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Circadian time- and sleep-dependent modulation of cortical parvalbumin-positive inhibitory neurons

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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 **11th May 2022**

Dear Dr. He,

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.

Given the referees' recommendations, I would like to invite you to submit a revised version of the manuscript, addressing the comments of all three reviewers. It is EMBO Journal policy to allow only a single round of revision, and acceptance of your manuscript will therefore depend on the completeness of your responses in this revised version. It would be good to discuss your plan for addressing the referees' concerns and I will be available to do so by zoom or by email in the coming weeks.

When preparing your letter of 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.embopress.org/page/journal/14602075/authorguide#transparentprocess

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 also 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.

Yours sincerely,

Kelly M Anderson, PhD
Editor
The EMBO Journal
k.anderson@embojournal.org

Referee #1:

This main focus of this manuscript is on time-of-day changes in inputs and output of parvalbumin (PV) neurons in the visual cortex. The main findings are that PV-evoked inhibition onto pyramidal neurons is stronger at ZT12 than at ZT0. They also show that synaptic inhibition onto PV neurons is stronger at ZT12 than at ZT0 due to increased mEPSC frequency and decreased mIPSC frequency onto PV neurons. They show across the light/dark cycle that spontaneous EPSCs (sEPSCs) onto PV neurons increase from ZT0 and are sustained until about ZT18 and that sIPSCs have the opposite pattern: they rapidly drop from ZT0 to ZT4 and remain low until ZT18. The change in sEPSCs appears to be experience dependent, while the change in sIPSCs is sleep dependent. These conclusions were supported by examining another brain region (motor cortex). The regulation of inhibitory synapses onto PV neurons is in part regulated by acetylcholine M1 receptors. Using 2-photon imaging, they report that identified PV neurons in V1 have higher activity at ZT12 than at ZT0, while putative pyramidal neurons have higher activity at ZT0 than at ZT12. They also showed that there is a diurnal rhythm across four times of day in which PV neurons rapidly increase activity during the light inactive cycle and decrease during the dark active cycle. Pyramidal neurons show the opposite pattern. In a final experiment to measure response gain, they optogenetically drive LGN neurons and measure the Ca2+ response in the same population of V1 neurons at ZT0 and 12. They found higher response fideltiy of pyramidal neurons at ZT0 (end of the dark cycle) than at ZT12 (end of the light cycle), presumably due to lower inhibition by PV neurons. Overall, this is a carefully conducted and well thought-out set of convincing experiments that address an important question about how diurnal changes in E:I balance are mediated. I have some suggestions for improvement in the clarity of the manuscript, and there are several places where the text does not refer to the correct figure.

1. Generally throughout the manuscript, it is unclear what synapses are being examined (onto PV neurons or from PV neurons

to pyramidal neurons). This should be more explicitly stated throughout both the Results section and in the Figure legends. This could also be shown within the Figure itself to make it much clearer for the reader.

2. The authors could comment on their prior work showing the same diurnal changes across several brain regions whereas here they show opposite changes in the motor cortex vs visual cortex.

3. There are several mismatches with figures that the text refers to and the description in the Results. This should be re-checked carefully throughout the manuscript. Some examples are:

a. Cort levels are shown in S2F (not S2E) and EEG characterization is 2E.
b. Line 289 refers to synaptic transmission but refers to Fig S2C, which is actually % time spent in wake.
c. Lines 255-260. Check all figures referred to here.
d. Figure S6 also has the incorrect figures referred to in the text.

4. They mention that CORT levels are not different, but there are only 4 animals and it is clear that 2 animals show an increase in CORT levels, so the conclusion is not strongly supported. Also, it is expected that CORT levels may increase during sleep deprivation.

5. Figure S6 is not clear - what are the non-PV cells here?

6. The discussion of 'Clock'-dependent regulation within the discussion section should be omitted, other than to reference other work or to speculate that their changes could be circadianally regulated, but to say PV transmission is dependent on 'Clock' is incorrect; one can say only that there are time-of-day changes or sleep-dependent changes from the data presented here.

Minor:
Line 186: Please state what nAChR antagonist was used here for Fig 3SF.
Line 250: "induced" should be "induce".
Line 268: "with" a variety of brain disorders should be "to" a variety..."
Line 294: "many evidences" should be replaced with "much evidence".
Line 302 "prolong" should be "prolonged'
Line 305 "increase" should be "increases".
Throughout, "evidences" should be "evidence".
Line 320 - what are "NDNF" interneurons?
Line 345: "finding" should be "findings".
Line 786: add "to" for "higher compared to those..."
Line 795: H. Mice "that" underwent sleep deprivation..."
Figure S3B2 "volume" is misspelled on the y-axis.
Figures that show "insert" should be "inset" throughout.

Referee #2:

General summary and opinion:

This study combines in vitro and in vivo experiments to explore how the activity of a class of cortical inhibitory interneurons, referred to as PV interneurons, changes over the 24-hour cycle. The general hypothesis is that changes in certain cellular parameters leads PV interneurons to be less active around the onset of light (ZT0), when entrained mice typically go to sleep. Whereas the same population of PV interneurons are more active around the offset of light (ZT12), when mice typically wake and are active in their environment.

The authors use a range of different techniques, including optogenetic mapping of synaptic outputs, whole cell recordings of spontaneous synaptic currents, immunohistochemistry, pharmacological manipulations, and in vivo calcium imaging. From the data, it does indeed appear as though there are daily changes in multiple aspects of PV interneuron physiology. Unfortunately however, I find the data on the underlying mechanisms confusing and the experiments on functional consequences require further control experiments in order to be interpreted.

Specific major concerns essential to be addressed:

1. In Figure 1, the authors show that there is more synaptic output from PV interneurons to pyramidal neurons at ZT12 compared to ZT0, when using light-evoked optogenetic stimulation in acute brain slices in vitro. This is an interesting observation and could be due to multiple factors including changes in the intrinsic excitability of PV interneurons, changes in the release probability at PV to pyramidal synapses, or changes in the strength of synapses from PV to pyramidal neurons, (or possible technical factors such as variation in the expression of ChR2 or changes in the light transmissibility of slices across the

24-hour cycle). These possibilities need to be tested to understand the underlying mechanism.

2. Rather than explore the mechanisms underlying the increased synaptic output of the PV interneurons onto pyramidal neurons at ZT12, the authors switch direction and ask whether synaptic inputs onto PV interneurons themselves shows variation between ZT0 and ZT12. In Figure 2, they use recordings of miniature post-synaptic currents, which are independent of action potential activity. In Figure 3 they record spontaneous EPSCs and IPSCs, which include action potential evoked currents onto the PV interneurons. In both cases, these data suggest that there is greater excitatory synaptic input and lower inhibitory synaptic input onto PV interneurons at ZT12. These are interesting observations, but it is not clear how they relate to the data in Figure 1. The authors should investigate how these are mechanistically linked.

3. The authors show that 12 h of dark rearing from ZT0 to ZT12 prevents the normal increase in excitatory input to PV interneurons, but not the decrease in inhibitory synaptic input to PV interneurons. The authors then show that sleep deprivation from ZT0 to ZT4 prevented the reduction in inhibitory synaptic input to PV interneurons, but not the increase in excitatory input to PV interneurons. However, the sleep deprivation caused an additional enhancement in the excitatory input to PV interneurons. The authors conclude that "synaptic excitation and inhibition of PV neurons is mainly regulated by experience and sleep, respectively." I find this a difficult conclusion because the experiments do not control for the effects of experience and sleep-wake history of the animal. For example, there is no control to rule out that the dark rearing manipulation has not affected sleep-wake patterns. And there is no control to support the conclusion that the additional enhancement in the excitatory input to PV interneurons reflects experience. These aspects would need to support the stated conclusions.

4. The authors consider underlying mechanisms in Figure 4. They focus on the increase in inhibitory input to PV interneurons, but omit investigation of the changes in excitatory input for some reason. Given that the variation in excitatory input to PV interneurons could alter PV interneuron physiology, the authors should provide an explanation why they do not investigate this aspect.

5. The authors' first investigation into underlying mechanisms reveals an increase in GAD65-positive presynaptic puncta onto PV interneurons at ZT12. This identifies a mechanism for changes in inhibitory input to PV interneurons, but the direction of effect is counterintuitive given the electrophysiological data. GAD65 puncta onto PV soma are larger at ZT12, when the functional data suggests a decrease in presynaptic interneuron input to the PVs at ZT12. The authors should account for this discrepancy, as it appears to be directly contradictory to previous work.

6. The authors then conduct a series of interesting experiments that build on previous evidence that levels of neuromodulators vary across the 24-hour cycle. They apply agonists of neuromodulatory receptor systems to brain slices for 1 hour, to test whether this is able to cause an increase in inhibitory conductance at ZT12, when mIPSCs are normally low. This leads to the conclusion that mAChR activation can induce an increase in mIPSC frequency, providing a potential mechanism by which inhibitory inputs to PV interneurons are increased during the dark period (towards ZT0). To demonstrate the physiological significance of this mechanism would require data showing that blocking mAChR activation prevents the normal increase in inhibitory input observed by ZT0. This would be most convincing if conducted in vivo.

7. The authors rightly acknowledge the importance of in vivo experiments. In Figure 5, they perform awake calcium imaging experiments to monitor the spontaneous activity of PV interneurons and putative pyramidal neurons. These calcium signals are widely accepted to report action potential (spiking) activity and the data reveal that PVs show more spiking activity at ZT12. This is consistent with the in vitro observations that PV interneurons at ZT12 receive less spontaneous inhibitory input and more spontaneous excitatory input. The only aspect that I find difficult to understand, given previous work in this field, is the lack of correlation between the calcium signals and the animal's level of locomotion. The authors should comment on this observation.

8. Finally, the authors conduct an in vivo experiment to investigate the functional consequences of PV interneuron activity at ZT0 and ZT12. In Figure 6, they use light activation of ChR2-expressing LGN neurons to test responses in V1 (presumably the data are from putative pyramidal neurons). The interpretation is that greater PV activity leads to reduced responses to thalamic stimulation at ZT12, compared to ZT0. However, there is currently nothing in the experiment to say that these differences in thalamic-evoked responses are actually due to cortical PV interneurons. Whilst direct stimulation of the thalamus bypasses potential differences in the retina, the authors cannot rule out potential differences at the level of the thalamus, especially as the activity of this structure is thought to vary over the 24-hour cycle. One would at least expect to see evidence that PVs are indeed more active to the LGN stimulation at ZT12, than at ZT0.

Referee #3:

General summary and opinion about the principal significance of the study, its questions and findings

Zong and colleagues investigate in mice how parvalbumin-positive (PV) neurons, the main class of inhibitory (inter-)neurons neocortex are modulated throughout the light-dark cycle and by sleep and wakefulness.
Performing in vitro whole cell recordings in acute brain slices as well as in vivo calcium imaging, combined with optogenetic

stimulation, the authors produced a consistent set of data showing a circadian modulation of PV neuron activity and - as previously shown - pyramidal cell activity in contrast to a lack of circadian modulation of other non-PV non-putative pyramidal neurons in mouse neocortex. The authors show that inhibition of (layer 2/3) pyramidal neurons by PV neurons is higher at the end of the light period (ZT12) compared to the end of the dark period (ZT0). Turning from the output to the input of PV neurons, the authors demonstrate that over the light period excitatory input to PV interneurons increases while inhibitor input decreases. The opposite changes occur during the dark period, when excitatory input decreases while inhibitory input increases. Together these inverse oscillations in inhibitor and excitatory inputs to PV neurons explain why PV neuron activity is higher at the end of the light period (ZT12) compared to the end of the dark period (ZT0). The authors move on to show that excitatory inputs to PV interneurons, at least in primary visual cortex V1, appear to undergo experience-dependent regulation while inhibitory inputs appear to be under sleep-dependent regulation. Looking in into the molecular regulation of PV neuron activity, the authors suggest a cholinergic regulation of PV neurons via M1Rs and KCNQ channels. The authors conclude with showing that differences in PV activity between ZT0 and ZT12 affect the response of excitatory (pyramidal) neurons in V1 to optogenetic dLGN stimulation, thereby providing some functional relevance of the observed circadian modulation.
In my view the experiments elegantly address the question of whether PV interneurons are modulated by circadian rhythms show clear and consistent results in respect to both output of and inputs to PV neurons. The experiments addressing molecular mechanisms and functional relevance are rather preliminary but provide some promising leads. My main concern is that the paper claims a sleep- and experience-dependent modulation of PV neurons but the data to support this statement is wake. Only one sleep deprivation experiment was performed when looking at excitatory and inhibitory inputs to PV neurons and the condition of 'reduced experience' is a change in the light-dark condition to constant darkness, which is an unusual way to test a lack of 'experience' and would widely be considered an interference with the circadian zeitgeber and hence affect the activity of the suprachiasmatic nucleus. Whether PV neurons are regulated by sleep and expericne as the authors states remains unresolved based on the data provided.
Overall, the paper is well structured and of good quality but the authors do not sufficiently disentangle circadian and sleep-dependent modulation of PV neurons. Their claims should be weekend or additional data provided. I enjoyed reading this paper but some improvements will be necessary for publication at the EMBO Journal.

