleep-influenced?

We have now changed this sentence (lines 140-141) to make it more precise: “*This demonstrates that part of the TNFα-dependent modulation of phosphorylation events (39.2% - 1150 out of 2932 phosphosites) is not dependent on sleep but is rather influenced by circadian cycle*”.

7. "The peptides threshold was decreased to 1 peptide across 3 biological replicates of a group to consider a phosphosite in the downstream analysis." Does this mean, the same peptide had to be found in 3 of 5 biological replicates?

Yes. For clarification, this information is now added in the material & methods (line 543).

8. Fig 1B: it took me a while to figure out why there were three columns instead of two. Could this information be presented more clearly, or could the figure legend explain it more?

A description was now included in the figure legend (lines 1038-1041). Graph 1B was also changed to make the difference between the 2nd and 3rd bars clearer.

9. So Fig 1B and 1C show to my eye that TNFα knockout in microglia leads to a 7-fold INCREASE in S/W phosphorylation events. Thus, TNFα signaling suppresses changes...

As suggested, this finding has now been clearly mentioned in the text as we now write (lines 107-108): *"…showing that microglial TNFα acts to suppress daily changes in phosphorylations*".

10. I don't understand this point - could you re-state it differently? "Strikingly, most of this modulation depends on microglial TNFα since 70.7% of these phosphosites showed no more significant changes between S and W in micTNFα-KO."

We have now written (lines 95-96): "*The majority of ZT6-ZT18 cycling phosphosites (70.7%) did not change between ZT6 and ZT18 in these mice (fig. 1B) and are thus dependent on microglial TNFα*". We hope these changes make this point clearer.

11. The changes in kinase phosphorylation is probably really interesting but I can't readily find the data. The sentence "We also found that phosphorylation sites known to control the activity of kinases are regulated by microglial TNFα during the sleep period, such as Thr286 on CaMKIIα, Tyr205/Tyr185 on MAPK3/1 and Ser21 on GSK3α (table S2)." I need help from the authors to find this data and see the quantitation that supports this sentence. Table S2 has 8 tabs, and is 583 kb in size. When I checked GSKa I see the result is less clearcut than the text "Q2NL51-Phospho:S21.S41" So is it S21, or S41? This should be checked with the commercially available very good anti-phosphoepitope antibodies.

Please find a revised version of the tables for easy accessibility of the data. For each table, we have now included a "table description" that describes the content of each tab and provides a description of the column headings. We hope this will guide readers to more easily locate the data of interest in the provided tables. Also, for simplicity, the number of tabs in each table was reduced. We now present the MS data in 5 rather than in 3 tables, so please note that the titles of the tables and their numbering has changed (sup material lines 128-141).

Concerning the quantification of the data presented in the tables, please note that the tables compile only the phosphosites that were considered significantly different in the indicated pairwise comparison (including phosphosites with an adjusted p-value ≤ 0.05 and unique phosphosites) (this concerns tables 1, 2 and 3). This is now clearly indicated in "table description" tab and explained in detail in the material & methods (lines 544-546). The adjusted p-value of each individual phosphosite is provided in the tables.

We are sorry for the misunderstanding. "Q2NL51-Phospho:S21.S41" means that both Serine 21 and Serine 41 on GSK-3α are phosphorylated in this peptide. A significant difference in the abundance of this phosphosite (adjusted p-value of 0.022 and log2 fold change of -0.839) means that both Serine 21 and Serine 41 on GSK-3α are downregulated on micTNF-KO vs. CTL at sleep time (ZT6 in the current version of the manuscript). As suggested, we have now validated this result by performing WB with an antibody specific for phosphorylated GSK3 (phospho-GSK3α/β at Ser21/9 antibody, cell signaling #9331). We further validated phosphorylation of MAPK3/1 with a phosphoepitope-specific antibody (phospho-p44/42 MAPK at Thr202/Tyr204 antibody, cell signaling #4370). This antibody detects phosphorylated Erk1 at Tyr204 (mouse MAPK3 phospho-Tyr205) and phosphorylated Erk2 at Tyr187 (mouse MAPK1 phospho-Tyr185). The obtained results validate the MS data and were included in this new version of the manuscript (figure 3D; lines 177-182).

12. Fig 3E and F are not very compelling. First, what are the sites they are probing? What was the prediction from the MS data? Should the p-value (which is borderline) be corrected for multiple testing, as I expect they tested more than these antibodies before deciding which data to use? I understand that the method casts a

wide net and the magnitude of the effect is diluted by other sites that might not change. But the authors should tell me this, and use it as an indirect indicator supporting the MS data.

