Protocol

Neural Stimulation during *Drosophila* Activity Monitor (DAM)-Based Studies of Sleep and Circadian Rhythms in *Drosophila melanogaster*

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Sleep is a fundamental feature of life for virtually all multicellular animals, but many questions remain about how sleep is regulated by circadian rhythms, homeostatic sleep drive that builds up with wakefulness, and modifying factors such as hunger or social interactions, as well as about the biological functions of sleep. Substantial headway has been made in the study of both circadian rhythms and sleep in the fruit fly *Drosophila melanogaster*, much of it through studies of individual fly activity using *Drosophila* activity monitors (DAMs). Here, we describe approaches for the activation of specific neurons of interest using optogenetics (involving genetic modifications that allow for light-based neuronal activation) and thermogenetics (involving genetic modifications that allow for temperature-based neuronal activation) so that researchers can evaluate the roles of those neurons in controlling rest and activity behavior. In this protocol, we describe how to set up a rig for simultaneous optogenetic or thermogenetic stimulation and activity monitoring for analysis of sleep and circadian rhythms in *Drosophila*, how to raise appropriate flies, and how to perform the experiment. This protocol will allow researchers to assess the causative role in the regulation of sleep and activity rhythms of any genetically tractable subset of cells.

MATERIALS

It is essential that you consult the appropriate Material Safety Data Sheets and your institution's Environmental Health and Safety Office for proper handling of equipment and hazardous materials used in this protocol.

RECIPES: Please see the end of this protocol for recipes indicated by <R>. Additional recipes can be found online at http://cshprotocols.cshlp.org/site/recipes.

A comprehensive list of required materials and equipment is provided below. Specific companies and catalog numbers are proposed for certain items, but equivalents can also be obtained.

Reagents

All-*trans*-retinal (ATR, 100 mM) <R> (optional; see Steps 15 and 24)

Wear gloves when handling ATR.

Bacto agar (Carolina Biological Supply 156783B)

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Bleach, 7.5% (v/v) sodium hypochlorite
Bloomington *Drosophila* Stock Center fly lines:
w [1118]; P{y[+t7.7] w[+mC] = 20XUAS-IVS-CsChrimson.mVenus} attP2 (BL 55136)
w [*]; P{y[+t7.7] w[+mC] = UAS-TrpA1(B). K} attP16w [1118]; P{y[+t7.7] (BL 26263)
w [+mC] = GAL4.1Uw} attP2 (BL 68384)
y [1] w [*]; PBac{y[+mDint2] w[+mC] = 20XUAS-TTS-shi[ts1]-p10} VK00005 (BL 66600)
D. melanogaster driver lines of desired genotypes (selected based on the desired target neurons)
Drosophila cornmeal–yeast food <R> with and without ATR (Juneau et al. 2019)

To prepare ATR+ food, add all-trans-retinal (ATR, 100 mM) <R> to a final concentration of 1 mM to fly food immediately before dispensing the food into vials.

ATR+ food can also be prepared by melting a known volume of cornmeal food in the microwave, mixing in the ATR solution at 1:100, and then pouring the resulting 1 mM ATR food into bottles and/or vials as needed.

Milli-Q-purified H_2O
Sucrose (Fisher Scientific)

Equipment

Aluminum foil
Autoclave
Caps (black, CAP5; TriKinetics)
CO_2 diffusion system
CO_2 regulator (Genesee Scientific 59-143)
CO_2 tank
Colander
"Daisy Chain" phone cable connectors (SPLT5; TriKinetics)
DAMSystem3 software (TriKinetics)
Data collection computer (Macintosh or Windows)
Dissection stereomicroscope
Drosophila bottles (6-oz, square-bottom, polypropylene; Genesee Scientific)
Drosophila activity monitors (DAMs) (TriKinetics)

We used DAM2, but other types, such as DAM1 or DAM5M, can also be used.