Major concerns

1) Figure 3B:
For sEPSC charge no data is plotted at ZT8 although this data was acquired according to the schematic and the legend in Figure 3A and as shown in the sIPCSC charge panel 3C. Please provide the data or explain why this data is missing!
- Why was no data at this time point collected/presented?
- If it was collected was it consistent or inconsistent with the otherwise very convincing oscillation of sIPSC charges?

2) Figure 1:
What is the effect of sleep deprivation on PV output?
Ideally, a sleep deprivation experiment would be performed to support the claim that the activity of PV neurons is modulated not only by circadian time but also by sleep. Alternatively, the authors should discuss this limitation and specifically mention that their experiments cannot disentangle circadian and sleep-dependent modulation of PV neuron activity?

3) Figures 5 and 6:
What is the effect of sleep deprivation on PV neuron activity and on dLGN-evoked responses in the in vivo experiments?
As for Figure 1 one would hope that the authors could disentangle circadian and sleep-dependent contributions to the very interesting changes in PV neuron activity and resulting differences in dLGN-evoked responses.

4) Statistics:
It appears from the statistics section and the figure legends that the authors might have treated recordings of different cells in the same slice as independent measurements. Could the authors justify the use of an ANOVA instead of a GLM?

Minor concerns

1) In the abstract, the authors do not mention the species used for their experiments nor the experimental method used for their in vitro experiments. This is crucial information and should be added to the abstract.

2) In the introduction the authors mention that "In mature brain, different sleep/wake stages and sleep history are reported to acutely affect the spontaneous firing of PV(Niethard, Hasegawa et al., 2016) or general inhibitory neurons(Miyawaki & Diba, 2016, Vyazovskiy, Olcese et al., 2009)". It is not clear what the authors mean with general inhibitory neurons. Importantly Vyazovksiy et al. 2009 demonstrated a change in firing rates of inhibitory and excitatory neurons. I therefore strongly suggest to replace the phrasing 'general inhibitory neurons' with 'neocortical excitatory and inhibitory neurons'.

3) Line 86: "pyramidal neurons": please specify cortical layer in which the pyramidal neurons were recorded. The figure suggests

these were L2/3 pyramidal neurons, the text suggests that these were L5 pyramidal neurons.

4) Figure 1 C,D,F:
• The y-axis goes to 125%. This makes little sense given that the maximum cumulative probability is 100%. I suggest ending the y-axis at 100%.
• The y-axis label of the inlays is missing. This should be added.

5) Section "Spontaneous activity of PV neurons oscillates naturally during the light/dark cycle": Please add the cortical area in which the imaging was performed in the main text. If possible, please further add the specific layer (I assume only L2/3 was reached with two-photon imaging) that was imaged.

6) Recently several papers suggest a cortical regulation of sleep. Both SOM+ GABAergic neurons (Tossell et al., BioRxiv, 2021) as well as pyramidal neurons (Krone et al., Nature Neuroscience, 2021) seem to be involved though the circuitry is still unclear. I would encourage the authors to mention that experience-dependent modulation of PV-interneurons might indirectly affect homeostatic sleep drive through modulation of (layer 5) pyramidal neurons by PV neurons. For example, this could be added in line 278.

7) Methodological details on EEG/EMG-based sleep analysis are missing. Please specify how the data was recorded and how vigilance states were assigned, e.g. if automatic or manual scoring was used and what were the criteria.

Non-essential suggestions

1) The title could be more specific. "Mechanism and consequence..." is very generic. I would suggest reconsidering a more meaningful title.

2) In my view the authors are overstating the clinical relevance of their findings in the introduction. This is a basic research study and it remains unclear how diurnal changes in PV activity and inputs "provides a potential explanation" for brain disorders involving PV disfunction. I suggest removing this statement.

3) Figure 4A: Why do the authors show a human brain? The research is conducted in mice. I suggest replacing this schematic (which is in principle correct) with a schematic of the species investigated in this study.

4) Line 125-127: "Specifically, sEPSCs and sIPSCs were increased and decreased respectively 4 hours after entering the light cycle, then they were maintained relatively stable between ZT4 and ZT12." This finding is particularly interesting because it reflects the time course of slow wave activity the most commonly used measure of homeostatic sleep drive. Under baseline conditions in most mouse strains decays over the first four ours of the light period and then remains relatively constant. The authors could consider adding a few words and a reference here, e.g. Huber et al., Brain Research, 2000 or one of the many other papers showing this time course of.

5) I suggest language-editing by a native speaker. The text still contains a few typos and some sentences are difficult to understand, e.g.
- Line 154: Typo boo sting. Please write boosting.
- Line 173: Word missing. I assume the authors mean circadian rhythms and sleep.
- Line 211: Word missing. I assume the authors mean may be altered.

6) The spelling should be made consistent throughout the text (e.g. 2-photon or two-photon).

7) Please specify the number and age of mice in the methods section

Dear editor and reviewers,

First of all, thank you very much for spending time and effort reviewing our manuscript and we truly appreciate all of your comments and suggestion. We are now providing our responses for each comment below.

(Reviewers' comments in black, responses in blue and red. Responses using italic font are those copied directly from the revised manuscript.)

Referee #1:

This main focus of this manuscript is on time-of-day changes in inputs and output of parvalbumin (PV) neurons in the visual cortex. The main findings are that PV-evoked inhibition onto pyramidal neurons is stronger at ZT12 than at ZT0. They also show that synaptic inhibition onto PV neurons is stronger at ZT12 than at ZT0 due to increased mEPSC frequency and decreased mIPSC frequency onto PV neurons. They show across the light/dark cycle that spontaneous EPSCs (sEPSCs) onto PV neurons increase from ZT0 and are sustained until about ZT18 and that sIPSCs have the opposite pattern: they rapidly drop from ZT0 to ZT4 and remain low until ZT18. The change in sEPSCs appears to be experience dependent, while the change in sIPSCs is sleep dependent. These conclusions were supported by examining another brain region (motor cortex). The regulation of inhibitory synapses onto PV neurons is in part regulated by acetylcholine M1 receptors. Using 2-photon imaging, they report that identified PV neurons in V1 have higher activity at ZT12 than at ZT0, while putative pyramidal neurons have higher activity at ZT0 than at ZT12. They also showed that there is a diurnal rhythm across four times of day in which PV neurons rapidly increase activity during the light inactive cycle and decrease during the dark active cycle. Pyramidal neurons show the opposite pattern. In a final experiment to measure response gain, they optogenetically drive LGN neurons and measure the Ca2+ response in the same population of V1 neurons at ZT0 and 12. They found higher response fideltiy of pyramidal neurons at ZT0 (end of the dark cycle) than at ZT12 (end of the light cycle), presumably due to lower inhibition by PV neurons. Overall, this is a carefully conducted and well thought-out set of convincing experiments that address an important question about how diurnal changes in E:I balance are mediated. I have some suggestions for improvement in the clarity of the manuscript, and there are several places where the text does not refer to the correct figure.
**We thank the reviewer for his/her positive feedback regarding our manuscript.

1. Generally throughout the manuscript, it is unclear what synapses are being examined (onto PV neurons or from PV neurons to pyramidal neurons). This should be more explicitly stated throughout both the Results section and in the Figure legends. This could also be shown within the Figure itself to make it much clearer for the reader.
**We thank the reviewer for pointing out this important issue. Now we have clarified the synapses throughout the manuscript including the results section and figure legends.

2. The authors could comment on their prior work showing the same diurnal changes across several brain regions whereas here they show opposite changes in the motor cortex vs visual cortex.
**We thank the reviewer for the suggestion. Our current results indicate that the diurnal changes in excitatory synapses onto cortical PV neurons are mainly regulated in an experience-dependent manner, which is consistent with previous finding (Kuhlman, Olivas et al., 2013). PV neurons in sensory cortices are heavily innervated by thalamocortical inputs (Kuhlman et al., 2013, Ma, Liu et al., 2021), which relay sensory information that oscillates with the animal's activity cycle. As nocturnal animal, PV neurons in mouse V1 versus M1 receive sensory inputs at different times of the day, which leads to the opposite changes in their sEPSC diurnal patterns.

In turns of pyramidal neurons, their sEPSCs share similar daily pattern across brain regions (Bridi, Zong et al., 2020). V1 L2/3 pyramidal neurons are known to receive majority of their excitatory inputs from the lateral intracortical connections that relay 'top-down' information from other cortices (Binzegger, Douglas et al., 2004, Douglas & Martin, 2007, Petrus, Rodriguez et al., 2015). Therefore, the diurnal rules for these intracortical synapses are likely similar to other cortices and the hippocampus, which have more experience-driven inputs during the active cycle (dark cycle) while reduced inputs during the inactive cycle (light cycle).

In summary, the major excitatory inputs and their relayed experience determine the daily patterns of the excitatory synaptic transmission of both PV and pyramidal neurons.

In contrast to the excitatory synapses, the inhibitory synapses are tightly regulated by sleep for both pyramidal (Bridi et al., 2020) and PV neurons via neuromodulatory signaling. The brain-wide changes in neuromodulatory tone along the sleep/wake cycle make such regulation a feasible universal mechanism. The inhibitory synapses of pyramidal neurons indeed share the same diurnal pattern. Although we have not examined inhibitory synapses of PV neurons in other cortices, we speculate that they may undergo similar diurnal modulation as those in V1.

3. There are several mismatches with figures that the text refers to and the description in the Results. This should be re-checked carefully throughout the manuscript. Some examples are:

a. Cort levels are shown in S2F (not S2E) and EEG characterization is 2E.
b. Line 289 refers to synaptic transmission but refers to Fig S2C, which is actually % time spent in wake.
c. Lines 255-260. Check all figures referred to here.
d. Figure S6 also has the incorrect figures referred to in the text.

**We thank the reviewer for pointing out these errors. Now we have corrected all these mistakes in the revised manuscript.

4. They mention that CORT levels are not different, but there are only 4 animals and it is clear that 2 animals show an increase in CORT levels, so the conclusion is not strongly supported. Also, it is expected that CORT levels may increase during sleep deprivation.

** We appreciate reviewer's keen observation. Indeed, SD mice had greater variability in plasma CORT level, although the comparison between the two groups is not statistically significant. We now have rephrased in Line 152 as following:

"SD was achieved by gentle handling and its efficacy was confirmed by electroencephalogram (EEG) and electromyogram (EMG) recordings (Fig S2E)".

We revised the figure legend for Fig S2F as '*4 hours of sleep deprivation mildly increased the plasma level of corticosterone*'.

CORT is reported to enhance glutamatergic synaptic transmission (Karst & Joels, 2005). However, we found that sleep deprivation in complete darkness (Dark-SD_ZT4) prevented the SD-induced increase in sEPSCs (Fig R1), suggesting visual experience but not other factors such as CORT that dictates PV's excitatory inputs. We now included Fig R1 as Fig S2G in the revised manuscript.

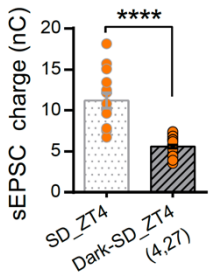

Fig R1. Sleep deprivation in complete darkness from ZT0 to 4 prevents the increase in sEPSCs induced by sleep deprivation alone (Added figure to Fig S2G).

5. Figure S6 is not clear - what are the non-PV cells here?
**We apologize for the confusion. Now we have changed them into '*dLGN-responsive neurons'.*

6. The discussion of 'Clock'-dependent regulation within the discussion section should be omitted, other than to reference other work or to speculate that their changes could be circadianally regulated, but to say PV transmission is dependent on 'Clock' is incorrect; one can say only that there are time-of-day changes or sleep-dependent changes from the data presented here.

** Thank you for the suggestion. We meant to propose the time-dependent regulation and now we have replaced all 'Clock' into 'time'.

Minor:
Line 186: Please state what nAChR antagonist was used here for Fig 3SF.

** We now have added the full name of MEC in both the result section in Line 190 '*Infusion with 50 μM non-selective mAChRs antagonist atropine (Atr) but not nAChRs antagonist mecamylamine (MEC) (Fig S3F) was sufficient to…*' and the legend of Fig 3SF as following: *'$F_{1\text{-}2}$. Washing on and out nAChR antagonist mecamylamine (MEC) caused no change in mIPSCs of PV neurons at ZT0'*.

Line 250: "induced" should be "induce".

** Thank you for pointing out this mistake. We now have corrected it to '*induce*'.

Line 268: "with" a variety of brain disorders should be "to" a variety..."
** Thank you for the suggestion. Since both "associated with" and "associated to" are acceptable, we decided to keep "with" here.

Line 294: "many evidences" should be replaced with "much evidence".

** Thank you for pointing out this mistake. We now have corrected it to '*much evidence*'.

Line 302 "prolong" should be "prolonged'

** Thank you for pointing out this mistake. We now have corrected it to '*prolonged*'.

Line 305 "increase" should be "increases".

** Thank you for pointing out this mistake. We now have corrected it to '*increases*'.

Throughout, "evidences" should be "evidence".

** Thank you for pointing out this mistake. We now have corrected it to '*evidence*'.

Line 320 - what are "NDNF" interneurons?

**We apologize for the confusion. Now we have added full name of NDNF in the manuscript as followed:

'A recent study reported that the neuron derived neurotrophic factor (NDNF)-expressing interneurons in V1 L1…'.

Line 345: "finding" should be "findings".

** Thank you for pointing out this mistake. We now have corrected it to '*findings*'.

Line 786: add "to" for "higher compared to those..."

