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Current Biology

Elevated sleep quota in a stress-resilient *Drosophila* species

Highlights

- Desert-adapted *Drosophila mojavensis* sleep more each day than *D. melanogaster*
- Increased sleep is shared across four different desert-dwelling *Drosophila* species
- Interspecies differences in sleep/wake neuromodulators correlate with sleep amount
- Sleep amount during starvation correlates with survival time in *D. mojavensis*

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In brief

Yano, Nave, et al. find increased sleep in desert-adapted fly species, including *Drosophila mojavensis*, when compared with *D. melanogaster*. Daily sleep of food-deprived *D. mojavensis* correlates with survival time, suggesting that elevated sleep may provide an adaptive behavioral strategy in extreme environments.

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Elevated sleep quota in a stress-resilient *Drosophila* species

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SUMMARY

Sleep is broadly conserved across the animal kingdom but can vary widely between species. It is currently unclear which selective pressures and regulatory mechanisms influence differences in sleep between species. The fruit fly *Drosophila melanogaster* has become a successful model system for examining sleep regulation and function, but little is known about the sleep patterns in many related fly species. Here, we find that fly species with adaptations to extreme desert environments, including *D. mojavensis*, exhibit strong increases in baseline sleep compared with *D. melanogaster*. Long-sleeping *D. mojavensis* show intact homeostasis, indicating that desert flies carry an elevated drive for sleep. In addition, *D. mojavensis* exhibit altered abundance or distribution of several sleep/wake-related neuromodulators and neuropeptides that are consistent with their reduced locomotor activity and increased sleep. Finally, we find that in a nutrient-deprived environment, the sleep patterns of individual *D. mojavensis* are strongly correlated with their survival time and that disrupting sleep via constant light stimulation renders *D. mojavensis* more sensitive to starvation. Our results demonstrate that *D. mojavensis* is a novel model for studying organisms with high sleep drive and for exploring sleep strategies that provide resilience in extreme environments.

INTRODUCTION

Although sleep is widely conserved across the animal kingdom, different species can exhibit dramatically different amounts of sleep. Koalas, sloths, and brown bats, for example, can sleep for roughly 20 h/day, while other mammals, like horses and elephants, only sleep for 3–4 h each day.[1] The basic functions and regulatory mechanisms that drive such wide differences in sleep between species are poorly understood. Previous studies and meta-analyses examined traits that correlate with interspecies variations in sleep, identifying trends in diet, body size, or life history that are associated with total sleep measurements in vertebrate species.[1–7] Although these correlations shed light on selective pressures influencing sleep evolution, the feasibility of systematic comparisons and mechanistic studies across many related vertebrate species is limited due to various practical constraints. By contrast, the *Drosophila* genus provides a diverse range of at least 1,600 species, including the genetic model species *D. melanogaster*, many of which can be cultured and behaviorally monitored in standard laboratory settings.[8]

Different *Drosophila* species thrive in a wide variety of environmental conditions across the planet, providing a set of natural experiments to explore the physiological adaptations that might be associated with variations in sleep. Exploiting this natural diversity to identify species with strongly elevated or reduced needs for sleep may provide new avenues to examine the fundamental functions fulfilled by sleep, neural signaling mechanisms that regulate sleep, and physiological tradeoffs that might be associated with different sleep strategies. Here, we examined differences in sleep between the genetic model species *D. melanogaster* and desert-adapted species, including *D. mojavensis*, a desert-dwelling species that shows heightened resilience to heat, starvation, and desiccation stresses. These features may contribute to their ability to thrive in harsh desert conditions,[9–11] but behavioral adaptations that accompany stress resilience in *D. mojavensis* remain unexplored.

We find that *D. mojavensis* exhibits increased sleep time across the day and night compared with *D. melanogaster* and that desert-adapted *D. mojavensis* flies respond to sleep loss with a homeostatic rebound. We observe several changes in sleep- or wake-related neuromodulator distribution: long-sleeping *D. mojavensis* flies exhibit high levels of serotonin (5-HT), decreased abundance of wake-promoting octopamine (OA), and reduced numbers of cells expressing the circadian

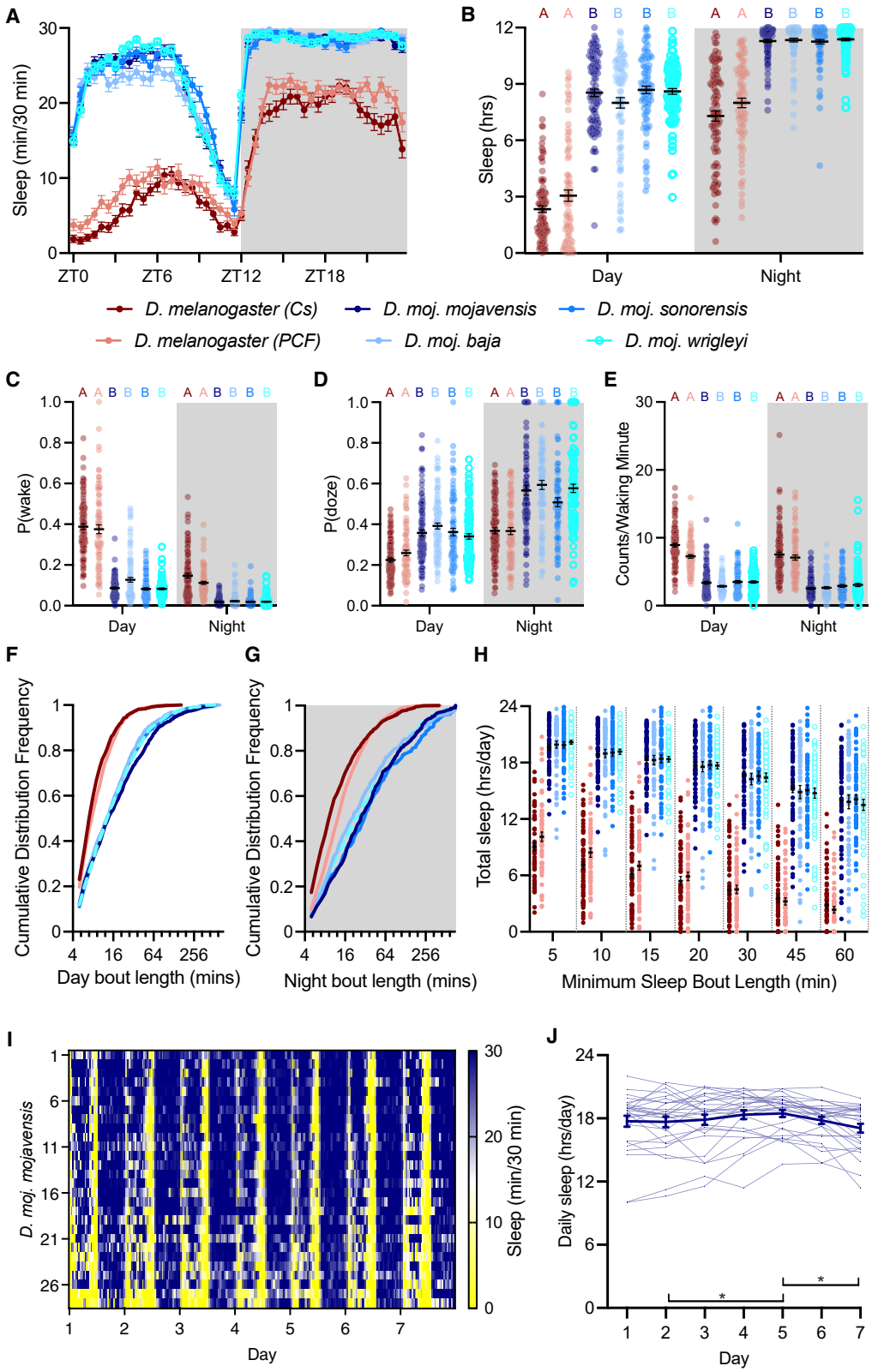

Figure 1. Elevated sleep time in desert-dwelling *Drosophila mojavensis*

(A) 24 h sleep time course for wild-type *D. melanogaster* (*Cs*, dark red; *Pcf*, light red) and four subspecies of *D. mojavensis* (blue). Two-way repeated measures ANOVA finds a significant genotype-by-time interaction ($F_{(235,27213)} = 16.99$, $p < 0.0001$).

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output peptide pigment dispersing factor (PDF). Finally, we examine contributions of elevated sleep to stress resilience in *D. mojavensis* by measuring starvation and dehydration responses. Long-sleeping *D. mojavensis* flies exhibit extended survival during food or food and water deprivation compared with *D. melanogaster*, and individual sleep time of *D. mojavensis* correlates positively with survival time while flies are starved and dehydrated. Together, these results indicate that *D. mojavensis* exhibits an increased internal sleep quota relative to *D. melanogaster* and that elevated sleep may contribute to increased stress resilience in desert-adapted flies.

RESULTS

Characterizing high sleep time in *Drosophila mojavensis*

D. melanogaster has become a popular genetic model system to study sleep and circadian rhythms.[12–14] Although focus on this model species permits the rapid development and proliferation of genetic tools and mechanistic frameworks, few studies have examined related species that are adapted to thrive in a variety of environmental conditions. Increased sleep is a behavioral adaptation that is hypothesized to support resistance to nutrient scarcity,[15] and artificial selection for starvation resistance in *D. melanogaster* can result in increased sleep time.[16] To test whether similar changes in behavior might correlate with interspecific changes in stress resistance, we compared sleep and starvation/dehydration responses in *D. melanogaster* and *D. mojavensis*.