In this version of the manuscript, please find several changes in figure 3 that we hope make the data more compelling. This includes showing the 5 replicates for CTL and micTNF-KO on the immunoblots and indicating the quantified signal with a dashed box (the latter applies to phosphorylated substrates of kinase candidates). Also, we have now indicated in the legends and along the test the phosphorylated sites probed by the antibodies used, and we clearly indicate in the text what was the result obtained from the MS data or the prediction by the RoKai tool (see adequate changes in the “kinases and phosphatases” section of the results, lines 147-207 and in the legends of figures 3, S4, S5, S6, S7 and S9).

Concerning the multiple testing, we acknowledge that this issue should be taken carefully. Besides the antibodies shown in the old version of the manuscript, we analyzed differences in our groups with only 1 more antibody, i.e. the phosphorylated substrates of AMPK, which we have now added to fig S6. Together with the new tested signals (phosphorylated GSK3, phosphorylated MAPK, phosphorylated NF-kB and caspase 3), a maximum total of 10 comparisons were made between CTL and micTNF-KO at each condition (ZT18, ZT6 or SD6). To correct for multiple testing, we have now performed a multiple t-test with Benjamini-Hochberg correction (GraphPad Prism).

13. In the methods, I got lost here: "For analysis of the amplitude of change, the log2 value of the fold change of unique phosphosites was imputed to 5 and -5 (according to the group in which the phosphosite was unique)." Is this imputation sort an extrapolation of missing data? Why 5 and -5? Please explain and provide some reference or justification for this step.

In a pairwise comparison, phosphosites that are unique to one group (found only in micTNFα-KO or only in CTL mice) do not have fold change. However, they are meaningful. In order to visualize them graphically (as in fig 1C, 2C and 2E) and perform subsequent analysis (kinase activity prediction with Rokai, fig 3E), an arbitrary value of log2 fold change (-5 or +5) was imputed. As 99.8% of the non-unique phosphosites significantly different in the comparisons herein performed have values of log2 fold change that fall within the 5 to -5 range (see fig. 2C for an appreciation of the distribution of log2 fold change values), we consider this as a reasonable window to the variations observed between our groups. We therefore attributed the arbitrary values of 5 and -5 to unique phosphosites in a pairwise comparison, representing up and downregulation, respectively. This explanation was added to the material & methods (lines 547-553).

Minor comments:

14. Methods: tree AttractSPE disk should be three?

Corrected (line 492).

15. There is always a space between numerals and units, e.g. 65 mph, not 65mph. likewise, 2 ug, not 2ug.

Corrected.

16. Standard writing: The first line of a paragraph should be indented, unless there is extra space between paragraphs. Microsoft Normal format screws this up.

Paragraphs are now separated by an extra space.

17. Plppr4 is not a phosphoprotein phosphatase. It is a lipid phosphatase.

To avoid misunderstandings, this is now clearly indicated in the text (lines 162) and a legend was included in table S4 on the phosphatases tabs.

18. Suggestion: "Taken together, these data show that during the sleep period, microglial TNFα modulates the cortical phosphoproteome in a manner dependent on the dark/light cycle and on sleep-intrinsic mechanisms.

Might be made more clear by saying "Taken together, these data show that during the sleep period, microglial TNFα modulates the cortical phosphoproteome both in a manner dependent on the dark/light cycle and in addition, on sleep-intrinsic mechanisms."

Corrected (lines 142-144).

Referee 2:

Major comments

1. Abstract (and elsewhere regarding 'time spent asleep' tracking 'sleep need'); it is written that 'time we spend asleep is adjusted to previous time spent awake, and therefore believed to be under tight homeostatic control'. This sentence should be revised given that sleep intensity (much more than time spent asleep) is tightly linked to previous wake/sleep duration as later adequately indicated by authors. Additionally, the abstract could point out to the exact kinases found to be more closely involved according to their findings (e.g., MAPK, CDK, PKC).

Accordingly, the following changes have now been made:

- In the abstract “The time we spend asleep is adjusted to previous time spent awake” has been changed by “Sleep intensity is adjusted to previous time spent awake” (line 21)

- In the discussion, line 307-309: “The homeostatic regulation of sleep sets the duration and intensity of sleep as a function of the preceding time spent awake.” Is now “*The homeostatic regulation of sleep sets the intensity of sleep as a function of the preceding time spent awake*”.

- In the abstract, please find examples of kinases previously shown to relate to sleep control that we now identify as substrates of microglial TNFα (line 25).

2. The introduction is missing the required information about the justification for the brain region investigated as well as information about findings in a given brain region. TNFalpha-derived from microglia should be expected to be influencing phosphoproteome in a sleep-relevant manner in specific brain regions. At the beginning of the Results, a justification can be found but this should be moved to the Introduction. Also, it seems important to expand the discussion in this regard (end of page 10) to address the likelihood of brain region-specific effects of microglia-derived TNFalpha.