** Thank you for pointing out this mistake. We now have corrected it to '*compared to…*'.

Line 795: H. Mice "that" underwent sleep deprivation..."

** Thank you for pointing out this mistake. We now have corrected it to '*Mice that underwent…*'.

Figure S3B2 "volume" is misspelled on the y-axis.

** Thank you for pointing out this mistake. We now have corrected it to '*volume*'.

Figures that show "insert" should be "inset" throughout.

** Thank you for pointing out this mistake. We now have corrected all 'insert' into 'inset'.

Referee #2:

General summary and opinion:

This study combines in vitro and in vivo experiments to explore how the activity of a class of cortical inhibitory interneurons, referred to as PV interneurons, changes over the 24-hour cycle. The general hypothesis is that changes in certain cellular parameters leads PV interneurons to be less active around the onset of light (ZT0), when entrained mice typically go to sleep. Whereas the same population of PV interneurons are more active around the offset of light (ZT12), when mice typically wake and are active in their environment.

The authors use a range of different techniques, including optogenetic mapping of synaptic outputs, whole cell recordings of spontaneous synaptic currents, immunohistochemistry, pharmacological manipulations, and in vivo calcium imaging. From the data, it does indeed appear as though there are daily changes in multiple aspects of PV interneuron physiology. Unfortunately however, I find the data on the underlying mechanisms confusing and the experiments on functional consequences require further control experiments in order to be

interpreted.

Specific major concerns essential to be addressed:

1. In Figure 1, the authors show that there is more synaptic output from PV interneurons to pyramidal neurons at ZT12 compared to ZT0, when using light-evoked optogenetic stimulation in acute brain slices in vitro. This is an interesting observation and could be due to multiple factors including changes in the intrinsic excitability of PV interneurons, changes in the release probability at PV to pyramidal synapses, or changes in the strength of synapses from PV to pyramidal neurons, (or possible technical factors such as variation in the expression of ChR2 or changes in the light transmissibility of slices across the 24-hour cycle). These possibilities need to be tested to understand the underlying mechanism.
**We thank the reviewer for raising this question. The altered PV-pyramidal inhibitory synaptic strength is indeed very interesting. We will address reviewer's concerns one by one below.

1) "changes in the intrinsic excitability of PV interneurons"
---We previously found that neither PV nor pyramidal cells have altered intrinsic excitability at different times of the day (Fig R2).

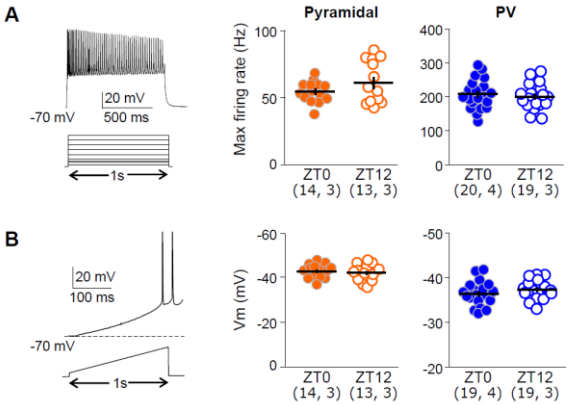

Figure R2. Intrinsic excitability of V1 layer 2/3 neurons does not change at different times of the day (Adapted from (Bridi et al., 2020)).

2) "changes in the release probability at PV to pyramidal synapses"
---We previously reported that the pair-pulse ratios of evoked IPSCs recorded from pyramidal neurons were not differed between ZT0 and ZT12 (Fig R3), indicating stable presynaptic release of the inhibitory synapses onto pyramidal neurons. Pyramidal cells receive major inhibitory inputs from PV, therefore, PV synapses onto pyramidal cells probably have stable presynaptic release between ZT0 and ZT12. However, it remains to be directly examined whether the presynaptic properties of PV onto pyramidal synapses indeed remain constant at different times of the day.

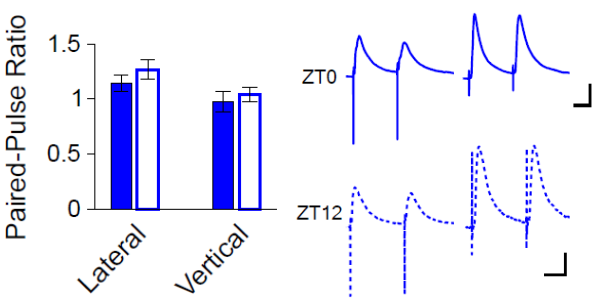

Figure R3. Time of the day did not affect the inhibitory paired-pulse ratio in both vertical and lateral synapses of V1 L2/3 pyramidal neurons. The inter-stimulus interval was 50 msec (scale bar: 200 pA, 20 msec) (Adapted from (Bridi et al., 2020)).

3) "changes in the strength of synapses from PV to pyramidal neurons"
---We previously found that mIPSCs of cortical pyramidal neurons recorded at ZT0 and ZT12 have similar amplitude (Fig R4), suggesting comparable postsynaptic GABA receptor function and hence synaptic strength. However, it remains to be

tested whether synapses from PV neurons maintain synaptic strength at different times of the day.

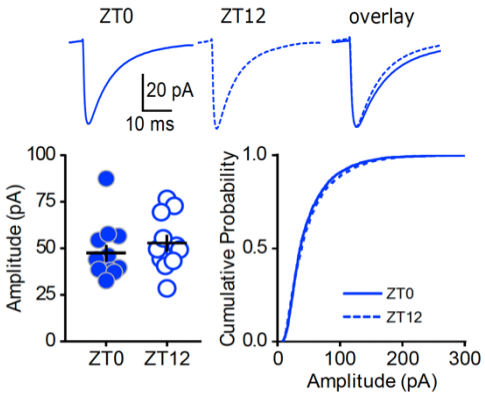

Figure R4. Time of the day did not affect the inhibitory synaptic strength reflected by unaltered mIPSC amplitude in V1 L2/3 pyramidal neurons (Adapted from (Bridi et al., 2020)).

4) "variation in the expression of ChR2“

---To make sure the change is not due to variation in ChR2 expression, we entrained mice from the same PV:Ai32 litter with opposite light/dark cycle. We then compared $_{LE}$IPSCs from littermate mice entrained in opposite light/dark cycle at around the same absolute time of the day. We observed consistently upregulated $_{LE}$IPSCs from mice sacrificed at ZT12 than at ZT0 across three different litters (Fig R5, Fig S1B). This result suggests the change is unlikely due to varied level of ChR2 expression.

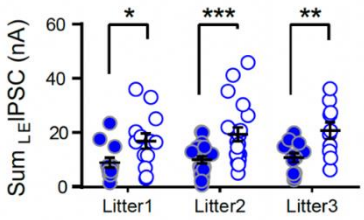

Figure R5. Comparison of the sum $_{LE}$IPSC amplitudes between ZT0 and ZT12. Mice from the same litter were randomly recorded at either time points and the results were compared within litter (Adapted from Fig S1B).

5) "changes in the light transmissibility of slices across the 24-hour cycle"

---We could not rule out the possibility of different light transmissibility of slice across the day. But we do think it is not likely since in our previous study, light-evoked E/I ratio of L4-L2/3 synapses recorded from L2/3 pyramidal neurons are not distinct between ZT0 and ZT12 (Fig R6), suggesting minimal effect of light transmissibility in our current result.

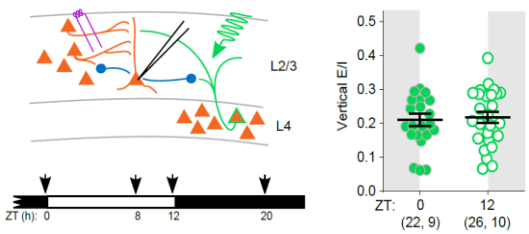

Figure R6. Comparable E/I ratio of L4-L2/3 synapses recorded at ZT0 and ZT12. Both E and I responses were evoked by blue light stimulation of ChR2 selectively expressed in L4 pyramidal neuron via aav injection (Adapted from (Bridi et al., 2020)).

Although mechanisms underlying the altered PV's output is not directly explored, we now added a discussion of the potential mechanism in Line 287 as following:

'*The exact mechanism underlying this daily modulation remains to be examined. Changes in PV and pyramidal neuron's intrinsic excitability or presynaptic properties unlikely contribute since both of them are found to remain unaltered between the light and dark cycle (Bridi et al., 2020). However, PV neurons receive distinct levels of inhibition at different times of the day (Fig 3C). Inhibition is known to gate neuronal output(Bridi et al., 2020, Gidon & Segev, 2012, Jelitai, Puggioni et al., 2016), the lower inhibition at ZT12 might enhance PV's output by*

reducing the activation threshold of PV neurons.'

2. Rather than explore the mechanisms underlying the increased synaptic output of the PV interneurons onto pyramidal neurons at ZT12, the authors switch direction and ask whether synaptic inputs onto PV interneurons themselves shows variation between ZT0 and ZT12. In Figure 2, they use recordings of miniature post-synaptic currents, which are independent of action potential activity. In Figure 3 they record spontaneous EPSCs and IPSCs, which include action potential evoked currents onto the PV interneurons. In both cases, these data suggest that there is greater excitatory synaptic input and lower inhibitory synaptic input onto PV interneurons at ZT12. These are interesting observations, but it is not clear how they relate to the data in Figure 1. The authors should investigate how these are mechanistically linked.

**We thank the reviewer for this great suggestion. We think the diurnal changes in PV's inhibitory synaptic input provide one explanation for the altered inhibitory synaptic efficacy between PV and pyramidal neurons reported in Figure1. PV neurons are less inhibited during the light cycle compared to the dark cycle (Fig 2B & 3C). Inhibition is well known to gate neuronal output (Gidon & Segev, 2012, Jelitai et al., 2016). Therefore, low inhibition at ZT12 may allow more PV neurons to be activated upon similar level of ChR2 stimulation, while high inhibition at ZT0 makes PV neurons harder to be excited. To address the link between synaptic transmission and PV output, we now added a paragraph of discussion in Line 287 as following:

'*These results imply that inhibitory synapses in adult brain are not as rigid as previously thought, but instead are flexibly modulated during the 24-hour day. The exact mechanism underlying this daily modulation remains to be examined. Changes in PV and pyramidal neuron's intrinsic excitability or presynaptic properties unlikely contribute since both of them are found to remain unaltered between the light and dark cycle (Bridi et al., 2020). However, PV neurons receive distinct levels of inhibition at different times of the day (Fig 3C). Inhibition is known to gate neuronal output(Bridi et al., 2020, Gidon & Segev, 2012, Jelitai et al., 2016), the lower inhibition at ZT12 might enhance PV's output by reducing the activation threshold of PV neurons.*'

3. The authors show that 12 h of dark rearing from ZT0 to ZT12 prevents the normal increase in excitatory input to PV interneurons, but not the decrease in inhibitory synaptic input to PV interneurons. The authors then show that sleep deprivation from ZT0 to ZT4 prevented the reduction in inhibitory synaptic input to PV interneurons, but not the increase in excitatory input to PV interneurons. However, the sleep deprivation caused an additional enhancement in the excitatory input to PV interneurons. The authors conclude that "synaptic excitation and inhibition of PV neurons is mainly regulated by experience and sleep, respectively." I find this a difficult conclusion because the experiments do not control for the effects of experience and sleep-wake history of the animal. For example, there is no control to rule out that the dark rearing manipulation has not affected sleep-wake patterns. And there is no control to support the conclusion that the additional enhancement in the excitatory input to PV interneurons reflects experience. These aspects would need to support the stated conclusions.

**We thank the reviewer for raising this concern. Previous study found that mice entrained to the regular 12h:12h light/dark cycle were able to maintain their activity pattern up to a week when exposed to dark rearing(Faradji-Prevautel, Cespuglio et al., 1990). Therefore, we speculate that the 12-hour acute dark exposure would not alter mice's sleep/wake pattern. We now provided this citation in Line 137 of our revised manuscript as following:

'To directly test the role of experience, we took advantage of the fact that mice entrained to regular light/dark cycle are able to maintain their activity pattern in complete darkness for several days (Faradji-Prevautel et al., 1990). We visually deprived a group of PV:Ai9 mice by dark exposure during the last light cycle prior to experimentation (Dark-ZT12, Fig 3D)….'

To further validate the role of experience in regulating PV's excitatory synaptic transmission and test whether the further enhanced sEPSCs post SD is really due to increased visual input, we now performed additional experiment by sleep depriving mice from ZT0 to ZT4 in complete darkness. In line with our speculation and other results, SD in dark abolished the increase (Fig R1, copy and paste below for reviewers' convenience), indicating that SD indeed promotes PV's excitatory input by indirectly enhancing visual experience. This data is now included as Fig S2G in the revised manuscript and we have modified the result section as following:

'Interestingly, SD failed to prevent the increase, and actually further enhanced sEPSCs (Fig 3H), which is likely due to an additional increase in visual experience indirectly caused by SD. Indeed, this increment was largely abolished by SD in darkness (Fig S2G), confirming the dominant role of experience in regulating excitatory synapses onto PV neurons.'

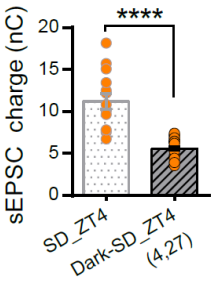

Fig R1. Sleep deprivation in complete darkness from ZT0 to 4 prevents the increase in sEPSCs induced by SD alone (Added figure to Fig S2G).

