Homeostatic Control of Deep Sleep in *Drosophila*: Implications for Discovering Correlates of Sleep Pressure

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Abstract

Homeostatic control of sleep is typically addressed through mechanical stimulation-induced forced wakefulness and the measurement of subsequent increases in sleep. A major confound attends this approach: biological responses to deprivation may reflect a direct response to the mechanical insult rather than to the loss of sleep. Similar confounds accompany all forms of sleep deprivation and represent a major challenge to the field. Here we describe a new paradigm for sleep deprivation in *Drosophila* that fully accounts for sleep-independent effects. Our results reveal that deep sleep states are the primary target of homeostatic control and establish the presence of multi-cycle sleep rebound following deprivation. Furthermore, we establish that specific deprivation of deep sleep state results in state-specific homeostatic rebound. Finally, by accounting for the molecular effects of mechanical stimulation during deprivation experiments, we show that serotonin levels track sleep pressure in the fly's central brain. Our results illustrate the critical need to control for sleep-independent effects of deprivation when examining the molecular correlates of sleep pressure and call for a critical reassessment of work that has not accounted for such non-specific effects.

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

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 (Dijk and von Schantz, 2005; Holding et al., 2020; Krause et al., 2017; Lane et al., 2022; Meyer et al., 2022). Sleep disruption is widespread in the modern age and contributes significantly to a wide array of negative health consequences in humans (Colten et al., 2006; Medic et al., 2017). 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 (Campbell and Tobler, 1984; Freiberg, 2020; Kanaya et al., 2020; Keene and Duboue, 2018; Nath et al., 2017; Webb, 1974). 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 (Davis and Raizen, 2017).

The vinegar fly *Drosophila melanogaster* has proved to be a useful model organism for understanding the molecular and cellular mechanisms regulating sleep (Hendricks et al., 2000; Shaw et al., 2000). The powerful genetic tools available in flies, 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 (De Jesús-Olmo et al.,

2020; Donlea et al., 2014; Ni et al., 2019; Seidner et al., 2015). Furthermore, such work in the fly has revealed mechanisms that also regulate sleep in mammals (Liu et al., 2016; Shafer and Keene, 2021). Just as in humans and other mammalian species, fly sleep is controlled by both a homeostat and a circadian clock, which, during normal sleep cycles, largely account for the timing and amount of daily sleep (Abhilash and Shafer, 2022; Borbély, 1982; Borbély et al., 2016; Deboer, 2018). Future work in the fly will undoubtedly continue to enrich our understanding of sleep regulation in all animals.

Nevertheless, there are significant challenges to the investigation of sleep regulation in *Drosophila.* For example, the magnitude of sleep rebound in flies is quite modest (Huber et al., 2004; Klose and Shaw, 2021; Oh et al., 2014; Vaccaro et al., 2020). Indeed, some forms of optogenetic or thermogenetic sleep deprivation produce no apparent homeostatic sleep rebound at all (Seidner et al., 2015; Vaccaro et al., 2020). 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 (Hendricks et al., 2000; Huber et al., 2004; Melnattur et al., 2020; Shaw et al., 2000). 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-h deprivation windows) and this is usually detectable only during the first few hours of the day after deprivation has ended (Geissmann et al., 2019, 2017; Hendricks et al., 2000; Huber et al., 2004; Klose and Shaw, 2021). Furthermore, homeostatic sleep rebound in *Drosophila* appears to repay a relatively small proportion of the sleep lost during deprivation; 800 minutes of

83 sleep loss in a full diurnal cycle was followed by only 100 minutes of recovery sleep (~12.5%)
84 (Huber et al., 2004). In contrast, mammalian species tend to display higher amounts of increased
85 sleep following deprivation that is observable over more than one circadian cycle (Berger and
86 Oswald, 1962; Mistlberger et al., 1983; Nakazawa et al., 1978; Tobler et al., 1983; Williams et al.,
87 1964), and such rebound usually accounts for a much larger proportion of lost sleep than the
88 rebound detectable in flies. For example, humans can reclaim around 40% of the sleep lost to
89 deprivation (Stroemel-Scheder and Lautenbacher, 2021). The relatively small rebound measured
90 in the fly is a major challenge to the study of sleep regulation in this species, as it provides a small
91 dynamic range over which to detect changes in homeostatic responses in experimental subjects.
92 These features of fly sleep are somewhat surprising, given the highly conserved nature of sleep in
93 the animal kingdom.
94
95 One possible cause of the relatively small homeostatic responses to sleep deprivation in flies are
96 the sleep-independent effects of the deprivation techniques used by the field. The most commonly
97 used method of fly sleep deprivation is frequent mechanical perturbation, typically shaking or
98 slamming (Hendricks et al., 2000; Melnattur et al., 2020; Shaw et al., 2000), to force movement
99 and prevent the attainment of sleep, which in *Drosophila* is defined as any bout of inactivity that
100 is five minutes or longer (Hendricks et al., 2000; Shaw et al., 2000). Though effective for the
101 prevention of sleep, these methods are likely to produce many physiological and behavioral effects
102 that are independent of sleep loss. Indeed, mechanical shaking is known to produce the
103 biochemical hallmarks of stress, even when delivered during times of wakefulness (Harbison and
104 Sehgal, 2009).
105

106 Recently, neurogenetic methods have been employed to deprive flies of sleep (Dubowy et al.,
107 2016; Vaccaro et al., 2020). This method involves the strong and chronic excitation of wake-
108 promoting neurons in the brain. Though this method avoids the sleep-independent effects of
109 physical perturbation, the strong non-physiological excitation of central brain neurons is likely to
110 be attended by its own sleep-independent effects. A fundamental challenge, therefore, is to
111 differentiate sleep-pressure-driven changes in behavior and physiology from sleep-independent
112 changes driven by the manipulation used to prevent sleep. We predict that accounting for such
113 sleep-independent effects would improve the sensitivity with which homeostatic sleep responses
114 can be detected in the fly.
115
116 In mammals, sleep consists of multiple, physiologically discrete stages, which differentially
117 respond to sleep deprivation (Achermann et al., 1993; Berger and Oswald, 1962; Endo et al., 1997;
118 Everson et al., 1989; Loomis et al., 1937; Rechtschaffen et al., 1999; Takahashi et al., 1978).
119 Following long bouts of wakefulness during normal sleep cycles, slow-wave Non-Rapid-Eye-
120 Movement (NREM) sleep stages dominate the initial bouts of sleep, suggesting that the daily rise
121 of sleep pressure is discharged first by slow-wave (i.e., deep) sleep (Borbély and Achermann,
122 1999; Webb, 1974). Slow-wave sleep is also preferentially increased following sleep deprivation
123 when compared to Rapid-Eye-Movement (REM) sleep (Berger and Oswald, 1962; Borbély and
124 Neuhaus, 1979; Dijk et al., 1990; Williams et al., 1964). Thus, differentiating between distinct
125 sleep stages is likely important for assessing the homeostatic responses to sleep deprivation.
126
127 A growing body of evidence indicates the existence of a deep sleep state in flies, which is
128 characterized by distinct patterns of brain signaling (Nitz et al., 2002; van Alphen et al., 2013),

129 reduced metabolic rate (Stahl et al., 2017), and waste clearance from the brain (van Alphen et al.,
130 2021). The physiological and metabolic correlates of this deep sleep state are associated with bouts
131 of inactivity that are significantly longer than the five-min inactivity criterion used to define fly
132 sleep (Nitz et al., 2002; Stahl et al., 2017; van Alphen et al., 2021, 2013). Furthermore, recent
133 studies have suggested that periods of inactivity briefer than the 5-min criterion may represent an
134 "active sleep" state in the fly (Niki et al., 2023; Tainton-Heap et al., 2021). The existence of
135 multiple sleep states in the fly suggests that treating sleep as a unitary state in this species might
136 obscure homeostatic sleep responses, particularly if, as in mammals, specific states more strongly
137 linked to homeostatic sleep pressure than shallower stages of sleep. Furthermore, our recent
138 analyses suggest that longer bouts of sleep are a better reflection of sleep homeostasis than
139 traditional definitions of sleep in the fly (Abhilash and Shafer, 2022).
140
141 In this study we address the sleep-independent behavioral effects of the most commonly used form
142 of sleep deprivation in the fly and develop a method of accounting for them. We also attempt to
143 address the extent to which the treatment of sleep as a single unitary state in the fly might explain
144 the apparently modest and brief nature of sleep rebound in the fly compared to such rebound in
145 mammalian species. We present evidence that the extent of mechanical perturbation employed
146 during sleep deprivation has significant effects on the amount of subsequent sleep displayed by
147 deprived flies. We describe the development of a yoked-control paradigm for flies, based on
148 previous work in rats (Rechtschaffen et al., 1983), that allows us to produce two sets of flies that
149 have experienced identical levels of mechanical perturbation while suffering significantly different
150 amounts of sleep restriction. Furthermore, by differentiating between long-bouts of sleep from the
151 traditional unitary definition of sleep, we show that flies display significant and lasting homeostatic

152 sleep increases following sleep deprivation that are only detectable when controlling for the sleep-
153 independent effects of mechanical deprivation. Finally, we illustrate the importance of yoked
154 controls for examining the molecular correlates of sleep pressure and identify serotonin as a
155 molecule that is increased within the brain in proportion to sleep loss but not mechanical
156 perturbation. Our work introduces methodological approaches that are likely to support the
157 discovery of new mechanisms of sleep regulation in the fly, calls for the reevaluation of previous
158 work identifying the molecular, physiological, and cellular correlates of sleep pressure, and
159 suggests that serotonin may act as a sleep substance within the central brain.
160

Results

Sleep rebound is shaped significantly by the frequency 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 (Nollet et al., 2020). Traditionally, sleep-deprived flies, which have undergone forced wakefulness by means of mechanical or neurogenetic perturbation, are compared to unperturbed flies (e.g., Hendricks et al., 2000). 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. A further complication in assessing sleep rebound is the duration and time of day during which forced wakefulness stimuli are applied (Huber et al., 2004) as both levels of sleep pressure and diurnal/circadian changes in physiology can have complex interactions with the perturbing stimuli.

Though the minimum criterion for a sleep-like state in *Drosophila* is five continuous 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 (Geissmann et al., 2019; Hendricks et al., 2000; Shaw et al., 2000). Once the permissive window of sleep opportunity is open after the period of sleep deprivation, usually timed to the beginning of the day, sleep is measured across next few hours and the amount of sleep

184 displayed by sleep-deprived flies is compared to unperturbed controls (Beckwith et al., 2017;
185 Geissmann et al., 2019, 2017). The amount of baseline sleep displayed by sleep-deprived and
186 control flies before deprivation is also often accounted for in the assessment of rebound to control
187 for small but significant differences in baseline sleep that are often observed between experimental
188 and control flies, even when the two groups are genetically identical (Geissmann et al., 2019)
189 (Figure 1A). Given the substantial discrepancy between the inactivity duration criterion for sleep
190 (five minutes or more) and the frequency of mechanical stimulation used to deprive flies of sleep
191 (multiple times per minute) we first asked how rebound sleep might differ between flies that were
192 sleep deprived with different frequencies of mechanical stimulation. Therefore, we varied the
193 duration of mechanical deprivation to compare our results with previous deprivation studies, which
194 have employed a various durations of sleep deprivation (Huber et al., 2004; Nall and Sehgal, 2013;
195 Seidner et al., 2015).
196
197 As previously described in multiple studies by others using the *Drosophila* Activity Monitor
198 (DAM) system, flies deprived of sleep for 6-, 12-, and 24-h using two seconds of mechanical
199 stimulation delivered at random times within each 20-s interval displayed immediate increases in
200 total sleep compared to undisturbed flies (Figure 1A, D, G) (Cirelli et al., 2005; Hendricks et al.,
201 2000; Huber et al., 2004). In each of these instances a significant 24-h rebound was observed when
202 compared to undisturbed flies (Figure 1C, F, I). This reproducibility with previous studies
203 confirmed that our lab population of *Canton-S* flies display homeostatic sleep increases like those
204 described in previous studies (Hendricks et al., 2000; Huber et al., 2004; Melnattur et al., 2020;
205 Nall and Sehgal, 2013; Shafer and Keene, 2021; Shaw et al., 2000). To ask how the extent of
206 mechanical stimulation shapes homeostatic sleep rebound, we decreased the frequency of such

stimulation while maintaining the three durations (6-h, 12-h, and 24-h) of sleep deprivation, and ensuring effective prevention of sleep. In order to examine the effects of stimulation frequency on sleep rebound, we decreased the mechanical simulation frequency to once every 220 seconds. This low-frequency trigger produced sleep deprivation for all the deprivation durations tested (Figure 1B, E, H). Surprisingly, no sleep rebound was observed for 6-h, 12-h, or 24-h deprivation experiments, despite the fact that the lower frequency stimulations effectively deprived sleep (Figure 1C, F, I). Lowering the intensity of mechanical stimulation therefore appeared to eliminate sleep rebound, despite having prevented sleep during deprivation.

Examination of the magnitudes of sleep rebound following high frequency mechanical deprivation revealed that the 6-h deprivation protocol produced higher sleep rebound than did 12-h, and 24-h deprivation, despite the fact that this duration produced the smallest loss of sleep (Figure 1J). To further confirm the role of mechanical disturbance in recovery sleep assessment, we added an additional trigger frequency of random shaking for two seconds within every 120 second interval for 24-h of sleep deprivation. We compared the sleep rebound produced by 24-h of sleep deprivation with stimulation frequencies of 20-s, 120-s, and 220-s using a two-way ANOVA and found that there was a significant interaction effect of treatment × trigger frequency on the amount of sleep rebound (total sleep post-deprivation minus total sleep pre-deprivation). Remarkably, we found significant rebound only for the flies deprived with 20-s trigger frequencies (Figure 1K). It is also important to note that although the higher trigger frequency produced a significant rebound, the amount of recovered sleep was very small (Figure 1L).