As suggested, this new version of the manuscript has a revised version of the introduction in which the relevance of the cortex to the regulation of sleep homeostasis is emphasized. Also, we describe in more detail the previous literature supporting TNFα as a sleep factor, including its sleep effect when manipulation was done at the cortex level (lines 54-63).

In addition, as suggested by the reviewer, we have now improved the discussion by addressing the likelihood of microglial TNFα acting in a brain region-specific manner (lines 385-391).

3. Some required methodological details need to be described in the Results section (which precedes the Materials and Methods). For instance, the very beginning of the Results should describe the animal model used (i.e., later life KO of microglial TNFalpha using tamoxifen in CX3CR1-CREert2 and TNF-f/f crossed mice). In addition, at the top of page 6, authors should specify from which brain region organotypic slices (even need to be indicated in the Methods on page 17 where only coronal slices are indicated) were prepared for the TNFalpha neutralization experiment and how the neutralization antibody was validated. These experimental details are required in the main text to understand the relevance of the conducted experiments.

As suggested, the required information was added in the Results (lines 84-86) and the Material and methods sections (lines 584-588).

Concerning the TNFα neutralizing antibody (from R&D systems, cat no. MAB4101), note that it is validated by the brand by measuring its ability to neutralize TNFα-induced cytotoxicity in a mouse fibroblast cell line.

4. The data in Fig. 3E are not convincing in showing a genotype effect for MAPK/CDK substrates in S, and blots do not appear to match the quantification. It is required that the bands quantified as indicative of

substrates be shown/indicated. Also, the apparent use of vinculin versus tubulin for data normalization does not seem to be reflected in quantifications of the right side of Fig. 3E.

In order to clearly show the genotype effect for MAPK/CDK substrates (and also the other tested phosphorylated substrates of kinase candidates), we now show the 5 replicates for CTL and micTNF-KO on the immunoblots and we indicate the quantified signal with a dashed box. These changes apply to figures 3, S4, S5, S6 and S9. We hope this will more convincingly show the differences between the genotypes. Please note that for the sake of space on figure 3, the immunoblots at the wake period (ZT18 in the current version of the manuscript) have been moved to fig S5 (clearly indicated in the legend of fig 3).

Also, concerning the vinculin vs tubulin issue, please find a corrected label on the y-axis of graph in fig 3F.

5. Middle of page 7; the authors write 'at the onset of the light (sleep) period, which is the time of highest accumulation of sleep need,..' whereas the highest level is reached during the dark phase, as can clearly been seen from the data in Fig. 5. Please revise the sentence.

The sentence has been revised as follow (lines 252-254) *'at the onset of the light (sleep) period, the amount of SWA was significantly lower in micTNFα-KO mice as compared to CTL mice (fig 5A)*'.

6. The use of 10-sec epochs for the identification of wake/sleep states is not adequate for interrogating wake/sleep variables in the mouse because states in the mouse change more rapidly (in comparison to humans). The use of shorter epoch length (e.g., 4-sec) is required to reliably determine the fine sleep-wake architecture (see Mang and Franken, 2012, reference #98 cited in the manuscript), and especially report on number of state bouts and their duration such as in Table S4.

There is no consensus in the sleep literature regarding the use of 4-sec vs. 10-sec epochs to describe the properties of SWA or sleep-wake states (e.g. 4-sec used in Funk et al., 2017; Vassalli and Franken, 2017; 10-sec used in Li et al., 2017- PMID: 28092659; Oishi et al., 2017 - PMID: 28963505; Niethard et al., 2018 - PMID: 30209214; Zielinski et al., 2019 - PMID: 31328777; Machado et al., 2022 - PMID: 35385692). However, we acknowledge that the use of 4-sec epochs for the identification of wake/sleep states may be more accurate to characterize changes in the fine sleep-wake architecture.

As suggested, we rescored and reanalyzed the EEG/EMG signals using the 4-sec instead of 10-sec epochs to characterize the effects of sleep deprivation which corresponds to the results presented in Figure 5B, C and Table S6. The material and method section has also been changed accordingly (lines 660; 673).
While the numbers of bouts and bouts mean durations slightly changed, the main effects we observed with the 10-sec epochs scoring remained similar.

7. The temporal resolution of the SWA time course is very poor (i.e., 2 time points during the baseline dark and 4 time points during the baseline light), and some differences could have been missed at other times with elevated homeostatic sleep need (e.g., end of the dark phase). For instance, the citation of the authors in support of the use of equal intervals (i.e., Mang and Franken, 2012, #98) is using 18 intervals (12 during the light and 6 during the dark), which should be implemented by the authors to ensure relevant times are not missed.

As suggested, by this referee, we have now reanalyzed the SWA time course with a more precise SWA time course that now includes 9 intervals (3 during the dark and 6 during the light period).
These results are now presented in Fig 5A, and confirmed that the main changes are observed during the first interval of the light period.

