Homeostatic control of deep sleep in *Drosophila*: Implications for Discovering Correlates of Sleep Pressure

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Abstract

A defining feature of sleep is its homeostatic control, which is most clearly expressed as increased sleep after forced wakefulness. *Drosophila* has served as a powerful model system for understanding the homeostatic control of sleep and ongoing work continues to be an important complement to studies in mammals and other vertebrate models. Nevertheless, there are significant challenges confronting investigators of sleep regulation in *Drosophila*. For example, the magnitude of sleep rebound in flies is relatively modest, providing a small dynamic range over which to detect changes in homeostatic responses in experimental subjects. In addition, the perturbation necessary to keep flies awake is associated with physiological and behavioral responses that may obscure homeostatic sleep responses. Furthermore, the analysis of fly sleep as a unitary state, without differentiation between shallow and deep sleep states, clouds our ability to fully characterize homeostatic sleep responses. To address these challenges, we describe the development of a yoked-controlled paradigm for flies that allows us to produce two sets of flies that have experienced identical levels of mechanical perturbation while suffering significantly different amounts of sleep deprivation. Moreover, by differentiating long bouts of sleep from all sleep, we show that flies display significant and lasting homeostatic increases in such long bouts following sleep deprivation, that are only detectable when controlling for the sleep-independent effects of mechanical deprivation. Finally, we illustrate the importance of yoked controls for examining the molecular correlates of sleep pressure. Our work introduces methodological approaches that are likely to support the discovery of new mechanisms of sleep regulation in the fly and calls for the reevaluation of previous work identifying the molecular, physiological, and cellular correlates of sleep pressure.

Keywords: *Drosophila*, sleep, Ethoscope, yoked controls, mechanical deprivation, sleep rebound, homeostasis, MALDI-TOF
Introduction

Sleep is a fundamental biological need and is homeostatically controlled: when sleep is disrupted, an elevated drive to sleep (i.e., sleepiness) is produced that is associated with decreased performance, well-being, and safety (1–5). Sleep disruption is ubiquitous in the modern age and contributes significantly to a wide array of negative health consequences in humans (6, 7). It is therefore critical to understand the homeostatic regulatory mechanisms of sleep. Sleep is ubiquitous in the animal kingdom and sleep-like states likely evolved at least 740 million years ago (8–13). Being so deeply conserved, mechanisms of sleep regulation uncovered in animals with relatively simple nervous systems can be highly relevant to sleep in mammalian species, including humans. The relative simplicity of invertebrate nervous systems and the existence of invertebrate models for which there are well-established methods of genetic and neuronal manipulations, make them amenable to experimental approaches that would be technically and logistically difficult or prohibitively expensive in mammalian model systems. Work in such model systems is therefore an important complement to studies in mammals and other vertebrate models (14).

The vinegar fly Drosophila has proved to be a useful model organism for understanding the molecular and cellular mechanisms regulating sleep (15, 16). The powerful genetic tools available in the fly, along with the ability to manipulate and measure neural signaling within small groups of defined cell types, have revealed genes and brain networks that mediate the homeostatic regulation of sleep (17–20). Furthermore, such work in the fly has revealed mechanisms that also mediate sleep regulation in mammals (21, 22). Just as in humans and other mammalian species, fly sleep is controlled by both a sleep homeostat and a circadian clock, which, during normal sleep
cycles, largely account for the timing and amount of daily sleep (23–26). Future work in the fly will undoubtedly continue to enrich our understanding of sleep regulation in all animals.

However, there are significant challenges to the investigation of sleep regulation in Drosophila. For example, the magnitude of sleep rebound in flies is quite modest (27–30). As for other animal models of sleep, the action of the fly’s sleep homeostat is assayed by depriving flies of sleep and measuring subsequent increases of sleep (compared to baseline), which reflect a homeostatic discharge of the sleep debt accrued during deprivation (15, 16, 27, 31). In contrast to mammalian species, however, sleep rebound following deprivation is often quite modest and brief. For example, male flies display variable levels of increased sleep following nighttime sleep deprivation of around 0 - 25 minutes, compared to 200-500 minutes of sleep lost (in 6- and 12-hour deprivation windows) and this is usually detectable only during the first few hours of the day after deprivation has ended (16, 27, 28, 32, 33). Furthermore, homeostatic sleep rebound in Drosophila appears to repay a relatively small proportion of the sleep lost during deprivation; 800 minutes of sleep loss in a full diurnal cycle saw only 100 minutes of recovery (~12.5%)(27). In contrast, mammalian species tend to display higher amounts of increased sleep following deprivation that is observable over more than one circadian cycle (34–38), and such rebound usually accounts for a much larger proportion of lost sleep than the rebound detectable in flies. For example, humans can reclaim around 40% of the sleep lost to deprivation (39). The relatively small rebound measured in the fly is a major challenge to the study of sleep regulation in this species, as it provides a small dynamic range over which to detect changes in homeostatic responses in experimental subjects. These features of fly sleep are somewhat surprising, given the highly conserved nature of sleep in the animal kingdom.
One possible cause of the relatively small homeostatic responses to sleep deprivation in flies is the sleep-independent effects of the deprivation techniques used by the field. The most commonly used method of fly sleep deprivation is frequent mechanical perturbation, typically shaking or slamming (15, 16, 31), to force movement and prevent the attainment of sleep, which in Drosophila is defined as any bout of inactivity that is five minutes or longer (15, 16). Though effective for the prevention of sleep, these methods are likely to produce many physiological and behavioral effects that are independent of sleep loss. Indeed, mechanical shaking is known to produce the biochemical hallmarks of stress, even when delivered during times of wakefulness (40).

Recently, neurogenetic methods have been employed to deprive flies of sleep (29, 41). This method involves the strong and chronic excitation of wake-promoting neurons in the brain. Though this method avoids the sleep-independent effects of physical perturbation, the strong non-physiological excitation of central brain neurons is likely to be attended by its own sleep-independent effects (reviewed in(22)). A fundamental challenge, therefore, is to differentiate sleep-pressure-driven changes in behavior and physiology from sleep-independent changes driven by the experimental intervention used to prevent sleep. We predict that accounting for such sleep-independent effects would improve the sensitivity with which homeostatic sleep responses can be detected in the fly.

In mammals, sleep consists of multiple, physiologically discrete stages, which differentially respond to sleep deprivation (35, 42–47). Following long bouts of wakefulness during normal sleep
cycles, slow-wave Non-Rapid-Eye-Movement (NREM) sleep stages dominate the initial bouts of sleep, suggesting that the daily rise of sleep pressure is discharged first by slow-wave (i.e., deep) sleep. Slow-wave sleep is also preferentially increased following sleep deprivation when compared to Rapid-Eye-Movement (REM) sleep (35, 38, 48, 49). Thus, differentiating between distinct sleep stages is likely important for assessing the homeostatic responses to sleep deprivation. A growing body of evidence indicates the existence of a deep sleep state in flies, which is characterized by slow-wave like neural signaling (50, 51), reduced metabolic rate (52), and waste clearance from the brain (53). The physiological and metabolic correlates of this deep sleep state are associated with bouts of inactivity that are significantly longer than the five-minute inactivity criterion used to define fly sleep (46, 47, 48). The existence of a distinct deep sleep state in flies suggests that treating sleep as a unitary state might obscure homeostatic sleep responses, particularly if, as in mammals, deep sleep is more strongly linked to homeostatic sleep pressure than shallower stages of sleep. Furthermore, our recent analyses suggest that longer bouts of sleep are a better reflection of sleep homeostasis than short bouts of sleep in the fly (26).