D. mojavensis are found in desert regions of Mexico and the southwestern USA and includes four geographically segregated subspecies: *D. moj. mojavensis*, *D. moj. baja*, *D. moj. sonorensis*, and *D. moj. wrigleyi* from the Mojave Desert, Baja California, Sonoran Desert, and Santa Catalina Island, respectively.[17–19] We measured sleep in all four *D. mojavensis* subspecies and in two wild-type stocks of *D. melanogaster* (*Cs*[20] and *Pcf*[21]) using multibeam *Drosophila* activity monitors. Each *D. mojavensis* subspecies exhibits significantly elevated sleep throughout the day and night compared with *D. melanogaster* (Figures 1A and 1B). To test whether elevated sleep in *D. mojavensis* can be attributed to an elevated pressure to maintain and/or to initiate sleep episodes, we quantified the likelihood that a sleeping fly would awaken (P(wake); Figure 1C) or that a waking fly would fall asleep (P(doze); Figure 1D).[22] Each of the four *D. mojavensis* subspecies exhibits reduced P(wake) and elevated P(doze) compared with *D. melanogaster*, consistent with both strengthened sleep maintenance and an elevated pressure to fall asleep. Along with increased sleep time, *D. mojavensis* also exhibits reduced waking locomotor activity (Figure 1E), consistent with previous reports.[23] We detected similar differences between *D. melanogaster* and *D. mojavensis* in male flies: sleep time is elevated in male *D. mojavensis* compared with *D. melanogaster* during the day and night, although daytime differences are dampened because male *D. melanogaster* sleep more during the day than females (Figure S1).

We analyzed the cumulative distribution of bout lengths during the day to better detail sleep architecture in *D. mojavensis* (Figure 1F) and night (Figure 1G). These analyses found that *D. mojavensis* flies exhibit an elevated frequency of longer sleep episodes than those observed in either wild-type line of *D. melanogaster*. Since *D. mojavensis* sleep consists of longer bouts, *D. moj. moj.* and *D. moj. baja* continue to exhibit elevated sleep amounts when we increase the minimum period of quiescence scored for sleep from 5 min, as most commonly used for *D. melanogaster*,[12,13] to at least 60 min (Figure 1H). Together, these results indicate that elevated sleep in *D. mojavensis* consists of increased drive to fall asleep and prolonged sleep episodes.

To test for variations in sleep across days, we measured locomotion in *D. moj. mojavensis* flies across a 7-day period and found that daily sleep varies between individuals but remains relatively stable over time for single flies (Figures 1I and 1J). Because our baseline data reveal nearly identical sleep amounts and architecture between the four *D. mojavensis* subspecies, we have narrowed our focus for many of our additional behavioral studies on *D. moj. mojavensis* and *D. moj. baja*.

Increased sleep in desert-adapted *Drosophila* across experimental conditions

Because the *D. mojavensis* stocks that we describe above were derived from wild populations more recently than either of our lab-reared wild-type *D. melanogaster* stocks, we tested whether fly lines isolated from the wild sleep more than those reared in lab conditions for longer periods of time. To test whether recently derived stocks show increased sleep, we examined a *D. melanogaster* stock that originated from flies collected in the Westwood area of Los Angeles in 2021. *D. melanogaster* descended from recently wild Westwood flies showed comparable sleep amounts to *Cs* and *Pcf* laboratory

(B) Day and night sleep totals for *D. melanogaster* (*Cs*, dark red; *Pcf*, light red) and *D. mojavensis* (blues). Two-way repeated measures ANOVA finds a significant genotype-by-time interaction ($F_{(5,577)} = 24.981$, $p < 0.0001$).
(C and D) P(wake) (C) and P(doze) (D) during the day and night for *D. melanogaster* (reds) and *D. mojavensis* (blues) stocks. Two-way repeated measures ANOVA detects a significant genotype-by-time interaction for P(wake) ($F_{(5,579)} = 75.43$, $p < 0.0001$) and for P(doze) ($F_{(5,553)} = 5.628$, $p < 0.0001$).
(E) Waking activity (position movements/waking minute) is decreased in *D. mojavensis* subspecies (blues) relative to *D. melanogaster* (reds). Two-way repeated measures ANOVA finds a significant main effect of genotype ($F_{(5,576)} = 139.4$, $p < 0.0001$).
For (A)–(E), $n = 101$ *Cs*, 82 *Pcf*, 100 *D. moj. moj.*, 100 *D. moj. baja*, 93 *D. moj. sonorensis*, 106 *D. moj. wrigleyi.*
(F–H) Cumulative distributions for the duration of sleep bouts of *D. melanogaster* (reds) and *D. mojavensis* (blues) during the day (F) and night (G). (H) Depicts total sleep over 24 h for individuals from (F) and (G) using increasing periods for the minimum sleep bout threshold. Kruskal-Wallis tests find significant effects of genotype for day bout lengths (F; Kruskal-Wallis statistic = 467.7, $n = 737–1,204$ sleep bouts/group from 60 to 64 flies/group, $p < 0.0001$) and night bout lengths (G; Kruskal-Wallis statistic = 499.7, $n = 398–1,380$ sleep bouts/group from 58 to 64 flies/group, $p < 0.0001$). Two-way repeated measures ANOVA finds a significant genotype-by-threshold interaction ($F_{(30,2244)} = 6.142$, $p < 0.0001$, $n = 62–64$ flies/group).
(I and J) Sleep time course heatmap (I) and daily sleep totals (J) for *D. moj. moj.* female flies across a 7-day experiment (Friedman test statistic = 18.47, $p = 0.0051$, $n = 28$ flies). * indicates $p < 0.05$ for sleep on day 2 vs. day 5 and day 5 vs. day 7 by Dunn's pairwise test for (J).
See also Figure S1. Group averages and error bars represent means and SEM for all panels.

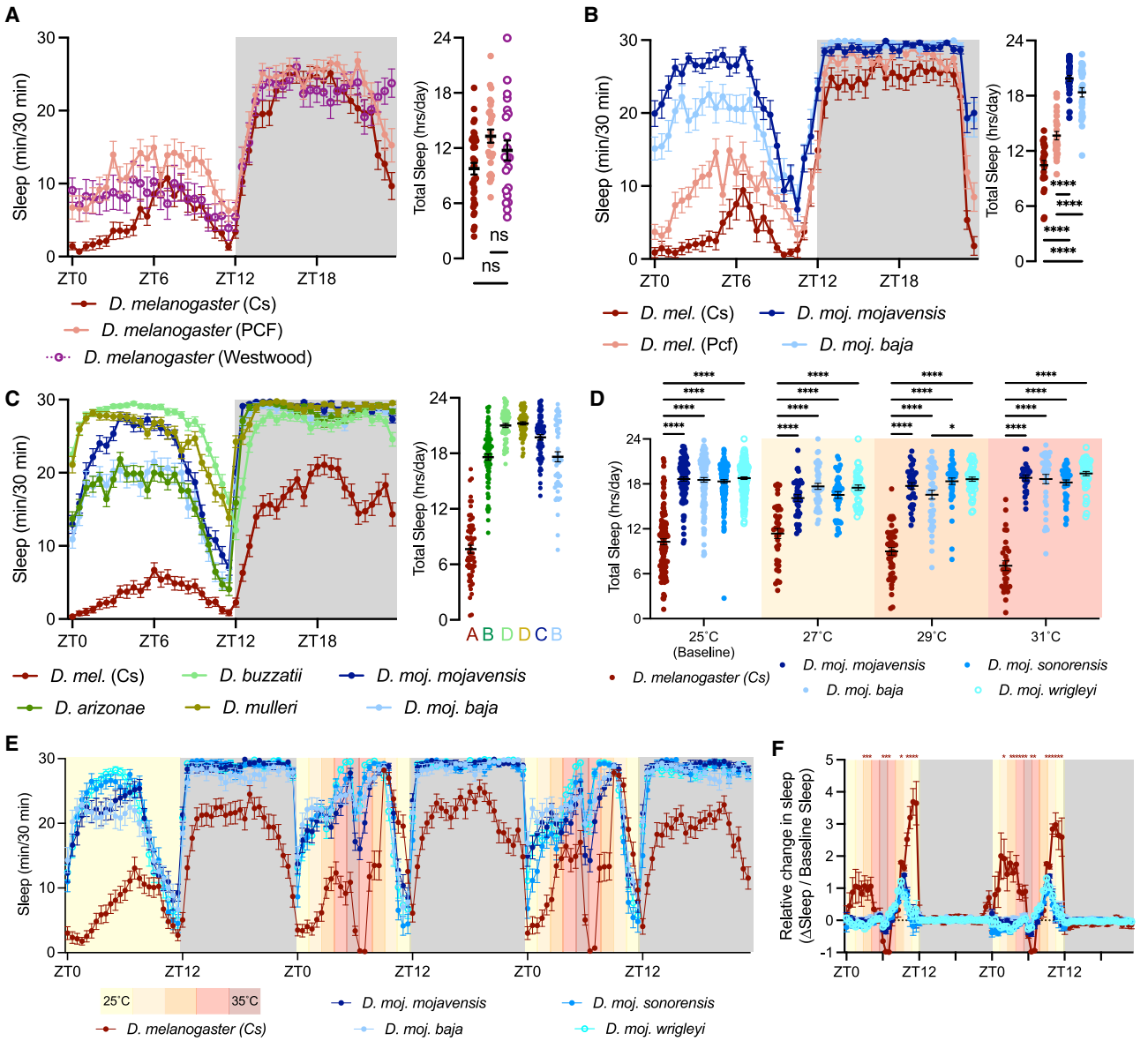

Figure 2. Elevated sleep time in *D. mojavensis* across conditions

(A) Sleep time course (left) and total sleep over 24 h (right) for *Canton-S* (dark red), *Pcf* (light red), and flies descended from *D. melanogaster* caught in Westwood, Los Angeles (open purple circles). ANOVAs detect a significant genotype-by-time interaction in sleep time course ($F_{(94,3995)} = 2.385$, $p < 0.0001$) and main effect of genotype for total daily sleep ($F_{(2,85)} = 5.793$, $p = 0.0044$; $n = 38$ *Canton-S*, 28 *Pcf*, and 22 wild-caught flies).

