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# Chronic Sleep Restriction in Mouse Pups By Means of Gentle Handling

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| **Author Comments:** | Dr. Ronald Myers<br>Science Editor, JoVE<br><br>Dear Dr. Myers,<br>I am attaching our manuscript entitled "Chronic sleep restriction in mouse pups by means of gentle handling" for submission as an original article to JoVE. 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.<br>This work has not been published elsewhere and is not under review by another journal. The manuscript has been read and approved by all of the authors.<br><br>We hope that you and the reviewers will find our manuscript acceptable for publication.<br><br>Yours sincerely,<br>Rachel Michelle Saré, Ph.D.<br>Section on Neuroadaptation & Protein Metabolism<br>National Institute of Mental Health |
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Dear Dr. Myers,

I am attaching our manuscript entitled “Chronic sleep restriction in mouse pups by means of gentle handling” for submission as an original article to *JoVE*. 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 manuscript has been read and approved by all of the authors.

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

Yours sincerely,
Rachel Michelle Saré, Ph.D.
Section on Neuroadaptation & Protein Metabolism
National Institute of Mental Health

**TITLE:**

# Chronic Sleep Deprivation in Mouse Pups by Means of Gentle Handling

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**KEYWORDS:**

Sleep, rodent, gentle handling, sleep deprivation, sleep disorders, development

**SHORT ABSTRACT:**

We describe a sleep deprivation technique known as gentle handling where investigators gently prod mice any time sleep behavior is observed. This method is a powerful tool that allows researchers to study the effects that chronic sleep restriction throughout development can have on future brain physiology and behavior.

**LONG ABSTRACT:**

Sleep is critical for proper development and neural plasticity. Moreover, abnormal sleep patterns are characteristic of many neurodevelopmental disorders. Studying how chronic sleep restriction during development can affect adult behavior may add to our understanding of the emergence of behavioral symptoms of neurodevelopmental disorders. While there are many methods that can be used to restrict sleep in rodents including forced locomotion, constant disruption, presentation of an aversive stimulus, or electric shock, many of these methods are very stressful and cannot be used in neonatal mice. Here, we describe gentle handling, a sleep deprivation technique that can be used chronically throughout development and into adulthood to achieve sleep restriction. Gentle handling involves close observation of the mice throughout the sleep deprivation period and requires the researcher to gently prod the animals whenever they are inactive or display behaviors associated with sleep. Coupled with EEG recordings, gentle handling could be used to selectively disrupt a specific phase of sleep such as rapid eye movement (REM) sleep. The technique of gentle handling is a powerful tool for the study of the effects of chronic sleep restriction even in neonatal mice that circumvents many of the more stressful procedures used for sleep deprivation.

**INTRODUCTION:**

Sleep plays a critical role in neuronal plasticity and synapse formation and elimination during brain development[1,2]. Specifically, rapid eye movement (REM) sleep is essential for forming stable synaptic circuits through both strengthening of specific synapses and synaptic pruning[2]. Pharmacological sleep deprivation early in life leads to many physiological and behavioral deficits[3,4]. In addition to pharmacological sleep deprivation, other forms of sleep deprivation such as constant shaking, forced locomotion, or presentation of an aversive stimulus have been associated with depression-like symptoms during adulthood, different neural activation patterns, and changes in sleep time and sleep continuity during a recovery period following deprivation[5-7]. When sleep is chronically restricted in mice for over five weeks during development, behavioral deficits were observed following a month of sleep recovery[8]. Taken together, these studies suggest that disruption of sleep in the neonatal rodent can affect later sleep patterns, brain function, and behavior. Highlighting the importance of sleep for normal brain development in humans, many patients with neurodevelopmental disorders, including Autism Spectrum Disorders, Tuberous Sclerosis Complex, Fragile X Syndrome, and Attention-Deficit/Hyperactivity Disorder, report abnormalities in sleep patterns in addition to a variety of behavioral deficits[1].

Given the number of neurodevelopmental disorders that are associated with abnormal sleep patterns, it is important to understand how lack of sleep affects brain function and behavior. However, despite the data that support the importance of sleep during development, most methodologies that restrict or deprive animals of sleep focus on adults. These techniques include a variety of tests that require forced locomotor activity (*e.g.,* constant treadmill), constant disruption (*e.g.,* rotating sweeper bar or continual novel object presentation), or disturbing the animal at the onset of REM sleep (*e.g.,* platform over water)[9,10]. Although these methods have effectively shown the problems associated with sleep deprivation in adults, none of these methods are appropriate for rodent pups because of their limited mobility, high sleep drive, and the adverse effects of stress early in life. Due to the importance of sleep during development, it is critical to understand how restricted sleep in neonatal pups affects future behavior during adulthood.