In this study we sought to address the sleep-independent behavioral effects of the most commonly used form of sleep deprivation in the fly and to develop a method of accounting for them. We also attempt to address the extent to which the treatment of sleep as a single unitary state in the fly might explain the apparently modest and brief nature of sleep rebound in the fly compared to such rebound in mammalian species. We present evidence that the extent of mechanical perturbation employed during sleep deprivation has significant effects on the amount of subsequent sleep displayed by deprived flies. We describe the development of a yoked-control paradigm for flies, based on previous work in rats, that allows us to produce two sets of flies that have experienced
identical levels of mechanical perturbation while suffering significantly different amounts of sleep restriction. Furthermore, by differentiating between long-bouts of sleep from the traditional unitary definition of sleep, we show that flies display significant and lasting homeostatic sleep increases following sleep deprivation that are only detectable when controlling for the sleep-independent effects of mechanical deprivation. Finally, we illustrate the importance of yoked controls for examining the molecular correlates of sleep pressure. Our work introduces methodological approaches that are likely to support the discovery of new mechanisms of sleep regulation in the fly and calls for the reevaluation of previous work identifying the molecular, physiological, and cellular correlates of sleep pressure.

**Results**

Sleep rebound is shaped significantly by the intensity of the mechanical stimulus used to prevent sleep.

The drive to sleep in the face of prolonged sleep deprivation is so strong that significant experimental intervention is necessary to keep animals awake. Such intervention is known to produce sleep-independent changes in behavior and physiology in addition to changes caused by increased sleep pressure (54). Traditionally, sleep-deprived flies, which have undergone forced wakefulness by means of mechanical or neurogenetic perturbation, are compared to unperturbed flies (16). Thus, it is not possible to differentiate between sleep-dependent and sleep-independent effects of deprivation, as the experimental flies have experienced both sleep deprivation and experimental insults (typically physical agitation), whereas control flies have experienced neither.
Though the criterion for a sleep-like state in *Drosophila* is five minutes of inactivity, the most common means of mechanical sleep deprivation in the field consists of the delivery of mechanical stimulation (shaking or slamming) for two seconds randomly within every 20 second interval (15, 16, 33). Once the permissive window of sleep opportunity is open after the period of sleep deprivation, rebound sleep is measured immediately during the next few hours by comparing the amount of sleep displayed by sleep-deprived flies compared to unperturbed controls (32, 33, 55). The amount of baseline sleep displayed by sleep-deprived and control flies before deprivation is also often accounted for in the assessment of rebound to control for small but significant differences in baseline sleep that are often observed between experimental and control flies, despite the two groups being genetically identical (33) (Figure 1A). Given the discrepancy between the criterion for sleep (five minutes or more of inactivity) and the frequency of mechanical stimulation used to deprive flies of sleep (multiple times per minute) we first asked how rebound sleep might differ between flies that were sleep deprived with different frequencies of mechanical stimulation.

As previously described many times by others using the *Drosophila* Activity Monitor (DAM) system, flies deprived of sleep for 24-h using two seconds of mechanical stimulation delivered at random times within each 20 second interval displayed immediate increases in total sleep compared to undisturbed flies(16, 27, 56). Furthermore, rebound sleep was most apparent during the first three hours following the end of the deprivation period (Figure 1A, B, and C top panels). To ask how the extent of mechanical stimulation shapes homeostatic sleep rebound, we decreased the frequency of such stimulation while maintaining 24-h of sleep deprivation. To this end we used two additional stimulation frequencies – two seconds of mechanical shaking delivered randomly
within every 120 second interval, and two seconds of random mechanical delivered within every 220 second interval. Both these frequencies produced significant sleep deprivation when five minutes of inactivity (300 seconds) of continuous inactivity was used as the definition of sleep (Figure 1A, middle, and bottom panels). Thus, all three stimulation frequencies (the standard 20s frequency and the 120s and 220s frequencies) were successful in producing significant sleep deprivation in experimental flies of sleep across the 24-hour deprivation window. Although there were small differences in total sleep during the deprivation window across all three conditions, it is important to note that this difference did not systematically change with the frequency of mechanical triggers (Figure 1A). The expected elevated sleep during the first six hours post deprivation was apparent for flies deprived with 20 and 120-sec triggers when compared to undisturbed controls (Figures 1B and C). Remarkably, such rebound was not clear for the 220 second triggers, despite a loss of sleep that was greater than that observed for the 120 second trigger. However, when post-deprivation sleep was normalized to baseline sleep, significant rebound was only detected for the flies deprived using 20 second triggers, with and no such rebound apparent for flies deprived with 120 second or 220 second triggers (Figure 1D).

To assess the effects of trigger frequency on sleep rebound, we performed a two-way ANOVA and found that there was a significant effect of treatment × trigger interaction on the amount of sleep rebound (total sleep pre-deprivation subtracted from total sleep post-deprivation). We found significant rebound only for the flies deprived with 20 second trigger frequencies (Figure 1D). Thus, lower frequencies of mechanical stimulation failed to produce significant sleep rebound, despite having produced similar extents of sleep deprivation as the commonly used 20 second frequency of perturbation (Fig. 1D). To address the extent to which these flies recovered lost sleep,
we estimated how much lost sleep was recovered over the 24-hour post-deprivation window (19, 57). We found that, as expected, the flies deprived of sleep using the most frequent mechanical stimulation (every 20s), recovered significantly higher percentages of their lost sleep compared to flies that experienced less frequent stimulation (Figure 1E). The fact that lower levels of mechanical stimulation failed to produce rebound, despite having resulted in similar levels of sleep deprivation, suggests that high frequency physical perturbation likely causes sleep-independent behavioral effects that might mask homeostatic sleep increases. Accounting for such effects might therefore reveal previously hidden characteristics of sleep homeostasis.

Accounting for mechanical stimulus using a yoked-controlled design uncovers significant sleep rebound in sleep-deprived flies when low-frequency triggers are used.

The mechanical shaking employed to sleep deprive flies loaded into the DAM system use near constant agitation without regard to the flies’ sleep state, delivering mechanical perturbations to both waking and sleeping flies. To further examine the potential sleep-independent effects of mechanical perturbation during sleep deprivation experiments, we adopted the Ethoscopes, a video-based system for recording fly behavior (32). In contrast to the DAM system, which requires the simultaneous mechanical perturbation of all flies within the same DAM monitor, the Ethoscope allows the user to deliver mechanical stimuli to individual flies only when a fly is inactive for a specified amount of time (32). The ability to track single flies and selectively stimulate individual flies to prevent sleep means that flies can be sleep deprived with significantly fewer mechanical perturbations.
We programmed Ethoscopes to track single flies and rotate their tubes at ~420 rpm for one second each time a fly had been inactive for 220 seconds, which resulted in 24-h of sleep deprivation (Figure 2A). When compared to undisturbed flies, these deprived flies failed to display significant increases in sleep during the immediate aftermath of deprivation, revealing an apparent absence of a rebound in sleep (Supplementary Figure 1A, 1B). Expanding our analysis to the full day following deprivation likewise revealed no apparent homeostatic sleep rebound (Figure 2B); a surprising result given the clear effectiveness of sleep deprivation (Figure 2A). The failure to produce clear homeostatic sleep increases following deprivation could be the product of two causes. As suggested by our observations above, the mechanical perturbations experienced by the deprived flies may exert sleep-independent effects on the behavior observed after deprivation. That is, the mechanical disturbance experienced by the sleep-deprived flies likely induce effects on the fly that could mask sleep-pressure-driven changes. Second, the 220s inactivity triggers used to deprive flies of sleep may have allowed brief sleep bouts that precluded the build-up of sufficient sleep pressure to engage a homeostatic response. This would suggest that the five-minute inactivity criterion for sleep may be too long. To begin to address these two possibilities, we first sought to produce flies that differed in the extent of sleep deprivation, despite experiencing identical mechanical perturbation, which would allow us to examine the specific contributions of sleep pressure to sleep behavior following deprivation.