4. The authors consider underlying mechanisms in Figure 4. They focus on the increase in inhibitory input to PV interneurons, but omit investigation of the changes in excitatory input for some reason. Given that the variation in excitatory input to PV interneurons could alter PV interneuron physiology, the authors should provide an explanation why they do not investigate this aspect.

**We thank the reviewer for this question. Since we are more interested in the neuronal function of sleep, we decided to focus on investigating the sleep-dependent regulation of the inhibitory synapses onto PV neurons. Indeed, the diurnal regulation of PV's excitatory input is also very important, which we found is highly dependent on experience that coincide with animals' light/dark cycle. We should keep the fact in perspective that, despite many years of research in the field, it is not yet clear what mechanisms during sleep/wake are responsible for the changes in excitation. The full range of mechanisms involved is likely to be complex and involve many processes, and exploring all possibilities is beyond the scope of our current study but a promising avenue for future exploration.

5. The authors' first investigation into underlying mechanisms reveals an increase in GAD65-positive presynaptic puncta onto PV interneurons at ZT12. This identifies a mechanism for changes in inhibitory input to PV interneurons, but the direction of effect is counterintuitive given the electrophysiological data. GAD65 puncta onto PV soma are larger at ZT12, when the functional data suggests a decrease in presynaptic interneuron input to the PVs at ZT12. The authors should account for this discrepancy, as it appears to be directly contradictory to previous work.

**We thank the reviewer for pointing out this mistake for us! We double-checked our IHC data and realized that by double-blindly analyzing the data, the student miss-matched the dataset of the two time points. Now we have corrected the figures in Fig S3B and posted below as Fig R7. We apologize for the confusion.

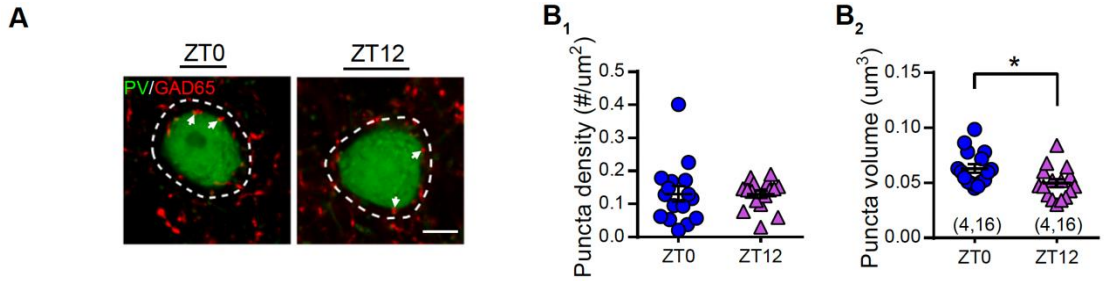

Figure R7. GAD65-positive presynaptic boutons onto PV neurons varies structurally at different times of the day.

In addition, we now provided an independent IHC dataset to further confirm such structural changes. PV neurons are known to form extensive inhibitory network with each other(Galarreta & Hestrin, 2002). We therefore used antibody ZNP-1 to label synaptotagmin-2(Fox & Sanes, 2007), which is a reliable presynaptic marker for PV-positive inhibitory boutons(Sommeijer & Levelt, 2012). By simultaneously label PV soma, we examined ZNP-1-positive boutons around PV+ soma from brain slices cut at ZT0 and ZT12. Our results show that ZNP-1 puncta density is unchanged (Fig R8B) while puncta volume (Fig R8C) is reduced at ZT12 compared to ZT0, which is consistent with GAD65 staining results.

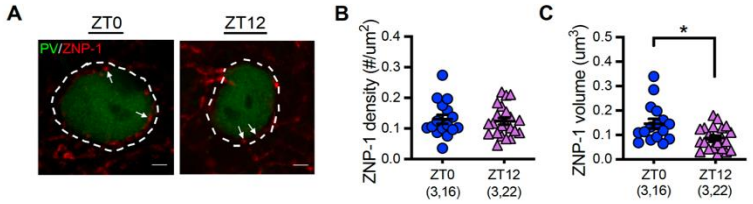

Figure R8. ZNP-1-positive puncta density was unchanged while puncta volume was reduced at ZT12 compared to ZT0.

Together, these results confirm that the size of the inhibitory presynapses onto PV neurons are altered at different times of day.

6. The authors then conduct a series of interesting experiments that build on previous evidence that levels of neuromodulators vary across the 24-hour cycle. They apply agonists of neuromodulatory receptor systems to brain slices for 1 hour, to test whether this is able to cause an increase in inhibitory conductance at ZT12, when mIPSCs are normally low. This leads to the conclusion that mAChR activation can induce an increase in mIPSC frequency, providing a potential mechanism by which inhibitory inputs to PV interneurons are increased during the dark period (towards ZT0). To demonstrate the physiological significance of this mechanism would require data showing that blocking mAChR activation prevents the normal increase in inhibitory input observed by ZT0. This would be most convincing if conducted in vivo.

**Thank you for the suggestion and we appreciate the comment. We did show in Fig 4F that blocking mAChR *ex vivo* by acutely infusing atropine reduced PV's mIPSC frequency recorded at ZT0. We copied and pasted these figures below as Fig R9.

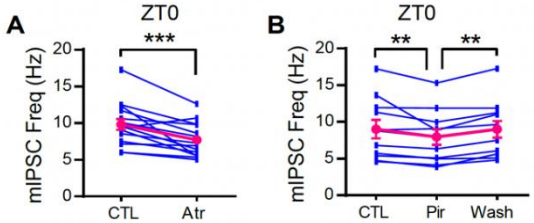

Figure R9. Blocking mAChR *ex vivo* by acutely infusing Atr (atropine, A) and Pir (pirenzepine, B) reduced PV's mIPSC frequency recorded at ZT0 (Adapted from Fig

4F & 4H).

The suggestion of in vivo validation is great! We have not carried out this experiment due to one major concern that mAChRs are expressed in most cell types. Application of mAChR antagonist or agonist will affect neuronal properties of all these cells, which will lead to network changes that may compromise PV's inhibitory synaptic changes. To test M1R regulation of PV's synaptic inhibition in vivo, we may need to cell type-selectively manipulate M1R activity, which will require much more effort that exceed our current research ability.

7. The authors rightly acknowledge the importance of in vivo experiments. In Figure 5, they perform awake calcium imaging experiments to monitor the spontaneous activity of PV interneurons and putative pyramidal neurons. These calcium signals are widely accepted to report action potential (spiking) activity and the data reveal that PVs show more spiking activity at ZT12. This is consistent with the in vitro observations that PV interneurons at ZT12 receive less spontaneous inhibitory input and more spontaneous excitatory input. The only aspect that I find difficult to understand, given previous work in this field, is the lack of correlation between the calcium signals and the animal's level of locomotion. The authors should comment on this observation.

**Thank you for the comments. We were concerned about the different locomotor activity of mice imaged at different times and did a detail cross correlation study between the neural activity and locomotor activity. We found that although mice imaged at ZT0 showed relatively higher locomotor activity (Fig S5D) (Fig R10A), on average their neural activity had no cross-correlation with locomotion at either time point for both PV and putative pyramidal neurons (Fig S5E) (Fig R10B). The distributions of the correlation of coefficient (r) were not different between ZT0 and ZT12 for PV neurons (Fig R10C). Even for those PV neurons showing a significant negative correlation with locomotion (cells within the red dotted box in Fig R10C), the detailed cross correlation analysis shows no delay or advance in the lag time, suggesting that locomotion unlikely drives the change in PV neural activity (Fig R10D). Together, these results indicate that the distinct spontaneous neural activity between ZT0 and ZT12 is not due to different levels of locomotion. This is consistent with previous findings showing that PV neurons in the superficial cortical layer are only minimally affected by locomotion (Dipoppa et al., 2018). To make it clearer, we now added the citation in Line 230 of the revised manuscript as following:

'*These distinct activity patterns are unlikely due to the relatively higher locomotor activity at ZT0 (Fig S5D) since no cross-correlation between neural activity and locomotion was found at either time point (Fig S5E). This is consistent with previous study showing that spontaneous activities of PV neurons in the superficial cortical layer are minimally affected by locomotion(Dipoppa, Ranson et al., 2018).*'

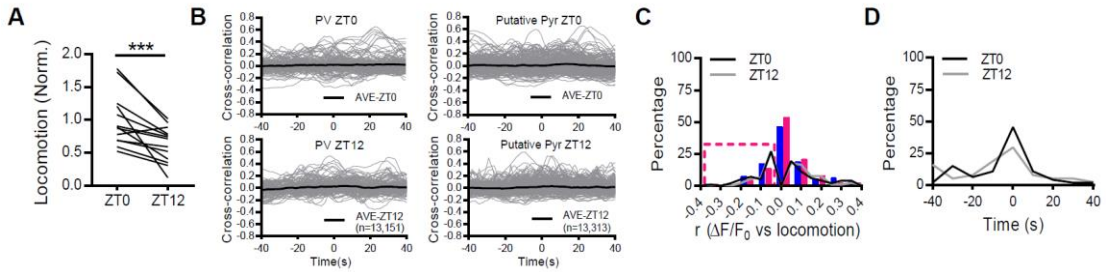

Figure R10. Daily oscillation in PV's spontaneous neural activity did not correlate with animals' locomotor activity.

8. Finally, the authors conduct an in vivo experiment to investigate the functional

consequences of PV interneuron activity at ZT0 and ZT12. In Figure 6, they use light activation of ChR2-expressing LGN neurons to test responses in V1 (presumably the data are from putative pyramidal neurons). The interpretation is that greater PV activity leads to reduced responses to thalamic stimulation at ZT12, compared to ZT0. However, there is currently nothing in the experiment to say that these differences in thalamic-evoked responses are actually due to cortical PV interneurons. Whilst direct stimulation of the thalamus bypasses potential differences in the retina, the authors cannot rule out potential differences at the level of the thalamus, especially as the activity of this structure is thought to vary over the 24-hour cycle. One would at least expect to see evidence that PVs are indeed more active to the LGN stimulation at ZT12, than at ZT0.

**Thank you for raising this question and we totally understand the concern. Unfortunately, we have not observed any superficial PV neurons directly responding to dLGN stimulation, we could not compare their activation levels upon dLGN stimulation at different times of the day. However, we did show data from two mice in Fig S6C-F (Fig R11) that local PV neurons were more spontaneously active when imaged at ZT12 compared to ZT0, while the dLGN-responsive neurons from the same field of view had larger response at ZT0 than ZT12. Since our data only provides a negative correlation between PV's activity and dLGN-evoked responses, we now rephrase the last sentence of this section as '*Thus, our data suggest that the daily modulation of PV neuronal function might be sufficient to impact the response gain in the visual cortex.*'

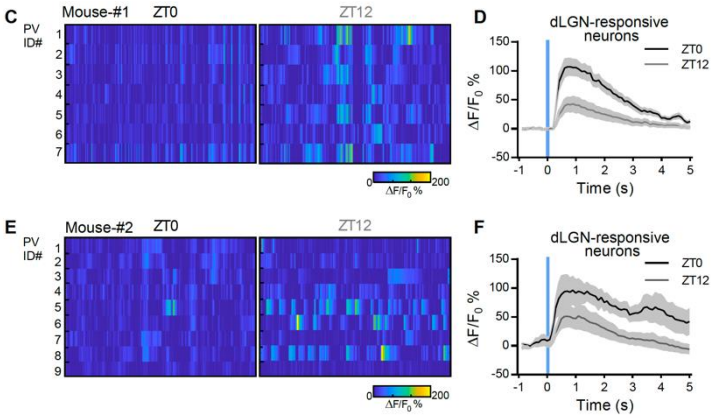

Fig R11. Local PV neurons were more active when imaged at ZT12 compared to ZT0, while the other dLGN-responsive neurons from the same field of view had larger response at ZT0 than ZT12 (Adapted from Fig S6C-F).