One technique to restrict sleep that can be used in rodent pups is gentle handling. Gentle handling involves the investigator directly interacting with the rodents any time sleep behavior is observed. Investigators can use their hands, a paintbrush, or presentation of novel objects to physically disrupt sleep in neonatal rodents[8,10-14]. Sleep can be behaviorally determined by lack of motor activity, myoclonic twitching, and eye closure (in older rodent pups). EEG validation confirms that disrupting sleep with gentle handling based on these behaviors reduces total sleep time by 91% in P12 rats[14]. Other techniques that have been used to deprive young mice include electric shock and presentation of unpleasant stimuli, but these techniques cannot be used for chronic sleep restriction and are more stressful than gentle handling[7,15]. Chronic sleep restriction can also be accomplished through a shaking protocol that can be used with or without EEG electrode implantation and associated computer software, but this protocol does not prevent all sleep and therefore is less controlled compared to gentle handling[4,16]. The ability to restrict or deprive mice of sleep for many days in a row without specific equipment, computer software, or electrode implantation is a benefit of the gentle handling technique. Chronic sleep restriction via gentle handling effectively limits sleep in neonatal rodents and produces a variety of behavioral

changes following the sleep deprivation period[8,11,12,17].

Here, we describe results in which chronic sleep deprivation for three hours per day from postnatal day 5 (P5) to P42 significantly affects sociability of mice following a 30-day recovery period in which sleep is not disturbed. These results highlight the long-term effects of chronic sleep restriction during development on future behavior.

**PROTOCOL:**

Note: All the procedures were approved by the National Institute of Mental Health Animal Care and Use Committee and performed in accordance with the National Institutes of Health Guidelines on the Care and Use of Animals.

**1. Sleep Deprivation Setup**

1.1 Use soft paintbrushes to gently poke mice when asleep.

1.1.1 Make sure the paintbrush can fit through the bars of the mouse cage feeder to ensure that mice have access to food and water *ad libitum* during sleep deprivation.

1.1.2 Do not use paintbrushes for multiple cages to prevent the transfer of scents between cages. We suggest that you label each brush with its assigned cage and restrict its use to that cage.

1.2 Designate a room where all sleep deprivation will occur.

**2. Animal Setup**

2.1 Randomly assign each cage to either sleep deprivation or control group.

2.2 Give mice access to food and water *ad libitum* during the experiment.

2.3 Make sure view of mice is not restricted by bedding, nest material, or enrichment objects in cage.

2.3.1 Remove extra bedding or enrichment objects that could restrict the view of the mice.

2.4 If food or water restricts view of mice, move mice to an area of cage where the view is not restricted.

2.4.1 If the food blocks a large portion of the cage, remove some food and place pellets at the bottom of the cage so that animals maintain access to food during the sleep deprivation period.

**3. Animal Conditions**

Note: We chose to begin the three hour sleep deprivation period at 11 AM, when mice are in the middle of their inactive phase, but the time of day and duration of sleep deprivation can be chosen based on the desired experimental conditions.

3.1 Control Group.

3.1.1 On P5, remove the sire from the breeder cage and leave the dam with the pups until weaning (P21).

3.1.2 Move mice assigned to the control group to the sleep deprivation room and gently prod the mice with a paint brush continuously for 10 minutes a day for the duration of the experiment (P5 to P42).

3.1.3 After these 10 minutes, return the mice to the animal holding room and do not disturb them

3.2 Sleep Deprivation Group.

3.2.1 On P5, remove the sire from the breeder cage and leave the dam with the pups until weaning (P21).

Note: Sleep deprivation prior to P5 may result in cannibalization of the pups. We did not observe significant cannibalization at P5 and later.

Note: In young pups, the drive to sleep is very high requiring almost constant stroking. As the mice develop, especially after weaning, the sleep pressure is lower and they do not need constant prodding.

3.2.2 Continue with sleep deprivation every day until P42.

Note: Sleep deprivation protocols can vary in length and extend past P42 if desired.

3.2.3 Move mice assigned to the sleep deprivation group to the sleep deprivation room daily during the light cycle where they will be monitored. The exact duration of sleep deprivation can be adjusted based on the specific research question.

Note: In neonatal mice sleep deprivation that lasts longer than 3 hours is difficult because of the increase in sleep pressure[15].

3.2.3.1 During this time, gently prod the mice with the paintbrush any time that sleep is evident. Specifics of sleep deprivation are explained in the next section.

4. **Sleep Deprivation**

4.1 Gently prod mouse with paintbrush if suspected of being asleep until a response is observed. Alternatively, invert mice and push over onto their backs to disrupt sleep.

4.1.1 Consider a mouse to be asleep if any of the following occur: inactivity, twitching (especially with pups), or if their eyes are closed (in mice older than P12).

4.1.2 Consider mice to be awake if they are moving around, trying to flip over after being on their backs, or grooming themselves.