To accomplish this, we adopted a paradigm of chronic sleep deprivation that was first introduced in the rat model. Employing what is referred to as yoked-controls for sleep deprivation, this approach produces pairs of rats that have experienced perfectly matched mechanical perturbation, in the form of a rotating platform over water, that nevertheless differed significantly in the amount
of sleep deprivation experienced (58). This approach involved a “focal” animal which was placed on one side of the platform and monitored via electroencephalogram recording. Whenever the focal animal displayed the physiological hallmarks of sleep, the platform would begin to rotate, thereby keeping the focal animal awake. A paired rat, the “yoked” control, was placed on the opposite side of the platform and could sleep when the focal rat was awake. This arrangement produced a pair of rats that experienced identical, time-matched mechanical stimulation while experiencing significantly different amounts of sleep loss (58).

We programmed the Ethoscope platform to produce a paired yoked-control fly for every sleep-deprived focal fly. That is, for each fly that was tracked and stimulated upon being inactive for 220s, a second fly received matched, time-locked tube rotations, which were independent of its sleep or wake state. This ensured that each focal and yoked pair received identical mechanical stimuli (Figure 2C). However, the yoked fly was able to attain sleep while the focal fly was active (Figure 2D-E), and suffered only partial sleep loss compared to focal flies (Figure 2F). This approach, therefore, succeeded in producing two sets of flies that had experienced the same level of physical perturbation while suffering significantly different levels of sleep loss. However, even when compared to these controls the focal sleep-deprived flies did not show a significant in sleep during the first six hours following 24 hours of total sleep deprivation (Figure 2F; Supplementary Figure 1C, 1D).

However, a comparison of total sleep across the entire 24 hours of the post-deprivation day revealed significantly higher levels of sleep in the focal flies compared to yoked controls (Figure 2G). Thus, the introduction of yoked controls revealed a homeostatic sleep response that was not
apparent when comparing sleep-deprived flies to unperturbed controls, supporting the notion that sleep-independent effects of mechanical perturbation can mask homeostatic sleep responses. Though yoked controls revealed significant sleep-pressure specific sleep increases, this homeostatic increase was quite modest, resulting in very little discharge of the sleep pressure built during the 24-h of sleep deprivation (Figure 2G). Given that most animals display homeostatic sleep increases over multiple sleep/wake cycles (34, 37, 46), we wondered if the rebound we detected using yoked controls might persist over multiple diurnal cycles.

Sleep rebound accumulates over multiple cycles in response to infrequent mechanical deprivation, but little sleep debt is paid off. The circadian control of sleep thresholds ensures that sleep is gated to occur at the appropriate/adaptive time of day. The action of the circadian system therefore prevents sleep debt from being discharged completely upon the first opportunity to sleep, ensuring that wakefulness will occur during the next diurnal cycle despite the presence of homeostatic sleep pressure. For this reason, sleep debt is typically discharged over several sleep/wake cycles (34, 37, 46). Current methods in the Drosophila sleep field do not typically produce multi-cycle rebound, but instead, reveal fairly modest sleep rebound during the first hours of the day-time following deprivation (32, 33, 55). Similarly, in our yoked-controlled experiment, we observed only modest sleep rebound that discharged very little of the sleep lost during the previous day’s deprivation, which may have been a product of the smaller but significant deprivation experienced by yoked controls. We wondered if the comparison of deprived flies with yoked controls might reveal additional increases of sleep in deprived flies across subsequent sleep/wake cycles. We therefore assessed the behavior of flies deprived of sleep using the Ethoscope for three full diurnal cycles following 24 hours of...
sleep deprivation (Figure 3A, 3B) and compared the sleep of deprived flies with sleep observed in both undisturbed and paired yoked controls.

When compared to yoked controls, focal sleep-deprived flies displayed modest but significant increases in total sleep for two successive cycles (Figure 3D). Once again, these increases were not detectable when deprived flies were compared to undisturbed controls (Figure 3C). In this case, total sleep displayed on baseline days before deprivation was not different between the three groups of flies (Figure 2B, 2G). We examined how much lost sleep was recovered across the three post-deprivation cycles by quantifying sleep gain in focal flies, which was calculated as the increases in total post-deprivation sleep compared to baseline sleep normalized to sleep in undisturbed controls to account for normal changes in sleep during the duration of the experiment (see methods). Despite this apparent two-cycle rebound, focal flies failed to discharge the substantial sleep debt accrued during deprivation (Figure 3E).

We wondered if the apparent absence of substantial homeostatic payback of sleep lost by focal flies during deprivation might be explained by treating fly sleep as a unitary state, in which all bouts of inactivity of five minutes are more are considered to be the same sleep state. Given the growing evidence of physiologically and metabolically distinct sleep states in the fly (46, 47, 48) and the fact that deep sleep stages are most immediately affected by sleep deprivation in mammals, we turned our attention to how the architecture of sleep might differ between focally deprived flies and their yoked controls across the three days following deprivation. Though the number of sleep bouts was not significantly different between focal and yoked flies across any of the three post-deprivation cycles (Supplementary Figure 2A), there was a significant increase in bout duration in
the focal flies compared to yoked controls across the first two days following deprivation (Supplementary Figure 2B). These two groups of flies displayed no differences in these metrics during baseline sleep before deprivation (Supplementary Figure 2C). This result suggested that focal flies, which had higher sleep pressures than yoked controls, were characterized by increased sleep bout durations.

The magnitude of homeostatic sleep rebound in the fly is masked by short-bout sleep.

Recent work in our lab revealed that long-bout sleep (~30 minutes or more of inactivity) is a better reflection of sleep homeostat action than shorter bouts of inactivity (26) and such long-bout sleep appears to represent a deep sleep stage that is physiologically and metabolically distinct from shorter bouts of sleep (51–53). We therefore hypothesized that the extent of sleep rebound and payback of lost sleep might be more readily apparent if we focused our analysis on long-bout sleep, eliminating the contribution of shorter, presumably shallower, bouts of sleep.

We reanalyzed the 24-hour sleep deprivation experiment reported in Figure 3, iteratively varying the inactivity duration criterion for sleep from one to 30 minutes, to ask which how varying the sleep definition might shape the apparent homeostatic recovery of lost sleep. We found that inactivity criteria of 25-min or more resulted in the largest payback of lost sleep when examined on the third day of recovery from deprivation (Figure 4B). This suggested that longer bouts of sleep might be most strongly influenced by homeostatic sleep regulation. With this inactivity criterion, sleep recovery approached ~50% of lost sleep, a much higher proportion than was apparent using currently employed unitary definition of sleep.
Based on this result, we re-examined our 24-h sleep deprivation data using 25 minutes of inactivity as our criterion for sleep, thereby focusing on longer bouts of sleep that likely correspond to the deep sleep state identified by others and omitting shorter, presumably shallower, bouts of activity from our analysis. Five-day sleep time-series for focal, yoked, and undisturbed flies are shown in Figure 4A using the 25-min inactivity criterion to focus on long-bout sleep. These plots reveal that focal flies were, as expected from 220s inactivity triggers, completely deprived of such sleep. Yoked flies also, as expected, displayed significantly reduced levels of long bout sleep compared to unperturbed controls, but such sleep was in fact attained by yoked controls, though at a level significantly reduced from that displayed by unperturbed controls (Figure 4A).