In addition, we could not rule out the diurnal change in thalamic activity that may also contribute to the altered responsiveness in V1. We now have added a discussion in Line 384 of our revised manuscript to reflect this possibility as following:

'*Recently, dLGN neural activity is found to be rhythmically regulated(Chrobok, Jeczmien-Lazur et al., 2021). It remains to be evaluated whether the rhythmic change in dLGN activity may also affect V1 responses at different times of the day.*'

Referee #3:

General summary and opinion about the principal significance of the study, its questions and findings

Zong and colleagues investigate in mice how parvalbumin-positive (PV) neurons, the main

class of inhibitory (inter-)neurons neocortex are modulated throughout the light-dark cycle and by sleep and wakefulness. Performing in vitro whole cell recordings in acute brain slices as well as in vivo calcium imaging, combined with optogenetic stimulation, the authors produced a consistent set of data showing a circadian modulation of PV neuron activity and - as previously shown - pyramidal cell activity in contrast to a lack of circadian modulation of other non-PV non-putative pyramidal neurons in mouse neocortex. The authors show that inhibition of (layer 2/3) pyramidal neurons by PV neurons is higher at the end of the light period (ZT12) compared to the end of the dark period (ZT0). Turning from the output to the input of PV neurons, the authors demonstrate that over the light period excitatory input to PV interneurons increases while inhibitor input decreases. The opposite changes occur during the dark period, when excitatory input decreases while inhibitory input increases. Together these inverse oscillations in inhibitor and excitatory inputs to PV neurons explain why PV neuron activity is higher at the end of the light period (ZT12) compared to the end of the dark period (ZT0). The authors move on to show that excitatory inputs to PV interneurons, at least in primary visual cortex V1, appear to undergo experience-dependent regulation while inhibitory inputs appear to be under sleep-dependent regulation. Looking in into the molecular regulation of PV neuron activity, the authors suggest a cholinergic regulation of PV neurons via M1Rs and KCNQ channels. The authors conclude with showing that differences in PV activity between ZT0 and ZT12 affect the response of excitatory (pyramidal) neurons in V1 to optogenetic dLGN stimulation, thereby providing some functional relevance of the observed circadian modulation.
In my view the experiments elegantly address the question of whether PV interneurons are modulated by circadian rhythms show clear and consistent results in respect to both output of and inputs to PV neurons. The experiments addressing molecular mechanisms and functional relevance are rather preliminary but provide some promising leads. My main concern is that the paper claims a sleep- and experience-dependent modulation of PV neurons but the data to support this statement is wake. Only one sleep deprivation experiment was performed when looking at excitatory and inhibitory inputs to PV neurons and the condition of 'reduced experience' is a change in the light-dark condition to constant darkness, which is an unusual way to test a lack of 'experience' and would widely be considered an interference with the circadian zeitgeber and hence affect the activity of the suprachiasmatic nucleus. Whether PV neurons are regulated by sleep and expericne as the authors states remains unresolved based on the data provided.
Overall, the paper is well structured and of good quality but the authors do not sufficiently disentangle circadian and sleep-dependent modulation of PV neurons. Their claims should be weekend or additional data provided. I enjoyed reading this paper but some improvements will be necessary for publication at the EMBO Journal.

Major concerns

1) Figure 3B:
For sEPSC charge no data is plotted at ZT8 although this data was acquired according to the schematic and the legend in Figure 3A and as shown in the sIPCSC charge panel 3C. Please provide the data or explain why this data is missing!
- Why was no data at this time point collected/presented?
- If it was collected was it consistent or inconsistent with the otherwise very convincing oscillation of sIPSC charges?

**We thank the reviewer for asking this question. We had only examined sIPSCs but not sEPSCs at ZT8 in order to see whether synaptic transmission remains stable between ZT4 and 12. Now we have collected sEPSCs at ZT8 and found that sEPSC is stable across ZT4 and ZT12. We now make the diurnal temporal profile of sEPSCs symmetrical to that of sIPSCs and update Fig 3B in the revised manuscript (Fig R12B, as in Fig 3B).

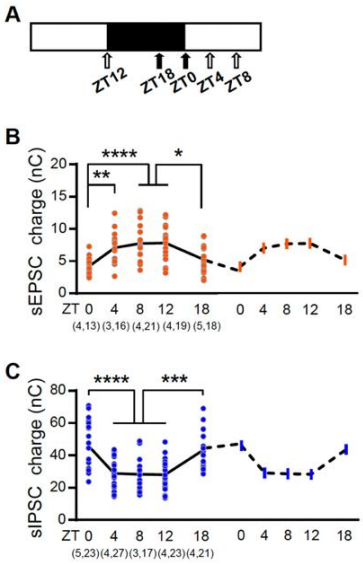

Fig R12. Diurnal changes in sEPSCs and sIPSCs of PV neurons. A. Schematics showing the timepoints of recordings. B-C. sEPSCs (B) and sIPSCs (C) of PV neurons recorded at different times of the day (Adapted from Fig 3A-C).

2) Figure 1:
What is the effect of sleep deprivation on PV output?
Ideally, a sleep deprivation experiment would be performed to support the claim that the activity of PV neurons is modulated not only by circadian time but also by sleep. Alternatively, the authors should discuss this limitation and specifically mention that their experiments cannot disentangle circadian and sleep-dependent modulation of PV neuron activity?
**Thank you for the question, which we have not directly tested in our current study. We agree with reviewer's opinion that our current experiment using sleep deprivation (SD) might not be sufficient to disentangle sleep versus time in the modulation of PV neuron activity. Our major concern is that SD may cause complicated network alteration via dysregulating a variety of neuromodulators and introducing stress signal, which may confound the sleep-dependent regulation of PV neural activity as well as PV's output.

However, we are also curious about how PV neurons may behave after SD. Therefore, we now have examined the spontaneous activity of PV neurons after 4 h of SD, which can also be used to deduce PV's output following SD. We found that PV neurons on average became more active compared to their baseline activities at ZT4 with normal sleep (Fig R13 A-B), suggesting that SD may enhance PV's output. Interestingly, this increase can be largely prevented by SD in complete darkness (Fig R13 C-D), suggesting the visual experience-dependency. The dominant role of experience in determining PV's spontaneous activity is in line with the experience-dependent regulation of PV's excitatory inputs (Fig 3E & Fig S2G), while the sleep-dependent downregulation of PV's inhibitory inputs seems to be not as important. Based on these results, we concluded that SD enhances PV's neural activity and likely PV's output as well in an experience-dependent manner. Sleep during the early light cycle but not circadian timing might modulate V1 PV's activity and output by indirectly controlling visual experience. However, we have no clue on how sleep and time may interact and contribute to the regulation of PV's neural activity during the late light cycle when PV's synaptic transmission (Fig 3B &C) and neural activity (Fig 5G) are stable. To disentangle the exact roles of sleep and circadian, more intensive research and more delicate experimental designs will be needed, which will be way beyond our current scope of study.

In order to keep our focus and also the brevity, we are not reporting Fig R13 in our revised manuscript. But we did discuss the potential involvement of sleep and time in the regulation of PV's spontaneous activity in the discussion section (Line 339) as following:

'*In V1, PV's opposite oscillations in E and I temporally coincide with their spontaneous activity in vivo, which is congruent with the role of E/I ratio in controlling somatic firing(Gidon &*

Segev, 2012). Therefore, PV's spontaneous activity likely shares similar time- and sleep-dependent regulation as their synaptic inputs during the day and the circadian rhythm of the cholinergic signaling may also be involved. Although this hypothesis remains to be tested, it…'

We further discuss the complex interaction between the sleep- and time-dependent regulation of neural activity in Line 355 as following:

'*All these studies suggest that sleep and time may interact dynamically, resulting in complex regulation of neural activity that will demand more in-depth investigation to disentangle the underlying mechanisms.*'

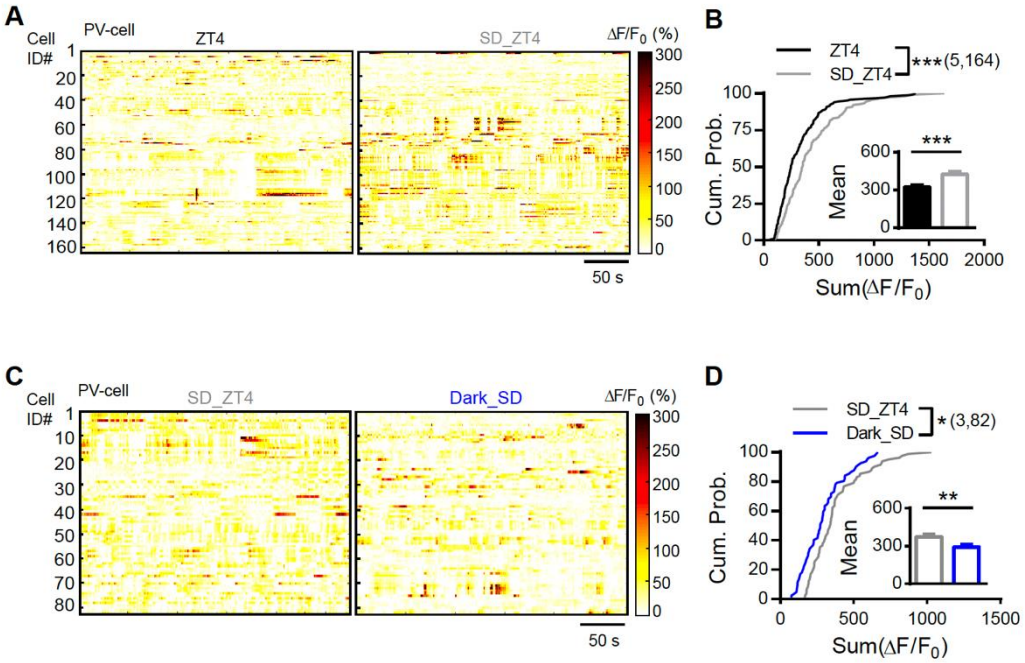

Fig R13. Effects of sleep deprivation on PV's spontaneous neural activity. (A-B).4 hours of sleep deprivation increased spontaneous activity of PV neurons in V1 L2/3 compared to their baseline levels recorded at ZT4 with normal sleep. (C-D). 4 hours of sleep deprivation in complete darkness prevents the SD-induced increased in PV's spontaneous activity.

3) Figures 5 and 6:
What is the effect of sleep deprivation on PV neuron activity and on dLGN-evoked responses in the in vivo experiments?
As for Figure 1 one would hope that the authors could disentangle circadian and sleep-dependent contributions to the very interesting changes in PV neuron activity and resulting differences in dLGN-evoked responses.
**We thank the reviewer for asking these interesting and important questions. As we addressed to question 2), our current experiments cannot disentangle circadian and sleep-dependent regulation. However, we are similarly curious as the reviewer and we now have conducted in vivo 2-photon experiments to examined both PV's spontaneous activity and dLGN-evoked responses after 4 hours of sleep deprivation (SD).

The spontaneous activity of PV neurons was elevated after SD (Fig R13 A-B) but prevented if SD in complete darkness (Fig R13 C-D). Our findings suggest that during the early light cycle, sleep modulates V1 PV's activity mainly by indirectly altering level of visual experience. Please refer to Question 2) for our detailed response.

As for dLGN-evoked responses in V1, most of the responsive neurons show upregulated responses after 4 hours of SD (Fig R14). In combine with the upregulated PV neural activity

after SD (Fig R13 A-B), these results suggest that under disturbed conditions such as sleep deprivation, modulation of dLGN-V1 connection may utilize complex mechanisms in addition to PV's inhibition. SD may enhance dLGN-V1 connections by promoting excitability of cortical neurons. This is supported by finding increased neural activity of putative pyramidal neurons after SD (Fig R15), which is consistent with the elevated excitatory(Liu, Faraguna et al., 2010) but reduced inhibitory(Bridi et al., 2020) inputs after SD reported previously. In addition, SD may directly facilitate dLGN-V1 synapses by recruiting neuromodulatory signal. For example, SD is known to increase brain-wide level of histamine (Soya, Song et al., 2008), which is found to strengthen the dLGN-V1 thalamocortical synapses(Kuo & Dringenberg, 2008). In summary, these data suggest that sleep may play a more potent role in modulating the dLGN-evoked V1 responses than circadian at least during the early light cycle by employing multiplex mechanisms.

Since SD causes complicated network alterations and disturbance, PV's modulation of pyramidal neurons as well as dLGN-evoked V1 responses may be overwhelmed by more potent mechanisms. Therefore, these results do not support or argue against the physiological consequence of PV's daily regulation. Since our current story focuses on PV's daily regulation and its consequence, we are not reporting these results in the revised manuscript for brevity. However, we will further investigate these interesting questions in another project that we study how visual circuit and visual perception is modulated by sleep and sleep disruption. We hope to present these results to readers soon.

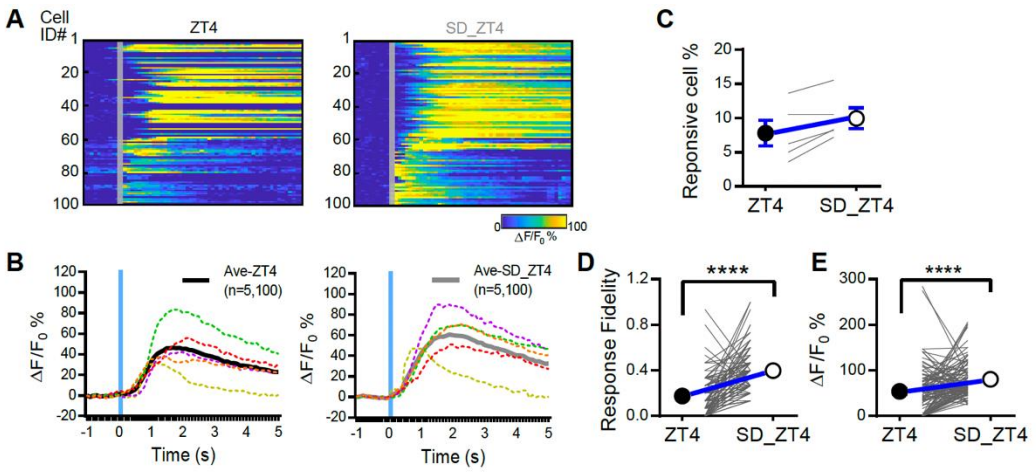

Fig R14. Sleep deprivation from ZT0 to ZT4 augments dLGN-evoked response in V1.