8. Moreover, a full spectral composition of states is important to incorporate to evaluate additional differences that could be associated with sleep homeostasis (e.g., wake theta/gamma activity, PMID 28630298). In this sense, a time course analysis of frequency bands other than SWA would be relevant to include, and also to further supplement the previous work conducted by the authors (https://www.biorxiv.org/content/10.1101/2022.02.21.481254v1.full). Importantly, the previous findings of delta peak activity shift and SW amplitude/duration changes in the same mouse model should be adequately discussed.

In agreement with this comment, wake theta/gamma activity during the sleep deprivation has been analyzed in control and mutant mice. These data are now included in the results (lines 274-278) and are shown in Fig

S8. The statistical analysis has revealed no change in waking oscillatory activities during SD in micTNFα-KO mice as compared to CTL mice.

In our previous report, we describe a shift in the delta peak activity together with changes in individual slow-waves duration in micTNFα-KO mice. In light of the findings in this manuscript on sleep homeostasis, these results were now properly addressed in the discussion (lines 333-349).

9. Nonsignificant interactions found in ANOVA should not be decomposed by post hoc or planned comparisons. The authors should thus avoid comparisons of Sham and SD for CTL in NREMS in Fig. 5B and NREMS bouts mean duration in Table S4 (and remove any reference to change between Sham and SD for these parameters in CTL).

As suggested by this referee, we removed the post-hoc analyses and changed the text and figure 5 accordingly (line 269). We now add panel 5B, which shows the delta change in wake and total sleep amounts between SD and sham in CTL and micTNFα-KO to show that the increased total sleep amount elicited by SD in CTL mice is abrogated in micTNFα-KO mice.

10. Please discuss the divergence between the large effects on the phosphoproteome under baseline conditions and the small effects on baseline sleep (e.g., page 10 highlights effect only under SD conditions for SWA whereas phosphorylated sites are importantly changed under baseline conditions and validated by SD).

As this referee pointed out, despite the large effect at the phosphoproteome level during the light period, microglial TNFα is not required to keep the structure of baseline sleep. We now explicitly propose that at steady state conditions, TNFα-controlled phosphorylation at sleep time is not determinant to keep sleep amounts at steady-state conditions, but instead may support intrinsic functions of sleep (now discussed lines 393-403).

11. Please discuss the potential for age-related effects of the KO. Given that tamoxifen was provided at adulthood (and young adulthood for some EEG-recorded mice), a developmental compensation should be avoided. Nevertheless, microglia activity/reactivity was shown to be impacted by age, and the choice of different ages (2 to 5 months) for experiments should be discussed.

We apologize for the misunderstanding. All animals used are males aged between 4-5 months old by the time of tissue collection or sleep recordings (now better explained lines 447-457). The timing of electrode implantation was adjusted according to the experimenter and/or set-up availability and differed by only 2 to 3 weeks between the different batches of mice (3 batches in total).

12. Methods, page 13; the middle paragraph indicates that the experiment were conducted 5-6 weeks after tamoxifen treatment while the last paragraph indicates 2 weeks. This apparent discrepancy should be clarified and corrected if necessary.

The apparent discrepancy results from bad wording in the first version of the manuscript. Experiments were indeed performed 5 to 6 weeks after tamoxifen treatment as indicated in the subsection “Conditional microglia-specific TNFα deletion” (line 457). The 2 weeks indicated on the following paragraph correspond to the time the mice are kept in isolation before tissue collection at ZT6, ZT18 or following sleep deprivation. Please find adequate corrections in the text (line 471).

13. Methods, page 15; please add relevant/applicable citations for the sentences 'The phosphosite localization accuracy was estimated by using the PtmRS node in PD, in PhosphoRS mode only.' and ' To estimate the significance of the change in protein abundance, a linear model (adjusted on peptides and biological replicates) was performed, and p-values were adjusted using the Benjamini-Hochberg FDR procedure.'. Please also define PD in the first of the two sentences.

The definition of PD was added (line 532) and citations have been added (lines 534-537).

14. Methods, page 19; the authors need to define on which signal was the EEG activity interrogated. Was only the frontal EEG electrode used or was the signal used a bipolar signal between the frontal and cerebellar sites.

This is now defined in the text. We used a monopolar derivation as the frontal electrode is compared to a neutral reference electrode placed over the cerebellum as defined in signal between the frontal and cerebellar sites (as in Mang and Franken, 2012) (lines 635-637).

Minor comments

15. Stars in Fig. 1E are not easily visible and a change in position is recommended (e.g., above datapoints instead of near average).

We have changed to make it more visible

16. Results description; changing wording to be more precise/appropriate; the word 'regulated' mostly represents an interpretation and the correct description would rather be 'changed'/'modified'/'altered' in micTNFalpha-KO (top of page 4)

We have now written "*X% of the phosphosites differed/changed between micTNFα-KO and CTL mice*" (lines 117 and 118) and whenever appropriate along the manuscript.