229 Thus, lower frequencies of mechanical stimulation failed to produce significant sleep rebounds,
230 despite having produced similar extents of sleep deprivation. To address the extent to which our
231 sleep deprived flies recovered lost sleep, we estimated how much lost sleep was recovered across
232 the 24-h following the end of the deprivation window (Donlea et al., 2014; Shaw et al., 2002). We
233 found that, as expected from previous work in many labs, the flies deprived of sleep using the most
234 frequent mechanical stimulation (every 20s) displayed apparent homeostatic sleep increases in the
235 hours immediately following deprivation (Figure 1A, D, G). In contrast, flies that experienced less
236 frequent stimulation displayed little if any such apparent rebound (Figure 1B, E, H). The fact that
237 lower levels of mechanical stimulation failed to produce rebound over one full cycle, despite
238 having produced similar amounts of sleep deprivation, suggests that high frequency physical
239 perturbation produces sleep-independent behavioral effects that might mask *bona-fide* homeostatic
240 sleep increases. Alternatively, it is also possible that during lower stimulation frequencies, flies
241 may increase their levels of the recently hypothesized "active sleep" state (inactivity of between
242 one to five minutes in duration) (Niki et al., 2023; Tainton-Heap et al., 2021) and that this may
243 prevent the build-up of sleep pressure and rebound. We examined the levels of such sleep during
244 and after deprivation using 220-s stimulation frequencies and found that the flies do display
245 substantial increases in this active sleep state throughout deprivation using 220-s stimulation
246 frequencies (Supplementary Figure 1). However, given the strong convergent evidence that
247 periods of inactivity of five minutes or more represent a physiologically distinct sleep-like state, it
248 is surprising to see the absence of rebound following deprivation of this sleep state in our
249 experiments using 220-s stimulation frequencies and standard sleep definitions. The absence of
250 rebound in these experiments supports the hypothesis that the apparent homeostatic rebound of fly
251 sleep is strongly shaped by the frequency of mechanical stimulation. Accounting for the sleep-

252 independent effects of mechanical deprivation on sleep and examining the homeostatic control of
253 distinct sleep states are likely to reveal previously hidden characteristics of sleep homeostasis.
254
255 **Accounting for mechanical stimulus using a yoked-controlled design uncovers significant**
256 **sleep rebound in sleep-deprived flies when low-frequency triggers are used.**
257
258 The mechanical shaking employed to sleep deprived flies loaded into the DAM system use near
259 constant agitation without regard to the flies' sleep state, delivering mechanical stimulation to both
260 active and inactive flies. To further examine the potential sleep-independent effects of mechanical
261 perturbation during sleep deprivation experiments, we adopted the Ethoscope, a video-based
262 system for recording fly behavior (Geissmann et al., 2017). In contrast to the DAM system, which
263 requires the simultaneous mechanical perturbation of all flies within the same DAM monitor, the
264 Ethoscope allows the user to deliver mechanical stimuli to individual flies only when a fly is
265 inactive for a specified amount of time (Geissmann et al., 2017). The ability to track single flies
266 and selectively stimulate individual flies to prevent sleep means that flies can be sleep deprived
267 with significantly fewer mechanical perturbations, and only when flies have been inactive for
268 defined durations.
269
270 We programmed Ethoscopes to track single flies and rotate their tubes at ~420 rpm for one second
271 each time a fly had been inactive for 220 seconds, which resulted in 24-h of sleep deprivation when
272 using the standard inactivity criterion for fly sleep (5-min or more) (Figure 2A, C). For both
273 *Canton-S* and w^{1118}, when compared to undisturbed flies, the sleep-deprived flies failed to display
274 significant increases in sleep during the 24-h following deprivation (Figure 2B, D); a surprising

275 result given the clear effectiveness of sleep deprivation (Figure 2A, C), but consistent with the lack
276 of rebound in similar situations using the vortexer (see Figure 1). This result is also surprising on
277 another account – the use of ethoscopes to measure sleep does not overestimate and saturate sleep
278 as much as the IR-beam crossing based method in the DAMs (compare Figures 1 and 2), thereby
279 providing a larger dynamic range wherein rebound could have been observed.
280
281 The failure to produce clear homeostatic sleep increases following deprivation using a 220-s
282 inactivity criterion could be the product of two causes. As suggested by our observations above,
283 the mechanical insult experienced by flies during sleep deprivation are likely to exert sleep-
284 independent effects on the behavior observed after deprivation. That is, the mechanical disturbance
285 experienced by the sleep-deprived flies may produce effects on the fly that could obscure sleep-
286 pressure-driven behavioral changes. Second, the 220-s inactivity triggers used to deprive flies of
287 sleep may have allowed brief inactivity bouts recently hypothesized to represent an "active sleep"
288 state (Niki et al., 2023; Tainton-Heap et al., 2021) and such a state prevented the build-up of
289 sufficient sleep pressure to produce a homeostatic response. This would suggest that the five-min
290 inactivity criterion for sleep may be too long to capture all forms of homeostatically-controlled fly
291 sleep. To begin to address these two possibilities, we first considered the effects of mechanical
292 perturbation and therefore sought to produce flies that differed in the extent of their sleep
293 deprivation, despite experiencing identical mechanical stimulation. Comparing sleep in such flies
294 would allow us to examine the specific contribution of sleep pressure to sleep behavior following
295 deprivation.
296

297 To accomplish this, we adopted a paradigm of "yoked" sleep deprivation that was first introduced
298 in the rat model, which produces pairs of rats that have experienced perfectly matched mechanical
299 stimulation, in the form of a rotating platform over water, that nevertheless differed significantly
300 in the amount of sleep deprivation experienced (Rechtschaffen et al., 1983). This approach
301 involved a "focal" animal which was placed on one side of the platform and monitored via
302 electroencephalogram recording. Whenever the focal animal displayed the physiological
303 hallmarks of sleep, the platform would begin to rotate, thereby keeping the focal animal awake. A
304 paired rat, the "yoked" control, was placed on the opposite side of the platform and could sleep
305 when the focal rat was awake. This arrangement produced a pair of rats that experienced identical,
306 time-matched mechanical stimulation while experiencing significantly different amounts of sleep
307 loss (Rechtschaffen et al., 1983).
308
309 We programmed the Ethoscope platform to produce a paired yoked-control fly for every sleep-
310 deprived focal fly. We conducted these experiments for a 24-h period commencing at lights ON
311 to avoid the circadian confounds of mechanical stimulations given at specific times of the day or
312 night. For each fly that was tracked and stimulated upon being inactive for 220-s, a second fly
313 received matched, time-locked tube rotations, which were delivered independently of its
314 sleep/wake state. This ensured that each focal and yoked pair received identical mechanical stimuli
315 (Figure 2E). 220-s inactivity criteria were necessary to provide sufficient time for our yoked
316 controls to attain substantial amounts of sleep, as defined by the standard criterion of 5-min or
317 more of inactivity. Indeed, using the 220-s inactivity trigger allowed yoked flies to attain sleep
318 while the focal fly was active (Figure 2F and G). Yoked controls, therefore, suffered only partial
319 sleep loss compared to focal flies (Figure 2H and J). This approach, therefore, succeeded in

320 producing two sets of flies that had experienced the same level of physical perturbation while
321 suffering significantly different levels of sleep loss.
322
323 A comparison of total sleep across the entire 24-h of the post-deprivation day revealed significantly
324 higher levels of sleep in the focal flies compared to yoked controls for both *Canton-S* and w^{1118}
325 (Figure 2I and K). Thus, the inclusion of yoked controls revealed a homeostatic sleep response
326 that was not apparent when comparing sleep-deprived flies to unperturbed controls, supporting the
327 notion that sleep-independent effects of mechanical perturbation can mask homeostatic sleep
328 responses. Both *Canton-S* (Figure 2H and I) and w^{1118} (Figure 2J and K) strains displayed such
329 rebound, exhibiting increases in both daytime and nighttime sleep during the 24-h following
330 deprivation. Mechanical stimulation during deprivation significantly increased the activity levels
331 of sleep-deprived flies a day after mechanical perturbation, when compared to undisturbed
332 controls. This increase was seen immediately after the light-to-dark transition, i.e., at the time
333 associated with the transition to night-time sleep (Supplementary Figure 2A and B). This
334 difference was not observed when comparing focal flies with yoked controls (Supplementary
335 Figure 2C and D). Though yoked controls revealed significant sleep-pressure specific increases in
336 sleep, this homeostatic increase was quite modest, resulting in very little discharge of the sleep
337 pressure built during the 24-h of sleep deprivation (Figure 2I and K). Given that most animals
338 display homeostatic sleep increases over multiple sleep/wake cycles (Mistlberger et al., 1983;
339 Nakazawa et al., 1978; Rechtschaffen et al., 1999), we wondered if the rebound we detected using
340 yoked controls might persist over multiple diurnal cycles.
341

342 **Sleep rebound accumulates over multiple cycles in response to infrequent mechanical**
343 **deprivation, but little sleep debt is paid off.**
344
345 The circadian control of sleep thresholds ensures that sleep is gated to occur at the
346 appropriate/adaptive time of day, even in the presence of increased sleep drive. The action of the
347 circadian system therefore prevents sleep debt from being discharged completely upon the first
348 opportunity to sleep, ensuring that wakefulness will occur during the next diurnal cycle despite the
349 presence of homeostatic sleep pressure. For this reason, sleep debt is typically discharged over
350 several sleep/wake cycles (Mistlberger et al., 1983; Nakazawa et al., 1978; Rechtschaffen et al.,
351 1999). Current methods in the *Drosophila* sleep field do not typically produce multi-cycle rebound,
352 but instead, reveal fairly modest sleep rebound during the first hours of the day-time following
353 deprivation (Beckwith et al., 2017; Geissmann et al., 2019, 2017). In our yoked-controlled
354 experiment, we observed only modest sleep rebound that discharged very little of the sleep lost
355 during the previous day's deprivation, which may have been a product of the smaller but significant
356 deprivation experienced by yoked controls. However, in this case, sleep rebound was apparent
357 across the diurnal cycle, rather than being limited to the first few hours of the recovery day (Figure
358 2H and J). We wondered if the comparison of deprived flies with yoked controls might reveal
359 persistent increases in sleep for deprived flies across subsequent sleep/wake cycles. We therefore
360 assessed the behavior of flies deprived of sleep using the Ethoscope for three full diurnal cycles
361 following 24-h of sleep deprivation (Figure 3A and B) and compared the sleep of deprived flies
362 with sleep observed in both undisturbed and paired yoked controls.
363

364 When compared to yoked controls, focal sleep-deprived flies displayed modest but significant
365 increases in cumulative sleep over two successive cycles (Figure 3D). Once again, these increases
366 were not detectable when deprived flies were compared to undisturbed controls (Figure 3C). In
367 this case, baseline sleep on the days before deprivation was not different between the three groups
368 of flies (see Figure 2B and G). We examined how much lost sleep was recovered across the three
369 post-deprivation cycles by quantifying sleep gain in focal flies, which we calculated as the increase
370 in total post-deprivation sleep compared to baseline sleep and normalized to sleep in undisturbed
371 controls to account for normal changes in sleep during the duration of the experiment (see
372 methods). Despite this apparent two-cycle rebound, focal flies failed to discharge a substantial
373 portion of the sleep debt accrued during deprivation (Figure 3E). This result is surprising because
374 when we estimated p(Doze), a recently introduced conditional probability metric for sleep pressure
375 (Wiggin et al., 2020), we found that the focal flies show sustained increases in sleep pressure for
376 at least three days post sleep deprivation (Supplementary Figure 3).
377
378 We wondered if the apparent absence of substantial homeostatic payback of sleep lost by focal
379 flies during deprivation might be explained by the fact that current sleep analysis methods do not
380 recognize distinct sleep states, treating sleep as a unitary state in which all bouts of inactivity of
381 five minutes or more are considered to be the same sleep state. Given the growing evidence of
382 physiologically and metabolically distinct sleep states in the fly (Stahl et al., 2017; van Alphen et
383 al., 2021, 2013) and the fact that deep sleep stages are most strongly and immediately affected by
384 sleep deprivation in mammals, we turned our attention to how the architecture of sleep might differ
385 between focally deprived flies and their yoked controls across the three days following deprivation.
386 Though the number of sleep bouts was not significantly different between focal and yoked flies

387 across any of the three post-deprivation cycles (Supplementary Figure 4A), there was a significant
388 increase in bout duration in the focal flies compared to yoked controls across the first two days
389 following deprivation (Supplementary Figure 4B). These two groups of flies displayed no
390 differences in these metrics during baseline sleep before deprivation (Supplementary Figure 4C).
391 This result suggested that focal flies, which had higher sleep pressures than yoked controls,
392 displayed increased sleep bout durations.
393
394 **The magnitude of homeostatic sleep rebound in the fly is masked by short-bout sleep.**
395
396 Recent work in our lab revealed that long-bout sleep (~30 minutes or more of inactivity) is a better
397 reflection of sleep homeostat action than shorter bouts of inactivity (Abhilash and Shafer, 2022).
398 Furthermore, such long-bout sleep has been shown by others to reflect a deep sleep stage that is
399 physiologically and metabolically distinct from shorter bouts of sleep (Stahl et al., 2017; van
400 Alphen et al., 2021, 2013). We therefore hypothesized that the extent of sleep rebound and payback
401 of lost sleep might be more readily apparent if we focused our analysis on long-bout sleep alone,
402 eliminating the contribution of shorter, presumably shallower, bouts of sleep.
403
404 We reanalyzed the 24-h sleep deprivation experiment reported in Figure 3, iteratively varying the
405 inactivity duration criterion for sleep from one to 30-min, a range, which includes "active sleep"
406 (defined as any bout of inactivity lasting one to five minutes), to ask how varying the sleep
407 definition might shape the apparent homeostatic recovery of lost sleep. We found that inactivity
408 criteria of 25-min or more resulted in the largest payback of lost sleep when examined on the third
409 day of recovery from deprivation (Figure 4B). This suggested that longer bouts of sleep are most

strongly influenced by homeostatic sleep regulation and that sleep bouts lasting one to 24 minutes are characterized by substantially lower rebound following deprivation. We found no evidence of post-deprivation increases in brief, "active sleep" (periods of inactivity lasting between one and five minutes). This was not surprising given our finding that 220-s inactivity triggers produce increases in such active sleep bouts during deprivation (Supplemental Figure 1). When we employed an inactivity criterion of 25-min, a duration that likely reflects a relatively deep sleep state (Stahl et al., 2017) sleep recovery approached ~50% of lost sleep by the third day of recovery, a much higher proportion than was apparent using currently employed unitary definition of sleep as bouts of inactivity lasting 5-min or more.

