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TITLE:

The Sleep Nullifying Apparatus: a highly efficient method of sleep depriving Drosophila

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Sleep; Drosophila; Homeostasis; Rebound; Sleep deprivation; Sleep restriction

SUMMARY:

Sleep deprivation is a powerful tool to investigate sleep function and regulation. We describe a protocol to sleep deprive *Drosophila* using the Sleep Nullifying Apparatus, and to determine the extent of rebound sleep induced by deprivation.

ABSTRACT:

Sleep homeostasis, the increase in sleep observed following sleep loss, is one of the defining criteria used to identify sleep throughout the animal kingdom. As a consequence, sleep deprivation and sleep restriction are powerful tools that are commonly used to provide insight into sleep function. Nonetheless, sleep deprivation experiments are inherently problematic in that the deprivation stimulus itself may be the cause of observed changes in physiology and behavior. Accordingly, successful sleep deprivation techniques should keep animals awake and, ideally, result in a robust sleep rebound without also inducing a large number of unintended consequences. Here, we describe a sleep deprivation technique for *Drosophila melanogaster*. The Sleep Nullifying Apparatus (SNAP) administers a stimulus every 10 s to induce negative geotaxis. Although the stimulus is predictable, the SNAP effectively prevents >95% of nighttime sleep even in flies with high sleep drive. Importantly, the subsequent homeostatic response is very similar to that achieved using hand-deprivation. The timing and spacing of the stimuli can be modified to minimize sleep loss and thus examine non-specific effects of the stimulus on physiology and behavior. The SNAP can also be used for sleep restriction and to assess arousal thresholds. The SNAP is a powerful sleep disruption technique that can be used to better understand sleep function.

INTRODUCTION

Sleep is near universal in animals, yet its function remains unclear. Sleep homeostasis, the compensatory increase in sleep following sleep deprivation, is a defining property of sleep, that has been used to characterize sleep states in a number of animals[1-5].

Sleep in the fly has many similarities with human sleep, including a robust homeostatic response to sleep loss[4,5]. Numerous studies of sleep in the fly have used sleep deprivation both to infer sleep function by examining the adverse consequences that accrue from extended waking, and to understand sleep regulation by determining the neurobiological mechanisms controlling the homeostatic regulation of sleep. Thus sleep deprived flies were shown to exhibit impairments in learning and memory[6-12], structural plasticity[13-15], visual attention[16], recovery from neuronal injury[17,18], mating and aggressive behaviors[19,20], cell proliferation[21], and responses to oxidative stress[22,23] to name a few. Further, investigations into the neurobiological mechanisms controlling rebound sleep have yielded critical insights into the neuronal machinery that constitutes the sleep homeostat[8,9,23-29]. Finally, in addition to revealing fundamental insights into sleep function in healthy animals, sleep deprivation studies have also informed insights into sleep function in diseased states[30,31].

While sleep deprivation is undeniably a powerful tool, with any sleep deprivation experiment, it is important to distinguish phenotypes that result from extended waking, from those induced by the stimulus used to keep the animal awake. Sleep deprivation by hand deprivation or gentle handling, is generally regarded as setting the standard for minimally disruptive sleep deprivation. Here we describe a protocol for sleep depriving flies using the Sleep Nullifying Apparatus (SNAP). The SNAP is a device that delivers a mechanical stimulus to flies every 10s, keeping flies awake by inducing negative geotaxis (**Figure 1**). The SNAP efficiently deprives flies of >98% of night-time sleep, even in flies with high sleep drive[8,32]. The SNAP has been calibrated on bang sensitive flies, agitation of flies in the SNAP does not harm flies; sleep deprivation with the SNAP induces a rebound comparable with that obtained by hand deprivation[7]. The SNAP is thus a robust method to sleep deprive flies while controlling for the effects of the arousing stimulus.

PROTOCOL

1. Experimental preparation

1.1. Collect flies as they eclose into vials, separating male and female flies.

NOTE: Sleep experiments are commonly conducted with female flies. It is important to collect virgin females. Mated females will lay eggs that hatch into larvae complicating the analysis of the data.

1.2. House flies of a single sex in groups of <20.

NOTE: Housing flies in a socially enriched environment (groups of >50) modulates sleep drive[6,13] potentially confounding measurements of rebound sleep. Further, following social enrichment, sleep will decline over a few days[6]. Thus, baseline sleep is not stable complicating analysis of rebound sleep. Keeping flies in groups of <20 avoids this potential confound.

1.3. Keep flies in vials for 3-5 days in a light and humidity controlled environment.

NOTE: Age and maturity of flies strongly influence sleep. Sleep is high in one day old flies and stabilizes by 3-5 days of age[4]. Flies are typically maintained on a 12 h light: 12 h dark schedule at 50% humidity.

2. Preparation of tubes for sleep recording

NOTE: Sleep is monitored using locomotor activity monitors. A monitor can hold 32 flies housed individually in 5 mm diameter tubes. Typically, genotypes are analyzed in groups of 16 or 32 flies.

2.1. Prepare an appropriate number of tubes with fly food at one end.

NOTE: Diet and metabolism are known to influence sleep[33,34], hence it is particularly important to place flies on the same food on which they were reared.

2.2. Seal the end of the tubes with wax.

NOTE: Sleep deprivation and rebound is a five day experiment, and food can dry out if not properly sealed. In properly sealed tubes, food can be maintained for 10 days or more. Thus, it is critical to ensure that the ends of the tubes are sealed well. Flies can also get stuck to wet food, however. Thus, it helps to make tubes 1-2 days before the start of the experiment.

2.3. Individually place wake, behaving flies into 65 mm long glass tubes for sleep recording using an aspirator and plug the end of the tubes with a foam stopper.

NOTE: Flies are never re-exposed to CO_2 anesthesia when placing flies into tubes for sleep recording. The aspirator is made from rubber tubing with one end covered with cheesecloth and inserted into a 1 mL pipette tip.

3. Recording sleep

3.1. Load flies in tubes into activity monitors to monitor sleep.

NOTE: The SNAP rocks monitors back and forth from -60° to +60° every ~10 s. The monitors are held at -60° for ~5.9s ; it takes ~2.9 s for the tray holding the monitors to move from -60° to +60° and ~1 s to move back from +60° to -60°. The cycle length can be altered as needed by adjusting the voltage supplied to the motor.

3.1.1. Take care to ensure that tubes are placed in activity monitors in the correct orientation. In the correct orientation, the end of the tube with food is at the top of the SNAP to ensure that flies do not get pushed into the food. In addition, the end with food is on the side of the monitor with the sleep recording jack. This allows activity monitors to be oriented correctly in the SNAP for efficient sleep deprivation while simultaneously monitoring activity.