(B) Sleep time course (left) and total sleep over 24 h (right) for *D. melanogaster* stocks (red) and two *D. mojavensis* subspecies (blue) from flies reared on Banana-Opuntia media. ANOVAs detect significant genotype-by-time interaction for the sleep time course ($F_{(141,4841)} = 8.838$, $p < 0.0001$) and a significant effect of genotype for total daily sleep ($F_{(3,103)} = 91.08$, $p < 0.0001$; $n = 24$ *Canton-S*, 27 *Pcf*, 28 *D. moj. moj.*, and 28 *D. moj. baja*).

(C) Sleep time course (left) and total sleep over 24 h (right) for *D. melanogaster* (red), *D. arizonae* (green), *D. buzzatii* (light green), *D. mulleri* (olive), and *D. mojavensis* (blues). ANOVA tests find significant genotype-by-time interaction for sleep time course ($F_{(235,17531)} = 18.07$, $p < 0.0001$) and a significant effect of genotype for total sleep ($F_{(5,373)} = 215.4$, $p < 0.0001$; $n = 63$ *Canton-S*, 78 *D. arizonae*, 62 *D. buzzatii, 57 D. mulleri*, 69 *D. moj. moj*, and 50 *D. moj. baja*). Letters below graph indicate statistically distinct groups by Holm-Sidak pairwise comparisons.

(D) Total sleep/day for flies housed at 25°C during one baseline day and then shifted to 27°C, 29°C, or 31°C for 3 days (daily sleep at 27°C, 29°C, and 31°C shows the 3-day mean). Mixed effects analysis finds a significant temperature-by-genotype interaction ($F_{(12,571)} = 4.076$, $p < 0.0001$, $n = 25$–131 flies/group). * $p < 0.05$, **** $p < 0.0001$ by pairwise Holm-Sidak's test. Sleep time courses shown in Figures S3A–S3C.

(E) Sleep time courses for *D. melanogaster* (Cs; red) and *D. mojavensis* (blues) flies that were housed at 25°C for 1 baseline day, then exposed to a temperature ramp from 25°C to 35°C and back to 25°C during the daytime (ZT0–12). Two-way repeated measures ANOVA finds a significant time-by-strain interaction ($F_{(568,26412)} = 9.054$, $p < 0.0001$; $n = 40$ *Cs*, 39 *D. moj. moj.*, 35 *D. moj. baja*, 35 *D. moj. sonorensis*, and 42 *D. moj. wrigleyi*).

(F) Relative sleep changes for experimental groups in (E) for temperature ramp days compared with baseline sleep. At each 30-min window, y axis values depict (sleep amount − baseline sleep amount at the same circadian time point)/(baseline sleep amount at the same circadian time point). Two-way repeated measures

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strains (Figure 2A). To test the impact of diet on *D. mojavensis*, we housed adult flies on media that included extract of opuntia cactus, a natural host for desert-adapted *D. mojavensis*. Their offspring developed in this media then continued to be fed the same diet as adults. Sleep in *D. mojavensis* remained elevated relative to *D. melanogaster* when both species were fed a banana-cactus diet (Figure 2B). In addition to *D. mojavensis*, several other related fly species, including *D. arizonae*, *D. buzzatii*, and *D. mulleri*, also live in deserts[24–26] (phylogeny schematic in Figure S2). As shown in Figure 2C, these three additional desert-adapted species sleep as much, or more, than *D. mojavensis*, suggesting that elevated sleep is not exclusive to *D. mojavensis* and could be conserved across the Repleta species that localize to desert regions.[27]

D. mojavensis can show a preference for warm temperatures,[28,29] so we also observed flies while they were housed at either 27°C, 29°C, or 31°C for 3 days after a baseline day at 25°C. As shown in Figures 2D and S3, average daily sleep at each of the three warmer temperatures remained strongly elevated in *D. mojavensis* compared with *D. melanogaster*. In their desert habitats, *D. mojavensis* are exposed to environmental stressors that include temperature variations and periods of sparse food and/or water availability. To measure sleep during desert-like temperature fluctuations, we exposed both *D. melanogaster and D. mojavensis* flies to daytime temperature ramps. Flies were held at 25°C overnight, then began to progressively increase the temperature across the first 6 h of daytime to a peak of 35°C before reducing back to 25°C by lights off at ZT12. Although *D. mojavensis* maintained higher amounts of sleep than *D. melanogaster* across most of the day during these conditions (Figure 2E), both species showed a brief period of arousal when temperature peaked at 35°C at mid-day (Figure 2F). As the temperature decreased afterward, *D. melanogaster* briefly increased their sleep to comparable levels as the desert-adapted *D. mojavensis* subspecies. These results indicate that sleep in both species can be altered by variations in temperature but that *D. mojavensis* retain elevated levels of daily sleep under naturalistic daytime temperature conditions.

Sleep homeostasis remains intact in *Drosophila mojavensis*

Elevated sleep in desert-adapted flies could indicate that this species has adapted an elevated need for basic functions that are fulfilled by sleep. To test whether desert-adapted *D. mojavensis* maintain an elevated sleep quota, we tested whether they respond to mechanical sleep deprivation with a homeostatic rebound. Vortex stimuli delivered for 3 s each minute were sufficient to strongly suppress sleep in *D. moj. moj.* (Figure 3A) and in *D. moj. baja* (Figure 3B). Following overnight deprivation, both *D. mojavensis* subspecies showed a recovery period of significantly increased sleep compared with baseline and regained approximately 20%–40% of their lost sleep after 24 h (Figure 3C). In the 24 h following deprivation, P(wake) is decreased during daytime on the first recovery day after deprivation, an indication of increased sleep depth (Figures S4A and S4B). Additionally, there was no decrease in locomotor activity per time awake (Figures S4C and S4D), indicating that waking locomotor activity is unimpaired by mechanical sleep deprivation. Following the first 24 h of recovery, *D. mojavensis* flies reduced their sleep nearly to baseline levels on the second recovery day (Figures S4E and S4F). Although *D. melanogaster* and *D. moj. baja* showed comparable sleep rebound after overnight deprivation, *D. moj. moj.* recovered a reduced amount of sleep relative to *D. melanogaster* (Figure 3C).

To test whether *D. moj. moj.* exhibit markers of increased sleep depth during recovery, we next probed arousability in recently deprived *D. moj. moj.* Flies were either left undisturbed, sleep-deprived for 12 h overnight (SD), or sleep-deprived and permitted 24 h of recovery (SD + 24 h) before they were exposed hourly to 60 s pulses of blue light. Light pulses were less likely to awaken sleep-deprived flies than rested controls; arousability returned to control levels in SD + 24 h flies (Figure 3D). After each light pulse, *D. moj. mojavensis* flies in the SD group had a reduced latency to fall back asleep compared with both the control and SD + 24 h groups (Figure 3E). These results indicate that long-sleeping *D. mojavensis* responds to mechanical sleep loss with homeostatic increases both in sleep time and intensity, consistent with the hypothesis that *D. mojavensis* have adapted an increased pressure for sleep.

To further probe responses of *D. mojavensis* to acute sleep loss, we also exposed *D. moj. moj.* and *D. moj. baja* flies to arousing blue light for 12 h overnight (ZT12-0). Overnight blue light disrupted sleep in both desert subspecies and was followed by prolonged rebound during the first recovery day (Figures 3F–3I). During light stimulation, *D. moj. moj.* lost 83.90% ± 3.50% (mean ± SEM, n = 35) of their sleep, while *D. moj. baja* reduced their sleep by 42.89% ± 3.74% (mean ± SEM, n = 53) (Figure 3H). Given that overnight light exposure significantly disrupted sleep, we next tested whether acute visual input bidirectionally influences sleep by housing *D. mojavensis* in 2 days of constant darkness. Both *D. moj. moj.* (Figure 3J) and *D. moj. baja* (Figure 3K) significantly increased their sleep when transferred to constant darkness after entrainment in a 12 h:12 h light-dark schedule. We found that in the absence of day-night light signals, the immediate increase in sleep during the subjective daytime persists across at least 2 days (Figure 3L). Previous observations of *D. melanogaster* have found either reduced or unchanged sleep when flies were housed in constant darkness,[30–33] indicating that light-dependent modulation of sleep differs between fly species.

Interspecies variation in sleep/wake-related neuromodulators correlates with sleep patterns

Research over the past 20 years identified several neuromodulators and neuropeptides that influence sleep/wake regulation in *D. melanogaster*,[33–38] but interspecies variation of these signals across fly species is not well studied. In particular, we

ANOVA detects a significant time-by-strain interaction ($F_{(376,17484)} = 11.30$, $p < 0.0001$). * represents time points at which Holm-Sidak pairwise comparisons find $p < 0.05$ between *D. melanogaster* and each of the four *D. mojavensis* subspecies.
See also Figures S2 and S3. Group averages and error bars represent mean and SEM for all panels.