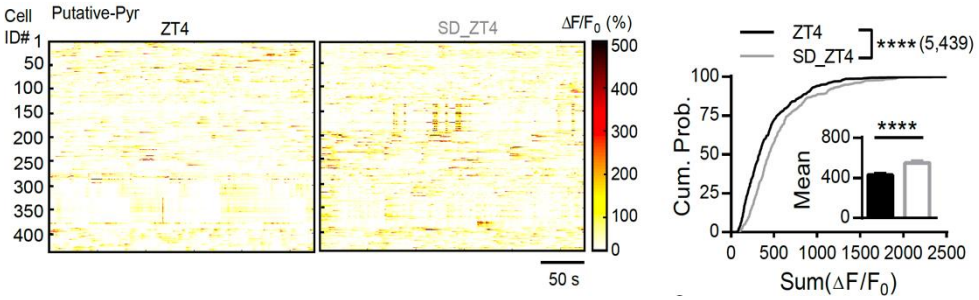

Fig R15. Putative pyramidal neurons show increased spontaneous neural activity after 4 hours of sleep deprivation compared to their basal activity with regular sleep.

4) Statistics:
It appears from the statistics section and the figure legends that the authors might have

treated recordings of different cells in the same slice as independent measurements. Could the authors justify the use of an ANOVA instead of a GLM?

**We apologize for the confusion about statistic in the manuscript. We are using nonparametric Mann-Whitney U test for two unpaired groups analysis and one-way ANOVA followed by Holm-Sidak's multiple comparison test for more than three groups unpaired analysis (Fig 4J-K). For acute drug wash-on and off recordings from the same neurons such as those in Fig 4H-I, we used repeated one-way ANOVA followed by Holm-Sidak's multiple comparison test. Now we have clarified the statistic in both figure legends and method section (Line 584).

'*Wilcoxon signed-rank test was used for two paired groups analysis. Mann-Whitney U test was used for two unpaired groups analysis. For more than three unpaired groups analysis, one-way ANOVA followed by Holm-Sidak's multiple comparison test was used. For acute drug wash-on and off recordings from the same neuron, one-way repeated measures ANOVA followed by Holm-Sidak's multiple comparison test was used.*'

Minor concerns

1) In the abstract, the authors do not mention the species used for their experiments nor the experimental method used for their in vitro experiments. This is crucial information and should be added to the abstract.

**Thank you for the suggestion. Now we have added the following sentence in the abstract:

'*By examining diurnal changes in PV's synaptic and neuronal properties in the supragranular layer of mouse's primary visual cortex, here we found that PV's neuronal function is modulated……*'

2) In the introduction the authors mention that "In mature brain, different sleep/wake stages and sleep history are reported to acutely affect the spontaneous firing of PV(Niethard, Hasegawa et al., 2016) or general inhibitory neurons(Miyawaki & Diba, 2016, Vyazovskiy, Olcese et al., 2009)". It is not clear what the authors mean with general inhibitory neurons. Importantly Vyazovksiy et al. 2009 demonstrated a change in firing rates of inhibitory and excitatory neurons. I therefore strongly suggest to replace the phrasing 'general inhibitory neurons' with 'neocortical excitatory and inhibitory neurons'.
**Thank you for the suggestion. Now we have rephrased this sentence as

'*In mature brain, different sleep/wake stages and sleep history are reported to acutely affect the spontaneous firing of both excitatory and inhibitory neurons(Miyawaki & Diba, 2016, Vyazovskiy, Olcese et al., 2009) including PV neurons(Niethard, Hasegawa et al., 2016),…*'

3) Line 86: "pyramidal neurons": please specify cortical layer in which the pyramidal neurons were recorded. The figure suggests these were L2/3 pyramidal neurons, the text suggests that these were L5 pyramidal neurons.
**Thank you for the suggestion. We were recording light-evoked IPSCs from the L2/3 pyramidal neurons. Now we added 'L2/3' in the sentence to make it clear.

'*……light-evoked inhibitory postsynaptic currents (LEIPSCs) were recorded from L2/3 pyramidal neurons voltage clamped at -50 mV.*'

4) Figure 1 C,D,F:
• The y-axis goes to 125%. This makes little sense given that the maximum cumulative probability is 100%. I suggest ending the y-axis at 100%.
• The y-axis label of the inlays is missing. This should be added.

**Thank you for the suggestion. Now we have modified the figures as suggested and added

y-axis label (Ampl (pA)) to the insets.

5) Section "Spontaneous activity of PV neurons oscillates naturally during the light/dark cycle": Please add the cortical area in which the imaging was performed in the main text. If possible, please further add the specific layer (I assume only L2/3 was reached with two-photon imaging) that was imaged.

**Thank you for the suggestion. Now we have modified the section title into '*Spontaneous activity of V1 L2/3 PV neurons oscillates naturally during the light/dark cycle*'. We further clarified the location of PV neurons in line 217 as '*Same group of L2/3 PV neurons in V1 were identified (Fig S5A) and we verified……*'.

6) Recently several papers suggest a cortical regulation of sleep. Both SOM+ GABAergic neurons (Tossell et al., BioRxiv, 2021) as well as pyramidal neurons (Krone et al., Nature Neuroscience, 2021) seem to be involved though the circuitry is still unclear. I would encourage the authors to mention that experience-dependent modulation of PV-interneurons might indirectly affect homeostatic sleep drive through modulation of (layer 5) pyramidal neurons by PV neurons. For example, this could be added in line 278.

**Thank you for the great suggestion! These two studies indeed provide evidences supporting a direct role of cortical neural activity in sleep regulation. It is possible that PV's daily modulation may participate as well via direct or indirect circuitry. Now we added a brief discussion in Line 387 as following:

'*Other consequences of PV's daily modulation remain to be investigated. Couple recent studies reported that both layer 5 cortical pyramidal neurons(Krone, Yamagata et al., 2021) and somatostatin neurons in PFC(Tossell, Yu et al., 2020) are directly involved in sleep regulation. It will be interesting to examine whether PV's daily changes may also have regulatory role for sleep via unknown circuitries.*'

7) Methodological details on EEG/EMG-based sleep analysis are missing. Please specify how the data was recorded and how vigilance states were assigned, e.g. if automatic or manual scoring was used and what were the criteria.
**Thank you for the suggestion. Now we have added representative EEG/EMG waveforms for wake, NREM, and REM sleep as Fig S2C (Fig R16), and provided detailed methods for EEG/EMG recording and analysis in the 'Supplementary Methods - Polysomnography recording and analysis' section as following:

'5-6 week old male PV:Ai9 mice were anesthetized by 1%-2% isoflurane vapor and head-fixed. The skull was exposed. Two epidural screws were implanted (B: -1--2 mm, L: 2-3 mm) as EEG electrodes. Two resin-insulated stainless-steel wires bared at the tip region were implanted into the dorsal neck muscles and sutured in place to record the electromyogram (EMG). All electrodes were connected to a 4-pin socket connector that was glued to the skull by dental cement. After surgery, the wound was treated with triple antibiotic ointment and mice were allowed to recover in their home cage for at least 7 days before the experiment. Mice were transferred to the customized chamber at least 48h before the recording for habituation.

During recording, EEG and EMG signals were sampled at 1024 Hz with 1000 times preamplifier gain and bandpass filtered at EEG: 0.3-1000 Hz, EMG: 1-5000 Hz. All signals were acquired by Spike Hound.

States of wakefulness, NREM, REM sleep were first automatically analyzed in 10-second epoch by Neuro Score (Data Sciences International) using the preconfigured protocol. EMG was used to discriminate between wake and sleep. EMG threshold was manually defined for each mouse. Epochs with more than 20% of the absolute EMG samples above the threshold were classified as wakefulness. In addition, slow wave ratio (δ power/total power) was used to discriminate between NREM sleep (with ratio ≥ 0.4) and wake/REM sleep (with ratio < 0.4). Theta ratio (θ power/total power) was used to discriminate REM sleep (with ratio ⩾ 3) from other sleep stages (with ratio < 3). All automatic analyses were visually confirmed. Amount of

time spent in each of three arousal states (NREM, REM, and wakefulness) was quantified. NREM and REM sleep amount were added up as total Sleep time. Time spend in wake and sleep were then compared between groups.'

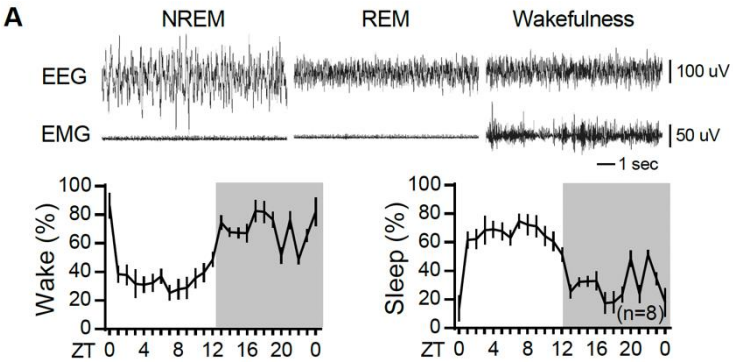

Figure R16. Sleep analysis based on EEG and EMG signals (Added figures to Fig S2C).

Non-essential suggestions

1) The title could be more specific. "Mechanism and consequence..." is very generic. I would suggest reconsidering a more meaningful title.

**Thank you for the suggestion. Now we have changed the title to '*Time- and sleep-dependent daily modulation of neuronal properties of cortical parvalbumin-positive inhibitory neuron*'.

2) In my view the authors are overstating the clinical relevance of their findings in the introduction. This is a basic research study and it remains unclear how diurnal changes in PV activity and inputs "provides a potential explanation" for brain disorders involving PV disfunction. I suggest removing this statement.

**Thank you for the suggestion. Now we have rephrased this sentence as following:

'*Here by showing how synaptic and neuronal properties of PV neurons are diurnally regulated, we provide new direction to investigate how AD-associated PV's dysregulation might be triggered*.'

3) Figure 4A: Why do the authors show a human brain? The research is conducted in mice. I suggest replacing this schematic (which is in principle correct) with a schematic of the species investigated in this study.
**Thank you for the suggestion. Now we have replaced it with a mouse brain in the schematic.

4) Line 125-127: "Specifically, sEPSCs and sIPSCs were increased and decreased respectively 4 hours after entering the light cycle, then they were maintained relatively stable between ZT4 and ZT12." This finding is particularly interesting because it reflects the time course of slow wave activity the most commonly used measure of homeostatic sleep drive. Under baseline conditions in most mouse strains decays over the first four hours of the light period and then remains relatively constant. The authors could consider adding a few words and a reference here, e.g. Huber et al., Brain Research, 2000 or one of the many other papers showing this time course of.

**Thank you for this great suggestion. We did quantify the slow wave activity (SWA) (Fig R17; Fig S2H-I) and observed the gradual decline of SWA during the first 4-6 hour which then stabilize during the rest of light cycle as previously reported. We now added a comment on

the potential correlation between SWA and the temporal pattern of PV's sIPSCs following reviewer's suggestion in Line 304:

'Indeed, we found that sleep is essential for regulating the transition of PV's inhibitory synaptic transmission during the early light cycle. *Interestingly, the gradually decreased sIPSCs during the light cycle temporally coincides with the reduction in slow wave activity (SWA) (Fig S2H-I), which is widely used to measure the sleep pressure(Huber, Deboer et al., 2000), further implying a connection between sleep and PV's synaptic modulation.* Therefore, the daily modulation of PV's synaptic transmission is dependent on both time and sleep.'

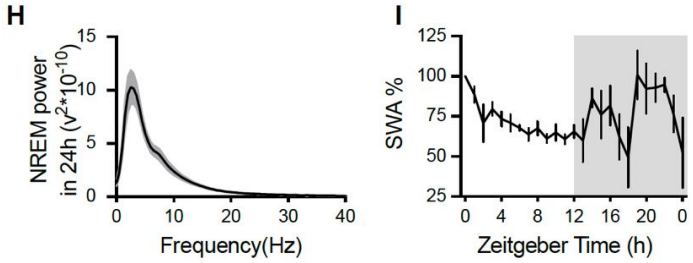

Figure R17. Hourly changes in slow wave activity (SWA) during the 24-hour day (Added figures to Fig S2H-I).

5) I suggest language-editing by a native speaker. The text still contains a few typos and some sentences are difficult to understand, e.g.
- Line 154: Typo boo sting. Please write boosting.
- Line 173: Word missing. I assume the authors mean circadian rhythms and sleep.
- Line 211: Word missing. I assume the authors mean may be altered.

**We apologized for the typos. We now have corrected all typos and had the manuscript proofread by native English speakers.

6) The spelling should be made consistent throughout the text (e.g. 2-photon or two-photon).
**Thank you for the suggestion. We now have uniformed the format of all abbreviations.

7) Please specify the number and age of mice in the methods section
**Thank you for the suggestion. Now we have highlighted the age of mice in the method sections and number of animals in figure legends.

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1st Revision - Editorial Decision **6th Oct 2022**

Dear Kai-Wen,

Congratulations on a great revision! Overall, the referees have been positive. However, they also ask to include several figures in the final version of the manuscript, and we feel this will greatly strengthen your important study.
When you submit your revised version, please also take care of the following editorial items and add this also to your point-by-point response:

1. We have received error messages when emailing the following authors: Yu-Ke Li - lyuke@sioc.mail.ac.cn. Please update the email address and advise as to whether each author is being informed of decision related to this manuscript.