When total long bout sleep across three successive cycles was quantified, focal flies displayed significant increases in such sleep when compared to yoked controls on days two and three of recovery (Figure 4D). The absence of a difference on day one is likely a product of the reduction in long-bout sleep that yoked flies accrued during the deprivation period. Thus, the comparison of long bout sleep between focal and yoked flies revealed a differential sleep rebound across three days of recovery. Remarkably, this rebound was not seen when focal flies were compared to unperturbed controls (Figure 4C). Once again, this was likely due to the sleep-pressure-independent effects of mechanical disturbance. To confirm that 25-minute bouts of inactivity were not an experimentally induced occurrence (i.e., that this state was not caused by the mechanical stimulation delivered by the Ethoscopes), we confirmed that such long bouts of sleep are present in unperturbed control flies. Indeed, all unperturbed control flies routinely displayed 25-minute or longer bouts of inactivity (Supplementary Figure 3A).
These results suggested that longer bouts of sleep are a more sensitive reflection of homeostatically-controlled sleep in flies. This conclusion is supported by the significant recovery of lost long bout sleep that continues across all three days of the observed recovery period. Thus, when the analysis focused on long, and presumably deep sleep states, a much larger percentage such sleep is recovered compared to a consideration of all epochs of inactivity lasting longer than five minutes. At least half of all sleep-deprived flies paid back more than 50% of the sleep they lost during the 24-h of deprivation, and over 25 percent of flies overshot their sleep-debt, thereby displaying a defining hallmark of homeostatic control (Figure 4E). These results lend further support for the idea that long bouts of inactivity represent a sleep state that is distinct from shorter bout sleep (51, 52, 59). Furthermore, the absence of robust homeostatic recovery of sleep using inactivity criteria of less than five minutes (Figure 4B) suggests that our initial failure to detect rebound when such mechanical triggers were used for deprivation (Figure 1), was not likely due to rebound enriched for brief sleep bouts with durations of fewer than five minutes.

Long Bout Sleep is Under Specific and Potent Homeostatic Control.

In mammals, both REM and NREM stages of sleep are controlled homeostatically, with stage-specific deprivation leading to stage-specific rebound (23, 44, 49, 60). If long-bout sleep indeed reflects a discrete sleep stage in Drosophila, selectively depriving flies of it should produce a homeostatic rebound in long-bout sleep, despite the presence of abundant short-bout sleep. To test this prediction, we set the Ethoscope’s inactivity-dependent trigger to rotate tubes only when focal flies had been immobile for 22 minutes. When using the standard inactivity duration criterion for sleep, this long trigger latency appeared to support largely normal sleep rhythms in both focal and yoked flies (Figure 5A).
Employing a 25-minute inactivity duration criterion to remove short bout sleep from our analysis revealed that 22-minute triggers effectively deprived flies of long-duration sleep while permitting yoked flies to achieve appreciable amounts of such sleep during focal deprivation (Figure 5B). When behavior was analyzed using the standard five-minute inactivity criterion, which would include short-bout sleep, focal flies displayed no significant increases in total sleep when compared to yoked controls (Figure 5C). In contrast, when short-bout sleep was eliminated from the analysis through the use of a 25-minute inactivity criterion, focal flies displayed significantly higher levels of long-bout sleep across all three days following deprivation (Figure 5D). Remarkably, the relative levels of post-deprivation sleep displayed by the focal flies appeared to increase over the course of the three days of observation, suggesting that homeostatic responses to long-bout sleep deprivation may persist for an extended period of time (Figure 5D). This rebound produced a significant recovery of lost sleep (Figure 5E), one that likely would have continued to build over additional sleep/wake cycles.

Long-bout-specific deprivation also produced significant effects on sleep architecture. Focal flies were characterized by an increased number of bouts that were longer than 25-minutes compared to yoked controls (Supplementary Figure 4B), although the duration of these longer sleep bouts did not increase (Supplementary Figure 4D). No differences in the number of long-bout sleep between focal and yoked flies were observed prior to the deprivation cycle (Supplementary Figure 4A). Our results suggest that long-bout sleep, previously shown by others to represent a relatively deep sleep state (51, 52, 59)), is under strong homeostatic control and highlights the importance of controlling for the sleep-independent effects of mechanical sleep deprivation on behavior.
Yoked controls are necessary to differentiate effects of sleep deprivation from effects driven by mechanical perturbation.

A major goal of sleep science is to identify the molecular, cellular, and physiological processes mediating the rise and fall of sleep pressure and how these changes operate in the brain to promote sleep or wakefulness. In the fly, previous work by others has described cellular (19, 21, 61) and physiological (53) correlates of sleep pressure, based on the effects of mechanical sleep deprivation. Given our behavioral results indicating significant sleep-independent effects of mechanical deprivation that appear to mask homeostatic sleep responses, we sought to examine the potential utility of yoking for differentiating sleep-pressure driven change from the sleep-specific effects of mechanical perturbation. Toward this end we conducted a simple molecular screen in the fly brain using matrix-assisted laser desorption/ionization – time-of-flight (MALDI-TOF) mass spectrometry. This method supports the mass spectrometric analysis of fly head sections (62) and consists of three major steps: the crystallization of a matrix and analytes within the tissue section, the laser ionization of analytes within this matrix, and time-of-flight mass spectrometry (MS) to analyze the molecules present (63). This method provides sufficient spatial resolution to detect the presence of molecules defined by their weight over charge ratios (m/z), specifically within the central brain (Figure 6B). As a test case for how the inclusion of yoked controls might aid in differentiating true molecular correlates of sleep pressure from direct responses to the insult incurred by mechanical deprivation, we examined general molecular responses within the brains of focally deprived flies, paired yoked controls, and unperturbed flies.
Using 2,5-dihydrobenzoic acid (DHB) as a matrix, which is considered useful for “universal analysis” and the detection of diverse molecular types (64), we sampled m/z value peaks between zero and 1,300, and detected 187 distinct peaks in the central brain regions of our head sections. Each m/z ratio peak measurement was made specifically for the central brain by registering the MALDI-TOF signal directly over the histological image of our tissue sections (dashed square, Figure 6B). When we compared the heights of these 187 m/z peaks between focal flies and unperturbed controls, we found that 129 displayed significantly higher amplitudes in sections from focal flies compared to those of unperturbed flies (Figure 6C). Thus, when compared to unperturbed flies, sleep deprivation in focal flies was accompanied by a large proportion of the molecule examined. However, it is highly likely that the mechanical perturbation associated with sleep deprivation causes many molecular changes within the brain that that may not be directly related to sleep loss or sleep pressure. We therefore asked the extent to which controlling for mechanical insult through yoked controls might allow us to differentiate increases explained by sleep pressure from those that were sleep-independent reflections.

If a molecule’s abundance is increased simply as consequence of mechanical insult during sleep deprivation, we would expect head sections from both focal and yoked flies to display significantly higher concentrations of that molecule compared to unperturbed controls. Furthermore, in this case, the focal and yoked sections would be expected to have statistically indistinguishable levels of the molecule in question, since they experience identical levels of mechanical insult (Figure 6D, top panel). In contrast, if a molecule’s abundance is increased specifically as consequence of sleep debt, we predict that it will be present at relatively low levels in sections from unperturbed controls, significantly higher levels in sections from focal flies, and with intermediate levels in yoked
controls that are statistically distinguishable from the other two conditions (Figure 6D, bottom panel). With the yoked design in place, just under half of the 187 discernable m/z value peaks (85) displayed statistically indistinguishable levels in the central brain across the undisturbed, yoked, and focal samples. 40 m/z value peaks displayed the pattern expected for changes driven by mechanical insult: high and indistinguishable levels in focal and yoked that are significantly higher than those detected in sections from unperturbed controls (for example amplitudes for the 104.1 m/z peak, shown in Figure 6D). Remarkably, only five m/z value peaks displayed the pattern expected of molecules driven by sleep pressure (Figure 6E) with focal samples displaying the highest levels, yoked sections at intermediate levels, and unperturbed at the lowest levels, with statistically significant differences across the three conditions (for example, the 175.1 m/z peak amplitudes shown in Figure 6D).

Thus, the standard method of comparing deprived flies to unperturbed controls produced 129 candidate molecules while the inclusion of yoked controls produced five such candidates, differentiating these from 40 other candidates that were more closely correlated with mechanical insult. Interestingly, 16 other m/z value peaks displayed a pattern, which we refer to as “Sleep Absence” in which the amplitude of m/z value peaks from focal flies were significantly higher than both the unperturbed and yoked controls, with the two controls being statistically indistinguishable. The remaining m/z value peaks (41) displayed differences between treatments that did not fit either “sleep pressure”, “mechanical deprivation,” or “sleep absence” patterns. These results suggest that yoking provides a powerful means of identifying molecular, cellular, and physiological correlates of sleep pressure in Drosophila.
Discussion

The impact of the sleep-independent effects of mechanical sleep deprivation on the measurement of homeostatic sleep rebound.