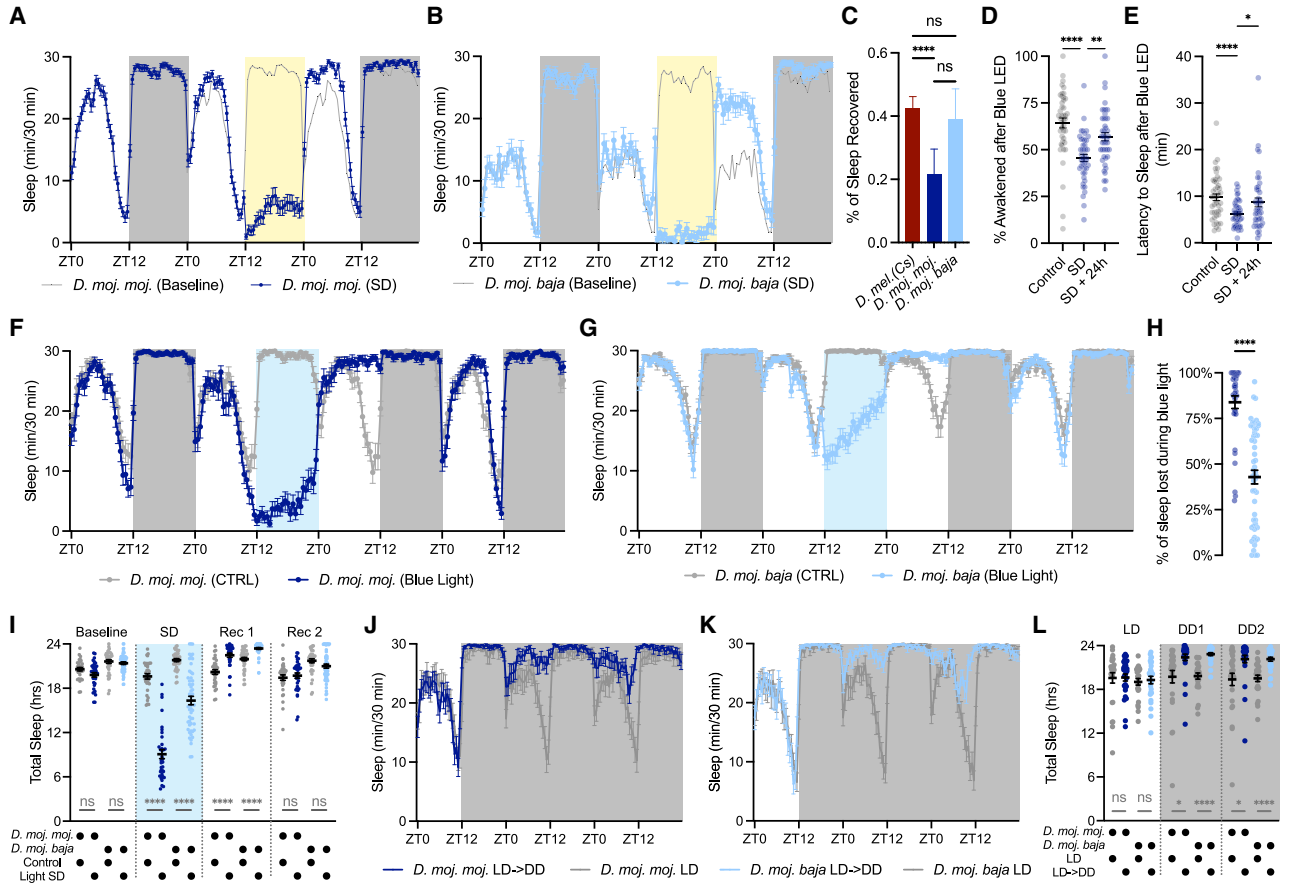

Figure 3. Homeostatic regulation of sleep and arousability in *Drosophila mojavensis*

(A and B) Sleep time course of *D. moj. moj.* (A) and *D. moj. baja* (B) across baseline, overnight mechanical sleep deprivation, and recovery days. Yellow shading indicates time of sleep deprivation. Gray lines show mean 24 h sleep time courses from the baseline day replotted on deprivation and recovery days for visual comparison (n = 77 flies in A, 38 in B).

(C) Percentage of sleep recovered within 24 h of recovery from mechanical sleep deprivation. *D. moj. moj.* shown in dark blue and *D. moj. baja* in light blue. Kruskal-Wallis test finds a significant effect of fly line (Kruskal-Wallis statistic = 24.60, $p < 0.0001$, n = 85 for *D. mel.*, 77 for *D. moj. moj.*, and 38 for *D. moj. baja* and flies/group). **** denotes $p < 0.0001$ by Dunn's multiple comparisons test.

(D) Portion of sleeping *D. moj. mojavensis* flies awakened by 60 s pulses of blue light. Individual data points represent group mean response rate from individual hourly light exposure trials. One-way repeated measures ANOVA finds a significant effect of condition ($F_{(1.874,80.56)}$ = 15.41, $p < 0.0001$, n = 44 trials/group).

(E) Mean sleep latency of *D. moj. mojavensis* flies after hourly 60 s pulses of blue light is reduced after mechanical sleep deprivation. Individual data points represent group mean sleep latency after individual hourly light exposure trials. One-way repeated measures ANOVA finds a significant effect of condition ($F_{(1.730,74.40)}$ = 7.342, p = 0.002, n = 44 trials/group).

(F and G) Sleep time course of *D. moj. mojavensis* (F) and *D. moj. baja* females (G) during baseline, overnight blue light exposure, and 2 recovery days. Blue shading shows the time of overnight light stimulation. Gray traces represent undisturbed controls, and blues depict sleep for flies exposed to blue light from ZT12 to ZT24 on day 2. Two-way repeated measures ANOVAs find significant time-by-condition interactions for (F) ($F_{(191,12606)}$ = 33.98, $p < 0.0001$, n = 33 control and 35 light SD) and for (G) ($F_{(191,16999)}$ = 15.98, $p < 0.0001$, n = 38 control and 53 light SD).

(H) Percentage of sleep lost during 12 h of overnight blue light exposure in *D. moj. moj.* (dark blue) and *D. moj. baja* (light blue). Unpaired t test: t = 7.593, df = 86, $p < 0.0001$; n = 35 *D. moj. moj.* and 53 *D. moj. baja*.

(I) Daily sleep for groups shown in (F) and (G). Two-way repeated measures ANOVA finds a group-by-day interaction ($F_{(9,465)}$ = 97.60, $p < 0.0001$; n = 33 control *D. moj. moj.*, 35 light SD *D. moj. moj.*, 38 control *D. moj. baja*, and 53 light SD *D. moj. baja*).

(J and K) Sleep time courses for *D. moj. moj.* (J) and *D. moj. baja* (K) during 1 day of 12 h:12 h light-dark followed by 2 days in constant darkness. Gray traces show controls that remain on 12 h:12 h light-dark (LD) schedule, and groups transferred to darkness are depicted in blues. Two-way ANOVAs find significant group-by-time interactions for (J) ($F_{(143,7436)}$ = 6.694, $p < 0.0001$, n = 26 LD and 28 LD → DD flies/group) and (K) ($F_{(143,7293)}$ = 10.40, $p < 0.0001$, n = 25 LD and 28 LD → DD flies/group).

(L) Daily sleep for groups shown in (J) and (K). Two-way repeated measures ANOVA finds a significant group-by-day interaction ($F_{(6,206)}$ = 12.02, $p < 0.0001$, n = 26 *D. moj. moj.* LD, 28 *D. moj. moj.* LD → DD, 25 LD *D. moj. baja*, and 28 LD → DD *D. moj. baja*).

See also Figure S4. Group averages and error bars represent mean and SEM for all panels.

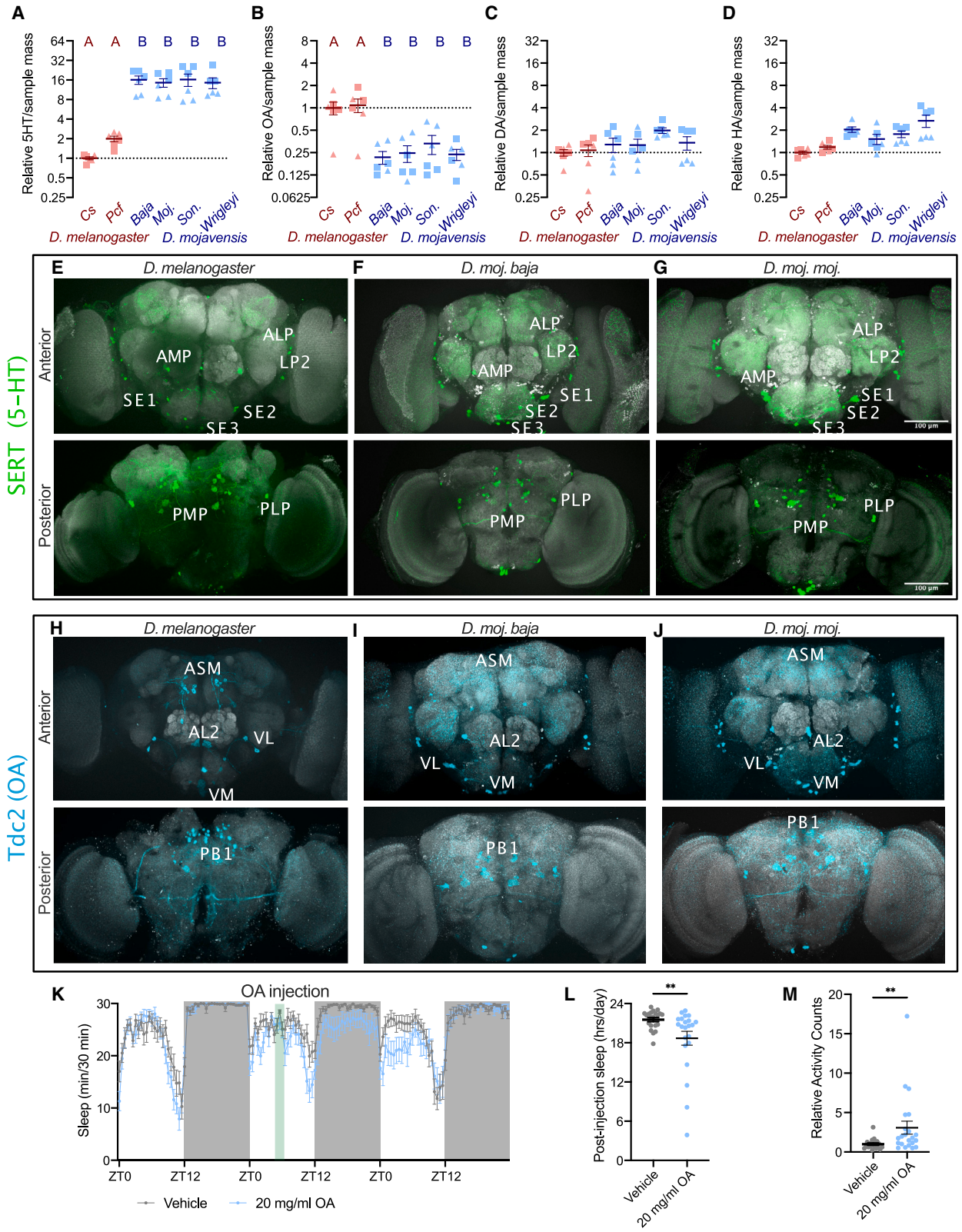

Figure 4. Interspecies variation of sleep- and wake-regulatory modulators between *D. melanogaster* and *D. mojavensis*