3.2. Place activity monitors in the recording chamber to monitor sleep.

3.3. Monitor sleep for at least two full days to estimate baseline sleep.

NOTE: The day flies are loaded into activity monitors is typically excluded as an adaptation day to allow flies to adapt to being housed in tubes. Baseline sleep is recorded for at least two full days (48hrs) beginning with the morning following the day flies are loaded.

3.4. Save locomotor activity counts of flies in 1 min bins from the time of lights on a given day to lights on the previous day using activity recording software (e.g., from 8 AM to 8 AM).

3.5. Estimate sleep from the locomotor activity data with custom macros using 5 min of inactivity as the threshold for a bout of sleep[35].

NOTE: A number of sleep metrics are computed from the locomotor activity counts. These include sleep in min/h over 24 h, total sleep time in 24 h, average and maximum daytime and nighttime sleep bout lengths[36].

4. Sleep deprivation and recovery

4.1. As flies can be sleep deprived for variable lengths of time (e.g., 12 h, 24 h and 36 h) and recovery sleep can also be evaluated at various intervals (e.g., 6 h, 12 h, 24 h and 48 h), determine the duration of recovery by experimental need. Sleep recovery can be visualized using a sleep gain/loss plot or by examining percent sleep recovered over a predetermined interval (e.g., 6 h).

4.2. If sleep is stable over the two baseline days, on the third day, place activity monitors into the SNAP for overnight sleep deprivation.

NOTE: Flies will exhibit a robust sleep rebound over a range of sleep times[8,32,37,38], but sleep has to be stable to reliably evaluate rebound sleep. Sleep is stable when the difference in sleep between baseline days is ± 100 min.

4.3. Make sure activity monitors are secured in place with monitor holder pins, monitor cords plugged in, and monitors oriented correctly with the end with food at the back, and plastic barriers in front (**Figure 1**).

NOTE: The SNAP is designed so the cam rotates once every 10 s (**Figure 1**). The plastic insert resets the tubes by pushing the tubes back when the apparatus is in the “up” position. Resetting the tubes is important to ensure that all tubes have the full range of motion at the beginning of each cycle.

4.4. Unplug activity monitors, and take monitors out of the SNAP immediately upon lights on following overnight sleep deprivation.

NOTE: It is critical that sleep deprivation is terminated, and flies are placed in recovery immediately upon lights on following 12 h of overnight sleep deprivation. Even a 20-30 min delay in placing flies into recovery can interfere with the extent of rebound sleep.

4.5. Place flies in a recording chamber where they will be undisturbed for two days (48 h) to monitor recovery sleep.

NOTE: If the recording chamber is being used for other experiments, extra care must be taken to avoid stimulating recovering flies.

4.6. Calculate the amount of sleep lost. For each individual fly, calculate the hourly difference between sleep obtained during sleep deprivation and the corresponding hour during baseline; sum the hourly differences to calculate total sleep lost.

4.7. Calculate the amount of sleep recovered. For each individual fly, calculate the hourly difference between sleep obtained during recovery and the corresponding hour during baseline; sum the hourly differences to calculate total sleep gained.

NOTE: Whether a fly is actually sleep deprived is empirical. Thus, the experimenter should examine percent sleep lost. If the fly has not lost a sufficient amount of sleep it can be excluded from the analysis. Although this might be required for other sleep deprivation approaches, it is rarely if ever required for the SNAP. More commonly, sleep may not be stable in a given fly prior to the initiation of sleep deprivation. If sleep is not stable, homeostasis cannot be calculated. We accept a maximum difference of ± 100 min of sleep calculated prior to the initiation of sleep deprivation as candidates for inclusion. On occasion, an individual fly's sleep is distributed unevenly across the 24 h day (e.g., some individuals may obtain 60-70% of their sleep quota during the day and thus only lose a small proportion of their 24 h sleep quota when deprived for 12 h at night). These flies can be evaluated separately.

4.8. Calculate the average percentage of sleep recovered (relative to baseline) over 12 h, 24 h and 48 h of the recovery period for each genotype.

4.9. From sleep data, compute the average and maximum daytime sleep bout length on baseline, and recovery days for each genotype.

NOTE: Rebound sleep in flies is characterized by increased sleep amount, and increased sleep depth in the recovery days. Sleep consolidation is used as measure of sleep depth. Arousal thresholds could also be used as a measure of sleep depth.

Representative Results

Canton S (Cs) was used as a wild-type strain. Flies were maintained on a 12 h light: 12 h dark schedule, and sleep deprived for 12 hours overnight. Inspection of the sleep profiles of Cs flies on the baseline day (bs), sleep deprivation day (sd), and two recovery days (rec1 and rec2) (**Figure 2A**) suggests that flies were effectively sleep deprived in the SNAP, and recovered sleep during the day consistent with observed reports in the literature[4,5]. The effectiveness of the SNAP in keeping flies awake is also seen in the high activity (300-350 counts/h) exhibited by flies during sleep deprivation (**Figure 2B**). Indeed, monitoring the activity counts of flies during sleep deprivation can be a useful barometer of the effectiveness of the deprivation protocol and/or an indirect measure of sleep drive. When the sleep deprivation is ineffective, flies are not as active during the period of deprivation. Flies that are under high sleep drive quickly fall asleep after each stimulus and do not traverse the tube as much[35].

Both the angle of tilt of the apparatus, and the speed of the drop are critical to ensuring that flies are effectively kept awake without harming them. Each lab can optimize the angle and velocity by adjusting the spring (**Figure 1B**) and/or the size and shape of the cam (**Figure 1C** and **Figure 1D**, right).

To quantitatively estimate the effectiveness of sleep deprivation and of recovery, sleep lost during deprivation and then regained in the recovery days was calculated for each individual fly (**Figure 2C**). Importantly, there was no significant change in baseline sleep between the deprivation day and the baseline day (see 0-12 h in **Figure 2C**) indicating that sleep is stable in these flies. A large difference in sleep in this 12 hour period (e.g., ± 100 min) would suggest that sleep was not stable. The SNAP effectively deprived flies of >98% of their night-time sleep. Flies recovered ~20% of their sleep in the first 12 h and did not recover additional sleep during the night, as previously reported. However, flies began to recover sleep the following day such that they recovered ~36% of their sleep over 48 h of recovery (**Figure 2D**). 30 – 40% recovered sleep over 48 h is fairly typical for wild-type flies sleep deprived using the SNAP.

