Dr. Phillip Steindel

Review Editor, JoVE

Dear Dr. Steindel,

I am attaching our revised manuscript entitled “Chronic sleep restriction in mouse pups by means of gentle handling”. This is a report of the method for restricting sleep in neonatal mice. This is a powerful tool to investigate the importance of sleep in development.

This work has not been published elsewhere and is not under review by another journal. The edited manuscript has been read and approved by all of the authors.

Following this letter, we have addressed all of the comments of the reviewers.

We hope that you and the reviewers will find our edited manuscript acceptable for publication.

Yours sincerely,

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**Reviewers' comments:**

**Reviewer #1:**  
Manuscript Summary:  
The authors wrote on the gentle handling method, which is used to cause total sleep deprivation during the experimental protocol. The method itself is well described, with several important topics being addressed. Specific details relating to sleep deprivation in rat pups are also provided. The methodological approaches are clear and are all present. I do have some comments:  
  
Minor Concerns:  
The role of the circadian timing in gentle handling could be clarified. Why the 11 AM time was chosen? Alternatively, the authors could explain in the manuscript the differences (or the lack of them) in starting protocols immediately after lights on/zeitgeber time 0 or in the middle of inactive phase.

Gentle handling can be started at any time and the protocol outlined in this manuscript would stay the same. We chose to start at 11AM, in the middle of the inactive phase, but any other time can be used. We have altered this point in the manuscript.  
  
Since the manuscript is about a method, a rationale for the 3 hour per day sleep deprivation (or any other duration) could be provided, it will enhance the manuscript and provides the reader with more information about the method. Sleep deprivation duration is also important to comment as the difficulty in carrying out the protocol increases with longer sleep deprivation duration.

Sleep deprivation can be sustained for any length of time that the experimenter wishes (which has now been added to the manuscript). It has been previously noted that, for mouse pups, achieving sleep deprivation past three hours is very difficult due to the increasing need for sleep. This explanation is included in the manuscript.   
  
Perhaps the second and third paragraph of the introduction might be changed. The literature has several examples of experiments which were carried with gentle handling (Hairston IS et al, 2001, 2004; Araujo P et al, 2014, 2018) to name a few. Thus, a non familiar reader might benefit more from a more detailed introduction of the method itself, for example method variations e.g novel objects along the paintbrush, or specific sleep features which are promoted or inhibited after the protocol. It is important to stress that rodent pups have special needs in regards to experimental manipulation and gentle handling should be preferred over other stressful methods. Nevertheless, as a methodology article, it would be welcome to the introduction to bring more references with the use gentle handling in rodent pups.  
  
References  
Araujo P et al. Sleep and pain: a relationship that begins in early life. Pain Physician. 2014 Nov-Dec;17(6):E787-98  
Araujo P et al. Neonatal Sleep Restriction Increases Nociceptive Sensitivity in Adolescent Mice. Pain Physician. 2018 Mar;21(2):E137-E148  
Hairston IS et al. Sleep deprivation elevates plasma corticosterone levels in neonatal rats. Neurosci Lett 2001;315:29-32  
Hairston IS, et al. Sleep deprivation effects on growth factor expression in neonatal rats: a potential role for BDNF in the mediation of delta power. J Neurophysiol 2004;91:1586-1595

These suggestions have been addressed in the introduction and these references have been added.  
  
**Reviewer #2:**  
Manuscript Summary:  
The manuscript by Lemons et al. describes the use of gentle handling via prodding with paintbrushes to conduct chronic sleep restriction in mouse pups. There are a few issues that authors can address to strengthen this manuscript.  
  
Major Concerns:  
1. Does this method of sleep restriction change corticosterone levels in mouse pups? If so, then this method of sleep restriction could also be interpreted as a stress paradigm and not a sleep restriction protocol.

Sleep restriction is inherently stressful. Unfortunately, it is impossible to separate out the effects of stress and sleep loss.

2. What is the significance of 10 minutes of prodding in control animals? Could it be longer or shorter? Added explanation of this length of time would help researchers understand the importance of handling control mice as well.  
When designing this protocol, we wanted to make sure that the control mice were handled daily, but we did not want to limit their sleep. Therefore, we chose 10 minutes of prodding for the control animals.

Minor Concerns:  
1. The phrasing of sleep restriction is confusing, particularly in lines 152-154. Sleep restriction in humans would refer to limiting sleep to a particular time period, not preventing sleep during that time period. Therefore, a 3-hr sleep restriction protocol in human studies would suggest that the subject is limited to sleeping only 3 hours, not experiencing 3 hours of sleep deprivation as written in this manuscript. Rephrasing throughout the manuscript would greatly enhance translatability for researchers using various subjects/models.  
We have rephrased sleep restriction throughout the manuscript to make this protocol clearer.  
  
**Reviewer #3:**  
Manuscript Summary:  
The authors discuss the application of the sleep restriction technique by gentle handling, commonly used in adult rodents, in newborn mice. The procedure is potentially very useful, given the difficulty of manipulating sleep in newborn mice, but there is no validation of of the effectiveness of the procedure in the pups  
  
Major Concerns:  
My major concerns relate to the fact that it is not possible to control the extent to which gentle handling reduces sleep in pups. In the adult this procedure is normally performed under EEG control, which allows to identify the appearance of sleep; furthermore, at the end of the procedure it is possible to quantify the reduction of NREM and REM sleep with respect to control periods. Without EEG it is impossible to say how much animals sleep. The behavioral criterion is not in itself sufficient to discriminate between waking and sleep. In fact, if it is reasonable to say that the mouse that moves is awake, it cannot be said with certainty that a mouse that does not actively move is sleeping. The behavioral criteria used for discrimination should be described in greater detail and, if possible, validated.

It is not possible to use standard EEG recordings in P5 mice, so specific behavior must be used to identify sleep. Others have used EEG recordings in neonatal rats beginning around P12 and have found that sleep behavior (myoclonic twitching, lack of locomotion) has correlated with sleep. Therefore, these same behaviors should be well correlated with sleep and useful to successfully perform sleep deprivation with gentle handling.

Furthermore, it seems to me difficult for a single operator to watch and simultaneously deprive different pups in the same cage. The number of animals present in each cage in this experiment must be specified.

When performing gentle handling in neonatal mice, the mice are grouped together in one location of the cage. Therefore, it is possible to observe many mice simultaneously and prod them to disrupt sleep.

Finally, the protocol used for the control group and the restricted sleep group is critically different: in fact, the animals of the control group are moved to a room for sleep restriction and subjected to an active interaction with the researcher for 10 minutes a day, while for animal of the other group this period is 3 hours long. It cannot therefore be ruled out that the results obtained depend on this difference rather than on the reduction of sleep. The animals of the two groups should remain in the sleep restriction room for the same time (3 hours) and the animals of the control group should in this period also be subjected to gentle handling, while however they are in waking conditions.

One limitation of sleep restriction is that it is impossible to separate interaction with the researcher from loss of sleep. The control group interacted with the researcher for only 10 minutes a day to limit the amount of sleep loss. Increasing the interaction with the researcher would inherently increase sleep deprivation in the control group.

Minor Concerns:  
in the introduction, the part about sleep deprivation techniques currently used in adults, and the limits of their application to studies on newborns, should be expanded and critically discussed

Additional information was added to expand this section.