In order to study the sleep homeostat, one must prevent sleep through the imposition of prolonged wakefulness (58, 65–67). However, the very act of sleep disruption is expected to cause not just the buildup of sleep pressure, but also other potentially confounding responses to the stimulus used to prevent sleep. The specific confounds attending sleep deprivation will depend on the method and duration of the sleep-depriving stimulus employed, but all forms of experimental sleep deprivation are expected to produce such confounds. For example, sleep deprivation methods employing forced locomotion result in increased muscular activity, which will be attended by sleep-independent effects. Chronic mechanical agitation produces stress (68–70), and even gentle handling of rodent subjects, a means of deprivation thought to cause little stress, has significant effects on feeding behavior following deprivation (71). Such confounding, sleep-independent effects can mask homeostatic sleep responses and therefore must be accounted for whenever possible. Here we have attempted to account for the sleep-independent effects of mechanical sleep deprivation in Drosophila, the most commonly used method in the field of sleep research.

To assess the degree to which the frequency of mechanical agitation affects subsequent sleep rebound in Drosophila, we examined flies that were sleep deprived for 24 hours with three different frequencies of mechanical perturbation. The less frequent agitation produced significantly less sleep rebound, despite having produced similar amounts of sleep loss. Thus, a sleep-pressure independent effect of mechanical perturbation appeared to be the primary determinant of post-deprivation sleep. An alternative explanation for this result was that short
bouts of inactivity may represent a brief but restorative sleep stage that the 120 and 220-second perturbation frequencies were not effective at depriving flies of this state. However, we found no evidence of homeostatic rebound in the sub-220 second range (Figure 4B).

A previous attempt to control for the sleep-independent effects of mechanical perturbation delivered mechanical stimuli matched to sleep deprived flies, but delivered during periods of high activity (i.e., wakefulness), thereby exposing the control flies to mechanical shaking while resulting in negligible amounts of sleep loss (40). However, mechanical shaking only during times of wakefulness increased molecular signatures of stress, even when given for relatively short periods(40). To minimize the extent of mechanical perturbation necessary to prevent sleep and to control for the sleep-independent effects of such perturbation, we adopted the Ethoscope, a video based system that tracks single flies and delivers a mechanical stimulus, the rapid rotation of the glass tube containing the fly, only when a fly meets a user-defined inactivity duration criterion (32). Thus, rather than agitating all the flies of the sleep-deprived condition at a given frequency throughout the entire deprivation period, the Ethoscope prevents single flies from attaining specific durations of inactivity, just before meeting a user defined duration. Previous work using this system has revealed remarkably low levels of homeostatic rebound and sleep deprivation-associated death (33).

Though the Ethoscope produces sleep deprivation using fewer mechanical perturbations, it nevertheless delivers significant mechanical perturbation whose sleep-independent effects might mask homeostatic sleep responses. For this reason, we introduced yoked controls to the Ethoscope platform, allowing us for the first time to disentangle effects associated with sleep pressure from...
those best explained by the mechanical insult delivered during sleep deprivation (Figure 2). When flies were prevented from attaining five-minute bouts of inactivity - the standard criteria for a sleeplike state in the fly - using 220s inactivity triggers in Ethoscopes, they failed to show significant homeostatic rebound when their sleep was compared to unperturbed controls. Comparison of these deprived flies to their yoked controls, in contrast, produced a significant, albeit small, increase in sleep following deprivation (Figures 2F, G). Thus, yoking revealed homeostatic rebound that was not apparent when deprived flies were compared to controls that had not experienced mechanical perturbation. However, when compared to yoked controls, the homeostatic response of sleep deprived flies was quite small, amounting to very little recovery of lost sleep (Figure 3E).

Sleep as a non-unitary state in Drosophila: Long-bout sleep is a distinctly regulated sleep stage.

In mammals, sleep is not a unitary state but rather consists of distinguishable sleep stages (42, 72). For example, REM and NREM sleep represent distinct physiological states that differ in their relationship to sleep pressure and the circadian system (23, 44, 49). Slow-wave NREM sleep appears to be the mammalian sleep state most strongly controlled by the daily homeostatic control of sleep: initial sleep cycles following prolonged wakefulness are rich in slow wave sleep, and this form of sleep is also prevalent following sleep deprivation (35, 73). For the majority of studies using Drosophila, sleep has been treated as unitary state, with all inactivity bouts of five minutes or more being treated as sleep. However, a growing body of works suggests the existence of distinct sleep stages in the fly. For example, in one of the very first papers describing sleep-like states in Drosophila, Hendricks et al., reported differences in durations of sleep bouts consisting
of epochs of inactivity lasting 5 min or more (16). The longest bout lengths occurred at the
beginning of the subjective night under constant conditions between circadian time 13.5 and 16.
More recently, quantitative approaches using hidden Markov modeling have provided evidence
for the existence of light and deep sleep states in the fly (74, 75).

Flies display both longer and deeper bouts of sleep at night (16, 51) and deeper sleep is associated
with distinct patterns of global brain activity when compared to brain activity during wakefulness
or during more brief and shallow bouts sleep (50, 59). Longer sleep bouts are also attended by
lower metabolic rates, where bout lengths of 60 min were shown to be associated with the lowest
rates (52), a phenomenon similar to that observed for deeper NREM sleep stages in humans (76).
Finally, flies display a stereotyped pattern of proboscis movements that appear to drive waste
clearance (53), reminiscent of the changes in fluid dynamics observed in the cerebral spinal fluid
of sleeping mammals, that occur at times corresponding to deep sleep stages (77). The time of
peak occurrence of these proboscis movements were characterized by a significant reduction of
brain wave activity compared to sleep at other times (53).

Physiological and metabolic signatures of deep sleep in the fly both suggest that they correspond
to sleep bouts that are significantly longer than the standard sleep definition of five minutes of
inactivity and multiple lines of evidence suggest that deeper sleep states are likely reached upon
attaining 15-30 minutes of inactivity (51, 59). Finally, recent work from our lab has suggested that
bouts of inactivity of between 30 and 60 minute are stronger reflections of the daily homeostatic
control sleep than shorter bouts of sleep (26). How might such long bouts of inactivity relate to
homoeostatic sleep rebound?
Homeostatic sleep rebound, as traditionally measured in flies, appears to differ from mammalian sleep rebound, in that it is relatively modest in magnitude and appears to largely run to completion during the first few hours following the offset of deprivation. In contrast, mammalian rebound appears to play-out over several sleep-wake cycles (34, 37, 46, 78). Remarkably, when we biased our analysis to bouts of inactivity lasting 25-min or more, clear and prolonged homeostatic rebound of sleep was observed following deprivation. This rebound lasted several days (Fig. 5D) and discharged a large proportion (>50%) of the long-bout sleep debt suffered during deprivation (compare Fig. 3E to 4E). This result further supports the notion that long durations of inactivity more sensitively reflect the action of the sleep homeostat.

When we deprived flies only of inactivity bouts that were longer than 22 minutes, we observed a homeostatic increase in long-bout sleep, while seeing no such homeostatic response in short-bout sleep (Figure 5). Moreover, the homeostatic rebound of long bout-sleep was apparent on the very first day of recovery (Figures 5B, D) as opposed to the rebound seen following deprivation using shorter inactivity triggers (Figures 4A, D). This is probably due to the fact that long bout sleep deprivation engaged mechanical triggers only 12-14 times on average over the 24 hours of deprivation, and thereby likely eliminated most nonspecific effects of mechanical perturbation.

Yoked controls are critical for the examination of molecular and physiological correlates of sleep pressure.