Sleep homeostasis is characterized both by increased sleep duration and by increased sleep depth during the recovery period following deprivation. Daytime sleep consolidation is commonly used as a readout of sleep depth. Sleep consolidation can be assessed as the average sleep bout duration over the entire day (**Figure 2E**). However, as sleep pressure is dissipated during recovery, the average sleep bout duration will be reduced as the day progresses. Thus, it is frequently helpful to also examine changes in the maximum sleep bout duration which can provide a more sensitive metric (**Figure 2F**).

FIGURE AND TABLE LEGENDS

Table 1: Survey of different methods of sleep deprivation used in the literature. Only 116 /254 papers used sleep deprivation. The number of papers using each method = “Total # of papers”. The fraction of papers using each method = “% papers / technique”. The mean length of recovery evaluated for each method = “Avg recovery evaluated”. SD – Sleep deprivation. SNAP – Sleep Nullifying Apparatus

Table 2. Length of sleep deprivation performed in different studies. SD – Sleep deprivation

Figure 1. The Sleep Nullifying APparatus (SNAP). A) Front view of the apparatus. The SNAP can accommodate 8 activity monitors in two rows; holder pins restrain the monitors in place. The legs can be adjusted to help position the apparatus at the correct orientation. **B**) Closeup view of the motor and spring that rock the apparatus back and forth. The motor turns a cam that tilts the apparatus back to the “up” position and compresses the spring. Release of the spring from compression snaps the apparatus back to the “down” position. **C**) Left - Side view of the apparatus in the “down” position. Holder pins restrain monitors; a monitor cord slot ensures that monitor cords are held in place. Pads help cushion the impact of the apparatus snapping to the ‘down’ position. Right – Close view up of the cam. **D**) Left Side view of the apparatus in the “up” position. Right – The counter-clockwise rotation of the cam tilts the apparatus into the ‘up’ position.

Figure 2. Experimental results. **A**) Sleep plots of *Cs* flies for the four days of the experiment:

the baseline day (bs), sleep deprivation day (sd), and two days of recovery (rec1 and rec2). **B**) Average locomotor activity counts of flies on the day of sleep deprivation. Flies were sleep deprived from hours 12-24. **C**) Time course of sleep deprivation and recovery. *Cs* flies were sleep deprived from hours 12 – 24, and allowed to recover from hour 24 – 72. The SNAP effectively deprived flies of >98% sleep, which was partially recovered over 48 h (n = 12 flies, Repeated Measures ANOVA for time, $F_{[70,1470]}=12.97$, $p < 10^{-15}$). D) Percentage of sleep recovered over 48 h. Flies recovered ~20% of their sleep over 12 h, and ~36% of their sleep over 48 h. **E**) Sleep consolidation for each day of the experiment as measured by average sleep bout duration during the day. Sleep is more consolidated on the first recovery day compared to baseline ($p < 0.05$, t-test). **F**) Sleep consolidation for each day of the experiment as measured by maximum sleep bout duration during the day. Sleep is more consolidated on the first recovery day compared to baseline ($p < 0.05$, t-test).

DISCUSSION

Sleep in *Drosophila* was independently characterized in 2000, by two groups[4,5]. In these pioneering studies, flies were deprived of sleep by gentle handling (i.e., hand deprivation) and shown to exhibit a robust homeostatic response to overnight sleep deprivation. Importantly, with any sleep deprivation experiment it is crucial to control for potential confounding effects of the method used to keep the animal awake. Hand deprivation studies set the benchmark for studies of fly homeostasis as a minimally disruptive means of sleep depriving flies. The SNAP efficiently deprives flies of sleep of >98% of nighttime sleep, and importantly induces a sleep rebound comparable to that obtained with hand deprivation[4,7].

Since the foundational studies defining sleep in flies, a number of methods have been developed to evaluate sleep homeostasis in flies in a high throughput manner[7,9,39-41]. We surveyed ~250 papers on sleep in flies and found ~46% of these published articles reported using sleep deprivation to evaluate sleep regulation or function (**Table 1**). A number of different methods effectively induced a sleep rebound in flies. Interestingly, of the studies that have evaluated sleep rebound, the protocols used for sleep deprivation and sleep rebound differed. Specifically, both the duration of sleep deprivation (**Table 2**) and duration for which rebound was evaluated (**Table 1**) varied substantially, potentially complicating comparisons of results obtained with different protocols. Sleep rebound in flies is known to persist for up to 48hours following sleep deprivation[5]. Accordingly, we think a thorough description of the effects of a given sleep manipulation on homeostasis are best obtained when homeostatic rebound is evaluated over a 48 h recovery period.

It is important to note that depriving flies of sleep during the day does not consistently increase sleep drive[4]. Hence, starting a 24 h sleep deprivation protocol at lights-on and continuing until the next day would not additionally enhance recovery sleep compared to a 12 h sleep deprivation protocol beginning at lights-off. In fact, the calculated sleep rebound may be lower since it will include non-homeostatically regulated daytime sleep in addition to nighttime sleep. The observation that daytime sleep deprivation does not induce a homeostatic rebound can however be used to control for potential confounding effects of the method of sleep deprivation. Thus, flies sleep deprived overnight in the SNAP are compared to flies that receive a comparable stimulus in the daytime[7].

In addition to being used for total sleep deprivation, by changing the frequency of the stimulus, the SNAP can also be used to chronically restrict and fragment sleep[7,42], thus mimicking conditions of chronic sleep loss in humans. Further, by delivering stimuli in steps of increasing frequency, the SNAP can also be used to measure arousal thresholds[8]. The SNAP is thus a facile way to effectively deprive and restrict sleep of flies, evaluate the homeostatic response, and measure other sleep characteristics.

The SNAP can fit in a standard laboratory fly incubator, but will definitely disturb flies in the incubator that are not part of the experiment. Fortunately, the SNAP can be placed in an isolated location to sleep deprive flies without disturbing other ongoing experiments. Since recovery sleep is fragile, care should be taken to ensure that recovery sleep takes place in a quiet location.

Complementing studies of sleep deprivation, genetic and pharmacological tools have been developed to enhance sleep in flies[8,43,44]. Thus, the ability to readily modulate sleep bidirectionally will allow fly sleep research to continue to provide deep insights into sleep regulation and function.

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D**ISCLOSURES**
The authors have nothing to disclose.