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 the longer bouts of sleep that likely correspond to a deep sleep state and omitting shorter, presumably shallower, bouts of inactivity from our analysis. Five-day long bout sleep time-series for focal, yoked, and undisturbed flies are shown in Figure 4A. Unperturbed controls displayed two daily peaks of long bout sleep, with approximately twice as much deep sleep taking place at night, as previously described (Hendricks et al., 2000; Stahl et al., 2017). Focal flies were, as expected from 220-s inactivity triggers, completely deprived of sleep bouts consisting of 25-min or more. Yoked flies also, as expected, displayed significantly reduced levels of such long bout sleep compared to unperturbed controls, but such sleep was in fact attained by these controls, though at levels significantly reduced from those displayed by unperturbed controls (Figure 4A).

432 When total long bout sleep across three successive cycles was quantified, focal flies displayed
433 significant increases in such sleep when compared only to yoked controls on days two and three
434 of recovery (Figure 4C and D). The absence of a difference with yoked flies on day one is likely a
435 product of the substantial reduction in long-bout sleep that yoked flies experienced during the
436 deprivation period. Nevertheless, focal and yoked flies revealed differential long-bout sleep
437 rebounds across three days of recovery. Remarkably, this rebound was not seen when focal flies
438 were compared to unperturbed controls (Figure 4C). Once again, this was likely due to the sleep-
439 pressure-independent effects of mechanical disturbance. To confirm that frequent 25-min bouts of
440 inactivity were not an abnormal occurrence induced by the Ethoscope (i.e., that this state was not
441 induced by the rotations delivered by the Ethoscopes), we confirmed that such long bouts of sleep
442 are frequently observed in unperturbed control flies. Indeed, all unperturbed control flies routinely
443 displayed 25-min or longer bouts of inactivity (Supplementary Figure 5A). The sleep architecture
444 of focal flies after sleep deprivation also changed significantly compared to yoked controls where
445 instances of longer and consolidated sleep bouts were significantly increased in the focal flies,
446 although long bout sleep durations were not significantly different (Supplementary Figure 5B, C).
447
448 These results suggested that longer bouts of sleep are a more sensitive reflection of
449 homeostatically-controlled sleep in flies. This conclusion is supported by the significant recovery
450 of lost long bout sleep that continues across all three days of the observed recovery period (Figure
451 4E). Thus, when the analysis focused on long and presumably deep sleep states, a much larger
452 percentage such sleep is recovered compared to a consideration of all epochs of inactivity lasting
453 longer than one minute. At least half of all sleep-deprived flies paid back more than 50% of the
454 sleep they lost during the 24-h of deprivation, and over 25 percent of flies overshot their sleep-

455 debt, thereby displaying a defining hallmark of homeostatic control (Figure 4E). These results lend
456 further support for the idea that long bouts of inactivity represent a sleep state that is distinct from
457 shorter bout sleep (Stahl et al., 2017; Tainton-Heap et al., 2021; van Alphen et al., 2013).
458 Furthermore, robust homeostatic recovery of long-bout suggests that our initial failure to detect
459 rebound when 220-s inactivity triggers were used for deprivation (see Figure 1) was not simply
460 due to increases in "active sleep" preventing the build-up of homeostatic sleep drive
461 (Supplementary Figure 1), as long bout sleep displays clear homeostatic rebound even when 220-
462 s triggers allow for substantial levels of "active sleep" during deprivation.
463
464 **Long Bout Sleep is Under Potent Homeostatic Control.**
465
466 In mammals, both REM and NREM stages of sleep are controlled homeostatically, with stage-
467 specific deprivation leading to stage-specific rebound (Borbély, 1982; Borbély and Neuhaus, 1979;
468 Endo et al., 1998, 1997). If long-bout sleep indeed reflects a distinct sleep stage in *Drosophila*,
469 selectively depriving flies of it should produce a homeostatic rebound in long-bout sleep, despite
470 the presence of abundant short-bout sleep. To test this prediction, we set the Ethoscope's
471 inactivity-dependent trigger to rotate tubes only when focal flies had been immobile for 22-min as
472 a means to prevent flies from attaining 25-min of uninterrupted inactivity. When using the standard
473 inactivity duration criterion for sleep (5-min or more), this long trigger latency supported what
474 appeared to be largely normal sleep rhythms in both focal and yoked flies (Figure 5A).
475 Unsurprisingly, normal sleep patterns were also observed in unperturbed flies (data not shown).
476

477 Employing a 25-min inactivity duration criterion to remove short bout sleep from our analysis
478 revealed that 22-min triggers effectively deprived flies of long-duration sleep while permitting
479 yoked flies to achieve appreciable amounts of such sleep during focal deprivation (Figure 5B).
480 Unperturbed control flies once again displayed the bimodal long-bout sleep rhythms described
481 above, with substantially more nighttime than daytime deep sleep (Supplementary Figure 6). When
482 behavior was analyzed using the standard five-min inactivity criterion, which would include short-
483 bout sleep, focal flies displayed no significant increases in total sleep when compared to yoked
484 controls (Figure 5C). In contrast, when short-bout sleep was eliminated from the analysis through
485 the use of a 25-min inactivity criterion, focal flies displayed significantly higher levels of long-
486 bout sleep across all three days following deprivation (Figure 5D). Remarkably, the relative levels
487 of post-deprivation sleep displayed by the focal flies appeared to increase over the course of the
488 three days of observation, suggesting that homeostatic responses to long-bout sleep deprivation
489 may persist for an extended period of time (Figure 5D). This rebound produced a significant
490 recovery of lost sleep (Figure 5E), one that may have continued to build over additional sleep/wake
491 cycles. No such patterns were observed when focal flies were compared to unperturbed controls
492 (data not shown), thereby once again highlighting the importance of yoked controls.
493
494 Long-bout-specific deprivation also produced significant effects on sleep architecture. Focal flies
495 were characterized by an increased number of bouts that were longer than 25-mins compared to
496 yoked controls (Supplementary Figure 7B), although the durations of these longer sleep bouts did
497 not increase (Supplementary Figure 7D). No differences in the number of long-bout sleep between
498 focal and yoked flies were observed prior to the deprivation cycle (Supplementary Figure 7A). Our
499 results suggest that long-bout sleep, previously shown by others to represent a relatively deep sleep

500 state, is under strong homeostatic control and highlights the importance of controlling for the sleep-
501 independent effects of mechanical sleep deprivation on behavior.
502
503 **Yoked controls are necessary to differentiate effects of sleep deprivation from effects driven**
504 **by mechanical perturbation.**
505
506 A major goal of sleep science is to identify the molecular, cellular, and physiological processes
507 mediating the rise and fall of sleep pressure and how these changes operate in the brain to promote
508 sleep or wakefulness. In the fly, previous work by others has described cellular (Blum et al., 2021;
509 Donlea et al., 2014; Liu et al., 2016) and physiological (Pimentel et al., 2016; Tainton-Heap et al.,
510 2021) correlates of sleep pressure, based on the effects of mechanical sleep deprivation. Given our
511 behavioral results indicating significant sleep-independent effects of mechanical deprivation that
512 appear to mask homeostatic sleep responses, we sought to examine the potential utility of yoking
513 for differentiating sleep-pressure driven changes from the sleep-independent effects of mechanical
514 perturbation. Toward this end we conducted a simple molecular screen in the fly brain using
515 matrix-assisted laser desorption/ionization – time-of-flight (MALDI-TOF) mass spectrometry
516 (Veerasammy et al., 2020). This method supports the mass spectrometric analysis of fly head
517 sections (Figure 6A; Salisbury et al., 2013) and consists of three major steps: the crystallization of
518 a matrix and analytes within the tissue section, the laser ionization of analytes within this matrix,
519 and time-of-flight mass spectrometry (MS) to analyze the molecules present (Veerasammy et al.,
520 2020). This method provides sufficient spatial resolution to detect the presence of molecules
521 defined by their weight over charge ratios (m/z), specifically within the central brain (Figure 6B).
522 Using our behavioral sleep deprivation approach (Figure 2E-G), we examined the molecular

523 responses within the central brains of focally deprived flies, paired yoked controls, and
524 unperturbed flies following 24-h of sleep deprivation using 220-s inactivity triggers.
525
526 Using 2,5-dihydrobenzoic acid (DHB) as a positive-ion matrix, which is considered useful for
527 "universal analysis" and the detection of diverse molecular types (Snovida et al., 2006), we
528 sampled m/z value peaks of up to 1,300 and detected 188 distinct peaks (in the central brain regions
529 of *Drosophila* head sections. Each m/z ratio peak measurement was made specifically for the
530 central brain by registering the MALDI-TOF signal directly over the histological image of our
531 tissue sections (dashed square, Figure 6B). When we compared the heights of these peaks between
532 focal, sleep-deprived flies and unperturbed controls, we found 127 peaks that displayed
533 statistically significantly higher amplitudes in sections from focal flies compared to those of
534 unperturbed flies (Figure 6C). Thus, when compared only to unperturbed flies, sleep deprivation
535 in focal flies was accompanied by increases in a large proportion of the molecules examined.
536 However, it is highly likely that the mechanical stimulation associated with sleep deprivation
537 causes many of these molecular changes within the brain and such increases might not track sleep
538 pressure. We therefore asked the extent to which controlling for mechanical insult using yoked
539 controls might allow us to differentiate increases explained by sleep pressure from those that were
540 simply produced by mechanical stimulation.
541
542 If a molecule's abundance is increased simply as consequence of mechanical insult during sleep
543 deprivation, we would expect head sections from both focal and yoked flies to display similar
544 concentrations of that molecule that are elevated compared to unperturbed controls (Figure 6D,
545 left-top). In contrast, if a molecule's abundance is increased specifically as consequence of sleep

546 pressure, we predict that it will be present at relatively low levels in sections from unperturbed
547 controls, significantly higher levels in sections from focal flies, and with intermediate levels in
548 yoked controls that are statistically distinguishable from the other two conditions (Figure 6D, left-
549 bottom). Example m/z peaks that show these characteristic patterns in the central brain are shown
550 in Figure 6D (right; m/z values of 104.1 and 553). When yoked controls are included in the
551 analysis, approximately half of the discernable m/z value peaks displayed statistically
552 indistinguishable levels in the central brain across the undisturbed, yoked, and focal samples
553 (Figure 6E, "N.S.", 45%). Seventy-three of the m/z peaks displayed the relative abundances
554 expected of molecules tracking mechanical perturbation (38.8%), whereas only eight displayed the
555 relative abundances expected of molecules tracking sleep pressure (~4%, Figure 6E). Thus, the
556 standard method of comparing sleep-deprived flies to unperturbed controls produced 127
557 candidate sleep-tracking molecules in two replicate experiments, whereas the inclusion of yoked
558 controls produced only eight candidates. Thus, many of molecular differences between sleep-
559 deprived flies and unperturbed controls were likely explained as responses to mechanical
560 stimulation rather than to the loss of sleep.
561
562 We replicated this molecular screen in an independent MALDI-TOF experiment. This run detected
563 slightly fewer m/z peaks (170 compared to 188 in the first replicate). Despite the markedly lower
564 sensitivity of this replicate, we found that two of the eight molecules identified in the first screen
565 as tracking sleep pressure, once again displayed the relative abundances expected of a sleep
566 pressure tracking molecule. One of these had an m/z peak at ~553, and the other at 177.1 m/z in
567 the DHB matrix. The 177.1 m/z peak has previously been shown to correspond to serotonin (Cao
568 et al., 2019; Moriarty et al., 2011) and fits the molecular weight range of serotonin (176.2) in a

569 positive ion matrix like DHB, which adds a cation with a single positive charge to the m/z peak,
570 which for serotonin would result in a peak at ~177 m/z. Furthermore, we confirmed that this m/z
571 value reflected serotonin by spotting serotonin on slides imaged by MALDI-TOF MS and
572 confirmed strong peaks at m/z = 177 for regions of interest corresponding to these spots
573 (Supplementary Figure 8). Thus, serotonin would appear to be a strong candidate for a molecule
574 whose abundance reflects sleep pressure in the fly's central brain (see Discussion), that follows
575 the expected pattern across unperturbed, yoked and focal flies (Figure 6F). An unknown molecule
576 with an m/z value of ~553 is likewise also a strong candidate for another such molecule.
577

578 # Discussion

579

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

582

583 In order to study the sleep homeostat, one must prevent sleep through the imposition of prolonged
584 wakefulness (Bentivoglio and Grassi-Zucconi, 1997; Henri, 1895; Patrick and Gilbert, 1896;
585 Rechtschaffen et al., 1983). However, the very act of sleep disruption is expected to cause not just
586 the build-up of sleep pressure, but also other potentially confounding responses to the stimulus
587 used to prevent sleep. The specific confounds attending sleep deprivation will depend on the
588 method, duration, and time-of-day of the sleep-depriving stimulus employed, and all forms of
589 experimental sleep deprivation are expected to produce such confounds, including methods based
590 on chemo-, thermo-, or opto-genetics. Any sleep deprivation method that produces increased
591 locomotion will increase muscular activity, which will be attended by sleep-independent effects.
592 Chronic mechanical agitation also produces stress (Meerlo et al., 2008; Sgoifo et al., 2006; Tartar
593 et al., 2009), and even gentle handling of rodent subjects, a means of deprivation thought to cause
594 little stress, has significant effects on feeding behavior following deprivation (Đukanović et al.,
595 2022). Such confounding, sleep-independent effects can mask homeostatic sleep responses and
596 must therefore be accounted for whenever possible. Here we have attempted to account for the
597 sleep-independent effects of mechanical sleep deprivation in *Drosophila,* the most commonly used
598 method in the field of fly sleep research.

