**TITLE:**

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

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**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 development1,2. Specifically, rapid eye movement (REM) sleep is essential for forming stable synaptic circuits through both strengthening of specific synapses and synaptic pruning2. Pharmacological sleep deprivation early in life leads to many physiological and behavioral deficits3,4. 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 deprivation5-7. When sleep is chronically restricted in mice for over five weeks during development, behavioral deficits were observed following a month of sleep recovery8. 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 deficits1.

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 rodents8,10-14. 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 rats14. 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 handling7,15. 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 handling4,16. 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 period8,11,12,17.

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. Use soft paintbrushes to gently poke mice when asleep.
      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.
      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.
   2. Designate a room where all sleep deprivation will occur.
2. **Animal Setup**
   1. Randomly assign each cage to either sleep deprivation or control group.
   2. Give mice access to food and water *ad libitum* during the experiment.
   3. Make sure view of mice is not restricted by bedding, nest material, or enrichment objects in cage.
      1. Remove extra bedding or enrichment objects that could restrict the view of the mice.
   4. If food or water restricts view of mice, move mice to an area of cage where the view is not restricted.
      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.

* 1. Control Group.
     1. On P5, remove the sire from the breeder cage and leave the dam with the pups until weaning (P21).
     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. After these 10 minutes, return the mice to the animal holding room and do not disturb them
  2. Sleep Deprivation Group.
     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.

* + 1. Continue with sleep deprivation every day until P42.

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

* + 1. 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 pressure15.

* + - 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.

1. **Sleep** **Deprivation**
   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.
      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).
      2. Consider mice to be awake if they are moving around, trying to flip over after being on their backs, or grooming themselves.
   2. If dam is covering pups during sleep deprivation, gently prod her away from pups so the view of the pups is unrestricted.
2. **After Sleep Deprivation**
   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.
   2. Return cages to animal holding room until the next day of sleep deprivation.
3. **Subsequent Experiments**
   1. Depending on the goal of the sleep deprivation study, proceed with subsequent experiments.
      1. Harvest serum from animals to determine corticosterone levels 18.
      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 P428. 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.* 20168.

**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 time15.

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.

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**DISCLOSURES:**

The authors have nothing to disclose.

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