17. Top of page 6; please define 'activation loop of MARKs' (what is the loop in terms of components/cascades, and the relevance).

This information has been added (line 197) : "*MARKs are activated by phosphorylation of a conserved threonine in the activation loop (Timm et al, 2003). Changes in the activity of MARKs was evaluated by phosphorylation of their activation loop*".

18. Fig. 3C; please define the grey datapoints (similar to what has been done for red, blue and dark green dots). Grey color seems to be defined in Fig. 3D but no grey identification is used in this panel. Please revise accordingly. Also, in 3D, it could be interesting to show the behavior for wake of the same kinases represented for sleep (instead of showing an empty wake panel).

Figure 3 has been revised accordingly. Concerning the kinases predicted behavior on fig 3E (3D on the old version), please note that an empty wake panel means that no kinase candidates have been predicted at W (ZT8 in the current version of the manuscript) by the kinase activity inference tool used, RoKAI (Yilmaz et al 2021 Nat Com). RoKAI is a network-based algorithm that infers changes in kinase activity based on fold changes of a given pool of phosphorylation events. In order to infer which kinases would have altered activity between CTL and micTNF-KO at S and W (now ZT6 and ZT18), we used as input the lists of significantly different phosphosites between the two groups and correspondent log2 fold change (listed in table S2). At W (now ZT18), no significant kinase hits were obtained with RoKAI, supportive of a minimal effect of microglial TNF in phosphorylation cascades at this time of day. For clarification, a brief description was added in the results (line 186) and the figure legend (line 1096).

19. Given the role of microglia in shaping sex-dependent brain maturation and behavior, discussing the sole use of males in the current study could be of relevance.

We agree that sex-specific functions of microglia are extremely relevant. However, there are also known gender differences in sleep phenotypes and female sleep may be differentially regulated during the estrous cycle (e. g. Arthaud *et al* 2022 Sleep PMID: 35429396). To be rigorously carried out, females should be recorded over multiple days and sleep deprivation sessions should be done at different times during the 4-day estrous cycle. This would quadruple the number of mice to be studied. We agree that studying sleep and phosphoproteome changes in mutant females would be very interesting and that it should be adequately addressed in a future project. This aspect is now discussed as a limitation to our study (line 435)

Referee 3:

Major concerns:

1. The Introduction is too brief. There is not enough background information for the reader to be introduced to key topic necessary to understand the study. This should be significantly expanded.

We now include an extended version of the introduction, in which we describe in more detail the previous literature supporting TNFα as a sleep factor and the role of the cortex in sleep homeostasis (lines 53-63). We hope this provides the background information requested by this referee.

2. It is not accurate to say TNF-a is mostly, if not exclusively produced by microglia. This concept is overstated throughout the manuscript. There are several publications, more recent than the manuscripts cited in the Introduction, that confirm that other glia cells (e.g., astrocytes) and neurons make cytokines. TNF-R1 and TNF-R2 have been documented for decades on neurons, astrocytes, and microglia throughout the CNS.

We have not been able to find recent publications showing convincing expression of TNFα in other glia cells or neurons. Furthermore, in the latest release of the Cortex and Hippocampus of the Allen Brain Atlas (May 2021), no expression of TNFα is detected in Astrocytes or oligodendrocytes, but mostly, if not exclusively in microglia:

TNFα profile:

https://celltypes.brain-map.org/rnaseq/mouse_ctx-hpf_10x?selectedVisualization=Scatter+Plot&colorByFeature=Gene+Expression&colorByFeatureValue=Tnf

Microglia :

https://celltypes.brain-map.org/rnaseq/mouse_ctx-hpf_10x?selectedVisualization=Scatter+Plot&colorByFeature=Gene+Expression&colorByFeatureValue=Aif1

Astrocytes :

https://celltypes.brain-map.org/rnaseq/mouse_ctx-hpf_10x?selectedVisualization=Scatter+Plot&colorByFeature=Gene+Expression&colorByFeatureValue=Gfap

Oligodendrocytes :

https://celltypes.brain-map.org/rnaseq/mouse_ctx-hpf_10x?selectedVisualization=Scatter+Plot&colorByFeature=Gene+Expression&colorByFeatureValue=Mbp

This is in agreement with data from a study performing single-cell RNA sequencing in the mouse nervous system (Zeisel et al Linnarsson 2018 PMID: 30096314; online tool http://mousebrain.org/wheel/) clearly showing expression of TNFα in microglia cells but not in other cell types. For these reasons, we think that it is accurate to consider TNFα mostly as a microglial signaling molecule.

Finally, we do agree that TNFα receptors are expressed by many cell types but we believe that this does not impact on our results.