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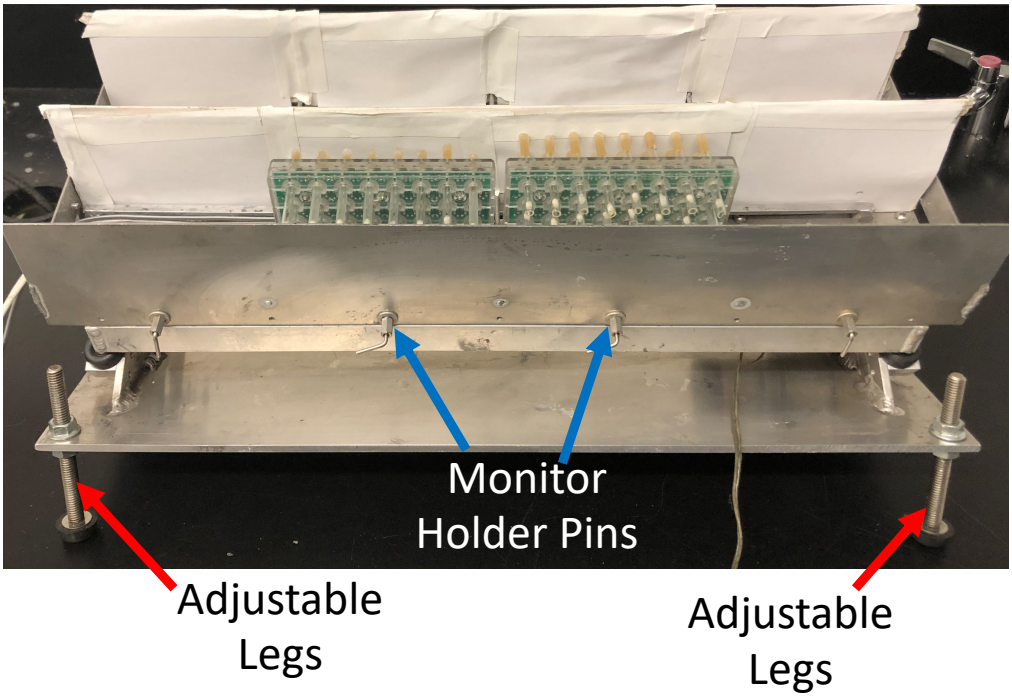
Figure 1

A



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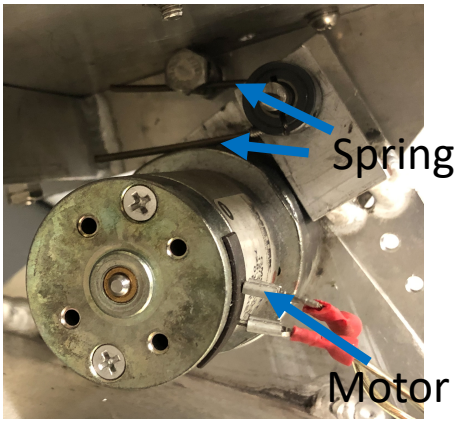
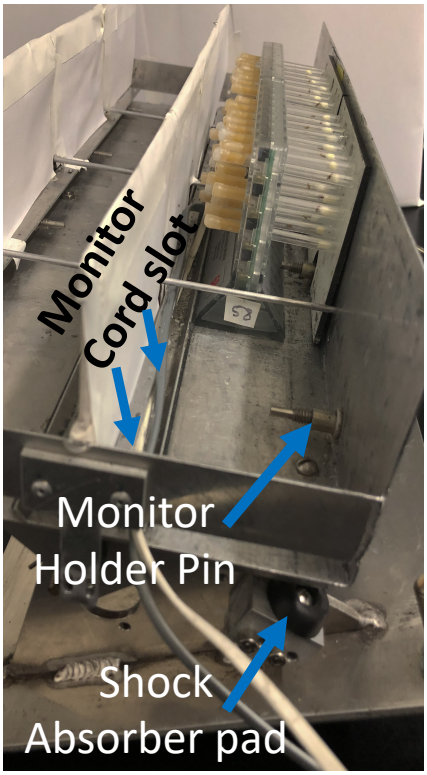
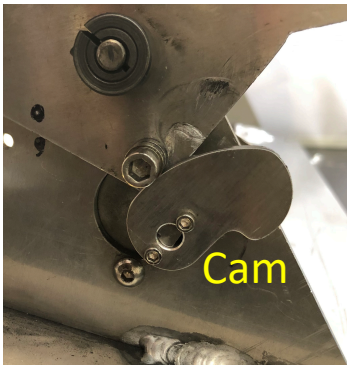
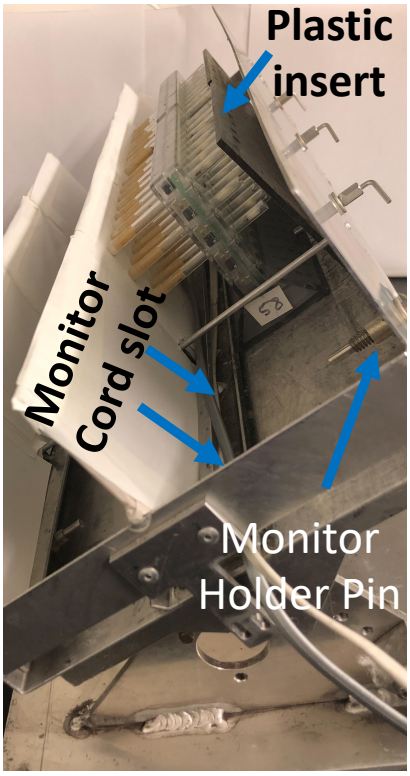