4.2 If dam is covering pups during sleep deprivation, gently prod her away from pups so the view of the pups is unrestricted.

**5. After Sleep Deprivation**

5.1 When the three-hour period of sleep deprivation is complete, put the bedding and any food that may have been removed back and put the lid back on the cage.

5.2 Return cages to animal holding room until the next day of sleep deprivation.

**6. Subsequent Experiments**

6.1 Depending on the goal of the sleep deprivation study, proceed with subsequent experiments.

6.1.1 Harvest serum from animals to determine corticosterone levels [18].

6.1.2 Do any of a number of behavioral tests, including Open field, RotaRod, Elevated Plus Maze, Social behavior testing, Marble Burying, *etc.*[8,19,20] These behavior tests will also prevent the animals from sleeping so that should be factored in when determining how many days of continuous sleep deprivation to employ.

**REPRESENTATIVE RESULTS:**

To investigate the effects of chronic sleep restriction on behavior, we sleep restricted mice for three hours daily between 11:00 AM and 2:00 PM from P5 to P42[8]. Following the sleep

restriction, mice were left undisturbed for four weeks and allowed to recover from sleep restriction. Mice were tested for behavior following the four-week recovery period. The three-chamber social behavior test was used to assess sociability (preference for a novel mouse compared to a novel object) and social novelty (preference for a novel mouse compared to a familiar mouse). In the sociability test, sleep restricted females spent more time sniffing the stranger mouse than control females ($p < 0.05$) (**Figure 1A**) and sleep-restricted males had a tendency to spend more time sniffing the object when compared to control males ($p = 0.077$) (**Figure 1A**). In the social novelty phase of testing, sleep-restricted female mice spent an increased the amount of time sniffing the novel mouse than female controls ($p < 0.01$) (**Figure 1B**).

**FIGURE AND TABLE LEGENDS:**

**Figure 1: Chronic sleep restriction alters social behavior following a 4-week sleep recovery period. (A)** Sociability test: Time spent sniffing reveals that male mice that were sleep restricted spent more time sniffing the novel object compared to the controls males ($p = 0.077$). Sleep-restricted female mice spent more time sniffing the stranger mouse compared to control females (*$p < 0.05$). Sleep-restricted female mice also tended to spend more time sniffing the object compared to control females ($p = 0.065$). **(B)** Social Novelty test: Time spent sniffing the novel or familiar mouse revealed that sleep restricted female mice spent statistically significant more time sniffing the novel mouse compared to female mice that were not sleep restricted (**$p < 0.01$). Bars represent the means ± SEM in 11 control male, 13 sleep-restricted male, 22 control female, and 17 sleep-restricted female mice. Data were analyzed by means of *post hoc t*-tests following an ANOVA. This figure has been modified with permission from Sare *et al.* 2016[8].

**DISCUSSION:**

Here we describe a method for sleep deprivation, gentle handling, that can be used in both rodent pups and in adults. Gentle handing requires researchers to observe mice for the duration of a sleep deprivation period and gently prod the animals whenever they are inactive or twitching to prevent sleep. Prior sleep deprivation studies have verified by means of simultaneous electroencephalogram (EEG) recordings that gentle handling prevents both REM and non-REM (NREM) sleep [10]. While gentle handling is one of the only sleep deprivation methods that can be used on young rodent pups, there are some limitations to its usage.

First, gentle handling relies heavily on the experimenter to be able to both recognize and promptly respond to pre-sleep behaviors. When depriving sleep in rodent pups, the pups must be almost constantly prodded or inverted to assure that they are awake for the entire sleep restriction period. Recognition of twitching in young pups is critical to properly prevent sleep [21]. Second, it should be acknowledged that sleep deprivation that occurs before weaning, when the pups are still with their mother, could affect the mother's sleep as a secondary effect; however, the extent of this effect has yet to be explored. Third, it is important to recognize that any sleep deprivation process will be inherently stressful, and it is not possible to separate the effects of sleep deprivation from the stress that is caused by the procedure. However, using the gentle handling protocol described here attempts to minimize the stress of sleep deprivation compared to other sleep deprivation protocols. And finally, whereas gentle handling is a useful technique for chronic neonatal sleep deprivation, it is a burdensome procedure for researchers as it is time

consuming and requires constant attention. Longer sleep deprivation protocols are also difficult to perform in neonatal mice due to the increases in sleep pressure over time[15].

Despite these limitations, gentle handing is a powerful tool to study the effects of chronic sleep restriction throughout development and into adulthood and how sleep restriction can affect both behavior and brain physiology. Most importantly, this method allows researchers to continue to explore the effects that sleep restriction at an early age can have on the development of the brain both immediately following sleep restriction and later in life.