3. The Results section should only contain results from experiments, per the journal instructions. Currently, each Results section has introductory information, study design details, and interpretation of data. The Results section should be strictly a presentation of data and results from the analyses. All interpretation of results should be in the Discussion.

We have now removed most of interpretations that were in the results section (e.g. lines 128; 207; 222; 273). We only kept some of the transition sentences at the end of the sections.

4. There are no study limitations listed in the Discussion. This is a relatively low-powered study (n=5/group) which is a weakness. There is no justification for not including female mice. There is no mention of limitations with methods.

We used n=5 / group for the phosphoproteomic analysis, which is comparable to the size of the groups used in other phosphoproteomic studies of sleep (n=5 mice in Diering et al. Science 2017; n=3-4 – Wang et al. Nature 2018; n=4 – Bruning et al. Science 2019). However, and as it is now better explained in the legend of figure 5, we used n = 12-15 mice for sleep analysis (line 1135).

Concerning the use of females, please see our answer to referee 2 comment 19. In addition, and as suggested, we have now added a limitation (line 435): "*The phosphoproteomic analyses and sleep recordings described in this study were exclusively performed on male mice. It is now acknowledged that microglia display sex-specific functions (e.g. VanRyzin et al., 2019) and the mechanisms that are described here may not be extrapolated to female brains*".

We have now discussed the fact that we only studied the cortex phosphoproteome while we used a mouse in which microglial TNFα is depleted in the whole brain (line 386).

5. It is unclear how many mice are used in each experiment. There is no number of mice listed for the entire study. There are inconsistencies with animal details. In "Animals and housing" the mice are males 4-5 months old. In "Electrode Implantation" mice are listed as 2-4 months of age, and no sex is specified. What mice are used for what experiment? A study design figure would be helpful that included animal numbers and what outcome measures were derived from what mice.

All animals used are males aged between 4-5 months old by the time of tissue collection or sleep recordings. Please find this information now clearly indicated in "Animals and housing" (line 447). Indeed, indicating the age of the mice at the beginning of the study and not at the time of the sleep recordings makes the text confusing. We now indicate the age of the mice at the time of the sleep recordings, which corresponds to the age of 4-5 months (line 645).

As suggested, a study design figure was added (fig S11) in which the number of mice used in each experiment is indicated. We further included in the study design the figures and tables that show the data generated with the indicated experiment.

6. That statistics are incomplete. All F and p values should be included in the main document (not supplemental material). These values are pertinent for the reader to make inferences about the data.

The statistical test used and the F and p-values are now indicated in the figure legends.

7. The ARRIVE guidelines for reporting animal research have not been followed, as requested by scientific journals. Were any investigators scoring sleep blinded to the experimental conditions? How were animals randomized in the sleep disruption studies? What were the exclusion criteria for each experiment? Based on the degrees of freedom listed in the supplementary table, there were analyses where mice were excluded. Were these outliers? What outlier test was used?

We agree, and we now provide more details for the sleep study:

Randomization of mice to groups is now indicated in the material and methods (line 471 and line 640).

The scorer was blind to the genotype as now indicated (line 662).

Three independent studies were conducted each including control and mutant mice (line 656). For each study, control and mutant mice were randomly assigned to recording chambers (line 640). Sleep recordings were designed as follows: baseline sleep, sham sleep, and sleep deprivation. This order was chosen to end the protocol with the most invasive procedure (line 654). For each study, mice were sleep deprived on the same day. Thus, there is no randomization during the sleep recordings.

Some mice were excluded from the spectral analysis. This is related to technical issues that are common in polygraphic studies. For some mice, the EEG signal can be disturbed by movements or by water drops in the connector. If these disturbances are too frequent, we excluded the mouse from the spectral analysis (SWA analysis for example). This is indicated in line 675.

8. Most of the text on the figures is not legible. At 300% zoom they can barely be seen. Figures should be updated and size of text when printed on a journal page should be considered.

Please find a revised version of all figures in which the text was increased. We believe the text will now be legible at 100% zoom.

Minor concerns:

9. There are grammatical errors throughout, and the manuscript should be read by all authors and edited for grammar and typographical errors.

We are sorry about that. All the authors have read the manuscript and we have done our best to edit grammar and typographical errors

10. TNF-a abbreviation is not defined in first paragraph of the Introduction.

Corrected (line 49).

11. ZT is not defined in the Results.

Corrected (line 89).

12. S and W are not explicitly defined in the Results section.

There are inconsistencies with using S-W cycle and sleep-wake cycle.

Please see our response to comment 13 of this referee.

13. The term "sleep period" is misleading. There is a light and dark period. During the light period the mice sleep more but there is 20-40% of this period that the mice are not sleeping.