C "Down" position





D "Up" position



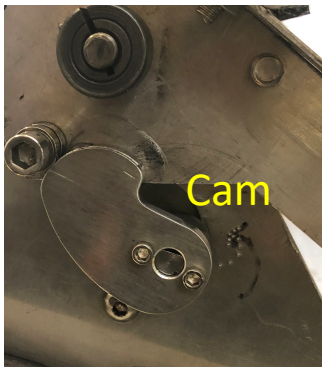
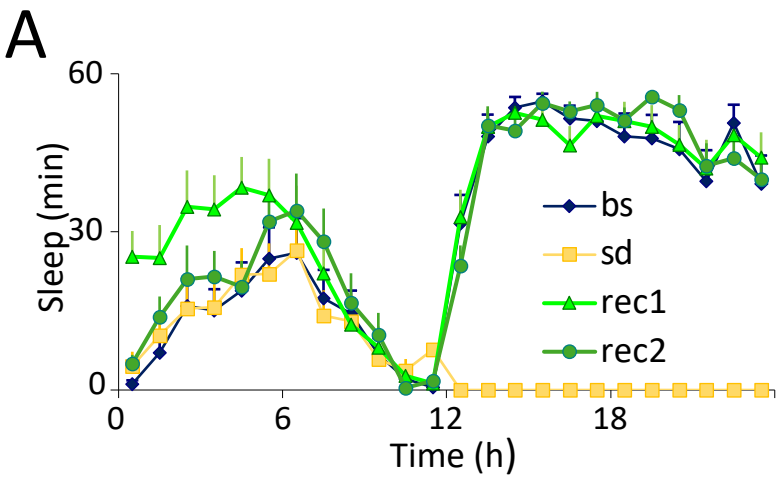
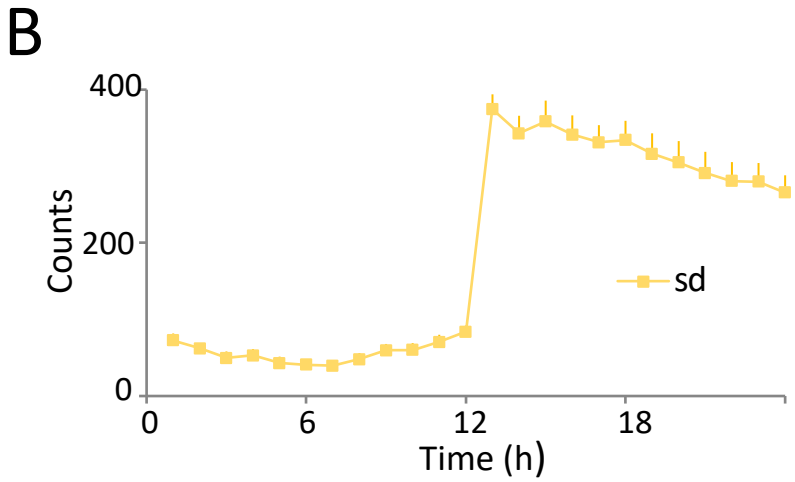
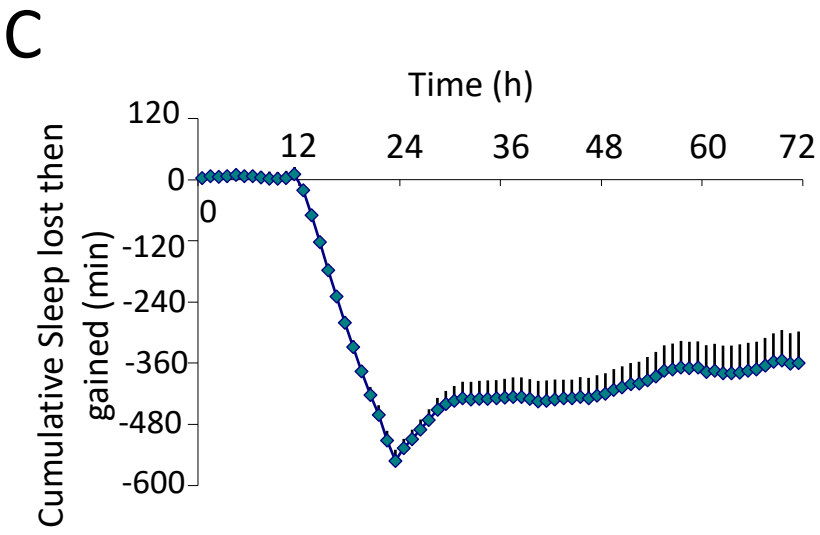
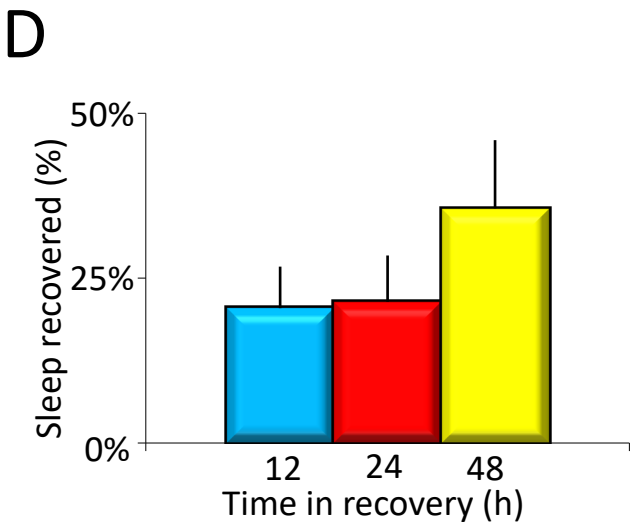


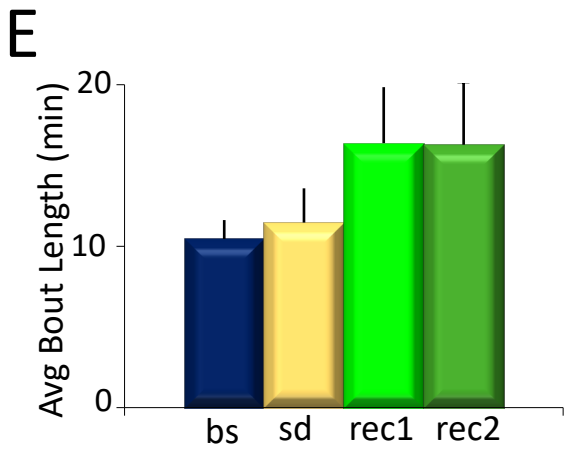
Figure 2


A
60
30
0
Sleep (min)
0
6
12
18
Time (h)
bs
sd
rec1
rec2


B
400
200
0
Counts
0
6
12
18
Time (h)
sd


C
Time (h)
120
0
-120
-240
-360
-480
-600
0
12
24
36
48
60
72
Cumulative Sleep lost then gained (min)


D
50%
25%
0%
Sleep recovered (%)
12
24
48
Time in recovery (h)


E
20
10
0
Avg Bout Length (min)
bs
sd
rec1
rec2

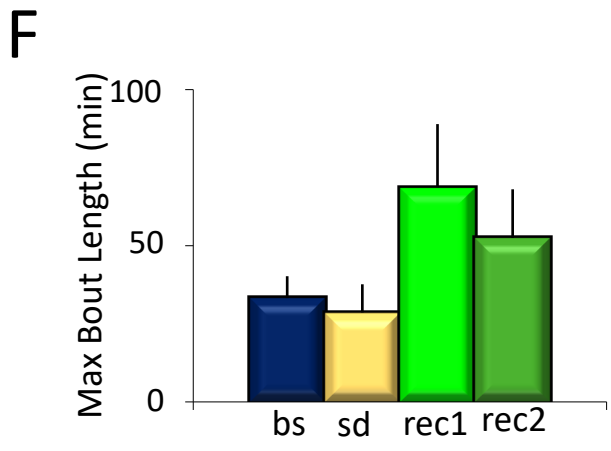

F
100
50
0
Max Bout Length (min)
bs
sd
rec1
rec2

Table1

Click here to access/download;Table;Melnattur_et_al_Table1_nov20.xlsx

Method of Sleep Deprivation	Total # of papers	% papers / technique	Avg recovery evaluated
SNAP	52	37.14%	33 ± 3
Vortexer/Random Shaking	49	35.00%	18 ± 3
Hand-Deprivation	9	6.43%	36 ± 11
Thermogenetic SD	15	10.71%	36 ± 12
Unspecified	15	10.71%	29 ± 10