(A–D) Relative LC-MS/MS quantification of 5-HT (A), octopamine (B), dopamine (C), and histamine (D) in heads of *D. melanogaster* wild-type stocks (reds) and *D. mojavensis* subspecies (blues). Data represent two independent experiments, each with three biological replicates per group (n = ~100 heads/biological

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hypothesized that elevated sleep time in *D. mojavensis* may be correlated with an upregulation of sleep-promoting signals and a decrease in arousal pathways. To identify relevant neuromodulators, we conducted liquid chromatography-mass spectrometry (LC-MS) assays of fly heads from both *D. melanogaster* and *D. mojavensis*. We found that long-sleeping *D. mojavensis* flies from all four subspecies contain a significant increase in 5-HT and decrease of OA (Figures 4A and 4B), indicating a correlation in the abundance of these two neuromodulators with sleep time. No uniform change in dopamine (DA) or histamine (HA) was measured between species (Figures 4C and 4D). 5-HT signaling promotes sleep in *D. melanogaster*[35,39–41] and in vertebrates,[42–44] while OA, a paralog of norepinephrine,[45] drives arousal.[36,46] Changes in the abundance of 5-HT and OA between species may either indicate: (1) that altered numbers of neurons produce these modulators or (2) that conserved populations of cells have changed their rates of 5-HT and OA synthesis and/or release.

We observed the distribution of 5-HTergic cells by staining for the serotonin transporter (SERT) in *D. melanogaster* (Figure 4E), *D. moj*. *baja* (Figure 4F), and *D. moj*. *moj*. (Figure 4G). Images of the anterior and posterior cell bodies indicate that both species show similar overall patterns of 5-HTergic neurons, but it is possible that projection targets or cell numbers within specific clusters may vary. Similarly, we stained *D. melanogaster, D. moj*. *baja*, and *D. moj*. *moj.* brains for the OA synthesis enzyme Tdc2 (Figures 4H–4J) to observe the number and organization of OAergic cells. Our images reveal weak Tdc2-immunostaining in the anterior superior medial protocerebrum (ASM) neurons of the anterior protocerebrum of *D. mojavensis* flies (Figures 4H and S5A–S5C), a population of cells that underlies the wake-promoting role of OA.[46] Together, these results indicate that the distribution of 5-HTergic neurons is similar between species but that *D. mojavensis* may contain either weak signal or only a subset of the OAergic cells that are observed in *D. melanogaster*.

We next sought to test whether arousal circuitry might retain sensitivity to OA in long-sleeping species by microinjecting *D. moj*. *baja* females with 18.4 nL of either 20 mM OA or vehicle control. During the first 24 h after OA injections, we found that *D. moj*. *baja* females showed reduced sleep and increased locomotor activity (Figures 4K–4M) compared with vehicle-treated siblings. Although OA abundance is decreased in *D. mojavensis*, the wake-promoting effect of OA injection suggests that OA-sensitive arousal circuitry is likely conserved in desert-adapted flies.

To examine whether the distribution of other wake-promoting signals might differ between these two fly species, we performed immunostaining for the arousing circadian output peptide PDF.[49] Although *D. melanogaster* brains contain eight PDF-positive ventrolateral neurons (LNvs) in each hemisphere, four small LNvs (s-LNvs) and four large LNvs (l-LNvs) (Figure 5A),[50] careful analysis reveals inconsistent PDF-expression patterns between *D. melanogaster* and *D. mojavensis*. Specifically, *D. mojavensis* retained three to four PDF-positive l-LNvs but showed no s-LNv cell bodies or dorsal protocerebrum projections that were labeled with anti-PDF (Figures 5B and 5C). A loss of PDF immunostaining in s-LNvs has also been reported in other *Drosophila* species, indicating that selective pressures may drive reconfiguration of clock circuits as species adapt to different environments.[51–54] Together, these results indicate that elevated sleep of desert-adapted *D. mojavensis* correlates with both an increase in sleep-promoting 5-HT and reductions of arousing OA and PDF.

Sleep in *Drosophila mojavensis* supports resilience to nutrient deprivation

D. mojavensis sleeps more than *D. melanogaster* and responds to prolonged waking with a homeostatic rebound, indicating that this species may have an increased drive for sleep relative to *D. melanogaster*. To further test the functional relevance of heightened sleep pressure in desert-adapted flies, we also measured sleep and survival while flies were deprived of food alone or both food and water. Both Baja and Mojavensis subspecies of *D. mojavensis* survive longer than wild-type *D. melanogaster* when housed in glass tubes with non-nutritive agar media (Figure 6A) or in empty, dry glass tubes (Figure 6B), as described previously.[10] Although wild-type *D. melanogaster* suppress their sleep during food deprivation,[55,56] *D. mojavensis* instead show subspecies-specific changes. *D. moj. baja* exhibit moderate increases in sleep time during several days of food deprivation and awaken when both food and water are unavailable (Figure 6C). By contrast, *D. moj*. *moj.* show no significant sleep changes when food deprived and only a transient increase in sleep on the first day of food and water deprivation (Figure 6D). These trends are consistent with the hypothesis that elevated sleep in *D. mojavensis* is associated with prolonged survival during nutrient deprivation. We tested this relationship by depriving *D. mojavensis* females of food alone or both food and water, then housing them either in 12 h:12 h LD light or in constant blue light (LL) to disrupt sleep. Although constant light did not increase mortality in fed flies (Figure S6A), food-deprived *D. moj*. *baja* that were housed in constant blue light die from food deprivation more rapidly than siblings housed in LD (Figures 6E and S6B).

replicate; squares represent data from experiment #1, triangles are from experiment #2). One-way ANOVAs find a significant effect of genotype for 5-HT ($F_{(5,30)}$ = 10.26, $p < 0.0001$), octopamine ($F_{(5,30)}$ = 9.488, $p < 0.0001$), and histamine ($F_{(5,30)}$ = 5.950, $p = 0.0006$) but no significant effect of genotype for dopamine ($F_{(5,30)}$ = 2.465, $p = 0.055$).

(E–G) Immunostaining for SERT (green) in brains from *D. melanogaster* (E), *D. moj*. *baja* (F), and *D. moj*. *moj.* (G). Z-projections showing cell bodies from anterior of brain in top row, from posterior in bottom row. Scale bars in (G) also depict dimensions for (E) and (F). Cell cluster labels based on Kim et al.[47]

(H–J) Immunostaining for Tdc2 (cyan) in whole brains from *D. melanogaster* (H), *D. moj*. *baja* (I), and *D. moj*. *moj.* (J). Z-projections showing cell bodies from anterior of brain in top row, from posterior in bottom row. Cell cluster labels based on Donelson et al.[48]

(K) Sleep time course for *D. moj*. *baja* flies that were microinjected with 18.4 nL of 20mM octopamine (blue) or vehicle (gray). Green shading denotes the time of OA injection. Two-way repeated ANOVA finds a significant time-by-treatment interaction ($F_{(143,5863)}$ = 1.651, $p < 0.0001$).

(L and M) Total sleep (L) and normalized activity counts (M) during 24 h post-injection for groups shown in (K). At least one distribution in (L) and (M) fail D'Agostino and Pearson test for normality; Mann-Whitney tests find U = 125, $p = 0.0092$ for (L) and U = 110.5, $p = 0.0051$ for (M).

For (K)–(M), n = 21 vehicle control and 22 OA-injected flies.

See also Figure S5. Group averages and error bars represent mean and SEM for all panels.

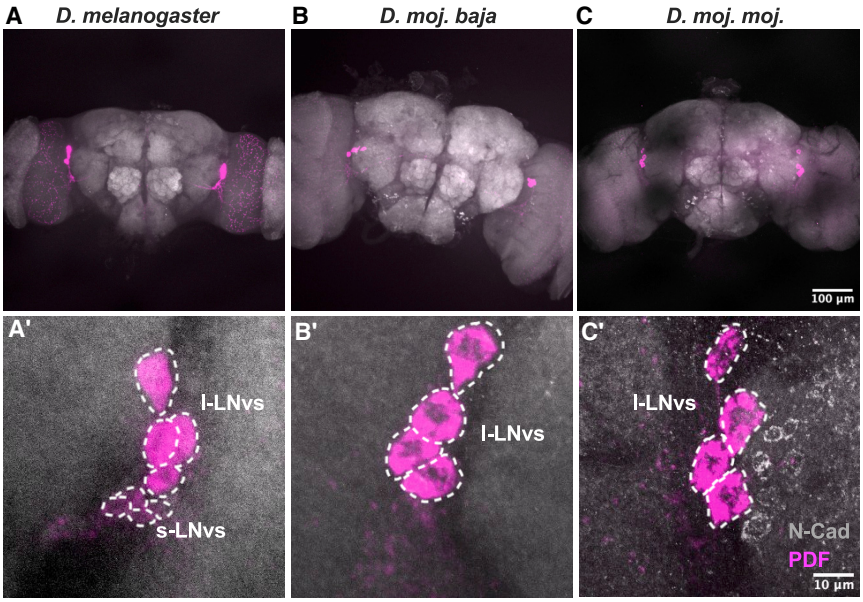

Figure 5. Interspecies variability in PDF distribution

(A) Confocal projection of PDF immunostaining in a whole brain from *D. melanogaster*. (A′) Higher magnification confocal micrograph of LNv cell bodies in *D. melanogaster*.