Please find in this new version of the manuscript, the terms “sleep period” and “wake period” replaced by light period (ZT6) and dark period (ZT18), respectively. Changes in this regard are in grey. We further mention in the text (line 90) and in figure 1 legend (lines 1036) that in the light and dark periods mice are mostly asleep and awake, respectively.

14. In the Results, figure parts are referenced out of order.

Reference in order, or re-order the figures.

Appropriate changes were made to figure 1 and in the text.

15. Figure 1 you cannot see the asterisks on panel E.

Changed to make it more visible.

Dear Alain,

Congratulations on a great revision! Overall, the referees are positive, and we are happy to move forward with your manuscript.

However, there remain the following editorial items to take care of in a revised version and also add this to your point-by-point response:

1. Please provide key words in the manuscript, after the abstract.

2. Please move the data accessibility section to the end of the Materials and Methods section and if you do not have any data in external repositories please use the following statement: "This study includes no data deposited in external repositories"

3. Please review our new policy on conflict of interests on the EMBO author guide website, the title of this section should be: Disclosure and competing interests statement.

4. Please remove author credit/contributions from the manscript file.

5. In the manuscript, Maria Pinto is not listed as a corresponding author. Please advise whether this is correct.

6. Please rename the appendix and dataset figures as "Appendix Figure S1" etc. or "Dataset EV1" etc. Please also add page numbers to the table of context in the appendix file and remove the table information.

7. In the manuscript there is a reference to Table S6, but this has not been uploaded.

8. The acknowledgements and conflicts of interest statement should come before the reference list.

9. We include a synopsis of the paper (see http://emboj.embopress.org/). Please provide me with a general summary statement and 3-5 bullet points that capture the key findings of the paper.

10. We also need a summary figure for the synopsis. The size should be 550 wide by 200-440 high (pixels). You can also use something from the figures if that is easier.

Thank you for the opportunity to consider your work for publication. I look forward to your revision.

Yours sincerely,

Kelly

Kelly M Anderson, PhD
Editor
The EMBO Journal
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Referee #1:

The authors have done a lot of work responding to the critiques, and have adequately addressed my concerns.

Referee #3:

This is a convincing data set that identifies microglia as a critical cell for controlling sleep homeostasis via TNF alpha. The manuscript has been significantly improved and the authors addressed most comments from the reviewers. The edits to the figures greatly enhances the readability.

The authors performed the requested editorial changes.

2nd Revision - Editorial Decision **11th Oct 2022**

Dear Alain,

Congratulations on an excellent manuscript, I am pleased to inform you that your manuscript has been accepted for publication in the EMBO Journal. Thank you for your comprehensive response to referee concerns. It has been a pleasure to work with you to get this to the acceptance stage.

I will begin the final checks on your manuscript before submitting to the publisher. Once at the publisher, it will take about 3 weeks for your manuscript to be published online. As a reminder, the entire review process, including referee concerns and your point-by-point response, will be available to readers.

I will be in touch throughout the final editorial process until publication. In the meantime, I hope you find time to celebrate!

Kind regards,

Kelly

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EMBO Press Author Checklist

Corresponding Author Name: Alain Bessis
Journal Submitted to: Embo Journal
Manuscript Number: EMBOJ-2022-111485

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This checklist is adapted from Materials Design Analysis Reporting (MDAR) Checklist for Authors. MDAR establishes a minimum set of requirements in transparent reporting in the life sciences (see Statement of Task: 10.31222/osf.io/9sm4x). Please follow the journal's guidelines in preparing your manuscript.
Please note that a copy of this checklist will be published alongside your article.

Abridged guidelines for figures

1. Data
The data shown in figures should satisfy the following conditions:
- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- ideally, figure panels should include only measurements that are directly comparable to each other and obtained with the same assay.
- plots include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- if n<5, the individual data points from each experiment should be plotted. Any statistical test employed should be justified.
- Source Data should be included to report the data underlying figures according to the guidelines set out in the authorship guidelines on Data Presentation.

2. Captions
Each figure caption should contain the following information, for each panel where they are relevant:
- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements.
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.
- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple χ2 tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

Please complete ALL of the questions below.
Select "Not Applicable" only when the requested information is not relevant for your study.