Table 2

Length of SD	Total studies
< 6 h	12
6 h	23
>6 h & < 12 h	17
12 h	69
>12 h & <24 h	7
24 h	19
> 24 h	9
Chronic SD	4
Any SD	160

Table of Materials

Click here to access/download;Table of Materials;Melnattur_et_al_table_materials_nov18.xlsx

Name of Material	Company	Catalog Number	Comments
Locomotor activity tubes			
Fisher Tissue Prep Wax	Thermo Fisher	13404-122	Wax used for sealing tubes
Glass tubes	Wale Apparatus	244050	We cut 5mm diameter Pyrex glass tubes into 65mm long tubes to record sleep. Pre-cut tubes can also be purchased.
Nutri Fly Bloomington Formulation fly food	Genesee Scientific	66-113	Labs might use their own fly food recipe. It is important that sleep be recorded on the same food that flies were reared in.
Rotary glass cutting tool	Dremel Multi Pro	395	Used to cut 65mm long glass tubes
Monitoring Sleep			
DAM System and DAMFileScan software	Trikinetics		Software used to acquire data from DAM monitors and save the acquired data in an appropriate format
Data acquisition computer	Lenovo	Idea Centre AIO3	A equivalent computer from any manufacturer can substitute
Drosophila Activity Monitors	Trikinetics	DAM2	These monitors are used to record flies' locomotor activity
Environment Monitor	Trikinetics	DEnM	Not essential, but an easy way to monitor environmental conditions in the chamber where sleep is recorded
Light Controller	Trikinetics	LC4	A convenient way to control the timing of when the SNAP is turned on and off
Power Supply Interface Unit for DAM	Trikinetics	PSIU-9	Required for data acquisition computers to record Trikinetics locomotor acitvity data
RJ11 connector	7001-64PC	Multicomp	DAM monitors accept RJ11 jacks
Splitters	Trikinetics	SPLT5	Used to connect upto 5 DAM monitors
Telephone cable wire	Radioshack	278-367	Phone cables to acquire data from

			DAM monitors

Sleep Deprivation

Power supply	Gw INSTEK	GPS-30300	Power supply for the SNAP
Sleep Nullifying Apparatus	Washington University School of Medicine machine shop		

We thank the editor and reviewers for their comments and suggestions to improve the manuscript. We have copied the original comments in black below, and included our responses below the comments in red.

Editorial comments:

Changes to be made by the Author(s):

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. Please define all abbreviations at first use.

We have proofread the manuscript and corrected typos.

2. JoVE policy states that the video narrative is objective and not biased towards a particular product featured in the video. The goal of this policy is to focus on the science rather than to present a technique as an advertisement for a specific item. To this end, we ask that you please reduce the number of instances of "the SNAP" within your text. The term may be introduced but please use it infrequently and when directly relevant. Otherwise, please refer to the term using generic language.

We apologize for the confusion. The SNAP is not a product it is the name of an apparatus. The apparatus was given a name in 2002. It is as simple as that. We are aware of other groups building their own versions of this apparatus and referring to it as a SNAP to ensure the readers understand what type of apparatus it is.

3. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents.
For example: Drosophila Activity Monitors (Trikinetics); DamFileScan etc

We have removed commercial language in the manuscript, and replaced commercial language with generic terms as appropriate e.g. 'Drosophila Activity Monitors' has been replaced with 'activity monitors'

However, this request doesn't make sense. The SNAP was designed to be used with Trikinetics Monitors; the data is analyzed accordingly. We think explicitly saying "Drosophila Activity Monitors" and "DamFileScan" etc will help make the protocol clearer to readers.

4. Please revise the text, especially in the protocol, to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).

We have revised the text as suggested e.g. in lines 127-129 we have changed the text
"We place wake, behaving flies into recording tubes" to now read
"*Individually place wake, behaving flies into 65mm long glass tubes for sleep recording*"
in lines 173-174, we have changed the text
"*We typically consider sleep as stable when the difference in sleep between baseline days is ± 100 min.*" to now read
"*Sleep is stable when the difference in sleep between baseline days is ± 100 min.*"
and in lines 186 we changed the text

" In our experience, it is critical that sleep deprivation is terminated" to now read
"*It is critical that sleep deprivation is terminated,"*

5. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., "Do this," "Ensure that," etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as "could be," "should be," and "would be" throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a "Note." However, notes should be concise and used sparingly. Please include all safety procedures and use of hoods, etc.

We have edited the text in the protocol section to ensure that it is written in the imperative tense. For example, we have modified the text in lines 139:
"*The end of the tube with food **should be** at the top of the SNAP to ensure that flies do not get pushed into the food. In addition, the end with food **should be** on the side of the monitor with the sleep recording jack. This will allow DAM monitors to be oriented correctly in the SNAP for efficient sleep deprivation while monitoring activity*"
to now read
"*In the correct orientation, the end of the tube with food **is** at the top of the SNAP to ensure that flies do not get pushed into the food. In addition, the end with food **is** on the side of the monitor with the sleep recording jack*"

6. Please note that your protocol will be used to generate the script for the video and must contain everything that you would like shown in the video. Please add more details to your protocol steps. Please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. Please add more specific details (e.g., button clicks for software actions, numerical values for settings, etc) to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol.