(B) PDF immunostaining in *D. moj. baja* brain, high magnification image of l-LNv soma in (B′).

(C) Distribution of PDF in *D. moj. moj.* brain; (C′) depicts the high magnification view of l-LNv cell bodies. (A)–(C) use identical scales (see 100 μm scale bars in C), (A′)–(C′) also use matching scales (see 10 μm scale bars in C′).

To test whether disrupted sleep might render *D. mojavensis* flies more sensitive to starvation, we examined the relationship between average daily sleep and survival time using the individuals shown in Figure 6E and found a highly significant positive correlation (Figure 6F). Similar trends arose when *D. moj. baja* flies were exposed to constant light to suppress sleep while deprived of both food and water (Figure S6C); constant light exposure reduced survival time (Figure 6G), and daily sleep strongly correlated with survival (Figure 6H). Although LL exposure significantly reduced survival time in food-deprived *D. moj. moj*. (Figure 6I), the effect of constant light was less pronounced than in *D. moj. baja*. Interestingly, individual *D. moj. moj.* flies showed a wide variety of sleep responses to constant light during starvation (Figure 6J) and subdividing the *D. moj. moj*. flies that were housed in LL during food deprivation revealed that the half of that group with the lowest sleep time during starvation died earlier than the half with the weakest sleep disruption (Figure 6K).

To more broadly examine whether sleep loss might render *D. mojavensis* flies more sensitive to starvation, we tested the correlation between average daily sleep and survival time in *D. moj. moj.* (Figure 6L) and, as with *D. moj. baja*, found highly significant positive correlations between daily sleep and starvation survival time. When *D. moj. moj.* were denied both food and water, LL exposure alone had no significant effect on survival time (Figure 6M). When the LL group of *D. moj. moj.* flies were sorted by daily sleep, we found that the half with the lowest amount of daily sleep during desiccation showed reduced survival time (Figures 6N–6O). Further, plotting individual daily sleep against desiccation survival time for *D. moj. moj.* that were housed in LD (filled dots) or in constant light (open dots) revealed a significant positive correlation across both experimental groups (Figure 6P). These data indicate that high amounts of sleep may confer desert-adapted flies with resistance to periods of insufficient food or water. We also detected significant negative correlations between survival and daily activity counts, indicating that the effect of sleep could, in part, be linked with decreased energy consumption during locomotion (Figure S6). Due to the significant correlation between waking activity intensity (counts/waking minute) and survival only for food-deprived *D. moj. baja* (Figure S6E) and not for food- and water-deprived *D. moj. baja* or either *D. moj. moj.* condition (Figures S6H, S6K, and S6N), it is likely that the influence of activity on survival can be linked with sleep amount and not necessarily changes in intensity of waking activity.

DISCUSSION

Periods of adaptive sleep loss have been reported in several vertebrate species, especially in birds[57] and marine mammals.[58,59] During these periods, it is thought that animals can acutely defer or offset the costs that accumulate from sleep loss. Here, we find that *D. mojavensis* exhibits an opposing behavioral strategy: they chronically show an elevated intrinsic sleep quota, even during periods of insufficient food. This adaptive strategy confers a survival advantage in conditions of hunger or thirst, supporting a functional role for sleep in maintaining efficient energy usage.[60–62] Similarly, recent studies found that flies show reduced metabolic rate while asleep[63] and that sleep is elevated in *D. melanogaster* artificially selected for starvation resistance.[16] Along these lines, it is possible that increasing daily sleep quotas, including during starvation, could protect *D. mojavensis* by slowing the usage of energy stores when food is not available. Alternatively, high amounts of sleep may allow desert-adapted *D. mojavensis* to allocate energy reserves specifically to necessary functions most efficiently fulfilled during sleep,[62,64,65] such as clearing metabolic waste,[66,67] managing oxidative stress,[68–70] or scaling synaptic connectivity.[71–74] The high sleep quota in *D. mojavensis* could indicate that increased metabolic investment in these sleep-restricted functions may be required to offset the costs of physiological adaptations made by desert-adapted flies that allow them to thrive in the desert environment.[9,11,18,75–78] In either case, consistently investing large amounts of time to sleep suggests that *D. mojavensis* likely trade behavioral flexibility for starvation resilience. Because of their reliably high daily sleep quotas, *D. mojavensis* may provide new opportunities to examine beneficial functions of sleep at times of insufficient food or other

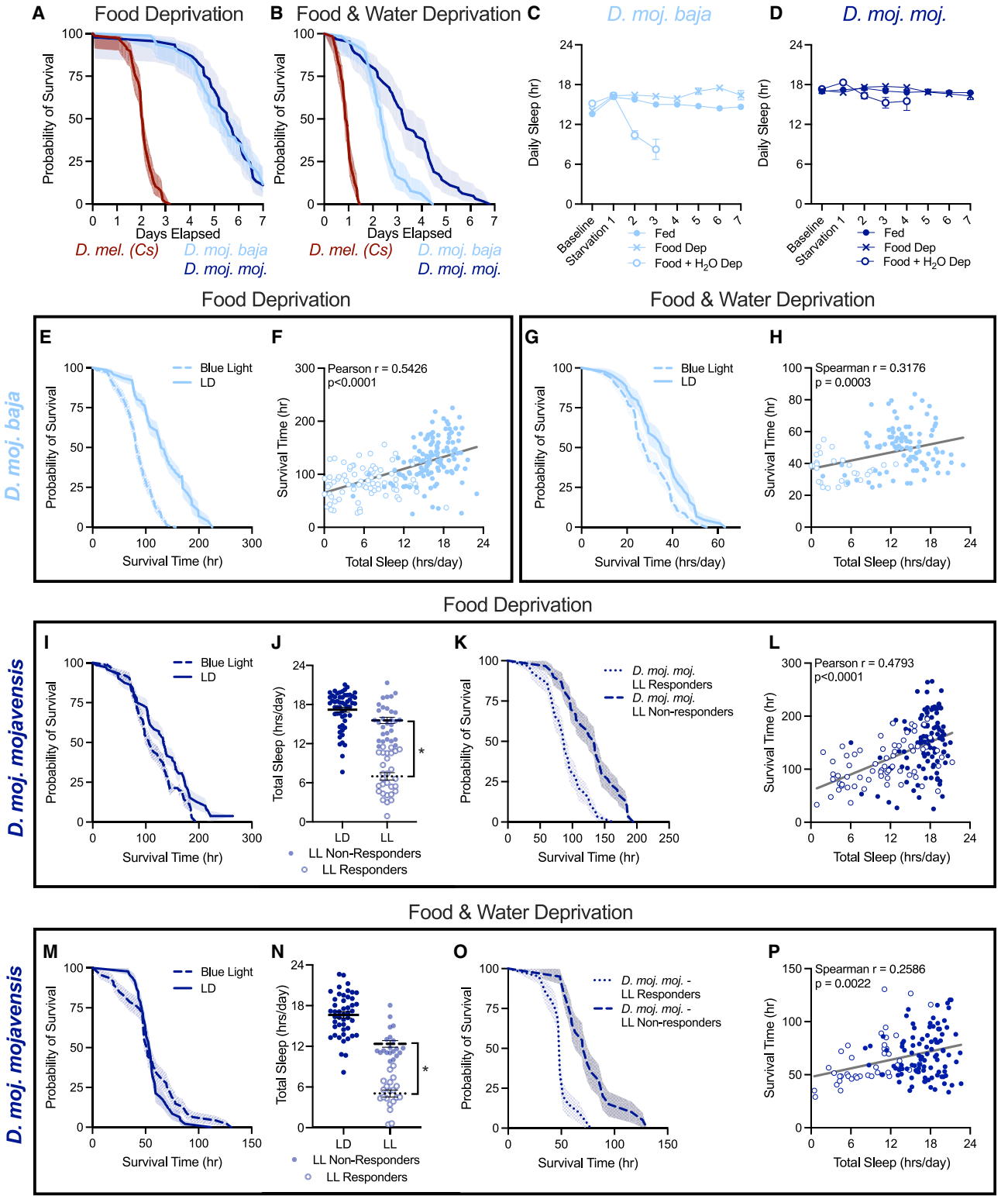

Figure 6. Sleep responses of *D. mojavensis* to nutrient deprivation correlate with survival time

(A and B) Survival times for *D. melanogaster* (*Cs*; red) and *D. mojavensis* (blues) females when housed on starvation agar (A) or dry tubes (B). Mantel-Cox test finds significant effects for (A) (χ^2 = 250.6, df = 2, $p < 0.0001$; n = 70 *Cs*, 45 *D. moj. moj.*, and 77 *D. moj. baja*) and for (B) (χ^2 = 232.6, df = 2, $p < 0.0001$; n = 64 *Cs*, 63 *D. moj. moj.*, and 64 *D. moj. baja*).

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physiological stressors. Interestingly, another recent study found that other *Drosophila* species exhibit a range of homeostatic responses to sleep loss,[79] indicating that broad studies of *Drosophila* evolution could uncover interspecific adaptations in sleep need or function.