A major goal of sleep science is to identify the molecular, cellular, and physiological mechanisms underlying the build-up of sleep pressure and how such pressure promotes sleep. As has been
recognized since the founding of sleep science (79), inescapable confounds attend sleep deprivation experiments. The increased activity caused by most forms of experimental sleep deprivation is likely to have myriad behavioral and physiological effects. Mechanical sleep deprivation through agitation, the most commonly used method of sleep deprivation in *Drosophila* research, is particularly problematic in this regard, as it appears to cause significant physical stress (40). Neurogenetic methods of sleep deprivation, wherein wake-promoting/sleep-preventing neurons are constitutively driven at high rates of firing, are also likely to produce both sleep-dependent and sleep-independent changes in flies (reviewed in (22)). A fundamental challenge for the sleep scientist working in the fly system therefore is to differentiate between the effects of increased sleep pressure from the sleep-independent effects of the stimuli used to prevent sleep. The use of yoked controls and longer duration inactivity triggers in our study appears to be a useful means of accounting for the sleep-independent effects of mechanical sleep disruption on behavior (Figures 3-5). We therefore wondered if this approach would allow us to differentiate molecular changes that were specific to sleep pressure from changes produced directly by the effects of mechanical perturbation.

Given the extent of disturbance necessary to keep flies awake during prolonged sleep deprivation, we predicted that a large number of molecules whose abundance increases in response to sleep deprivation would reflect responses to mechanical perturbation rather than sleep pressure driven increases. Indeed, when we compared the brains of sleep-deprived files to unperturbed controls, 129 molecules of distinct molecular weights were detected that showed a significant increase in abundance. However, when we employed yoked controls as an additional control to identify which molecular weights were higher in deprived flies compared to controls that had experienced
identical mechanical perturbation but significantly less sleep deprivation, we found only five distinct molecular weights whose abundance reflected sleep pressure (Figure 6). Thus, the majority of molecular changes in response to sleep deprivation were explained by the effects of mechanical disturbance and not by sleep pressure.

This result supports the general utility of yoked controls for confirming that identified molecules, genes, and cells mediate sleep homeostasis rather than the behavioral or physiological responses to mechanical perturbation. Such controls will be critical, in particular, for the vetting of physiological or cellular correlates of sleep pressure. More specifically, the small molecular screen we describe here supports the feasibility of using yoked controls to discover sleep substances within the fly brain, molecules whose abundance rises and falls with sleep pressure that are used by sleep control centers to exert homeostatic control of sleep.

The work described here provides both new insights into sleep homeostasis in flies and new methodologies that are likely to promote the ability to detect homeostatic sleep responses. Our work indicates that the mechanical perturbation most commonly used to deprive flies of sleep produces both behavioral and molecular changes that potentially mask the sleep-pressure-specific changes that underly homeostatic sleep control. The results of our work also reveal that long-bouts of sleep, which have been shown by several investigators to represent a deep sleep state (51, 59), are a more sensitive indicator of sleep pressure and rebound than the previously used unitary definition of sleep as five minutes or more of inactivity. Remarkably, when analysis is focused on longer (~30-min) bouts of inactivity, homeostatic control of sleep in the fly appears much more similar to that seen in mammals, in that a larger proportion sleep debt is repaid and is discharged.
over multiple sleep wake cycles. We predict that this larger magnitude of sleep rebound will be of
great utility to the field, increasing the dynamic range over which the effects of molecular and
physiological alterations can be examined and thereby increasing the likelihood of discovering
new molecular and cellular components of sleep homeostasis.

Methods

Fly stocks and Husbandry
Flies were reared on Corn Syrup/Soy media made by Archon Scientific (Durham, North Carolina)
under a 12h:12h light:dark (LD) cycle at 25 °C and 60-70% humidity. Male wild-type Canton-S
(CS; BDSC stock number: 64349) flies were used for all experiments.

Sleep assay and analysis
One- to three-day old male flies were collected in groups of 30 into Corn Syrup/Soy containing
vials and were subsequently isolated under CO₂ anesthesia and loaded into glass tubes [70 mm ×
5 mm × 3 mm (length × external diameter × internal diameter)] containing 5% sucrose and 2% agar
when they were five to seven days old. The loading was done at least 24-hour prior to the beginning
of behavioral experiments. Flies were allowed to adjust to the tubes for the remainder of the
loading day and no data from this day was subject to analysis, which began using data from the
next LD cycle. Locomotor activity was measured using two independent methodologies:  
*Drosophila* Activity Monitors (DAM, TriKinetics, Waltham, MA), and Ethoscopes built in our lab
based on resources provided by the Gilestro Lab (Imperial College London) (32).
The DAM system employs an infrared beam and a sensor for each tube and records beam crossing at the tube’s mid-point as a measure of locomotor activity. Beam crossings were recorded every minute and from these data, inactivity bouts of five minutes or more were used as the standard definition of sleep (15, 16). For Ethoscope experiments, continuous video tracking via infrared cameras were done for individual flies using Raspberry Pi (https://github.com/raspberrypi/documentation/). Ethoscopes quantified maximal velocity in mm/s in 10s epochs. The maximal velocity data was analyzed using a custom R package (available on request) to quantify sleep. Maximal velocities of <1 mm/s for any epoch were considered an instance of immobility (32), and sleep was then computed using the standard five-minute inactivity criterion, with immobility lasting 300 seconds (30 epochs) or more considered a bout of sleep. This criterion was subsequently adjusted to bias our analysis toward longer bouts of sleep.

Homeostatic sleep rebound analysis: Total sleep displayed by sleep-deprived flies on days one, two, and three following sleep deprivation (see below) were calculated across each 24-hour day, subtracted from the baseline sleep displayed on the day before deprivation. Normalization was carried out using the following formula [(FocalPostDeprivationSleep – FocalPreDeprivationSleep) – (UnperturbedPostDeprivationSleep – UnperturbedPreDeprivationSleep)]. Sleep debt accrued in sleep deprived flies was quantified by subtracting baseline 24-hour sleep on the day before deprivation from total sleep measured during the 24-hour deprivation window.

Mechanical Sleep deprivation
Mechanical deprivation was always carried out for 24 hours under 12/12 L:D cycles starting at ZT00 (lights-on) following one day of baseline sleep measurement. The temperature was kept constant at 25 °C with 60-70% humidity.

Mechanical sleep deprivation using DAM and mechanical shakers:

Shakers (Fisherbrand™ Analog MultiTube Vortexer, Catalog # 02-215-450) were used as previously described to sleep deprive animals in DAM monitors (80). DAM monitors housing 32 flies within glass tubes were shaken randomly for 2s every 20-seconds, 120-seconds, or 220-seconds across the entire day of deprivation. The Multitube Vortexer was set at a shaking intensity of four.

Inactivity dependent sleep deprivation using Ethoscopes:

Using the rotational module, we sleep deprived flies with a 220 second inactivity threshold. DAM tubes were rotated at ~420 rpm for one second every time an individual fly was inactive for 220 seconds.

Yoked-controlled mechanical deprivation in the Ethoscope:

The Ethoscope rotational module (32) was used to develop a yoked-controlled mechanical deprivation platform. Traditionally, the Ethoscope has been used to rotate single tubes only when the animal has been inactive for specific durations of time (as opposed to shaking randomly and without regard to behavioral state as is the case of vortexing DAMs). We altered the closed loop feedback control (see below) in the Ethoscope to rotate two tubes at the same time based on the inactivity state of only one of the two flies. The fly whose behavior determined the rotation of the
tubes is referred to here as the focal fly, whereas the paired fly that received identical time-matched rotations without regard to its behavioral state is called the yoked fly. This approach was adapted from one used for the sleep deprivation of rats (58). Two inactivity dependent rotational triggers were used in this study: 220 seconds, which prevented the focal fly from attaining the standard definition of sleep (five minutes of inactivity or more) and 1320 seconds, which deprived focal flies of inactivity durations of 22 minutes or more.