We have added more details particularly in the section on recording sleep (page 5) and on calculating sleep rebound (page 6) reproduced below:

4. *Save locomotor activity counts of flies in 1min. bins from the time of lights on on a given day to lights on the previous day using activity recording software e.g. from 8am to 8am*

5 Estimate sleep from the locomotor activity data with custom macros using 5 min of inactivity as the threshold for a bout of sleep[35].
NOTE: A number of sleep metrics are computed from the locomotor activity counts. These include sleep in min/hr over 24h, total sleep time in 24h, average and maximum daytime and nighttime sleep bout lengths[36]

6. *Calculate the amount of sleep lost. For each individual fly, calculate the hourly difference between sleep obtained during sleep deprivation and the corresponding hour during baseline; sum the hourly differences to calculate total sleep lost*

7 Calculate the amount of sleep recovered. For each individual fly, calculate the hourly difference between sleep obtained during recovery and the corresponding hour during baseline; sum the hourly differences to calculate total sleep gained

7. Line 116: What is an “appropriate number”?

As we note (page 3) “ Typically, genotypes are analyzed in groups of 16 or 32 flies” – the appropriate number of flies would vary based on the number of groups to be evaluated.

8. Please move the information about the WT strain used (line 200) to the beginning of the protocol where you discuss using mainly females for the study etc.

We used CantonS wild-type flies in the experiment used to generate representative results. However, the protocol will work for any genotype, and thus we don’t think it is appropriate to include reference to a specific strain on flies in the protocol.

9. Please include a one line space between each protocol step and then highlight up to 3 pages of protocol text for inclusion in the protocol section of the video.

Each protocol step is separated by one line space as suggested. The protocol section is 3 pages in length.

10. As we are a methods journal, please add limitations of the technique to the Discussion.

In the original submission, we had included a small paragraph in the Discussion section (Page 9), where we discuss caveats of the technique which we reproduce below.

It is important to note that depriving flies of sleep during the day does not consistently increase sleep drive[4]. Hence, starting a 24 h sleep deprivation protocol at lights-on and continuing until the next day would not additionally enhance recovery sleep compared to a 12 h sleep deprivation protocol beginning at lights-off. In fact, the calculated sleep rebound may be lower since it will include non-homeostatically regulated daytime sleep in addition to nighttime sleep. The observation that daytime sleep deprivation does not induce a homeostatic rebound can however be used to control for potential confounding effects of the method of sleep deprivation. Thus, flies sleep deprived overnight in the SNAP are compared to flies that receive a comparable stimulus in the daytime[7]

11. Please do not abbreviate journal names in the reference list.

Journal names have been expanded in the reference list.

12. Please sort the Materials Table alphabetically by the name of the material.

The Materials Table is sorted alphabetically by name of material

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Reviewers' comments:

Reviewer #1:

Manuscript Summary:

This manuscript by Melnattur and colleagues on the sleep nullifying apparatus provides a very useful resource for properly performing sleep deprivation experiments in flies. As one key criterium of sleep, homeostatic rebound sleep is an important tool to understand underlying mechanisms of sleep regulation.

Minor Concerns:
* A potential source for noise or error of this sleep deprivation protocol is that flies would have to be relocated twice. After two days of baseline measurement in a recording chamber, flies are placed on the SNAP for overnight sleep deprivation and then moved again to the recording chamber. Please specify whether this happens in the same incubator and how to minimally disturb the flies in more detail. Would it be possible to down-size SNAP to fit in a small incubator (to potentially avoid relocating flies during the sleep experiment)?

The SNAP fits in a standard incubator and can be customized to accommodate different numbers of monitors. For example, we currently have SNAPs that fit 1 monitor, 6 monitors and 8 monitors respectively. Since all variety of SNAPs fit in an incubator there is no necessity to relocate the flies. However, many labs will not have dedicated space for both depriving flies and allowing flies to recover in the same space. Since resources are variable across labs, we prefer not to offer specific advice. However, as noted we emphasized that if the monitors must be moved, they should be moved immediately at the start of the recovery period.

* It is not entirely clear how quickly the SNAP set up moves from -60 to +60 degrees in the experiments shown. The 10s are a full cycle, right?

Yes, 10s are a full cycle. We have included a breakdown of the time for different steps in the text of the manuscript as a note in the section on recording sleep. The revised note reads as below.

The SNAP rocks monitors back and forth from -60° to +60° every ~10s. The monitors are held at -60° for ~5.9s; it takes ~2.9s for the tray holding the monitors to move from -60° to +60° and ~1s to move back from +60° to -60°. The cycle length can be altered as needed by adjusting the voltage supplied to the motor.

* Line 177, please comment on the typical length or span of time windows to consider when calculating sleep loss and rebound. Maybe an example calculation would help here?

We thank the reviewer for the suggestion. We have added a section to the protocol describing time windows, and have modified the text to be more explicit. Examples of calculations are shown in Fig. 2C. The revised section reads as below:

Sleep Deprivation and Recovery

1. *Flies can be sleep deprived for variable lengths of time (e.g. 12h, 24h and 36h). Recovery sleep can also be evaluated at various intervals (e.g. 6h, 12h, 24h and 48h). The duration of recovery is determined by experimental need. Sleep recovery can visualized using a sleep gain/loss plot or by examining percent sleep recovered over a predetermined interval (e.g. 6h).*

2. *If sleep is stable over the two baseline days, on the third day, place activity monitors into the SNAP for overnight sleep deprivation.*
   NOTE: Flies will exhibit a robust sleep rebound over a range of sleep times[8,32,37,38], but sleep has to be stable to reliably evaluate rebound sleep. Sleep is stable when the difference in sleep between baseline days is ± 100 min.

3. *Make sure activity monitors are secured in place with monitor holder pins, monitor cords plugged in, and monitors oriented correctly with the end with food at the back, and plastic barriers in front (Figure 1).*
   NOTE: The SNAP is designed so the cam rotates once every 10s (Figure 1). The plastic insert resets the tubes by pushing the tubes back when the apparatus is in the "up" position. Resetting the tubes is important to ensure that all tubes have the full range of motion at the beginning of each cycle.

4. *Unplug activity monitors, and take monitors out of the SNAP immediately upon lights on following overnight sleep deprivation.*
   NOTE: It is critical that sleep deprivation is terminated, and flies are placed in recovery immediately upon lights on following 12hrs of overnight sleep deprivation. Even a 20-30 min delay in placing flies into recovery can interfere with the extent of rebound sleep.

5. *Place flies in a recording chamber where they will be undisturbed for two days (48h) to monitor recovery sleep.*
   NOTE: If the recording chamber is being used for other experiments, extra care must be taken to avoid stimulating recovering flies.