Our characterization of increased sleep time in stress-resilient *D. mojavensis* provides a novel model species to examine the adaptive advantage(s) of elevated sleep and to investigate the evolution of sleep regulatory mechanisms across related species. Recent efforts to sequence the genomes of many *Drosophila* species have enabled the analysis of genetic correlates to environmental adaptations,[18,80–84] but the contributions of altered behavioral strategies as populations adapt to environmental niches remain to be explored. We anticipate that combining genomic approaches with behavioral phenotyping across many species could identify common mechanisms that drive changes in sleep regulation and in the underlying functions of sleep across the *Drosophila* genus. In this case, examining flies that have evolved to withstand high desert temperatures and periods of nutrient deprivation could inform our understanding of the tolls of changing global climates on physiology and identify possible behavioral approaches for animals to cope with a warming world.[85–87]

Our neurochemical and anatomical studies reveal correlations between sleep time and the abundance of two sleep/wake-related neuromodulators, 5HT and OA, in *D. melanogaster* and *D. mojavensis*. Similarly, we find a restricted distribution of the wake-promoting circadian output peptide PDF in long-sleeping *D. mojavensis* flies. The similar distributions of serotonergic and octopaminergic neurons within *D. melanogaster* and *D. mojavensis* suggest that sleep circuit organization may be conserved between the species but that mechanisms governing neural activity or signaling dynamics could be differentially tuned as populations evolve. The availability of sequenced genomes for many *Drosophila* species, including *D. mojavensis*,[47,88–90] may enable future studies to dissect neuromodulator signaling components with precise genetic tools similar to those already applied in *D. melanogaster*. These studies will be required to clearly test whether the altered 5HT and OA abundance directly alter sleep between species. Although the global organization of 5HT and Tdc2-expressing neurons appears largely similar between *D. melanogaster* and *D. mojavensis*, distribution of PDF expression in core circadian circuits differs. Similar to reports in several other *Drosophila* species,[52–54] we did not detect immunostaining for PDF in soma or axonal projections from s-LNvs, indicating that circadian circuit organization may commonly differ between fly species. Future studies will be required to examine the precise contributions of changes in each neuromodulator system to behavioral variations between species, and precisely examining each of these components may provide more insight into the functional importance of high sleep drive in *D. mojavensis*.

(C) Daily sleep time for *D. moj. baja* flies during 1 day of baseline conditions that were then fed standard fly media (closed blue circles), 1% agar in water (crosses), or dry tubes (open circles). Mixed effects analysis finds significant effect of time ($p < 0.0001$), but not of condition ($p = 0.0966$), $n = 153$ control, 108 food-deprived, and 110 food- and water-deprived flies at the beginning of the experiment.
(D) Daily sleep time for *D. moj. moj.* flies during 1 day of baseline followed by feeding either standard fly media (closed circles), 1% agar in water (crosses), or dry tubes (open circles). Mixed effects analysis detected no effect of condition ($p = 0.7460$) but a significant effect of time ($p = 0.0011$); $n = 140$ control, 95 food-deprived, and 100 food- and water-deprived flies at the beginning of the experiment.
(E) Survival time of food-deprived *D. moj. baja* was reduced for flies housed in constant blue light (dashed line) compared with siblings in 12 h:12 h LD (solid line). Log-rank (Mantel-Cox) test identifies a significant effect of constant light ($\chi^2 = 56.49$, $p < 0.0001$, $n = 76$ flies/group).
(F) Mean sleep/day for food-deprived *D. moj. baja* individuals that were housed in 12 h:12 h LD (filled dots) or in constant blue light (open dots) shows a positive association with survival time. Pearson r = 0.5128, $p < 0.0001$; $n = 60–76$ flies/group). Daily sleep was averaged across survival time for each fly.
(G) Survival time for *D. moj. baja* flies that were housed in LD (solid line) or constant blue light (dashed line) while deprived of both food and water. Mantel-Cox test $\chi^2 = 5.126$, $p = 0.0236$; $n = 44–48$ flies/group.
(H) Average daily sleep plotted against survival time for individual *D. moj. baja* flies housed in LD (filled dots) or constant blue light (open dots). Spearman r = 0.3176, $p = 0.0003$, $n = 29$ flies in constant light and 95 flies in LD. Total sleep was averaged across each day of survival for individual flies.
(I) Survival time for food-deprived *D. moj. moj.* females that were housed in 12 h:12 h LD (solid line) or constant blue light (dashed line). Log-rank (Mantel-Cox) test finds a significant effect of light exposure ($\chi^2 = 4.977$, $p = 0.0257$, $n = 72–75$ flies/group).
(J) Daily sleep of food-deprived *D. moj. moj.* housed in LD or constant light. Flies exposed to LL that responded with daily sleep in the lower half of the distribution marked with open circles, filled circles mark non-responders with the highest sleep amounts. Mann-Whitney test between blue light responders vs. non-responders; $p < 0.0001$; $n = 29$ LL responders, 30 LL non-responders, and 56 LD. Total sleep was averaged across each day of survival for individual flies.
(K) Starvation survival time for *D. moj. moj.* that responded to constant light with the lowest sleep amounts (dotted line) was reduced compared with siblings with higher sleep amounts during light exposure (dashed line). Mantel-Cox test $\chi^2 = 28.16$, $p < 0.0001$, $n = 29–30$ flies/group.
(L) Mean sleep/day during starvation for individual *D. moj. moj.* females housed in 12 h:12 h LD (filled dots) or constant blue light (open dots) correlates positively with survival time. Pearson r = 0.4523, $p < 0.0001$, $n = 56$ flies in LD and 59 flies in constant blue light. Total sleep was averaged across each day of survival for individual flies.
(M) Survival time for food- and water-deprived *D. moj. moj.* housed in LD (solid line) or constant blue light (dashed line). Mantel-Cox test $\chi^2 = 1.225$, $p = 0.2684$, $n = 47$ flies/group.
(N) Daily sleep during food and water deprivation for *D. moj. moj.* flies housed in LD or constant blue light. Flies exposed to LL that responded with daily sleep in the lower half of the distribution marked with open circles; filled circles mark non-responders with the highest sleep amounts. Mann-Whitney test between blue light responders vs. non-responders; $p < 0.0001$; $n = 20$ LL responders, 20 LL non-responders, and 47 LD. Total sleep was averaged across each day of survival for individual flies.
(O) Survival time during food and water deprivation for *D. moj. moj.* females housed in constant blue light. Short-sleeping responders represented by dotted line and longer-sleeping non-responders by dashed line. Mantel-Cox test $\chi^2 = 21.39$, $p < 0.0001$, $n = 20$ flies/group.
(P) Scatterplot of daily sleep vs. survival time for *D. moj. moj.* that were housed in LD (filled dots) or constant blue light (open dots) during food and water deprivation. Spearman r = 0.2586, $p = 0.0022$, $n = 98$ flies in LD and 40 flies in constant light. Total sleep was averaged across each day of survival for individual flies.
See also Figure S6. Group averages and error bars represent mean and SEM for all panels.

STAR★METHODS

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SUPPLEMENTAL INFORMATION

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AUTHOR CONTRIBUTIONS

J.Y., C.N., K.L., P.H., C.J., M.R., M.L.F., D.T., X.H., and J.M.D. performed the experiments and/or analyzed data. G.E. and J.P.W. provided consultations, completed LC-MS experiments, and analyzed the data. S.W. and J.M.D. initially discussed and designed the project. J.M.D. supervised the research. J.Y., C.N., and J.M.D. integrated the data, interpreted the results, and wrote the manuscript. All authors discussed the results and commented on the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Experimental models: *Drosophila* strains		
Canton-S (*D. melanogaster*)	Gero Miesenböck (University of Oxford)	N/A
Pcf (*D. melanogaster*)	Mark Frye (UCLA)	N/A
D. mojavensis mojavensis	Luciano Matzkin (University of Arizona)	N/A
D. mojavensis baja	Luciano Matzkin (University of Arizona)	N/A
D. mojavensis sonorensis	Luciano Matzkin (University of Arizona)	N/A
D. mojavensis wrigleyi	Luciano Matzkin (University of Arizona)	N/A
D. arizonae	NDSSC (Cornell University)	SKU: 15081-1271.36
D. buzzatii	NDSSC (Cornell University)	SKU: 15081-1291.02
D. mulleri	NDSSC (Cornell University)	SKU: 15081-1371.01
Antibodies		
Mouse anti-PDF C7	DSHB (University of Iowa)	DSHB Cat# PDF C7, RRID:AB_760350
Rat anti-CadN (DN-EX#8-C)	DSHB (University of Iowa)	DSHB Cat# DN-Ex #8, RRID:AB_528121
Rabbit anti-5HT (Serotonin) Transporter	Immunostar	ImmunoStar 24330, RRID:AB_572209
Rabbit anti-Tyrosine decarboxylase 2 (Tdc2)	Covalab	Ref. 00013520
Alexa Fluor488 Goat Anti-Rabbit IgG (H+L)	ThermoFisher Scientific	Cat # A-11008, RRID: AB_143165
Alexa Fluor488 Goat Anti-Mouse IgG (H+L)	ThermoFisher Scientific	Cat # A-11001, RRID: AB_2534069
Alexa Fluor488 Goat Anti-Rat IgG (H+L)	ThermoFisher Scientific	Cat # A-11006, RRID: AB_2534074
Software		
DAMSystem311	Trikinetics Inc. (www.trikinetics.com)	N/A
Visual Basic sleep analysis macros	Paul Shaw (Washington University in St. Louis)	N/A
SCAMP	Chris Vecsey (Colgate University)	N/A
MATLAB R2022b	MathWorks (www.mathworks.com)	N/A
Prism 10	Graphpad (www.graphpad.com)	N/A
FIJI	FIJI (https://fiji.sc)	N/A
Chemicals		
Phosphate buffered saline tablets	Sigma-Aldrich	P4417-100TAB
Triton X-100	Sigma-Aldrich	X100-100ML
VECTASHIELD Mounting Medium	Vector Laboratories	H-1000
Paraformaldehyde 32% Solution	Electron Microscopy Sciences	15710-S
2-(3,4-Dihydroxyphenyl)ethyl-2,2-d2-amine HCl	CDN Isotopes	D-1493
Histamine-alpha,alpha,beta,beta-d4 2HCl	CDN Isotopes	D-2270
(±)-p-Octopamine-alpha,beta,beta-d3 HCl	CDN Isotopes	D-1774
Serotonin-alpha,alpha,beta,beta-d4 Creatinine Sulfate Complex H_2O	CDN Isotopes	D-1550
Deposited data		
Source data	This paper	https://doi.org/10.5061/dryad.w9ghx3fxw

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Jeffrey Donlea (jdonlea@ucla.edu).