2. CRediT has replaced the traditional author contributions section because it offers a systematic machine readable author contributions format that allows for more effective research assessment. Please remove the Authors Contributions section from the manuscript and use the free text boxes beneath each contributing author's name in our system to add specific details on the author's contribution. More information is available in our guide to authors.

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

4. For references, the number of authors before et al. should be 10. Please correct.

5. For the appendix file, please add a table of contents.

6. Please add the supplemental material and methods to the main manuscript.

7. Please move figure legends underneath the corresponding figures.

8. Please update the nomenclature to "Appendix Figure S1" etc. and Key Resources Table should be "Appendix Table S1".

9. We encourage the publication of source data, particularly for electrophoretic gels and blots and graphs, with the aim of making primary data more accessible and transparent to the reader. It would be great if you could provide me with a PDF file per figure that contains the original, uncropped and unprocessed scans of all or key gels used in the figure or for graphs, an Excel spreadsheet with the original data used to generate the graphs. The PDF files should be labeled with the appropriate figure/panel number and should have molecular weight markers; further annotation could be useful but is not essential. The PDF files will be published online with the article as supplementary "Source Data" files.

10. 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.

11. 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 M Anderson, PhD
Editor
The EMBO Journal
k.anderson@embojournal.org

Referee #1:

The authors have now addressed nearly all of the concerns I had. There are just a few remaining minor changes needed as listed:

1. The particular synapses for some measurements are still unclear; I did not see where the authors added information about what neurons were measured in Figures 3 and 4.

2. I did not see reference to Fig S2F in the manuscript (corticosterone data). Also, in response to item #4, they changed in the Figure legend to Fig S2 to indicate that "4 hours of sleep deprivation mildly increased the plasma level of corticosterone", but this is not the case, since there was not a significant increase in CORT levels, so the authors could instead state "4 hours of sleep deprivation produced variable responses in corticosterone levels in mice, although the differences were not statistically significant."

3. Page 5, lines 140-141: The author's response to Reviewer 2's concern #3 regarding placing mice in the dark should be qualified that they measured sEPSCs (Fig 3E) within one day of the last light cycle and that this manipulation would thus only minimally disrupt entrainment to light. They instead state the following: "we took advantage of the fact that mice entrained to regular light/dark cycle are able to maintain their activity pattern in complete darkness for several days (Faradji-Prevautel, Cespuglio et al., 1990). However, mice begin to show phase advancement even after only a day or two when placed under constant darkness, so this statement and reference do not appear to be accurate.

4. Minor:
Line 393: please change "Couple recent studies" to: "Some recent studies".

Line 301: "Circadian has long been proposed" change to "Circadian rhythms". And line 306: "In addition to circadian"...add circadian "rhythms".

Referee #2:

This study combines in vitro and in vivo experiments to explore how the activity of a class of cortical inhibitory interneurons, referred to as PV interneurons, changes over the 24-hour cycle. The general hypothesis is that changes in certain cellular parameters leads PV interneurons to be less active around the onset of light (ZT0), when entrained mice typically go to sleep. Whereas the same population of PV interneurons are more active around the offset of light (ZT12), when mice typically wake and are active in their environment.

The authors use a range of different techniques, including optogenetic mapping of synaptic outputs, whole cell recordings of spontaneous synaptic currents, immunohistochemistry, pharmacological manipulations, and in vivo calcium imaging. From the data, it does indeed appear as though there are daily changes in multiple aspects of PV interneuron physiology.

The authors have revised aspects of the text and conducted some further experiments to address most of my points. There are a number of further text changes that I believe are important. These relate to being clearer about the actual experiments conducted, accurately representing the published literature, and things that are currently only disclosed to the reviewer but should be made available to the reader.

For each of the points raised:

1. Good response, thank you.

2. Good response, thank you.

3. Good text changes and the new Figure S2G is an important control, as it shows that performing sleep deprivation in the dark prevents the increase in sEPSCs that was previously observed.

4. The authors argue that their study is primarily focused on sleep effects and I think this is reasonable.

5. The authors correct a mistake that was made in the labelling of the two conditions. This now "flips" the data around and makes it consistent with the functional studies. The authors have also added new data with an additional marker of presynaptic inputs, which supports the conclusion.

6. The authors' set up the hypothesis that it is high levels of neuromodulatory (cholinergic) signaling during the wake period that is causing the build-up of higher inhibitory input to PV interneurons. The authors have not directly tested this hypothesis as they have performed an acute "reversal" experiment in vitro, by exposing slices to an antagonist for 1 hour after the effects of the wake period had exerted its effects (Figure 4F & 4H). The in vivo experiment I suggested would allow them to test the hypothesis directly by providing the chance to manipulate the cholinergic system during the waking period and then recording

the effects subsequently. The authors point out that there may be caveats associated with drug effects on different cell types. I acknowledge that these are difficult experiments, but the authors should be clearer in the text about what hypothesis their experiment actually tests.

7. The authors have changed the text to state that "consistent with previous study...spontaneous activities of PV neurons in the superficial cortical layer are minimally affected by locomotion". This misrepresents the literature. I recommend that the authors make text improvements to reflect the fact that spontaneous calcium signals in PV interneurons in L2/3 of visual cortex of mice have been shown to exhibit bimodal changes associated with locomotion: some PVs show a significant decrease in activity during locomotion, some show a significant increase in activity during locomotion, and there is an association with depth within L2/3. The most relevant references are: Dipoppa et al., Neuron, 2018 (already cited) and (Fu et al., Neuron, 2014).

8. I recommend that the authors make text improvements to provide important information to the reader. The authors should state in the text that the data in Figure S6D and S6F are from putative pyramidal neurons and not PV interneurons (as they imply in their response to the reviewer). The authors should also state in the text that they did not observe any superficial PV neurons responding to dLGN stimulation. This seems strange to me, but is important to report as it relates to the potential mechanism, as it would suggest that it is the differences in spontaneous PV activity that mediate the difference in dLGN-evoked responses.

Referee #3:

The authors have collected a set of new data that supports their initial findings suggesting a time-of-day (i.e. circadian) and sleep-dependent modulation of (neo-)cortical parvalbumin-positive (PV) inhibitory neurons.

While the previously provided data already clearly suggested that PV activity oscillates dependent on the time of day, I appreciate that the authors have filled the remaining gap and collected sEPSC data for ZT8, which is assuringly consistent with the proposed pattern.

The new sleep-deprivation experiments (figures R1 and R13-15 in the response to the reviewers) support the authors' claim that the excitatory inputs to PV-neurons are mostly experience-regulated. Unfortunately, the do not compare the change in sIPSCs after 4 hours of sleep deprivation in the light and 4 hours of sleep deprivation in the dark (SD_ZT4, Dark-SD_ZT4) as they do for sEPSC in Figure R1. If the authors were to show that independent of light or darkness, sleep deprivation prevents the rapid drop in sIPSCs that occurs between ZT0 and ZT4 (Figure 3I) I would be fully convinced by the distinction proposed by the authors that excitatory inputs to PV neurons are regulated by experience and inhibitory inputs by sleep. If the authors have conducted this experiment but missed to include the data in their revision, I strongly suggest adding this simple comparison to complete the picture and strengthen the conclusions.

While I'm overall satisfied with the response to the reviewers, I do not agree with the authors suggestion to show figures R13-15 only for the benefit of the reviewers and editor but to exclude this data from the manuscript in order to publish it in a separate manuscript. This essential data is needed to support the core conclusion of the present manuscript that "excitatory synapses (onto PV neurons) are predominantly regulated by experience, inhibitory synapses (onto PV neurons) are regulated by sleep". In summary, if the authors include figure R13-15 in the manuscript, I see my major concerns resolved and would support the publication of this manuscript in the EMBO Journal.

There're two minor concerns, I would like to add:
1) Comment #1 by reviewer #1 remains an important issue and should be resolved before publication of the manuscript. The manuscript is still hard to read because it is often unclear which synapses the authors are examining in a specific experiment. "1. Generally throughout the manuscript, it is unclear what synapses are being examined (onto PV neurons or from PV neurons to pyramidal neurons). This should be more explicitly stated throughout both the Results section and in the Figure legends. This could also be shown within the Figure itself to make it much clearer for the reader."

2. While several spelling and wording mistakes have been corrected, the manuscript should once more be proofread by a native speaker, especially the following two sentences:
Line 301: "Circadian has long been proposed..."
Line 393: "Couple recent studies reported that..."

Dear editor and reviewers,

First of all, we deeply thank the editor and reviewers for reviewing our manuscript and we highly appreciate all your feedbacks! Now we have addressed point by point below and revised our manuscript accordingly.

1. We have received error messages when emailing the following authors: Yu-Ke Li - lyuke@sioc.mail.ac.cn. Please update the email address and advise as to whether each author is being informed of decision related to this manuscript.

**We now have corrected this author's email as: liyuke@sioc.ac.cn.

2. CRediT has replaced the traditional author contributions section because it offers a systematic machine readable author contributions format that allows for more effective research assessment. Please remove the Authors Contributions section from the manuscript and use the free text boxes beneath each contributing author's name in our system to add specific details on the author's contribution. More information is available in our guide to authors.

**We now have removed the 'Author Contribution' section from the manuscript and updated this information in the submission system.

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

**We now have updated this section title to "Disclosure and competing interests statement".

4. For references, the number of authors before et al. should be 10. Please correct.

**We inserted all references using EMBO J format in Endnote.

5. For the appendix file, please add a table of contents.

**We now have added a table of contents for all appendix file.

6. Please add the supplemental material and methods to the main manuscript.

**We now have added the supplemental material and methods to the main manuscript.

7. Please move figure legends underneath the corresponding figures.

**We now have moved figure legends underneath the corresponding figures.

8. Please update the nomenclature to "Appendix Figure S1" etc. and Key Resources Table should be "Appendix Table S1".

**We now have updated the nomenclature.

9. We encourage the publication of source data, particularly for electrophoretic gels and blots and graphs, with the aim of making primary data more accessible and transparent to the reader. It would be great if you could provide me with a PDF file per figure that contains the original, uncropped and unprocessed scans of all or key gels used in the figure or for graphs, an Excel spreadsheet with the original data used to generate the graphs. The PDF files should be labeled with the appropriate

figure/panel number and should have molecular weight markers; further annotation could be useful but is not essential. The PDF files will be published online with the article as supplementary "Source Data" files.

**We will public our resource data (excel spreadsheets with the original data used to generate the graphs).

10. 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.

**Here is the synopsis for our article:

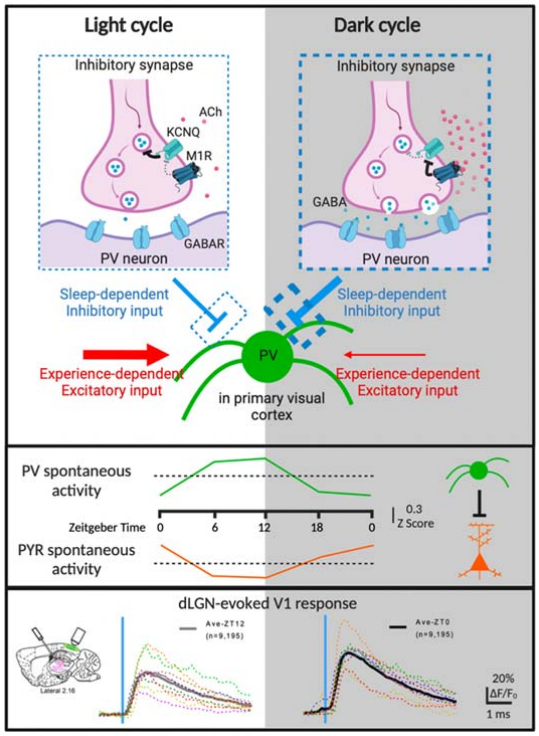
Synopsis

Unveiling the modulatory mechanism of cortical PV+ neurons in mature brain is critical for understanding how cognitive function is regulated in normal and diseased brains. Here PV neurons in V1 are found to be rhythmically regulated in time- and sleep-dependent manners during the natural light/dark cycle by utilizing synapse-specific cholinergic signaling. Such daily modulation is further shown to be physiologically important.

- The excitatory and inhibitory synaptic transmissions of PV neurons bidirectionally oscillate in opposite directions during the 24-hour day.
- Experience and sleep dominate the daily regulation of the excitatory and inhibitory synapses onto PV neurons respectively.
- ACh mediates the daily regulation of PV's inhibitory synaptic transmission via targeting the presynaptic M1Rs.
- In line with PV's synaptic modulation, PV's evoked output *ex vivo* and spontaneous activity *in vivo* are both altered at different times of the day.
- PV's functional oscillation negatively correlates with the spontaneous activity of surrounding pyramidal neurons and dLGN-evoked responses in V1.

11. 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.

**Here is the summary figure:



Referee #1:

The authors have now addressed nearly all of the concerns I had. There are just a few remaining minor changes needed as listed:

1. The particular synapses for some measurements are still unclear; I did not see where the authors added information about what neurons were measured in Figures 3 and 4.