**ACKNOWLEDGMENTS:**

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**DISCLOSURES:**

The authors have nothing to disclose.

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Figure

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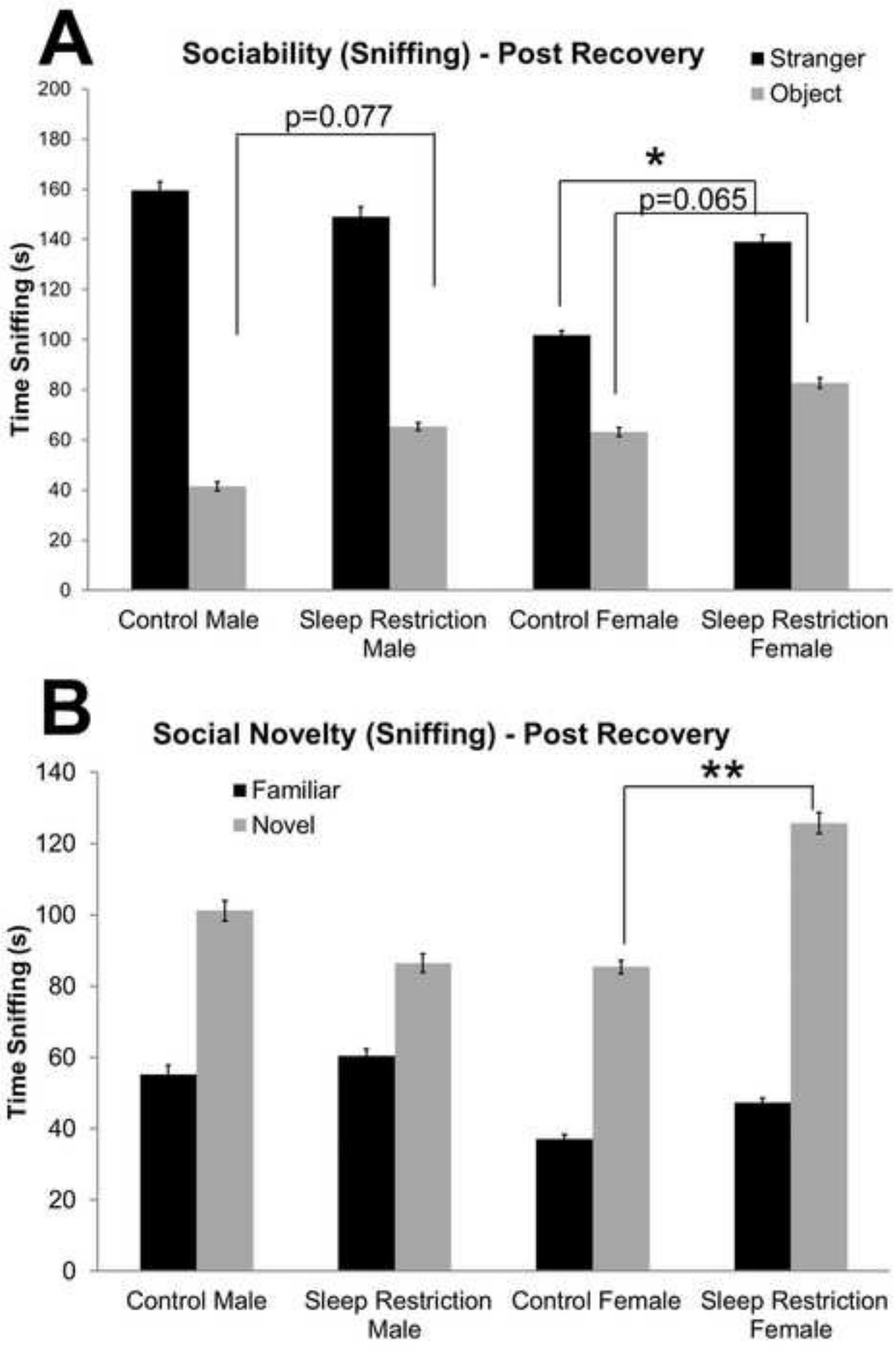

Materials Table

Click here to download Materials Table Sleep Restriction JoVE Materials.xlsx

| Name of Material/ Equipment | Company | Catalog Number |
|---|---|---|
| C57BL/6J mice | Jackson Labs | 000664 |
| Super Mouse 750 Mouse Cage | Lab Products, Inc. | |
| SANI-Chips Bedding | PJ Murphys | |
| S&S Bristle Brush Assortment Pack | Staples | Item # 13764, Model AB37100 |

**Comments/Description**

Wildtype mice used

Cages for the mice

Bedding for the mice

Paint brushes are used to gently poke mice

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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,
Rachel Michelle Saré, Ph.D.
Section on Neuroadaptation & Protein Metabolism
National Institute of Mental Health

**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.

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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
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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.

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