599

600 To assess the degree to which the frequency of mechanical agitation affects subsequent sleep
601 rebound in *Drosophila*, we examined flies that were sleep deprived for 24-h with different
602 frequencies of mechanical perturbation. The less frequent agitation produced significantly less
603 sleep rebound, despite having produced similar amounts of sleep loss. Thus, a sleep-pressure
604 independent effect of mechanical perturbation appeared to be the primary determinant of post-
605 deprivation sleep. An alternative explanation for this result was that short bouts of inactivity may
606 have provided brief but restorative sleep that the longer perturbation frequencies were not effective
607 at depriving. Indeed, 220-s inactivity triggers produced increases in brief (between one and five
608 minute) periods of inactivity during deprivation (Supplementary Figure 1). Such "active sleep" is
609 proposed to be a distinct sleep state from the standard sleep traditionally measured in flies (Niki et
610 al., 2023; Tainton-Heap et al., 2021). However, based on our estimates of p(Doze), a metric of
611 sleep pressure (Supplementary Figure 3), which is persistently high for three cycles post sleep
612 deprivation, we do not think that this "active sleep" prevented homeostatic sleep drive, at least in
613 this context. Furthermore, based on a large body of work conducted on humans and other
614 mammals, we would not necessarily expect increases in one sleep state to prevent the homeostatic
615 regulation of another.
616
617 Though it is possible that increases in the amount of brief "active sleep" bouts may have reduced
618 the build-up of sleep pressure during deprivation experiments employing 220-s inactivity triggers,
619 our results strongly support the conclusion that mechanical stimulation produces sleep-
620 independent effects that can mask homeostatic sleep rebound. Six, 12-, and 24-h of nighttime sleep
621 deprivation using frequent (20-s) triggers failed to produce the expected relationship between the
622 amount of sleep lost and the magnitude of rebound during the recovery day (Figure 1J). The failure

623 of rebound to track the amount of sleep loss indicates that the comparison of sleep deprived flies
624 and unperturbed controls is not sufficiently sensitive to detect the differential sleep rebounds
625 expected from flies that have suffered significantly different levels of sleep loss (Figure 1).
626
627 A previous attempt to control for the sleep-independent effects of mechanical perturbation
628 delivered mechanical stimuli matched to sleep deprived flies, but delivered during periods of high
629 activity (i.e., wakefulness), thereby exposing the control flies to mechanical shaking while
630 resulting in negligible amounts of sleep loss (Harbison and Sehgal, 2009). However, mechanical
631 shaking only during times of wakefulness increased molecular signatures of stress, even when
632 given for relatively short periods (Harbison and Sehgal, 2009). To minimize the extent of
633 mechanical perturbation necessary to prevent sleep and to control for the sleep-independent effects
634 of such perturbation, we adopted the Ethoscope, a video based system that tracks single flies and
635 delivers a mechanical stimulus, the rapid rotation of the glass tube containing the fly, only when a
636 fly meets a user-defined inactivity duration criterion (Geissmann et al., 2017). Thus, rather than
637 agitating all the flies of the sleep-deprived condition at a given frequency throughout the entire
638 deprivation period, the Ethoscope prevents single flies from attaining specific durations of
639 inactivity, just before meeting a user defined duration. Previous work using this system has
640 revealed remarkably low levels of homeostatic rebound and no sleep deprivation-associated death
641 (Geissmann et al., 2019).
642
643 Though the Ethoscope produces sleep deprivation using fewer mechanical perturbations, it
644 nevertheless delivers significant mechanical perturbation whose sleep-independent effects might
645 mask homeostatic sleep responses (Figure 2A-D). For this reason, we introduced yoked controls

646 to the Ethoscope platform, allowing us for the first time to disentangle effects associated with sleep
647 pressure from those best explained by the mechanical insult delivered during sleep deprivation
648 (Figure 2E-G). When flies were prevented from attaining five-min bouts of inactivity - the standard
649 criteria for a sleeplike state in the fly – using 220-s inactivity triggers in Ethoscopes, they failed to
650 show significant homeostatic rebound when their sleep was compared to unperturbed controls.
651 Comparison of these deprived flies to their yoked controls, in contrast, revealed a significant, albeit
652 small, increase in sleep following deprivation (Figure 2F, G). Thus, yoking revealed homeostatic
653 rebound that was not apparent when deprived flies were compared to controls that had not
654 experienced mechanical stimulation. However, when compared to yoked controls, the homeostatic
655 response of sleep deprived flies was quite small, amounting to very little recovery of lost sleep
656 (Figure 3E).
657
658 **Sleep as a non-unitary state in *Drosophila*: Long-bout sleep is a distinctly regulated sleep**
659 **stage.**
660
661 In mammals, sleep is not a unitary state but rather consists of distinguishable sleep stages (Davis
662 et al., 1937; Loomis et al., 1937). For example, REM and NREM sleep represent distinct
663 physiological states that differ in their relationship to sleep pressure and the circadian system
664 (Borbély, 1982; Borbély and Neuhaus, 1979; Czeisler et al., 1980; Endo et al., 1997). Slow-wave
665 NREM sleep appears to be the mammalian sleep state most strongly controlled by the daily
666 homeostatic control of sleep: initial sleep cycles following prolonged wakefulness are rich in slow
667 wave sleep, and this form of sleep is also prevalent following sleep deprivation (Berger and
668 Oswald, 1962; Ursin, 1971). For the majority of studies using *Drosophila*, sleep has been treated

669 as unitary state, with all inactivity bouts of five minutes or more being treated as sleep. However,
670 a growing body of works suggests the existence of distinct sleep stages in the fly. For example, in
671 one of the very first papers describing sleep-like states in *Drosophila*, Hendricks et al., reported
672 differences in durations of sleep bouts consisting of epochs of inactivity lasting five minutes or
673 more (Hendricks et al., 2000). The longest bout lengths occurred at the beginning of the subjective
674 night under constant conditions between circadian time 13.5 and 16. More recently, quantitative
675 approaches using Hidden Markov models have suggested the existence of light and deep sleep
676 states in the fly (Wiggin et al., 2020; Xu et al., 2021).
677
678 Flies display both longer and deeper bouts of sleep at night (Hendricks et al., 2000; van Alphen et
679 al., 2013) and deeper sleep is associated with distinct patterns of global brain activity when
680 compared to brain activity during wakefulness or during more brief and shallow bouts sleep (Nitz
681 et al., 2002; Tainton-Heap et al., 2021). Longer sleep bouts are also attended by lower metabolic
682 rates, where bout lengths of 60-min were shown to be associated with the lowest rates (Stahl et al.,
683 2017), a phenomenon similar to that observed for deeper NREM sleep stages in humans (Berger
684 and Phillips, 1995). Finally, flies display a stereotyped pattern of proboscis movements that appear
685 to drive waste clearance (van Alphen et al., 2021), reminiscent of the changes in fluid dynamics
686 observed in the cerebral spinal fluid of sleeping mammals, that occur at times corresponding to
687 deep sleep stages (Fultz et al., 2019). The time of peak occurrence of these proboscis movements
688 were characterized by a significant reduction of brain wave activity compared to sleep at other
689 times (van Alphen et al., 2021).
690

691 Physiological and metabolic signatures of deep sleep in the fly both suggest that they correspond
692 to sleep bouts that are significantly longer than the standard sleep definition of five minutes of
693 inactivity and multiple lines of evidence suggest that deeper sleep states are likely reached upon
694 attaining 15-30 minutes of inactivity (Tainton-Heap et al., 2021; van Alphen et al., 2013). Finally,
695 recent work from our lab has suggested that bouts of inactivity of between 30 and 60 minute are
696 stronger reflections of the daily homeostatic control sleep than shorter bouts of sleep (Abhilash
697 and Shafer, 2022). How might such long bouts of inactivity relate to homoeostatic sleep rebound?
698
699 Homeostatic sleep rebound, as traditionally measured in flies, appears to differ from mammalian
700 sleep rebound, in that it is relatively modest in magnitude and appears to largely run to completion
701 during the first few hours following the offset of deprivation (Huber et al., 2004). In contrast,
702 mammalian rebound appears to play-out over several sleep-wake cycles (Coborn et al., 2019;
703 Mistlberger et al., 1983; Nakazawa et al., 1978; Rechtschaffen et al., 1999). Remarkably, when we
704 biased our analysis to bouts of inactivity lasting 25-min or more, clear and prolonged homeostatic
705 rebound of sleep was observed following deprivation. This rebound lasted several days (Figure 4)
706 and discharged a large proportion (>50%) of the long-bout sleep debt suffered during deprivation
707 (compare Figures 3E to 4E). This result further supports the notion that long durations of inactivity
708 more sensitively reflect the action of the sleep homeostat.
709
710 When we deprived flies only of inactivity bouts that were longer than 22 minutes (see methods), a
711 duration that we chose based on its effectiveness at reliably preventing 25-min epochs of inactivity,
712 we observed a homeostatic increase in long-bout sleep, while seeing no such homeostatic response
713 in short-bout sleep (Figure 5). Moreover, the homeostatic rebound of long bout-sleep was apparent

714 on the very first day of recovery (Figure 5B, D) as opposed to the rebound seen following
715 deprivation using shorter inactivity triggers (Figure 4A, D). This is probably due to the fact that
716 long bout sleep deprivation engaged mechanical triggers only 12-14 times on average over the 24
717 hours of deprivation, and thereby likely reduced significantly the sleep-independent effects of
718 mechanical perturbation.
719
720 **Yoked controls are critical for the examination of molecular and physiological correlates of**
721 **sleep pressure.**
722
723 A major goal of sleep science is to identify the molecular, cellular, and physiological mechanisms
724 underlying the build-up of sleep pressure and how such pressure promotes sleep. As has been
725 recognized since the founding of sleep science (Kleitman, 1923, 1939), inescapable confounds
726 attend sleep deprivation experiments. The increased activity caused by most forms of experimental
727 sleep deprivation is likely to have myriad behavioral, physiological, and metabolic effects.
728 Mechanical sleep deprivation through vortexing, the most commonly used method of sleep
729 deprivation in *Drosophila* research, is particularly problematic in this regard, as it appears to cause
730 significant physical stress (Harbison and Sehgal, 2009).
731
732 The sleep-independent effects of deprivation protocols represent a significant confound and pose
733 a major challenge to the identification of the molecular and physiological correlates of sleep
734 pressure. If a molecular or physiological response to deprivation truly represents an increase in
735 sleep pressure, it must be established that it is not simply a response to the stimuli used to prevent
736 sleep. A fundamental challenge for the sleep scientist working in the fly system therefore is to

737 differentiate between the effects of increased sleep pressure from the sleep-independent effects of
738 the stimuli used to prevent sleep. The use of yoked controls and longer duration inactivity triggers
739 in our study appears to be a useful means of accounting for the sleep-independent effects of
740 mechanical sleep disruption on behavior (Figures 2-5). We therefore wondered if this approach
741 would allow us to differentiate molecular changes that were specific to sleep pressure from changes
742 produced directly by the effects of mechanical perturbation.
743
744 Given the extent of disturbance necessary to keep flies awake during prolonged sleep deprivation,
745 we predicted that a large number of molecules whose abundance increases in response to sleep
746 deprivation would reflect responses to mechanical stimulation rather than sleep pressure driven
747 increases. Indeed, when we compared the brains of sleep-deprived files to unperturbed controls,
748 around 70% of all detected molecules showed a significant increase in abundance. However, when
749 we employed yoked controls as an additional control to identify which molecular weights were
750 higher in deprived flies compared to controls that had experienced identical mechanical
751 perturbation but significantly less sleep deprivation, we found that only approximately four percent
752 of detectable molecules reflected sleep pressure (Figure 6). Thus, many of the molecular changes
753 in response to sleep deprivation can likely be explained by the effects of mechanical disturbance
754 and not by sleep pressure. This result highlights the importance of yoked controls for confirming
755 that specific molecules, genes, and cells mediate sleep homeostasis rather than the behavioral or
756 physiological responses to prolonged mechanical stimulation. Such controls will be critical for the
757 vetting of physiological or cellular correlates of sleep pressure. More specifically, the limited
758 molecular screen we describe here supports the feasibility of using yoked controls to discover sleep