Materials

Newly Created Materials	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
New materials and reagents need to be available; do any restrictions apply?	Not Applicable	

Antibodies	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
For **antibodies** provide the following information: - Commercial antibodies: RRID (if possible) or supplier name, catalogue number and or/clone number - Non-commercial: RRID or citation	Yes	Materials and Methods

DNA and RNA sequences	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Short novel DNA or RNA including primers, probes: provide the sequences.	Not Applicable	

Cell materials	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Cell lines: Provide species information, strain. Provide accession number in repository **OR** supplier name, catalog number, clone number, and/**OR** RRID.	Not Applicable	
Primary cultures: Provide species, strain, sex of origin, genetic modification status.	Yes	Materials and Methods
Report if the cell lines were recently **authenticated** (e.g., by STR profiling) and tested for mycoplasma contamination.	Not Applicable	

Experimental animals	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Laboratory animals or Model organisms: Provide species, strain, sex, age, genetic modification status. Provide accession number in repository **OR** supplier name, catalog number, clone number, **OR** RRID.	Yes	Materials and Methods
Animal observed in or captured from the field: Provide species, sex, and age where possible.	Not Applicable	
Please detail **housing and husbandry conditions**.	Yes	

Plants and microbes	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Plants: provide species and strain, ecotype and cultivar where relevant, unique accession number if available, and source (including location for collected wild specimens).	Not Applicable	
Microbes: provide species and strain, unique accession number if available, and source.	Not Applicable	

Human research participants	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
If collected and within the bounds of privacy constraints report on age, sex and gender or ethnicity for all study participants.	Not Applicable	

Core facilities	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
If your work benefited from core facilities, was their service mentioned in the acknowledgments section?	Yes	Acknowledgement

Design

Study protocol	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
If study protocol has been **pre-registered, provide DOI in the manuscript**. For clinical trials, provide the trial registration number **OR** cite DOI.	Not Applicable	
Report the **clinical trial registration number** (at ClinicalTrials.gov or equivalent), where applicable.	Not Applicable	

Laboratory protocol	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Provide DOI OR other citation details if **external detailed step-by-step protocols** are available.	Not Applicable	

Experimental study design and statistics	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Include a statement about **sample size** estimate even if no statistical methods were used.	Yes	Figure legends and Materials and Methods
Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. **randomization procedure**)? If yes, have they been described?	Yes	Materials and Methods
Include a statement about **blinding** even if no blinding was done.	Yes	Materials and Methods
Describe **inclusion/exclusion criteria** if samples or animals were excluded from the analysis. Were the criteria pre-established? If sample or data points were omitted from analysis, report if this was due to attrition or intentional exclusion and provide justification.	Yes	Materials and Methods
For every figure, are **statistical tests** justified as appropriate? Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it. Is there an estimate of variation within each group of data? Is the variance similar between the groups that are being statistically compared?	Yes	Materials and Methods

Sample definition and in-laboratory replication	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
In the figure legends: state number of times the experiment was **replicated** in laboratory.	Yes	
In the figure legends: define whether data describe **technical or biological replicates**.	Yes	

Ethics

Ethics	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Studies involving **human participants**: State details of **authority granting ethics approval** (IRB or equivalent committee(s), provide reference number for approval.	Not Applicable	
Studies involving **human participants**: Include a statement confirming that **informed consent** was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	Not Applicable	
Studies involving **human participants:** For publication of **patient photos**, include a statement confirming that consent to publish was obtained.	Not Applicable	
Studies involving experimental **animals**: State details of **authority granting ethics approval** (IRB or equivalent committee(s), provide reference number for approval. Include a statement of compliance with ethical regulations.	Yes	Materials and Methods
Studies involving **specimen and field samples:** State if relevant **permits** obtained, provide details of authority approving study; if none were required, explain why.	Not Applicable	

Dual Use Research of Concern (DURC)	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Could your study fall under dual use research restrictions? Please check biosecurity documents and list of **select agents and toxins** (CDC): https://www.selectagents.gov/sat/list.htm	Not Applicable	
If you used a select agent, is the security level of the lab appropriate and reported in the manuscript?	Not Applicable	
If a study is subject to dual use research of concern regulations, is the name of the **authority granting approval and reference number** for the regulatory approval provided in the manuscript?	Not Applicable	

Reporting

The MDAR framework recommends adoption of discipline-specific guidelines, established and endorsed through community initiatives. Journals have their own policy about requiring specific guidelines and recommendations to complement MDAR.

Adherence to community standards	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
State if relevant guidelines or checklists (e.g., **ICMJE, MIBBI, ARRIVE, PRISMA**) have been followed or provided.	Not Applicable	
For **tumor marker prognostic studies,** we recommend that you follow the **REMARK** reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	Not Applicable	
For **phase II and III randomized controlled trials**, please refer to the **CONSORT** flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	Not Applicable	

Data Availability

Data availability	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Have **primary datasets** been deposited according to the journal's guidelines (see 'Data Deposition' section) and the respective accession numbers provided in the Data Availability Section?	Yes	Materials and Methods
Were **human clinical and genomic datasets** deposited in a public access-controlled repository in accordance to ethical obligations to the patients and to the applicable consent agreement?	Not Applicable	
Are **computational models** that are central and integral to a study available without restrictions in a machine-readable form? Were the relevant accession numbers or links provided?	Not Applicable	
If publicly available data were reused, provide the respective **data citations in the reference list**.	Not Applicable	