6. *Calculate the amount of sleep lost. For each individual fly, calculate the hourly difference between sleep obtained during sleep deprivation and the corresponding hour during baseline; sum the hourly differences to calculate total sleep lost.*

7. *Calculate the amount of sleep recovered. For each individual fly, calculate the hourly difference between sleep obtained during recovery and the corresponding hour during baseline; sum the hourly differences to calculate total sleep gained.*
   NOTE: Whether a fly is actually sleep deprived is empirical. Thus, the experimenter should examine percent sleep lost. If the fly hasn't lost a sufficient amount of sleep it can be excluded from the analysis. Although this might be required for other sleep deprivation approaches, it is rarely if ever required for the SNAP. More commonly, sleep may not be stable in a given fly prior to the initiation of sleep deprivation. If sleep is not stable, homeostasis cannot be calculated. We accept a maximum difference of ± 100 min of sleep calculated prior to the initiation of sleep deprivation as candidates for inclusion. On occasion, an individual fly's sleep is distributed unevenly across the 24h day (e.g. some individuals may obtain 60-70% of their sleep quota during the day and thus only lose a small proportion of their 24 h sleep quota when deprived for 12 h at night). These flies can be evaluated separately.

8. *Calculate the average percentage of sleep recovered (relative to baseline) over 12hr, 24 hrs and 48hrs of the recovery period for each genotype*.

* Line 181: From this section, it is not entirely clear to me how often these different cases happen. How many flies in a sleep deprivation experiment are typically excluded following such criteria? This seems particularly relevant regarding the first exclusion criteria (exclusion of flies whose sleep loss is less than 90%). Could you provide

guidelines for 'typical' experiments?

Prior to the invention of the SNAP, when we used other protocols, we found that sleep deprivation was commonly ineffective. This rarely occurs with the SNAP. However, it is important for the reader to know that the effectiveness of the sleep deprivation apparatus is empirical and must be taken into account. Although we do not want to include this example in the manuscript, the SNAP easily induced sleep loss of >95% in the clock mutant *cycle*.
We have clarified this point and modified the protocol as follows:

Whether a fly is actually sleep deprived is empirical. Thus, the experimenter should examine percent sleep lost. If the fly hasn't lost a sufficient amount of sleep it can be excluded from the analysis. Although this might be required for other sleep deprivation approaches, it is rarely if ever required for the SNAP. More commonly, sleep may not be stable in a given fly prior to the initiation of sleep deprivation. If sleep is not stable, homeostasis cannot be calculated. We accept a maximum difference of ± 100 min of sleep calculated prior to the initiation of sleep deprivation as candidates for inclusion. On occasion, an individual fly's sleep is distributed unevenly across the 24h day (e.g. some individuals may obtain 60-70% of their sleep quota during the day and thus only lose a small proportion of their 24 h sleep quota when deprived for 12 h at night). These flies can be evaluated separately.

* It is not clearly stated in the legend of Table 1 what 'Percentage of all SD' refers to (% of recovered sleep?). It is also not clear how they obtained the values from the 116/254 papers on % of recovered sleep. Please provide more details for a clearer understanding on how SNAP compares to other methods.

We apologize for not being clearer. We have modified the titles in the Table legend. The new table legend is as follows:

Table 1: Survey of different methods of sleep deprivation used in the literature. Only 116 /254 papers used sleep deprivation. The number of papers using each method = "Total # of papers" using each method. The fraction of paper using each method = "% papers / technique". The mean length of recovery evaluated for each method = "Avg recovery evaluated". SD – Sleep deprivation. SNAP – Sleep Nullifying Apparatus

* There are full stops missing at the end of the sentences (for example, line 146).

We have corrected these typos.

Reviewer #2:
Manuscript Summary:
This protocol describes methodology for using the Sleep Nullifying Apparatus (SNAP) a method used to deprive fruit flies of sleep. The apparatus can be used for general sleep deprivation, as well as measuring arousal threshold, a critical and likely underutilized sleep readout. This protocol is well-written, timely, and should be of broad interest to the community.

Major Concerns:

None noted

Minor Concerns:
1. Line 79. Sleep deprivation by hand deprivation or gentle handling, is generally regarded as setting the standard for minimally disruptive sleep deprivation.
I am surprised by this statement as it seems to convey a mammalian-centric view of sleep deprivation. There are many reasons to think gentle handling is far from ideal (e.g. lack of consistency). There are no references cited for this in the discussion, but my understanding is the approach has not been widely used.

The first use of hand deprivation in flies was in 2000 (Shaw et al., 2000); we now include this reference. In any event, hand deprivation is not mammalian centric. It should be noted that hand deprivation refers to the experimenter disturbing the animal based upon its behavior; hands are not required and the system is not automated. Specifically, when using hand deprivation, the experimenter only disturbs the animal when the animal goes to sleep and leaves them undisturbed otherwise. Moreover, the stimulus used is calibrated to provide the least required perturbation for each intervention and for each animal. This is a big advantage for flies in which upto 32 animals can be sleep deprived at the same time.

2. Line 146: The representative results suggest the degree and velocity are critical. It may be worth putting a note in here about how these can be adjusted. If the formatting allows, it would be good to provide more information on how to analyze sleep, or at least a reference.

We have included a sentence clarifying that adjusting the spring and size and shape of the cam can help with altering the velocity of the SNAP. The sentence now reads:

Each lab can optimize the angle and velocity by adjusting the spring (Fig 1B) and/or the size and shape of the cam (Fig 1C and 1D, right).

We tried to focus the paper on the sleep deprivation technique *per se*. However, we now include a reference for sleep analysis and have expanded the description of sleep analysis as below:

Save locomotor activity counts of flies in 1min. bins from the time of lights on on a given day to lights on the previous day using activity recording software e.g. from 8am to 8am.

Estimate sleep from the locomotor activity data with custom macros using 5 min of inactivity as the threshold for a bout of sleep[35].
NOTE: A number of sleep metrics are computed from the locomotor activity counts. These include sleep in min/hr over 24h, total sleep time in 24h, average and maximum daytime and nightime sleep bout lengths[36]

3. One significant limitation I see is a lack of description of the SNAP. It would be helpful to provide information as a supplementary document about how to construct the device (if this is at all possible, and the authors are willing to do so). Are there SNAPs that are built outside of U. Wash and how similar are they?

Our machine shop will not provide the blueprints for the SNAP. However, we hope that the pictures will allow other machine shops to easily replicate this apparatus. This is not a complex apparatus to construct. Indeed, we have seen custom built SNAPs based upon our

design and they look good. It should be noted that the custom built SNAPs made by others were made using only the description without pictures.

4. The Table are extremely useful. One limitation of lumping all vortexter/thermogenetic forms of sleep deprivation together is that the results can differ dramatically depending on protocol.

We are glad the reviewer found the tables helpful. We agree that there is large variability in the protocols used for sleep deprivation and evaluation of rebound. We categorized the different methods as best we could to provide a heuristic for what is possible. To our surprise the results have been fairly consistent across methods with a few caveats.