Materials availability

This study did not generate new unique reagents.

Data and code availability

- Source data have been deposited at Dryad and are publicly available as of the date of publication. DOI is listed in the key resources table.
- This paper does not report original code.
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

Fly rearing and stocks

Fly stocks were cultured on standard cornmeal molasses media (per 1L H_2O: 12 g agar, 29 g Red Star yeast, 71 g cornmeal, 92 g molasses, 16mL methyl paraben 10% in EtOH, 10mL propionic acid 50% in H_2O) at 25°C with 60% relative humidity and entrained to a daily 12h light, 12h dark schedule. Experiments with Banana-Opuntia media used a recipe from the National *Drosophila* Species Stock Center (NDSSC; Cornell University): per 1L H_2O: 14.16g agar, 27.5 g yeast, 2.23g methyl paraben, 137.5g blended bananas, 95g Karo Syrup, 30g Liquid Malt Extract, 22.33g 100% EtOH, 2.125g powdered opuntia cactus.

Canton-S were provided by Dr. Gero Miesenböck (University of Oxford) and *Pcf* were shared by Dr. Mark Frye (UCLA). Primary stocks of *D. moj*. *mojavensis* (collected February 2020, North Joshua Tree National Park, CA), *D. moj*. *baja* (collected March 2020, La Paz, Mexico), *D. moj*. *wrigleyi* (collected November 2017, Catalina Island, CA), and *D. moj*. *sonorensis* (collected March 2020, Guyamas, Mexico) were a gift from Dr. Luciano Matzkin (University of Arizona), and additional stocks of *D. moj*. *mojavensis* and *D. moj*. *baja* were shared by Dr. Paul Garrity (Brandeis University). *D. arizonae* (SKU: 15081-1271.36), *D. mulleri* (SKU: 15081-1371.01), and *D. buzzatii* (SKU: 15081-1291.02) were ordered from the NDSSC. Wild caught *D. melanogaster* descended from a single pair of flies trapped in Los Angeles, CA in spring, 2021.

METHOD DETAILS

Behavior

4-8 day old female flies were housed individually in borosilicate glass tubes (65mm length, 5mm diameter) containing fly food coated with paraffin wax at one end and a foam plug in the other. Locomotor activity was recorded using DAM5M or DAM5H multibeam *Drosophila* Activity Monitors from Trikinetics Inc. (Waltham MA, USA) and sleep was analyzed in Matlab (MathWorks Inc) with the SCAMP script package.[48] Locomotor activity was measured as the number of movements between beams per one-minute bins. Periods of sleep were defined by at least 5 minutes with no change in position within the multibeam activity monitors. Sleep time courses display 30-min time bins and X-axis time labels denote zeitgeber time (ZT) in hours after lights-on

Sleep deprivation and arousability

Sleep deprivations were performed mechanically by mounting DAM5M activity monitors onto platform vortexers (VWR 58816-115). Individual tubes were plugged with food at one end and 3D-printed PLA plastic caps at the other. Monitors were vortexed at an intensity of 2.5g for 3-second pulses every minute through the duration of the 12-hour dark period. Arousability was tested in a darkened incubator with 60 seconds of blue light (luminance 0.048 Lv) every hour for 24 hours following sleep deprivation.

Food- and water-deprivation assays

All flies were put in DAM5H activity monitors on standard food for baseline recording. After 2-3 days, control flies were transferred to tubes containing fresh food, food-deprived flies to tubes containing a 1% agar gel, and food-and-water-deprived flies to empty tubes plugged with foam at both ends. Flies immobile for at least 24 hours were defined as dead and data subsequent to their last full day alive was removed from sleep analysis.

Pharmacological microinjections

4–8 day old female flies were loaded into behavior tubes and monitored in DAM5M Activity Monitors to obtain baseline sleep and locomotor activity under 12h light: 12h dark (25°C). After 1-2 days of baseline in DAM5M monitors, flies housed in borosilicate tubes were placed on ice for anesthetization prior to injection using Drummond Nanoject II. For injection of exogenous neuromodulators, the anteriormost ocelli of *D. mojavensis baja* were injected with 18.4nl of 20mg/mL of Octopamine (Sigma-Aldrich, Catalog # O0250). For each round of injections, new OA is solubilized using Schneider's *Drosophila* Medium with L-Glutamine (Genesee Scientific, Catalog # 25-515). Following each individual injection, flies are returned back into individual borosilicate tubes, and placed in respective DAM5M Activity Monitors to continue sleep and activity surveillance for >48h.

Immunohistochemistry

Female *D. melanogaster* and *D. mojavensis* were reared in 12h light:12h dark schedule at 25°C in normal fly food. Individual fly brains were dissected 5-7 days post-eclosion between a ZT0-ZT3 window to minimize time-of-day variation to antibody targets. All dissections, antibody staining, and preparation for imaging were carried out in the exact same manner to minimize variability when comparing between species. Flies are anesthetized using ice. Brains were dissected in chilled 1X PBS then placed in 4.0% paraformaldehyde/1X PBS (PFA) for 30 mins. in room temperature on a benchtop rotator. PFA from brains were removed by washing with 1.0% Triton-X in 1X PBS 3 times for 10 mins. each. Once brains were free of PFA, the brains were placed in 1x Sodium Citrate (10mM, pH=6.0, 15 mins. at 80°C) for antigen retrieval. Brains were then placed in a blocking buffer (5.0% normal goat serum in 0.5% Triton-X/1X PBS) and incubated at room temperature for 1.5h on a rotator. Brains were incubated with one the following primary antibodies (diluted using blocking buffer): 1:1000 Mouse anti-PDF (Developmental Studies Hybridoma Bank). Primary antibodies were incubated for two days in 4°C on a rotator. After incubation, brains were washed using 0.5% Triton-X in 1X PBS five times, 10 mins. each. Fly brains were then incubated in AlexaFluor secondary antibodies (1:1000 Goat anti-Mouse AlexaFluor 633nm; Molecular Probes) overnight at 4°C. Brains were washed using 0.5% Triton-X in 1X PBS five times, 10 mins. After washing, brains were mounted on glass slides in Vectashield mounting media, sealed with a coverslip and nail polish. Brains were imaged using a Zeiss LSM 780 laser scanning confocal microscope using a z-slice thickness of 1um and saved as CZI files. Maximum intensity projections were created from CZI files using FIJI/ImageJ (https://imagej.net/software/fiji/).[91]

Neurochemical quantifications

Sample preparation protocol

Fly brain samples were stored at -80°C then treated with 99.9/1 Water/Formic Acid. An internal standard (IS) of each targeted compound was added to every sample to account for compound loss during sample processing. The samples are vortexed, homogenized for 30 sec in a bead beater using 2.0 mm zirconia beads, and centrifuged at 16.000xg for 5 min. The supernatant is transferred to new microcentrifuge test tubes and dried in a vacuum concentrator. The samples are reconstituted in 40 μl of water, vortexed, and centrifuged. The supernatant is transferred to HPLC vials and 10 μl is injected to an HPLC - triple quadrupole mass spectrometer system for analysis.

Liquid chromatography-tandem mass spectrometry

A targeted LC-MS/MS assay was developed for each compound using the multiple reaction monitoring (MRM) acquisition method on a triple quadrupole mass spectrometer (6460, Agilent Technologies) coupled to an HPLC system (1290 Infinity, Agilent Technologies) with an analytical reversed phase column (GL Sciences, Phenyl 2 μm 150 x 2.1 mm UP). The HPLC method utilized a mobile phase constituted of solvent A (100/0.1, v/v, Water/Formic Acid) and solvent B (100/0.1, v/v, Acetonitrile/Formic Acid) and a gradient was used for the elution of the compounds (min/%B: 0/0, 10/0, 25/75, 27/0, 35/0). The mass spectrometer was operated in positive ion mode and fragment ions originating from each compound was monitored at specific LC retention times to ensure specificity and accurate quantification in the complex biological samples (Octopamine OA 159-136, Histamine HA 112-95, Dopamine DA 154-137, Serotonin 5HT 177-160). The standard curve was made by plotting the known concentration for each analyte of interest (CDN Isotopes) against the ratio of measured chromatographic peak areas corresponding to the analyte over that of the labeled standards. The trendline equation was then used to calculate the absolute concentrations of each compound in fly brain tissue.

QUANTIFICATION AND STATISTICAL ANALYSIS

Statistical analysis

Statistical tests were completed as described in the figure legends using Prism 9 (GraphPad Software, Boston MA, USA). Statistical comparisons primarily consist of one- or two-way ANOVAs followed by pairwise Holm-Sidak's multiple comparisons test when experiments include at least three experimental groups or two-tailed Student's T-test for experiments that include two groups; specific tests used are described in each figure legend. All data figures pool individual data points from at least two independent replicates.