759 substances within the fly brain, molecules whose abundance rises and falls with sleep pressure that
760 are used by sleep control centers to exert homeostatic control of sleep (Borbély, 1986).
761
762 **Serotonin is a candidate sleep substance in the *Drosophila* central brain.**
763
764 The inclusion of yoked controls in our experiments revealed that very few of the molecular changes
765 produced by mechanical sleep deprivation tracked sleep loss. One molecule that reliably tracked
766 sleep pressure in independent experiments was serotonin, which, based on our results, can be
767 considered a strong candidate sleep substance in the fly's central brain. A previous study using
768 HPLC to examine serotonin levels in dissected brains failed to observe serotonin increases in sleep
769 deprived flies (Davla et al., 2020). However, the spatial resolution provided by MALDI-TOF
770 imaging suggests that sleep loss driven serotonin increases take place in the central brain and not
771 the optic lobes (e.g., Figure 6B, F). We therefore suspect that serotonin does not build-up
772 throughout the optic lobes and that whole-brain assays might therefore fail to detect the sleep loss
773 induced changes we describe here. The identification of serotonin as a candidate sleep substance
774 is notable, as convergent evidence in the fly strongly supports the conclusions that serotonin
775 signaling promotes sleep in *Drosophila*.
776
777 The identification of serotonin as a candidate sleep substance is notable, as convergent evidence
778 in the fly strongly supports the conclusion that serotonin signaling promotes sleep in *Drosophila*.
779 Pharmacological increases in serotonin and the excitation of serotonergic neurons produce
780 increases in baseline sleep in the fly (Haynes et al., 2015; Pooryasin and Fiala, 2015; Yuan et al.,
781 2006). Similarly, blocking synaptic transmission in single pairs of serotonergic neurons have been

782 reported to reduce baseline sleep (Alekseyenko et al., 2014). Genetic loss of serotonin synthesis
783 decreases baseline sleep, as does the loss of 5HT-R-1A and 5HT-R-2B serotonin receptors (Qian
784 et al., 2017; Yuan et al., 2006). Loss of serotonin and the serotonin receptor 5HT-R-2B also results
785 in a reduction in sleep rebound following deprivation (Qian et al., 2017). Furthermore, the genetic
786 loss of arylalkylamine N-acetyltransferase (ANAAT), which normally functions to inactivate
787 serotonin and dopamine, is accompanied by both increased homeostatic sleep rebound and brain
788 serotonin levels following sleep deprivation, consistent with serotonin acting as a sleep substance
789 during mechanical deprivation (Davla et al., 2020). Finally, loss of the serotonin transporter
790 dSERT, which normally functions to remove extracellular serotonin, is associated with significant
791 increases in sleep (Knapp et al., 2022). Thus, convergent evidence supports the conclusion that
792 serotonin promotes both basal sleep and homeostatic increases in sleep following deprivation.
793
794 According to the two-process model of sleep regulation, basal seep and homeostatic increases in
795 sleep are driven by "process S," which is thought to be mediated by somnogens, molecules that
796 rise and fall with sleep pressure (Borbély, 1986, 1982; Borbély and Achermann, 1999). Our
797 MALDI results, in conjunction with previous work establishing a sleep-promoting role for
798 serotonin in the fly, suggest that serotonin levels build in the central brain during sleep deprivation
799 and increase the pressure to sleep through actions of serotonin receptors. Remarkably, this very
800 model was proposed by Jouvet to reconcile conflicting results regarding serotonin's role in the
801 control of mammalian sleep (Jouvet, 1999). A significant body of work has established that
802 increasing serotonin in the mammalian brain produces sleep increases and that the loss of serotonin
803 results in insomnia. These observations were the basis of the serotonergic theory of sleep (Jouvet,
804 1999). Serotonergic neurons were subsequently shown to be active during wakefulness and this

805 was taken as evidence against the hypothesis that serotonin promotes sleep (Jouvet, 1999).
806 However, neurons producing a sleep substance would be expected to be active during wakefulness
807 and to thereby promote sleep pressure during prolonged bouts of wakefulness. Serotonin neurons
808 were hypothesized to act in this way by Jouvet in order to reconcile the wake-active nature of
809 serotonin neurons with the serotonergic theory of sleep (Jouvet, 1999). Our work strongly
810 implicates serotonergic signaling as a component of process S in the *Drosophila* brain and sets the
811 stage for future work to test the predictions of the serotonergic theory of sleep in the fly.
812
813 **Conclusion**
814
815 The work described here provides both new insights into sleep homeostasis in flies and new
816 methodologies that are likely to improve our ability to detect homeostatic sleep responses and
817 identify molecular correlates of sleep pressure in this species. Our work indicates that the
818 mechanical perturbation most commonly used to deprive flies of sleep produces both behavioral
819 and molecular changes that potentially mask the sleep-pressure-specific changes that underly
820 homeostatic sleep control. The results of our work also reveal that long-bouts of sleep, which have
821 been shown by several studies to represent a deep sleep state (Tainton-Heap et al., 2021; van
822 Alphen et al., 2013), are a more sensitive indicator of sleep pressure and rebound than the
823 previously used unitary definition of sleep as five minutes or more of inactivity. Remarkably, when
824 analysis is focused on longer (~30-min) bouts of inactivity, homeostatic control of sleep in the fly
825 appears much more similar to that seen in mammals, in that a larger proportion sleep debt is repaid
826 and is discharged over multiple sleep wake cycles. We predict that this larger magnitude of sleep
827 rebound will be of great utility to the field, increasing the dynamic range over which the effects of

828 molecular and physiological alterations can be examined and thereby increasing the likelihood of

829 discovering new molecular and cellular components of sleep homeostasis.

830

831 # Methods

832 **Fly stocks and Husbandry**

833 Flies were reared on Corn Syrup/Soy media made by Archon Scientific (Durham, North Carolina)
834 under a 12h:12h light:dark (LD) cycle at 25 °C and 60-70% humidity. Male wild-type *Canton-S*
835 (*CS*; BDSC stock number: 64349) and w^{1118} (BDSC stock number: 3605) flies were used for the
836 experiments.

837

838 **Sleep assay and analysis**

839 One- to three-day old male flies were collected in groups of 30 into Corn Syrup/Soy containing
840 vials and were subsequently isolated under CO_2 anesthesia and loaded into glass tubes [70 mm ×
841 5mm × 3 mm (length × external diameter × internal diameter)] containing 5% sucrose and 2% agar
842 when they were five to seven days old. The loading was done at least 24-h prior to the beginning
843 of behavioral experiments. Flies were allowed to adjust to the tubes for the remainder of the
844 loading day and no data from this day was subject to analysis, which began using data from the
845 next LD cycle. Locomotor activity was measured using two independent methodologies:
846 *Drosophila* Activity Monitors (DAM, TriKinetics, Waltham, MA), and Ethoscopes built in our lab
847 based on resources provided by the Gilestro Lab (Imperial College London) (Geissmann et al.,
848 2017).

849

850 The DAM system employs a single infrared beam and a sensor for each tube and records beam
851 crossing at the tube's mid-point as a measure of locomotor activity. Beam crossings were recorded
852 every 20-s and from these data, inactivity bouts of five minutes or more were used as the standard
853 definition of sleep (Hendricks et al., 2000; Shaw et al., 2000). For Ethoscope experiments,

854 continuous video tracking via infrared cameras were done for individual flies using Raspberry Pi
855 (https://github.com/raspberrypi/documentation/). Ethoscopes quantified maximal velocity in
856 mm/s in 10-s epochs. The maximal velocity data was analyzed using a custom R package (available
857 on request) to quantify sleep. Maximal velocities of <1 mm/s for any epoch were considered an
858 instance of immobility (Geissmann et al., 2017), and sleep was then computed using the standard
859 five-min inactivity criterion, with immobility lasting 300 seconds (30 epochs) or more considered
860 a bout of sleep. This criterion was subsequently adjusted to bias our analysis toward longer bouts
861 of sleep.
862
863 *Homeostatic sleep rebound analysis:* Total sleep displayed by sleep-deprived flies on days one,
864 two, and three following sleep deprivation (see below) were calculated across each 24-h day,
865 subtracted from the baseline sleep displayed on the day before deprivation. Normalization was
866 carried out using the following formula $[(\text{Focal}_{\text{PostDeprivationSleep}} - \text{Focal}_{\text{PreDeprivationSleep}}) -$
867 $(\text{Unperturbed}_{\text{PostDeprivationSleep}} - \text{Unperturbed}_{\text{PreDeprivationSleep}})]$. Sleep debt accrued in sleep deprived
868 flies was quantified by subtracting baseline 24-h sleep on the day before deprivation from total
869 sleep measured during the 24-h deprivation window.
870
871 **Mechanical Sleep deprivation**
872 24-h mechanical deprivation was carried under 12/12 L:D cycles with mechanical deprivation
873 starting at ZT00 (lights-on) following one day of baseline sleep measurement. 12-h deprivation
874 commenced at lights-off (ZT12) following one day of baseline sleep measurement. 6-h
875 deprivation commenced at ZT 18 (six hours after lights-off) following one day of baseline sleep
876 measurement. The temperature was kept constant at 25 °C with 60-70% humidity.

877

878 *Mechanical sleep deprivation using DAM and mechanical shakers:*

879 Vortexers (Fisherbrand™ Analog MultiTube Vortexer, Catalog # 02-215-450) were used as
880 previously described to sleep deprive animals in DAM monitors (Kayser et al., 2015). DAM
881 monitors housing 32 flies within glass tubes were shaken randomly for 2-s every 20-s, 120-s, or
882 220-s across the entire period of deprivation. The Multitube Vortexer was set at a shaking intensity
883 of four.

884

885 *Inactivity dependent sleep deprivation using Ethoscopes:*

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

888

889 *Yoked-controlled mechanical deprivation in the Ethoscope:*

890 The Ethoscope rotational module(Geissmann et al., 2017) was used to develop a yoked-controlled
891 mechanical deprivation platform. The Ethoscope has been used previously to rotate single tubes
892 only when the animal has been inactive for specific durations of time (as opposed to shaking
893 randomly and without regard to behavioral state as is the case of vortexing DAMs). We altered the
894 closed loop feedback control (see below) in the Ethoscope to rotate two tubes at the same time
895 based on the inactivity state of only one of the two flies. The fly whose behavior determined the
896 rotation of the tubes is referred to here as the focal fly, whereas the paired fly that received identical
897 time-matched rotations without regard to its behavioral state is called the yoked fly. This approach
898 was adapted from one used for the sleep deprivation of rats (Rechtschaffen et al., 1983). Two
899 inactivity dependent rotational triggers were used in this study: 220 seconds, which prevented the

900 focal fly from attaining the standard definition of sleep (five minutes of inactivity or more) and
901 1320 seconds, which deprived focal flies of inactivity durations of 22 minutes or more. Due to
902 logistical issues relating to video-based feedback, the inactivity duration was set to 22 minutes –
903 this was to make sure that no bouts of inactivity that are longer than 25 minutes are allowed to
904 occur.
905
906 *Development of Ethoscope code to Support Yoked-Controls:*
907 Yoking was implemented at the level of the Ethoscope's "TrackingUnit," which is where the
908 connection between the video-based motion tracker and the stimulator (i.e., tube rotator) is
909 located [self._stimulator.bind_tracker(self._tracker)]. The Ethoscope's program was modified so
910 that the stimulator controlling the tubes containing yoked controls was bound to the tracker of
911 their paired focal flies. This results in the stimulator of the yoked animals responding to the
912 behavior of the focal animals at the same time as the stimulators of the focal flies. The code
913 supporting yoked controls is available at https://github.com/shaliulab/shaferlab-ethoscope (a fork
914 of https://github.com/gilestrolab/ethoscope) with tag v1.0.999, which can be acquired via pip
915 with the command pip install shaferlab-ethoscope.
916
917 **MALDI-TOF analysis**
918 After 24-h of sleep deprivation using 220-s immobility triggers for focal flies with their yoked and
919 unperturbed controls, heads from all three conditions were embedded in Optimal Cutting
920 Temperature (Tissue-Tek, Product code: 4583, Sakura Finetek USA Inc.) compound, frozen with
921 liquid nitrogen, cryo-sectioned, and prepared and assayed by MALDI-TOF as previously described
922 (Veerasammy et al., 2020). 2,5-Dihydrobenzoc acid (Sigma-Aldrich, Cat #149357) was used as

923 our matrix, which supports the ionization of metabolites and peptides, and is considered a useful
924 matrix for "universal analysis" of a diverse variety of molecular types (Snovida et al., 2006)
925 MALDI mass spectra were acquired in a Bruker Autoflex Speed TOF Machine (Bruker, Germany)
926 and peaks were identified in SCiLS MALDI Imaging software (Bruker Daltonics, Germany) by a
927 manual peak walk across the spectrum of m/z values between 0 and 1300 for each discernible m/z
928 ratio for unperturbed, focal, and yoked flies.
929
930 **Statistical analysis**
931 In order to test the effect of trigger frequency and mechanical disturbance on the amount of
932 rebound sleep, we used a two-way fixed factor ANOVA. We used trigger frequency as one fixed
933 factor with three levels, i.e., 20-s, 120-s and 220-s. The second fixed factor was treatment with two
934 levels, i.e., mechanically disturbed fly and unperturbed controls. Whether the difference in total
935 sleep post-deprivation between unperturbed controls and sleep deprived flies was dependent on
936 trigger frequency was inferred based on the interaction effect between the two factors described
937 above. In all other analyses reported throughout the manuscript, we either used the Mann Whitney-
938 U (also referred to as the Wilcoxon's Rank Sum test) or the Kruskal-Wallis test, depending on the
939 number of groups being compared. In all cases involving more than two groups, multiple
940 comparisons were done using a Bonferroni correction. In case of the MALDI-TOF experiment,
941 owing to the large number of peaks detected, all the *p*-values from individual tests were treated to
942 a Benjamini-Hochberg correction to adjust for inflated False Discovery Rates. All statistical
943 analyses were done and figures made using custom R scripts. Specific tests, sample sizes and *p*-
944 values are reported in the figure legends.
945