Development of Ethoscope code to Support Yoked-Controls:

Yoking was implemented at the level of the Ethoscope’s “TrackingUnit,” which is where the connection between the video-based motion tracker and the stimulator (i.e., tube rotator) is located [self._stimulator.bind_tracker(self._tracker)]. The Ethoscope’s program was modified so that the stimulator controlling the tubes containing yoked controls was bound to the tracker of their paired focal flies. This results in the stimulator of the yoked animals responding to the behavior of the focal animals at the same time as the stimulators of the focal flies. The code supporting yoked controls is available at https://github.com/antortjim/shaferlab-ethoscope with tag v1.0.999, which can be acquired via the Ethoscope repository (https://github.com/gilestrolab/ethoscope).

MALDI-TOF analysis

After 24-h of sleep deprivation using 220s immobility triggers for focal flies with their yoked and unperturbed controls, heads from all three conditions were embedded in Optimal Cutting Temperature (Tissue-Tek, Product code: 4583, Sakura Finetek USA Inc.) compound, frozen with liquid nitrogen, cryo-sectioned, and prepared and assayed by MALDI-TOF as previously described (63). 2,5-Dihydrobenzoc acid (Sigma-Aldrich, Cat #149357) was used as our matrix, which
supports the ionization of metabolites and peptides, and is considered a useful matrix for “universal analysis” of a diverse variety of molecular types (64) MALDI mass spectra were acquired in a Bruker Autoflex Speed TOF Machine (Bruker, Germany) and peaks were identified in SCiLS MALDI Imaging software (Bruker Daltonics, Germany) by a manual peak walk across the spectrum of m/z values between 0 and 1300 for each discernible m/z ratio for unperturbed, focal, and yoked flies.

**Statistical analysis**

In order to test the effect of trigger frequency and mechanical disturbance on the amount of rebound sleep, we used a two-way fixed factor ANOVA. We used trigger frequency as one fixed factor with three levels, i.e., 20s, 120s and 220s. The second fixed factor was treatment with two levels, i.e., mechanically disturbed fly and unperturbed controls. Whether the difference in total sleep post-deprivation between unperturbed controls and sleep deprived flies was dependent on trigger frequency was inferred based on the interaction effect between the two factors described above. In all other analyses reported throughout the manuscript, we either used the Mann-Whitney-U or the Kruskal-Wallis test, depending on the number of groups being compared. In all cases of more than two groups, multiple comparisons were done using a Bonferroni correction. In case of the MALDI-TOF experiment, owing to the large number of peaks detected, all the p-values from individual tests were treated to a Benjamini-Hochberg correction to adjust for inflated False Discovery Rates. All statistical analyses were done and figures made using custom R scripts. Specific tests, sample sizes and p-values are reported in the figure legends.
Acknowledgements

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Figure legends

**Figure 1.** Homeostatic sleep rebound is driven by the frequency of mechanical agitation when the amount of sleep deprivation is held constant. A. 24 hours of sleep deprivation was induced with three different mechanical trigger frequencies using vortexers: 2s randomized shaking every 20s, 120s, or 220s (each producing similar levels of sleep deprivation). B. The largest increases of normalized rebound sleep were observed in ZT0-3 when compared to undisturbed controls and were much lower for 220s frequencies compared to 120s and 20s triggers. C. Sleep rebound for long trigger (220s) disappears after normalization, which accounts for differences between controls and deprived flies in baseline sleep. Rebound is clear for the two shorter triggers, but is greatest for the 20s trigger condition. D. Short (20s) triggers produce immediate, persistent and statistically significant increases in sleep for both 6 hours post-deprivation as well as 24 hours post-deprivation analysis, but 120 second, and 220 second intervals do not. (6-hour ANOVA $F_{2,250}^{} = 3.56, p = 0.03$; 24-hour ANOVA $F_{2,250}^{} = 3.93, p = 0.02$). E. The amounts of sleep during the 24-h following deprivation result in very low levels of recovery relative to total sleep lost. Previously identified *Drosophila* (27) and Human sleep (39) recovery amounts have been marked with dashed lines. (Control$_{20\text{ sec}}^{}$ n = 32, Sleep-Deprived$_{20\text{ sec}}^{}$ n = 32; Control$_{120\text{ sec}}^{}$ n = 64, Sleep-Deprived$_{120\text{ sec}}^{}$ n = 64; Control$_{220\text{ sec}}^{}$ n = 64, Sleep-Deprived$_{220\text{ sec}}^{}$ n = 64). * < 0.05, and N.S. (Not Significant).

**Figure 2.** Accounting for sleep-depriving mechanical stimulation with Yoked-controls are necessary to observe 24-hour sleep rebound in low frequency mechanical trigger. A. Inactivity dependent sleep deprivation in Ethoscopes with 220s trigger fails to generate post-deprivation sleep rebound when compared with undisturbed controls. B. Focal flies do not show...
increases in total sleep compared to undisturbed flies on baseline day (Wilcoxon’s $W = 2523, p = 0.54$) and on post deprivation day (Wilcoxon’s $W = 2340, p = 0.87$). C. The Ethoscope platform was modified to include “yoked-controls” where inactivity dependent sleep depriving triggers for experimental (focal) flies are matched to their yoked control (as opposed to undisturbed controls, thereby generating paired flies with identical mechanical experience but significantly different amounts of sleep loss. D. Sleep and mechanical perturbation in an example of focal/yoked pair of flies: Sleep episodes for one baseline day and the day of deprivation are shown. Sleep episodes are indicated by red (focal) and blue (yoked) hash marks. Black hashmarks represent tube rotations triggered by the focal fly (Left panel). E. 10 focal-yoked paired flies on baseline, deprivation, and post-deprivation days shows how yoked flies (blue) were able to sleep during the deprivation cycle, whereas focal (red) flies lost all sleep (right panel). F. Average traces of focal and yoked flies 24-h before, during and after sleep deprivation. G. When compared to yoked flies that have undergone same levels of mechanical perturbation, focal flies show significant rebound post 24-h (Wilcoxon’s $W = 4074.5, p = 0.01$) while not showing any baseline differences (Wilcoxon’s $W = 3829, p = 0.12$). $n = 82$ each for focal and yoked categories, and $n = 58$ for unperturbed controls * $< 0.05$, and N.S. (Not Significant).

**Figure 3. Sleep pressure is discharged as cumulative sleep recovery across multiple cycles.**

A. Average sleep time-courses of baseline, deprivation (220 s inactivity dependent trigger), and 3 post deprivation days in focal and undisturbed flies, and B. focal-yoked pairs. C. Focal flies do not show significant differences in cumulative sleep gain across 3 post-deprivation days when compared to undisturbed controls (Post1: Wilcoxon’s $W = 2323.5, p = 0.81$; Post 2: Wilcoxon’s $W = 2351, p = 0.91$; Post 3: Wilcoxon’s $W = 2460.5, p = 0.72$). D. When compared to paired yoked
controls, focal animals showed significant increases in cumulative sleep across two cycles (Post1: Wilcoxon’s $W = 4088, p = 0.01$; Post 2: Wilcoxon’s $W = 4009, p = 0.03$). In the third post-deprivation day, even though the focal flies showed a tendency to have higher cumulative sleep gain, it was not statistically significant (Wilcoxon’s $W = 3932, p = 0.06$). All comparisons are made using the Wilcoxon rank sum test, and $n = 82$ each for focal and yoked categories. * < 0.05, and N.S. (Not Significant).

E. Compared to total sleep loss, focal flies do not pay back lost sleep. All focal data points are normalized to undisturbed controls (see methods). $n = 82$ each for focal and yoked categories, and $n = 58$ for unperturbed controls * < 0.05, and N.S. (Not Significant).