**Thank you for pointing it out. We now have added labels in both Figure 3 and 4 to clarify the synapses

2. I did not see reference to Fig S2F in the manuscript (corticosterone data). Also, in response to item #4, they changed in the Figure legend to Fig S2 to indicate that "4 hours of sleep deprivation mildly increased the plasma level of corticosterone", but this is not the case, since there was not a significant increase in CORT levels, so the authors could instead state "4 hours of sleep deprivation produced variable responses in corticosterone levels in mice, although the differences were not statistically significant."

**Thanks for the suggestion. We now have included a brief description of Fig S2F in line157 as the following:

"SD was achieved by gentle handling that caused variable but not significant

increase in plasma corticosterone level (Fig S2F), whose efficacy was confirmed by electroencephalogram (EEG) and electromyogram (EMG) recordings (Fig S2E).”

3. Page 5, lines 140-141: The author's response to Reviewer 2's concern #3 regarding placing mice in the dark should be qualified that they measured sEPSCs (Fig 3E) within one day of the last light cycle and that this manipulation would thus only minimally disrupt entrainment to light. They instead state the following: "we took advantage of the fact that mice entrained to regular light/dark cycle are able to maintain their activity pattern in complete darkness for several days (Faradji-Prevautel, Cespuglio et al., 1990). However, mice begin to show phase advancement even after only a day or two when placed under constant darkness, so this statement and reference do not appear to be accurate.

**We thank the reviewer for the advice. Mice indeed maintain their inactivity during the first dark-exposed light cycle and then start to show phase advancement after one or two days in constant dark(Colwell, Michel et al., 2003). So we are confident that our manipulation will not alter mouse’s sleep/wake cycle. We therefore modified our statement into

‘To directly test the role of experience, we visually deprived a group of PV:Ai9 mice by dark exposure during the last light cycle prior to experimentation (Dark-ZT12, Fig 3D). Such brief dark exposure barely affects activity pattern of mice (Faradji-Prevautel, Cespuglio et al., 1990) but completely abolished the upregulation of sEPSCs (Fig 3E), suggesting that experience plays’

4. Minor:

Line 393: please change "Couple recent studies" to: "Some recent studies".

**Thank you for the suggestion. We now have replaced “Couple” with “Some”.

Line 301: "Circadian has long been proposed" change to "Circadian rhythms". And line 306: "In addition to circadian"...add circadian "rhythms".

**Thank you for the suggestion. We now have replaced “circadian” with “circadian rhythm”

Referee #2:

This study combines in vitro and in vivo experiments to explore how the activity of a class of cortical inhibitory interneurons, referred to as PV interneurons, changes over the 24-hour cycle. The general hypothesis is that changes in certain cellular parameters leads PV interneurons to be less active around the onset of light (ZT0), when entrained mice typically go to sleep. Whereas the same population of PV interneurons are more active around the offset of light (ZT12), when mice typically wake and are active in their environment.

The authors use a range of different techniques, including optogenetic mapping of synaptic outputs, whole cell recordings of spontaneous synaptic currents, immunohistochemistry, pharmacological manipulations, and in vivo calcium imaging. From the data, it does indeed appear as though there are daily changes in multiple aspects of PV interneuron physiology.

The authors have revised aspects of the text and conducted some further experiments to address most of my points. There are a number of further text changes that I believe are important. These relate to being clearer about the actual experiments conducted, accurately representing the published literature, and things that are currently only disclosed to the reviewer but should be made available to the reader.

**We thank the reviewer for the positive feedbacks.

For each of the points raised:

1. Good response, thank you.

2. Good response, thank you.

3. Good text changes and the new Figure S2G is an important control, as it shows that performing sleep deprivation in the dark prevents the increase in sEPSCs that was previously observed.

4. The authors argue that their study is primarily focused on sleep effects and I think this is reasonable.

5. The authors correct a mistake that was made in the labelling of the two conditions. This now "flips" the data around and makes it consistent with the functional studies. The authors have also added new data with an additional marker of presynaptic inputs, which supports the conclusion.

6. The authors' set up the hypothesis that it is high levels of neuromodulatory (cholinergic) signaling during the wake period that is causing the build-up of higher inhibitory input to PV interneurons. The authors have not directly tested this hypothesis as they have performed an acute "reversal" experiment in vitro, by exposing slices to an antagonist for 1 hour after the effects of the wake period had exerted its effects (Figure 4F & 4H). The in vivo experiment I suggested would allow them to test the hypothesis directly by providing the chance to manipulate the

cholinergic system during the waking period and then recording the effects subsequently. The authors point out that there may be caveats associated with drug effects on different cell types. I acknowledge that these are difficult experiments, but the authors should be clearer in the text about what hypothesis their experiment actually tests.

**We appreciate reviewer's consideration. In figure 4F and 4H, we did an acute wash-on experiment to test the effect of blocking M1R on PV's mIPSCs and we found that the maintenance of high inhibition at ZT0 requires the persistent activation of M1R. We further confirmed that the elevation of PV's synaptic inhibition also requires M1R activation by showing the carbachol-induced increase in mIPSCs at ZT12 is abolished by co-incubating slices with M1R antagonist (Figure 4J). We therefore hypothesize that cortical ACh has to be kept above a certain level to increase and maintain the high inhibitory inputs onto PV neurons. We discussed this hypothesis in the discussion section as following:

"Interestingly, the working dynamic of the same cholinergic signaling seems to be different for up and down regulation of PV's mIPSCs. High mIPSCs at ZT0 can be rapidly reduced by blocking the ACh-M1R signal (Fig 4F&H), while low mIPSCs at ZT12 requires prolonged activation of this pathway (Fig 4B & Fig S3D). This distinction suggests that chronic high level of cortical ACh is critical for maintaining the inhibitory synaptic efficacy onto PV neurons during the dark cycle. However, when cholinergic tone is low during the late light cycle, these inhibitory synapses are more resistant to abrupt increases in ACh induced by interspersed wake and rapid eye movement (REM) sleep episodes (Ma, Zhang et al., 2020, Marrosu, Portas et al., 1995)."

7. The authors have changed the text to state that "consistent with previous study...spontaneous activities of PV neurons in the superficial cortical layer are minimally affected by locomotion". This misrepresents the literature. I recommend that the authors make text improvements to reflect the fact that spontaneous calcium signals in PV interneurons in L2/3 of visual cortex of mice have been shown to exhibit bimodal changes associated with locomotion: some PVs show a significant decrease in activity during locomotion, some show a significant increase in activity during locomotion, and there is an association with depth within L2/3. The most relevant references are: Dipoppa et al., Neuron, 2018 (already cited) and (Fu et al., Neuron, 2014).

**We thank the review for the suggestion. We now modified this section and included paper from Fu et al. as following:

"This is consistent with previous studies showing that the spontaneous activities of cortical PV neurons are either heterogeneously affected(Fu, Tucciarone et al., 2014) or minimally affected especially for PV neurons localized in superficial cortical layers(Dipoppa, Ranson et al., 2018) by locomotion."

8. I recommend that the authors make text improvements to provide important information to the reader. The authors should state in the text that the data in Figure S6D and S6F are from putative pyramidal neurons and not PV interneurons (as they imply in their response to the reviewer). The authors should also state in the text that

they did not observe any superficial PV neurons responding to dLGN stimulation. This seems strange to me, but is important to report as it relates to the potential mechanism, as it would suggest that it is the differences in spontaneous PV activity that mediate the difference in dLGN-evoked responses.

**We thank the review for this advice. Now we have added a statement to clarify the cell type of dLGN-responsive cells in Line 272 as following:

“The dLGN-responsive cells are mostly putative pyramidal neurons. No PV neuron was found to be responsive likely due to the small sample size or limited dLGN inputs were recruited.”

Referee #3:

The authors have collected a set of new data that supports their initial findings suggesting a time-of-day (i.e. circadian) and sleep-dependent modulation of (neo-)cortical parvalbumin-positive (PV) inhibitory neurons.

While the previously provided data already clearly suggested that PV activity oscillates dependent on the time of day, I appreciate that the authors have filled the remaining gap and collected sEPSC data for ZT8, which is assuringly consistent with the proposed pattern.

**We thank the reviewer for the positive feedback.

The new sleep-deprivation experiments (figures R1 and R13-15 in the response to the reviewers) support the authors' claim that the excitatory inputs to PV-neurons are mostly experience-regulated. Unfortunately, the do not compare the change in sIPSCs after 4 hours of sleep deprivation in the light and 4 hours of sleep deprivation in the dark (SD_ZT4, Dark-SD_ZT4) as they do for sEPSC in Figure R1. If the authors were to show that independent of light or darkness, sleep deprivation prevents the rapid drop in sIPSCs that occurs between ZT0 and ZT4 (Figure 3I) I would be fully convinced by the distinction proposed by the authors that excitatory inputs to PV neurons are regulated by experience and inhibitory inputs by sleep. If the authors have conducted this experiment but missed to include the data in their revision, I strongly suggest adding this simple comparison to complete the picture and strengthen the conclusions.

**We thank the reviewer for the feedback. We have not examined sIPSCs after 4 hours of SD in dark. Since we have already shown that dark exposure has no effect on the reduction of sIPSCs in the light cycle (Fig 3F), while 4 hours of SD completely abolished the reduction (Fig 3I). Therefore, we speculate that SD in dark will similarly prevent the downregulation of sIPSCs as SD in light.

While I'm overall satisfied with the response to the reviewers, I do not agree with the authors suggestion to show figures R13-15 only for the benefit of the reviewers and

editor but to exclude this data from the manuscript in order to publish it in a separate manuscript. This essential data is needed to support the core conclusion of the present manuscript that "excitatory synapses (onto PV neurons) are predominantly regulated by experience, inhibitory synapses (onto PV neurons) are regulated by sleep".

**We thank the reviewer for the suggestion and we agree to include Fig R13-15 into the current manuscript. Now we have added Fig R13 as main Figure 6, and Fig R14-15 as supplementary Figure 8.

In summary, if the authors include figure R13-15 in the manuscript, I see my major concerns resolved and would support the publication of this manuscript in the EMBO Journal.

There're two minor concerns, I would like to add:

1) Comment #1 by reviewer #1 remains an important issue and should be resolved before publication of the manuscript. The manuscript is still hard to read because it is often unclear which synapses the authors are examining in a specific experiment. "1. Generally throughout the manuscript, it is unclear what synapses are being examined (onto PV neurons or from PV neurons to pyramidal neurons). This should be more explicitly stated throughout both the Results section and in the Figure legends. This could also be shown within the Figure itself to make it much clearer for the reader."

**We thank the reviewer for this important feedback and we apologize again for not being clear enough. Now we have clarified the synapses and neurons throughout the manuscript including the result sections, figure and figure legends.

2. While several spelling and wording mistakes have been corrected, the manuscript should once more be proofread by a native speaker, especially the following two sentences:

Line 301: "Circadian has long been proposed..."

Line 393: "Couple recent studies reported that..."

**We thank the reviewer for the suggestion. We now have corrected these mistakes in the revised manuscript.

References

Colwell CS, Michel S, Itri J, Rodriguez W, Tam J, Lelievre V, Hu Z, Liu X, Waschek JA (2003) Disrupted circadian rhythms in VIP- and PHI-deficient mice. *Am J Physiol Regul Integr Comp Physiol* 285: R939-49

Dipoppa M, Ranson A, Krumin M, Pachitariu M, Carandini M, Harris KD (2018) Vision and Locomotion Shape the Interactions between Neuron Types in Mouse Visual Cortex. *Neuron* 98: 602-615 e8

Faradji-Prevautel H, Cespuglio R, Jouvet M (1990) Circadian rest-activity rhythms in the anophthalmic, monocular and binocular ZRDCT/An mice. Retinal and serotoninergic (raphe) influences. *Brain Res* 526: 207-16

Fu Y, Tucciarone JM, Espinosa JS, Sheng N, Darcy DP, Nicoll RA, Huang ZJ, Stryker MP (2014) A cortical circuit for gain control by behavioral state. *Cell* 156: 1139-1152

2nd Revision - Editorial Decision **4th Nov 2022**

Dear Kai-Wen,

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 the referee concerns and for providing detailed source data. 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 next week. 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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The EMBO Journal
k.anderson@embojournal.org

EMBO Press Author Checklist

Corresponding Author Name: Kai-Wen He
Journal Submitted to: The EMBO Journal
Manuscript Number: EMBOJ-2022-111304R

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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	Key Resources Table, 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.	Not Applicable	
Report if the cell lines were recently **authenticated** (e.g., by STR profiling) and tested for mycoplasma contamination.	Not Applicable	

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Animal observed in or captured from the field: Provide species, sex, and age where possible.	Not Applicable	
Please detail **housing and husbandry conditions**.	Yes	Methods

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Microbes: provide species and strain, unique accession number if available, and source.	Not Applicable	

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If collected and within the bounds of privacy constraints report on age, sex and gender or ethnicity for all study participants.	Not Applicable	

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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	
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Provide DOI OR other citation details if **external detailed step-by-step protocols** are available.	Yes	Methods

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Include a statement about **sample size** estimate even if no statistical methods were used.	Yes	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	Methods, Result section, Figure 1, Figure S1, Figure 5, Figure S5
Include a statement about **blinding** even if no blinding was done.	Not Applicable	
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	Methods-Whole cell recording; Calcium image analysis
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	Methods,Figure legends

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.	Not Applicable	
In the figure legends: define whether data describe **technical or biological replicates**.	Yes	Figures and figure legends

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	
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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	

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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.

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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	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	