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956 # Figure legends

957

958 **Figure 1. Homeostatic sleep rebound is driven by the frequency of mechanical agitation when**
959 **the amount of sleep deprivation is held constant. A-B.** 6-h of sleep deprivation was induced
960 with two different mechanical trigger frequencies using vortexers: 2-s randomized shaking every
961 20-s or 220-s (each producing similar levels of sleep deprivation). **C.** A significant increase in
962 sleep was observed for the 20-s trigger, but the 220-s trigger failed to show such increases when
963 compared with undisturbed controls (*Kruskal-Wallis critical value* = 40.43, *df* = 2, $p = 1.66 \times 10^{-}$
964 ^{9}, followed by Bonferroni corrections for pairwise comparisons). **D-E.** Time course of 12-h of
965 nighttime sleep deprivation using two trigger frequencies (20-s, 220-s) results in nighttime sleep
966 loss. **F**. Sleep rebound using for 20-s trigger produced significant increases compared to controls,
967 but we did not observe any significant changes when the 220-s trigger was used (*Kruskal-Wallis*
968 *critical value* = 24.59, *df* = 2, $p = 4.5 \times 10^{-6}$, followed by Bonferroni corrections for pairwise
969 comparisons). **G-H.** We increased the vortexing induced sleep deprivation to 24-h using 20-s and
970 220-s trigger frequencies and calculated sleep rebound for 24 hours post deprivation window
971 represented in the time course. **I.** Once again, the 20-s trigger produced significant sleep rebound
972 compared to control flies whereas the 220-s trigger induced deprivation did not, even though both
973 trigger frequencies effectively deprived sleep (*Kruskal-Wallis critical value* = 23.63, *df* = 2, $p =$
974 7.41×10^{-6}, followed by Bonferroni corrections for pairwise comparisons). **J.** We compared the
975 amount of sleep rebound during the first recovery day caused by different durations of deprivation
976 using 20-s (left) and 220-s (right) inactivity triggers and found significant rebound only for the 20-
977 s trigger. However, despite producing significantly different amounts of sleep loss, we observed
978 no increases in rebound between associated with increased duration of deprivation. Surprisingly,

979 the 6-h deprivation condition, which produced the least amount of sleep loss displayed
980 significantly higher rebound sleep compared to the 12-h, and 24-h deprivation conditions (20-s
981 trigger: *Kruskal-Wallis critical value* = 13.71, *df* = 2, *p* = 0.0011, followed by Bonferroni
982 corrections for pairwise comparisons; 220-s trigger: *Kruskal-Wallis critical value* = 1.96, *df* = 2, *p*
983 = 0.37, followed by Bonferroni corrections for pairwise comparisons). **K.** We included an
984 intermediate trigger frequency of 120-s for sleep deprivation and found that like the 220-s trigger,
985 it failed to produce statistically significant increases in sleep for 24 hours post-deprivation analysis
986 (ANOVA $F_{2,250}$ = 3.93, *p* = 0.02). **L.** Sleep levels observed during the 24-h following deprivation
987 reveal that flies recover very little of total sleep lost. Previously measured *Drosophila* (Huber et
988 al., 2004) and Human sleep (Stroemel-Scheder and Lautenbacher, 2021) recovery amounts are
989 indicated by dashed lines. 6-H SD Control n = 44, Sleep-Deprived$_{20\ sec}$ n = 62, Sleep-Deprived$_{220}$
990 $_{sec}$ n = 57; 12-H SD Control n = 32, Sleep-Deprived$_{20\ sec}$ n = 64, Sleep-Deprived$_{220\ sec}$ n = 64; 24-
991 H SD Control n = 64, Sleep-Deprived$_{20\ sec}$ n = 32, Sleep-Deprived$_{220\ sec}$ n = 64, Sleep-Deprived$_{120}$
992 $_{sec}$ n = 64; * < 0.05, and N.S. (Not Significant).
993
994 **Figure 2. Controlling for mechanical stimulation is necessary to observe 24-h sleep rebound**
995 **when low frequency mechanical triggers are used for deprivation. A.** Inactivity dependent
996 sleep deprivation in Ethoscopes with 220-s inactivity triggers fails to generate post deprivation
997 sleep rebound in wild-type (*Canton-S*) flies when compared with undisturbed controls. **B.** Focal
998 flies do not show differences in total sleep compared to undisturbed flies across the baseline day
999 (Wilcoxon's *W* = 2523, *p* = 0.54) or across the first post deprivation day (Wilcoxon's *W* = 2340,
1000 *p* = 0.87). **C.** w^{1118} flies also failed to display post-deprivation sleep rebound compared to
1001 undisturbed controls. **D.** Comparison of total sleep on the day before and after sleep deprivation

1002 for w^{1118} flies. There were no significant differences in sleep amount on the baseline day (Baseline
1003 sleep: Wilcoxon's W = 351, p = 0.07) or on the post deprivation day (Post deprivation sleep:
1004 Wilcoxon's W = 519, p = 0.71). **E.** The Ethoscope platform was modified to include "yoked-
1005 controls" where inactivity dependent sleep depriving triggers for experimental (focal) flies are
1006 matched to their yoked controls, thereby generating paired flies with identical mechanical
1007 experience but significantly different amounts of sleep loss. **F.** Sleep and mechanical perturbation
1008 in an example of a focal/yoked pair of flies: Sleep episodes for one baseline day and the day of
1009 deprivation are shown. Sleep episodes are indicated by red (focal) and blue (yoked) hash marks.
1010 Black hashmarks represent tube rotations triggered by the focal fly (Left panel). **G.** Averaged sleep
1011 profiles for ten focal-yoked paired flies on baseline, deprivation, and post-deprivation days shows
1012 how yoked flies (blue) were able to sleep during the deprivation cycle, whereas focal (red) flies
1013 lost all sleep. **H.** Average traces of focal and yoked flies 24-h before, during and after sleep
1014 deprivation for wild-type *Canton-S* flies. **I**. When compared to yoked flies that have undergone
1015 same levels of mechanical perturbation, focal flies increased sleep after 24-h deprivation
1016 (Wilcoxon's W = 4074.5, p = 0.01) while not showing any baseline differences on the day before
1017 deprivation (Wilcoxon's W = 3829, p = 0.12). **J.** To confirm reproducibility of yoking we used the
1018 w^{1118} line, a common genetic background, to replicate the sleep rebound finding observed for
1019 *Canton-S*. **K.** Compared to yoked flies that have undergone same levels of mechanical
1020 perturbation, focal flies show increased sleep following 24-h deprivation (Post deprivation sleep:
1021 Wilcoxon's W = 277, p = 0.037) while not showing any baseline differences on the deay before
1022 deprivation (Baseline sleep: Wilcoxon's W = 176, p = 0.53). For *Canton-S*, n = 82 each for focal
1023 and yoked categories, and n = 58 for unperturbed controls. For w^{1118}, n = 20 for focal and yoked
1024 categories, and n = 49 for unperturbed controls * < 0.05, and N.S. (Not Significant).

1025

1026 **Figure 3. Sleep pressure is discharged as cumulative sleep recovery across multiple cycles.**
1027 **A.** Average sleep time-courses of baseline, deprivation (220-s inactivity dependent trigger), and 3
1028 post deprivation days in focal and undisturbed flies, and **B.** focal-yoked pairs for the same days
1029 shown in A. **C.** Focal flies do not show significant differences in cumulative sleep gain across 3
1030 post-deprivation days when compared to undisturbed controls (Post1: Wilcoxon's $W = 2323.5$, p
1031 $= 0.81$; Post 2: Wilcoxon's $W = 2351$, $p = 0.91$; Post 3: Wilcoxon's $W = 2460.5$, $p = 0.72$). **D**.
1032 When compared to paired yoked controls, focal animals showed significant increases in
1033 cumulative sleep across two cycles (Post1: Wilcoxon's $W = 4088$, $p = 0.01$; Post 2: Wilcoxon's W
1034 $= 4009$, $p = 0.03$). In the third post-deprivation day, even though the focal flies showed a tendency
1035 to have higher cumulative sleep gain, this was not statistically significant (Wilcoxon's $W = 3932$,
1036 $p = 0.06$). All comparisons are made using the Wilcoxon rank sum test, and $n = 82$ each for focal
1037 and yoked categories. * < 0.05, and N.S. (Not Significant). **E.** Compared to total sleep lost, focal
1038 flies do not pay back a significant amount of lost sleep, perhaps owing to normalization with
1039 unperturbed controls (see methods). $n = 82$ each for focal and yoked categories, and n = 58 for
1040 unperturbed controls * < 0.05, and N.S. (Not Significant).

1041

1042 **Figure 4. Examination of long-bout sleep reveals a multi-cycle sleep rebound that discharges**
1043 **a significant proportion of lost sleep. A.** Averaged sleep time-series of long-bout sleep (25-min
1044 inactivity criterion) in baseline, deprivation (220-s inactivity dependent trigger), and 3 post-
1045 deprivation windows in focal, yoked, and undisturbed flies. **B.** Sleep recovery as a function of
1046 sleep definition (minutes of inactivity) visualized as a heatmap. When sleep is defined as
1047 progressively longer bouts of inactivity, deprived flies paid back a majority of their sleep debt

1048 cumulatively over three days as the inactivity criterion approaches 25-min. When either the
1049 standard sleep definition (5 min or more of inactivity) or active sleep criteria (1- to 5-min) are
1050 used, little to no sleep debt is repaid. **C.** In three post-deprivation days, focal flies showed trends
1051 of increased cumulative sleep recovery in 25-min sleep but were not significantly different from
1052 undisturbed controls (Post1: Wilcoxon's $W = 2458$, $p = 0.73$; Post 2: Wilcoxon's $W = 2689$, $p =$
1053 0.18; Post 3: Wilcoxon's $W = 2766$, $p = 0.10$). **D.** Accounting for mechanical stimulation resulted
1054 in significant and substantial multi-cycle total sleep increases for focal flies. Interestingly, the first
1055 cycle after deprivation did not show significant increases in long bout sleep in focal flies (Post1:
1056 Wilcoxon's $W = 3847$, $p = 0.11$; Post 2: Wilcoxon's $W = 3987$, $p = 0.03$; Post 3: Wilcoxon's $W =$
1057 4001, $p = 0.03$). **E.** Normalized sleep rebound for sleep bouts of 25-min or longer across three days
1058 shows the discharge of sleep debt accrued during the 24 hours of deprivation. Median recovery
1059 was more than 50 percent, while 25 percent of flies overshoot total sleep loss, thereby showing
1060 hallmarks of homeostatic control. $n = 82$ each for focal and yoked categories, and $n = 58$ for
1061 unperturbed controls * < 0.05, and N.S. (Not Significant).
1062
1063 **Figure 5. Deprivation of only long bout sleep is sufficient to produce homeostatic responses**
1064 **and elicit multiday sleep rebound. A.** 5-day somnogram using the standard inactivity criterion
1065 for sleep (5-min or more) in focal-yoked flies during baseline, long-bout sleep deprivation (22-
1066 min inactivity triggers), and three days of recovery. There were no discernable changes in the
1067 average standard sleep measurements between experimental and control flies. **B**. 5-day
1068 somnograms of long-bout sleep (inactivity durations of 25-min or more) during baseline,
1069 deprivation, and three post deprivation days in focal and yoked flies. **C.** In the three post-
1070 deprivation days there were no significant differences in standard sleep between focal and yoked

1071 flies (Post1: Wilcoxon's W = 269, p = 0.06; Post 2: Wilcoxon's W = 258, p = 0.12; Post 3:
1072 Wilcoxon's W = 263, p = 0.09). **D.** Long bout sleep displayed significant increases in the total
1073 amount of sleep over three post-deprivation cycles when compared to yoked controls (Post1:
1074 Wilcoxon's W = 288, p = 0.01; Post 2: Wilcoxon's W = 284, p = 0.02; Post 3: Wilcoxon's W =
1075 290, p = 0.01). **E.** Long bout sleep rebound showed over 25 percent of flies paying back total sleep
1076 debt and overshooting in three post SD cycles. Focal sleep debt reflects the total sleep lost during
1077 deprivation, which was normalized to sleep amounts in undisturbed controls. n = 20 each for focal
1078 and yoked categories * < 0.05, and N.S. (Not Significant).
1079
1080 **Figure 6. Identifying sleep substances using MALDI-TOF. A**. Representative hematoxylin-
1081 eosin stained cryosection of a fly head section. **B**. Distribution of two representative molecules
1082 (104.1 m/z and 175.1 m/z) within a single head section. Note the near zero relative intensity outside
1083 the brain region. Images are overlays of the high intensity signals on the cryosection used to detect
1084 the signal and highlights brain specific distribution of these molecules. The white dotted box
1085 represents the area, corresponding to the central brain, from which signal intensity was quantified.
1086 **C.** Post-deprivation increases in the relative abundance of molecules in focal (deprived) flies when
1087 mechanical perturbations are not accounted for yields 127 positive hits (out of 188 molecules –
1088 68%) in first replicate. We also found 59 peaks that were not significantly different between focal
1089 and undisturbed flies, and 2 peaks that were significantly different, but lower in the focal than in
1090 undisturbed flies, which we termed as "Others". **D**. Search patterns of molecules tracking
1091 mechanical perturbation signals (top) and sleep pressure specific signals (bottom). Example peaks
1092 (m/z 104.1 and m/z 553) showing each of the two categories. Values plotted are mean±SEM. **E.**
1093 Pie chart representing the number molecules in various categories when yoking is employed. The

number of molecules reflecting sleep pressure was eight (4%) for the first replicate. We identified an additional category of molecules that may track sleep and refer to it as "Sleep Absence". Molecules that track the complete absence of sleep (as in the case of focal flies, but not in partial sleep loss situations as in the yoked flies) are classified under this category, and we found nine such candidates. These nine molecules had indistinguishable values between yoked and undisturbed flies which were both significantly lower than focal flies. Focal brain sections $n = 6$; Yoked brain sections $n = 3$; Unperturbed brain sections $n = 4$. **F.** One of the detected sleep-tracking molecules was serotonin, detected at 177.1 m/z, which showed significant sleep pressure tracking changes in both replicate runs (Replicate 1: *Kruskal Wallis critical value* = 9.32, $df = 2$, $p = 0.009$; Replicate 2: *Kruskal Wallis critical value* = 11.56, $df = 2$, $p = 0.003$). Bars with the same letters are not significantly different from each other.