**Figure 4. Elimination of short bout sleep reveals a multi-cycle sleep rebound that discharges a significant proportion of lost sleep.**

A. Averaged sleep time-series of long-bout sleep (25 min) in baseline, deprivation (220s inactivity dependent trigger), and 3 post-deprivation windows in focal, yoked, and undisturbed flies. B. Sleep recovery as a function of sleep definition (minutes of inactivity) is visualized as a heatmap. When sleep is defined as progressively longer bouts of inactivity, deprived flies paid back a majority of their sleep debt cumulatively over three days. When the standard sleep definition is used, little to no sleep debt is repaid. C. In three post-deprivation days, focal flies showed trends of increased cumulative sleep recovery in 25-min sleep but were not significantly different from undisturbed controls (Post1: Wilcoxon’s $W = 2458, p = 0.73$; Post 2: Wilcoxon’s $W = 2689, p = 0.18$; Post 3: Wilcoxon’s $W = 2766, p = 0.10$). D. Accounting for mechanical perturbations resulted in significantly high multi-cycle total sleep in focal flies. Interestingly, the first cycle after deprivation did not show significant increases in 24-h, long bout sleep in focal flies (Post1: Wilcoxon’s $W = 3847, p = 0.11$; Post 2: Wilcoxon’s $W = 3987, p = 0.03$; Post 3: Wilcoxon’s $W = 4001, p = 0.03$). E. Normalized sleep rebound for sleep
bouts of 25-min or longer across three days shows the discharge of sleep debt accrued during the 24 hours of deprivation. Median recovery was more than 50 percent, while 25 percent of flies overshoot total sleep loss, thereby showing hallmarks of homeostatic recovery. n = 82 each for focal and yoked categories, and n = 58 for unperturbed controls * < 0.05, and N.S. (Not Significant).

**Figure 5. Deprivation of only long bout sleep is sufficient to produce homeostatic responses and elicit multiday sleep rebound.**  
A. 5-day somnogram of standard sleep in focal-yoked flies during baseline, long-bout sleep deprivation, and three days of recovery. Notice no discernable changes in the average sleep between experimental and control flies.  
B. 5-day somnogram of long-bout sleep during baseline, deprivation, and three post deprivation days in focal and yoked flies.  
C. In the three post-deprivation days there were no significant differences in standard sleep in focal and yoked flies (Post1: Wilcoxon’s W = 269, p = 0.06; Post 2: Wilcoxon’s W = 258, p = 0.12; Post 3: Wilcoxon’s W = 263, p = 0.09).  
D. Long bout sleep showed significant increases in the total amount over three post-deprivation cycles, compared to yoked controls (Post1: Wilcoxon’s W = 288, p = 0.01; Post 2: Wilcoxon’s W = 284, p = 0.02; Post 3: Wilcoxon’s W = 290, p = 0.01).  
E. Long bout sleep rebound showed over 25 percent of flies paying back total sleep debt and overshooting in three post SD cycles. Focal sleep debt designates total sleep debt, and the focal flies are normalized to undisturbed controls. n = 20 each for focal and yoked categories * < 0.05, and N.S. (Not Significant).

**Figure 6. Identifying sleep substances using MALDI-TOF.**  
A. Representative hematoxylin-eosin stained cryosection of a fly head.  
B. Distribution of 104.1 m/z and 175.1 m/z molecules in a
head section (left). Note the near zero relative intensity outside the brain region. Overlay of the high intensity signals on the actual cryosection used to derive the signal (right) highlights brain specific distribution. The 104.1 \( m/z \) molecule displays broader distribution across the brain whereas the 175.1 \( m/z \) appears to be concentrated in the central brain. The white dotted box represents the area of the central brain from which signal intensity has been quantified.

C. Post deprivation increases in the relative abundance of molecules in focal flies when mechanical perturbations are not accounted for finds 129 positive hits (out of 187 molecules- 68.9%). D. Search patterns of molecules tracking mechanical perturbation and of molecules tracking sleep pressure (left). Two representative molecules that track mechanical perturbation (\( m/z \) 104.1; Kruskal-Wallis \( \chi^2 = 7.71, p = 0.02; \) Focal = Yoked > Unperturbed) and sleep pressure (\( m/z \) 175.1; Kruskal-Wallis \( \chi^2 = 10.38, p = 0.005; \) Focal > Yoked > Unperturbed), respectively. Bars with the same letter are not statistically different from each other.

E. Identified 187 \( m/z \) peaks summarized in a pie chart representing the number molecules in various categories when yoking is employed. The number of positive hits is five (2.6%). Focal brain sections n = 6; Yoked brain sections n = 3; Unperturbed brain sections n = 4. Bars with the same letters are not significantly different from each other.

Supplementary Figure 1: A. Both total sleep and normalized rebound B. in 6-h post-release windows don’t show significant differences compared to undisturbed controls. C. 6-hour post deprivation windows for total sleep, as well as normalized sleep D. don’t show rebound signatures.

Supplementary Figure 2. A. Bout numbers of standard sleep between focal and yoked flies in three post-deprivation cycle did not show significant differences (Post1: Wilcoxon’s \( W = 3385, p \)
Post 2: Wilcoxon’s $W = 2962.5, p = 0.18$; Post 3: Wilcoxon’s $W = 3273, p = 0.77$). **B.** Bout duration of standard sleep across three post deprivation cycles show focal flies to be significantly higher for the first two recovery cycles (Post 1: Wilcoxon’s $W = 4077, p = 0.01$; Post 2: Wilcoxon’s $W = 4269, p = 0.002$; Post 3: Wilcoxon’s $W = 3624, p = 0.38$). $n = 82$ each for focal and yoked categories. Undisturbed control $n = 58 * < 0.05$, and N.S. (Not Significant).

**C.** Pre-deprivation bout numbers (Wilcoxon’s $W = 3358, p = 0.99$) and bout durations (Wilcoxon’s $W = 3705, p = 0.25$) were not significantly different between focal and yoked pairs). $n = 82$ each for focal and yoked categories, and $n = 58$ for unperturbed controls * $< 0.05$, and N.S. (Not Significant).

**Supplementary Figure 3.** **A.** Frequency distribution of 25-min sleep bouts in undisturbed flies collected from 5 cycles. Every fly shows at least one bout of sleep that is at least 25-min long. **B.** Total bout numbers in 25-min, long bout sleep across two post-deprivation cycles showed significant increases in focal flies compared to yoked flies (Post 1: Wilcoxon’s $W = 4029, p = 0.02$; Post 2: Wilcoxon’s $W = 4186.5, p = 0.006$; Post 3: Wilcoxon’s $W = 3779.5, p = 0.16$). **C.** Duration of long bouts are not significantly different between focal and yoked flies in three post-deprivation days (Post 1: Wilcoxon’s $W = 3422, p = 0.53$; Post 2: Wilcoxon’s $W = 3229.5, p = 0.66$; Post 3: Wilcoxon’s $W = 3888, p = 0.08$). $n = 82$ each for focal and yoked categories, and $n = 58$ for unperturbed controls * $< 0.05$, and N.S. (Not Significant).

**Supplementary Figure 4.** **A.** Long-sleep bouts prior to deprivation were not different between focal and yoked flies (Wilcoxon’s $W = 263, p = 0.09$). **B.** On post-deprivation day 1 focal flies showed significantly increased long bout numbers (Wilcoxon’s $W = 304, p = 0.004$). On second day even though a trend was seen, it was not significant (Wilcoxon’s $W = 244.5, p = 0.23$), before
returning to significant levels on post-deprivation day 3 (Wilcoxon’s $W = 295, p = 0.009$). C. Long bout duration on baseline days was not different between focal and yoked flies (Wilcoxon’s $W = 225.5, p = 0.32$). D. On three post derivation days, focal and yoked pairs did not show significant differences in long bout durations (Post1: Wilcoxon’s $W = 210.5, p = 0.57$; Post 2: Wilcoxon’s $W = 231.5, p = 0.24$; Post 3: Wilcoxon’s $W = 242.5, p = 0.14$). $n = 20$ each for focal and yoked categories. * $< 0.05$, and N.S. (Not Significant).
References


Figure 1.
Figure 2.
Figure 3.
Figure 4.
Figure 5.
Figure 6.