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Figure 1.

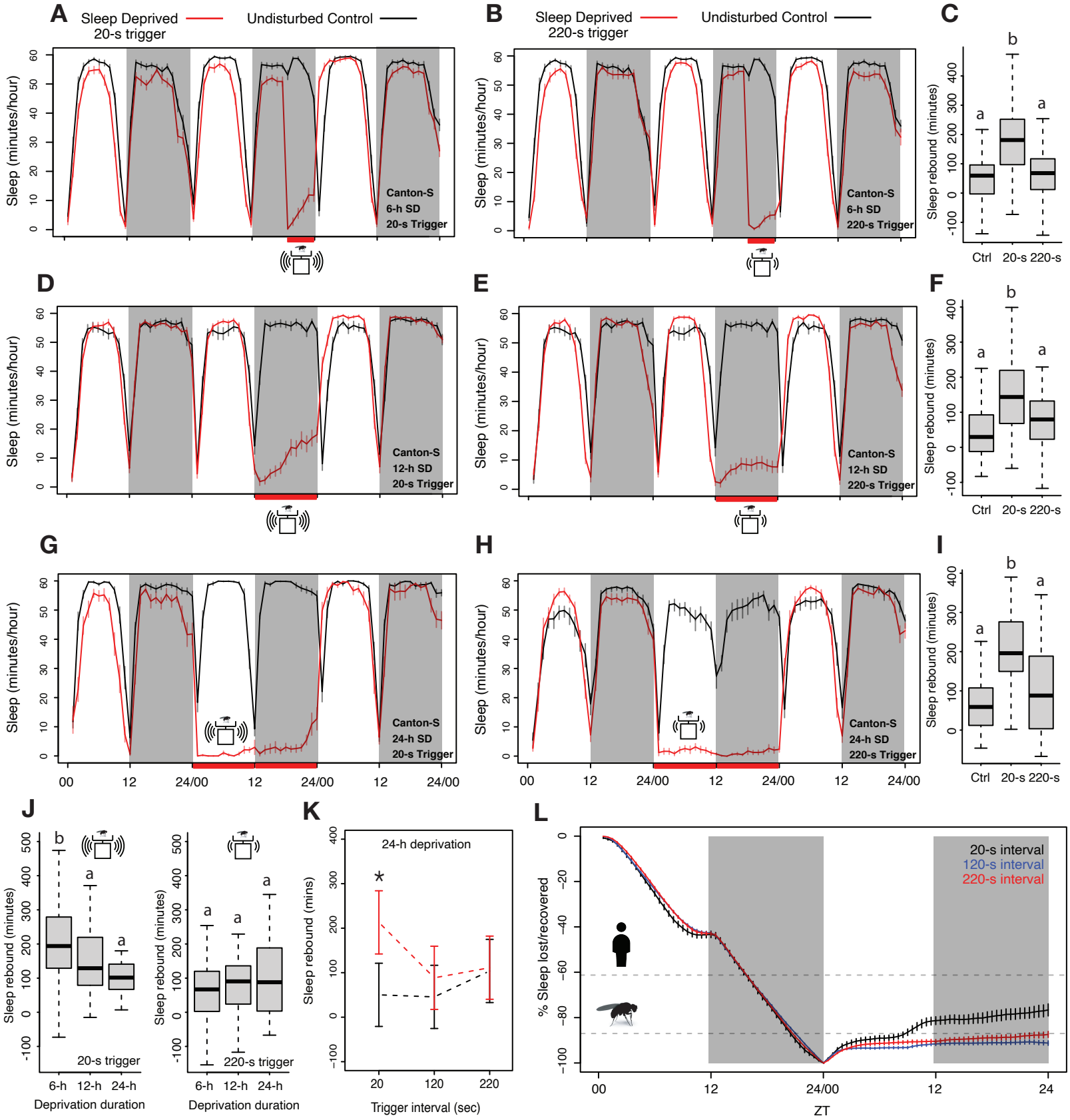

A
Sleep Deprived
20-s trigger
Undisturbed Control
Sleep (minutes/hour)
Canton-S
6-h SD
20-s Trigger
B
Sleep Deprived
220-s trigger
Undisturbed Control
Canton-S
6-h SD
220-s Trigger
C
Sleep rebound (minutes)
Ctrl
20-s
220-s
D
Canton-S
12-h SD
20-s Trigger
E
Canton-S
12-h SD
220-s Trigger
F
G
Canton-S
24-h SD
20-s Trigger
00
12
24/00
H
Canton-S
24-h SD
220-s Trigger
I
J
Sleep rebound (minutes)
20-s trigger
220-s trigger
6-h
12-h
24-h
Deprivation duration
K
24-h deprivation
Sleep rebound (mins)
Trigger interval (sec)
L
% Sleep lost/recovered
20-s interval
120-s interval
220-s interval
ZT

Figure 2.

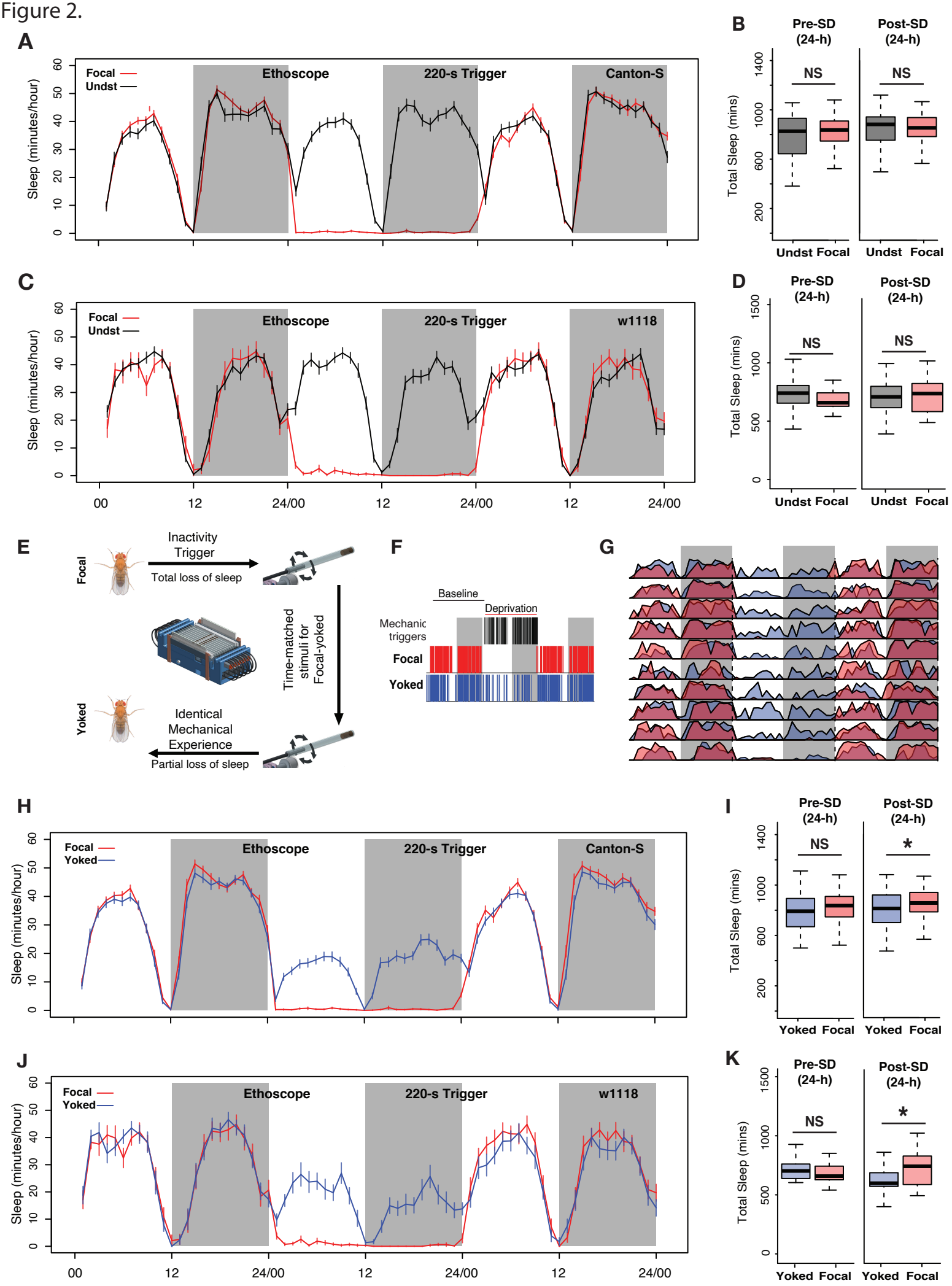

A
Focal
Undst
Ethoscope
220-s Trigger
Canton-S
Sleep (minutes/hour)
B
Pre-SD (24-h)
Post-SD (24-h)
NS
NS
Total Sleep (mins)
Undst Focal
Undst Focal
C
Focal
Undst
Ethoscope
220-s Trigger
w1118
Sleep (minutes/hour)
00
12
24/00
12
24/00
12
24/00
D
Pre-SD (24-h)
Post-SD (24-h)
NS
NS
Total Sleep (mins)
Undst Focal
Undst Focal
E
Focal
Inactivity Trigger
Total loss of sleep
Time-matched stimuli for Focal-yoked
Yoked
Identical Mechanical Experience
Partial loss of sleep
F
Baseline
Deprivation
Mechanic triggers
Focal
Yoked
G
H
Focal
Yoked
Ethoscope
220-s Trigger
Canton-S
Sleep (minutes/hour)
I
Pre-SD (24-h)
Post-SD (24-h)
NS
*
Total Sleep (mins)
Yoked Focal
Yoked Focal
J
Focal
Yoked
Ethoscope
220-s Trigger
w1118
Sleep (minutes/hour)
00
12
24/00
12
24/00
12
24/00
K
Pre-SD (24-h)
Post-SD (24-h)
NS
*
Total Sleep (mins)
Yoked Focal
Yoked Focal

Figure 3.

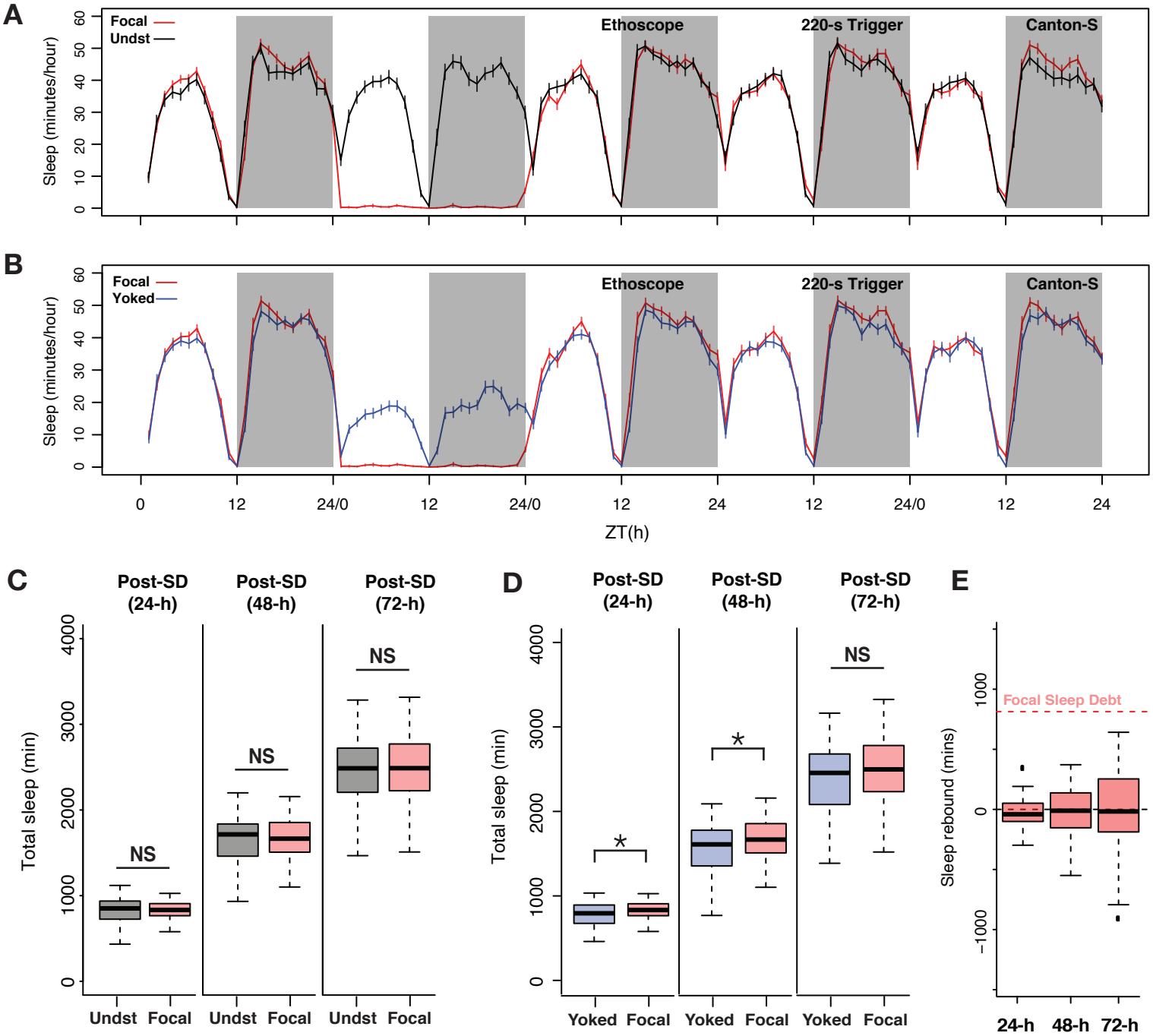

A
Focal
Undst
Ethoscope
220-s Trigger
Canton-S
Sleep (minutes/hour)
B
Focal
Yoked
Ethoscope
220-s Trigger
Canton-S
Sleep (minutes/hour)
ZT(h)
C
Post-SD (24-h)
Post-SD (48-h)
Post-SD (72-h)
NS
Total sleep (min)
Undst
Focal
D
Post-SD (24-h)
Post-SD (48-h)
Post-SD (72-h)
*
NS
Total sleep (min)
Yoked
Focal
E
Focal Sleep Debt
Sleep rebound (mins)
24-h
48-h
72-h

Figure 4.

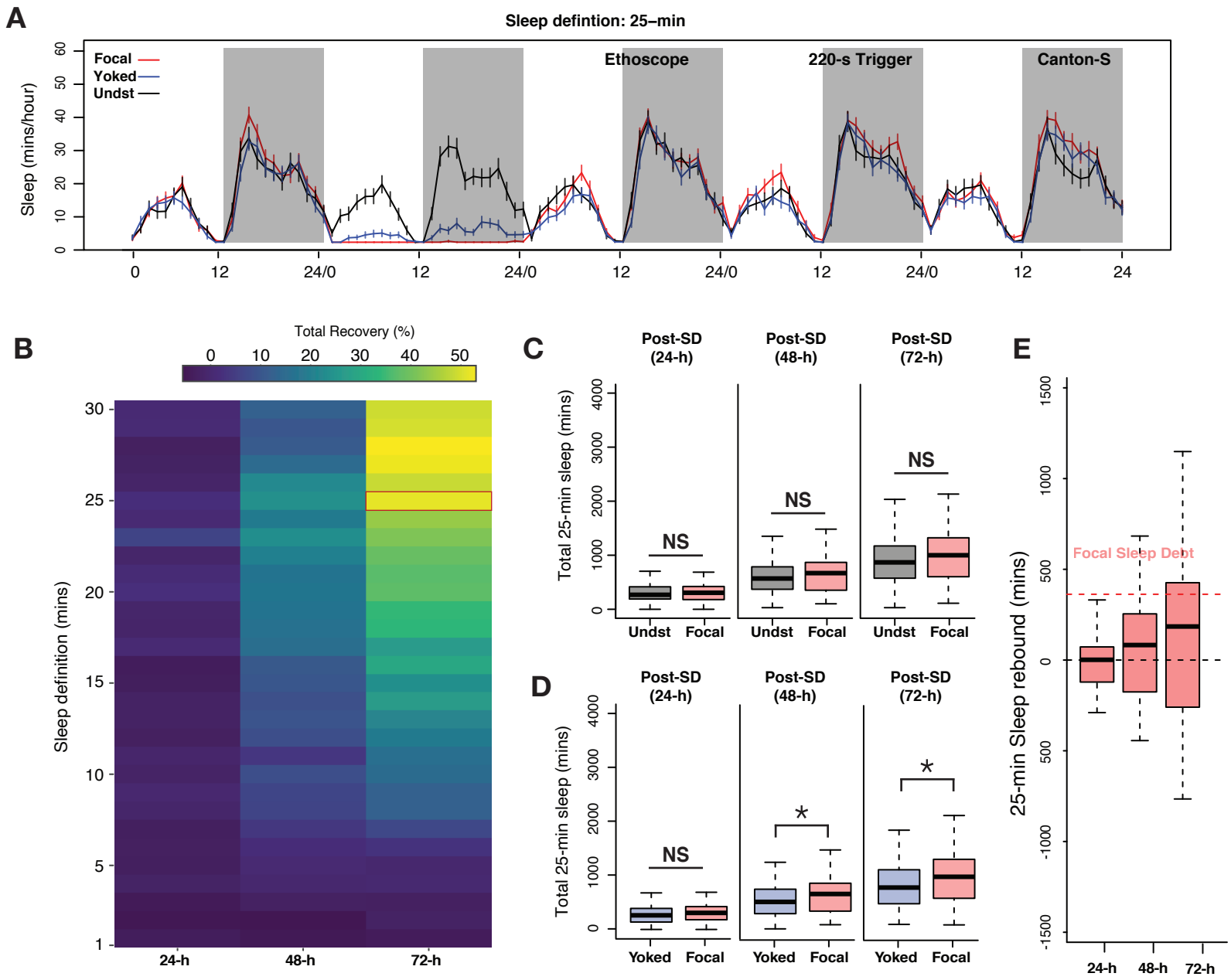

A
Sleep defintion: 25–min
Focal
Yoked
Undst
Ethoscope
220-s Trigger
Canton-S
Sleep (mins/hour)
B
Total Recovery (%)
Sleep definition (mins)
24-h
48-h
72-h
C
Post-SD (24-h)
Post-SD (48-h)
Post-SD (72-h)
Total 25-min sleep (mins)
NS
Undst
Focal
D
Yoked
*
E
Focal Sleep Debt
25-min Sleep rebound (mins)

Figure 5.

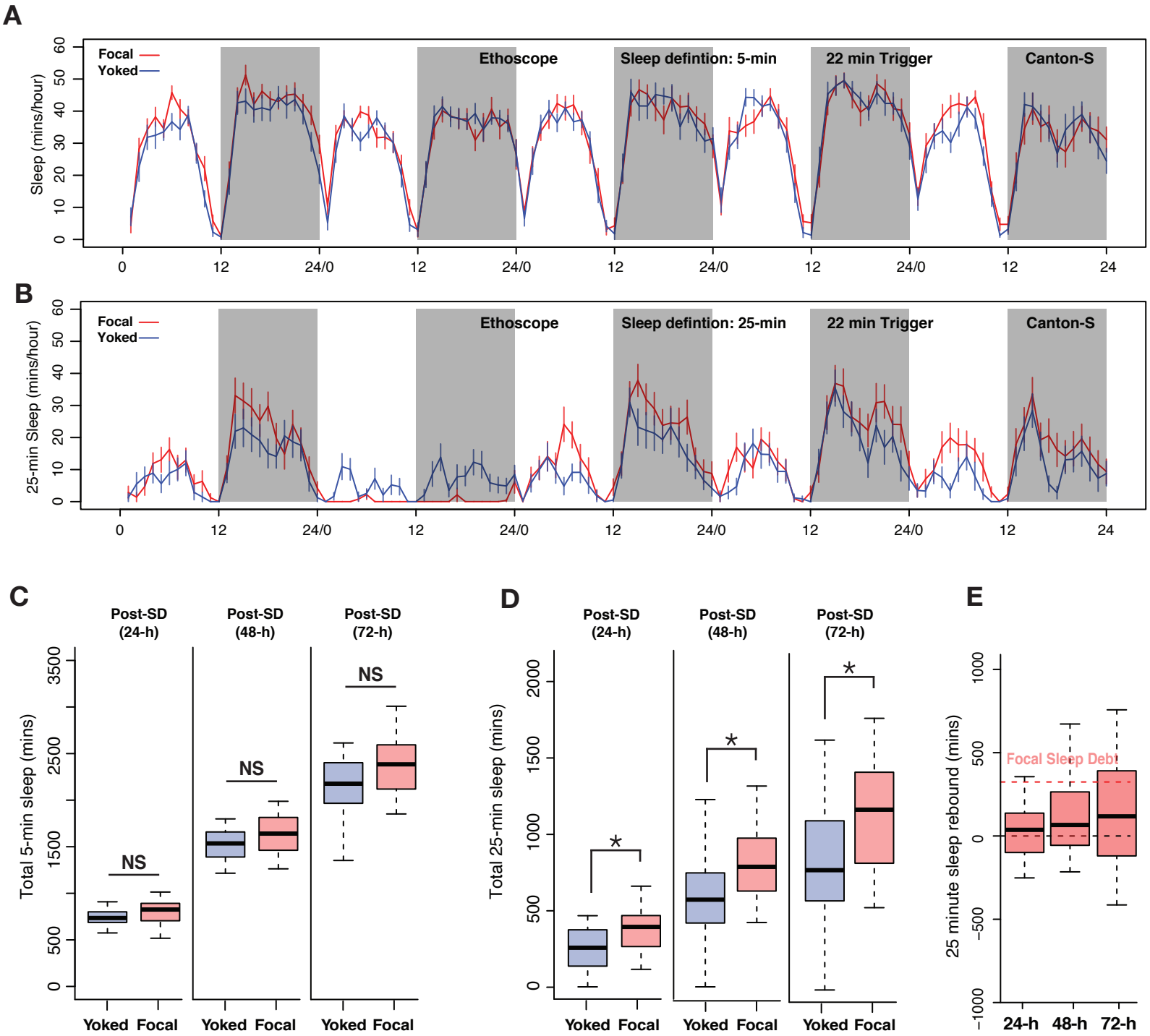
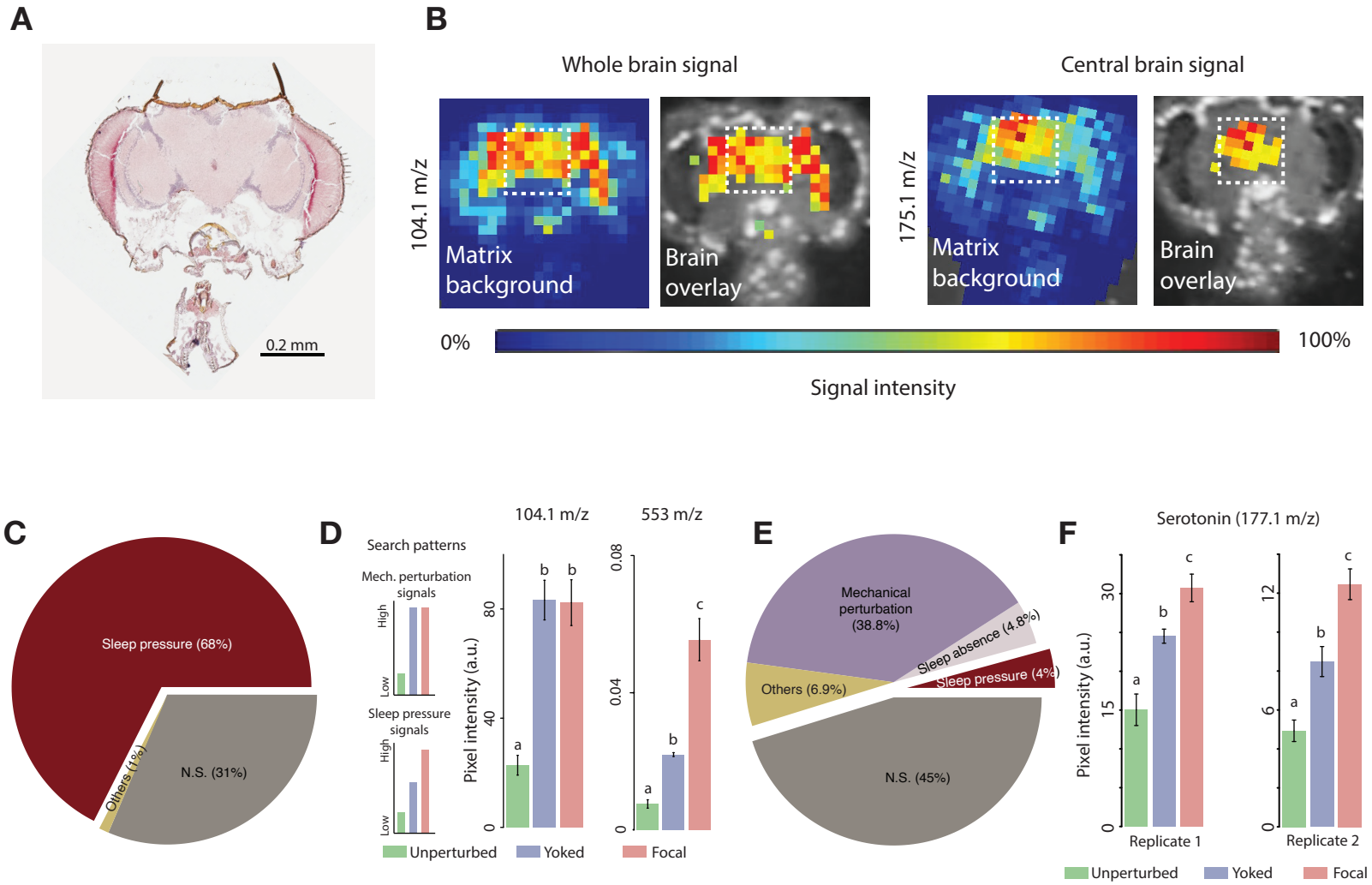

A
Focal
Yoked
Sleep (mins/hour)
Ethoscope
Sleep defintion: 5-min
22 min Trigger
Canton-S
B
25-min Sleep (mins/hour)
Sleep defintion: 25-min
C
Post-SD (24-h)
Post-SD (48-h)
Post-SD (72-h)
Total 5-min sleep (mins)
NS
Yoked
Focal
D
Total 25-min sleep (mins)
*
E
25 minute sleep rebound (mins)
Focal Sleep Debt
24-h
48-h
72-h

Figure 6.


A
0.2 mm
B
Whole brain signal
Central brain signal
104.1 m/z
175.1 m/z
Matrix background
Brain overlay
Matrix background
Brain overlay
0%
100%
Signal intensity
C
Sleep pressure (68%)
Others (1%)
N.S. (31%)
D
104.1 m/z
553 m/z
Search patterns
Mech. perturbation signals
Sleep pressure signals
High
Low
Pixel intensity (a.u.)
Unperturbed
Yoked
Focal
E
Mechanical perturbation (38.8%)
Sleep absence (4.8%)
Sleep pressure (4%)
Others (6.9%)
N.S. (45%)
F
Serotonin (177.1 m/z)
Pixel intensity (a.u.)
Replicate 1
Replicate 2
Unperturbed
Yoked
Focal