Drosophila environmental monitor (DEnM) (TriKinetics)
Drosophila vials (narrow, polystyrene; Genesee Scientific)
Erlenmeyer flask (500-mL)
Flypad (Genesee Scientific 59-114)
Glass beakers (500- and 1000-mL)
Incubator (e.g., Percival Scientific I-30-BLL)
Kimwipes
LC4 Light Controller (TriKinetics)
Microwave
Paintbrushes (small)
Parafilm (Fisher Scientific S37440)
Petri dishes, 60-mm × 15-mm (Fisher Scientific FB0875713A)
Phone cables (2-ft, CAB2 and 10-ft, CAB10; TriKinetics)
Pint-sized plastic container for humidification
Plastic wrap
Polycarbonate tubes (PPT5 × 65; TriKinetics)
PSIU9 Power Supply Interface Unit (TriKinetics)
PVC tubing, 0.125-in-ID × 0.25-in-OD (Sigma-Aldrich Z280356)
Red-light grid (HQRP 225 Red LED Indoor Garden Plant Grow Light Panel, 14-W)
Rubber bands

Safety pin or other dissection pin
Scissors
Uninterruptible power supply (UPS) with sufficient capacity (1500-VA, 940-W; Tripp Lite)
USB cable
Ziplock bags

METHOD

Incubator Setup

Overall, refer to the DAMSystem Overview document available on the TriKinetics website (https://www.trikinetics.com).

1. Fill a pint-sized plastic container halfway with H_2O and place it in the bottom of the incubator to keep the inside humidified.

   In our experience, 50%–70% humidity is ideal for the long-term health of the flies.

2. Place the red-light grid so that it is either hanging directly above or supported directly below where the DAMs will be placed. Run the power cable for the red-light grid out of the incubator (ideally through a side port that can be blocked to prevent light entry) and connect it to one of the power outputs on the LC4 unit.
3. Connect CAB2 phone cables to daisy chains, run the final daisy chain cable out of the incubator (ideally through the same side port as in Step 2), and connect it to the PSIU9 unit using a CAB10 phone cable.
4. Plug one CAB2 phone cable into the DEnM. Place the DEnM unit in the incubator where it can accurately assess light, temperature, and humidity conditions in the incubator.
5. Connect the LC4 unit to AC power and to the PSIU9 unit.
6. Plug all data-collection and light-control power cables, including for the data-collection computer, into a UPS. Next, plug the UPS into a wall socket.

   This will prevent data loss and experiment disruptions in the case of brief power outages. Check to make sure that the UPS unit has the capacity to support the hardware connected to it. For this reason, we do not connect the incubator itself to a UPS, as overloading the UPS can be a fire risk.

Setup of the Data-Collection Computer

Overall, refer to the DAMSystem3 QuickStart Guide document available on the TriKinetics website (https://www.trikinetics.com).

7. Download and install the appropriate USB driver from the TriKinetics website.
8. Install the appropriate version of the DAMSystem3 software based on your operating system.
9. Establish the serial port being used by the PSIU9. Once the power source is connected and the driver is installed, the DAMSystem3 program identifies a serial port with the prefix "COM."
10. Under "Preferences," select the monitor range so that it includes the numbers of all monitors being used (including data monitors and the DEnM).
11. Under "Preferences," set the reading interval to be 1 min.

    This is necessary for appropriate measurements of sleep behavior during analysis.

12. Under "Light Controller," create settings to output to the appropriate light channel to turn on the red-light grid at the desired time and for the desired duration.

    In many of our optogenetic experiments, we used a 1-h light pulse that was set ahead of time to turn on at a specific time point during the second full day of data collection. Note that the system can be set up to elicit

more complicated intermittent light-pulse patterns, although the light grid described in this protocol is not designed to turn off and on rapidly. More information about establishing these settings can be found in the LC4 data sheet document available on the TriKinetics website.

13. Connect the PSIU9 unit by USB cable to the data-collection computer.

Drosophila Preparation

For basic information about fruit fly husbandry, genetics, and mating schemes, please see Roote and Prokop (2013), including the Supplemental Material.

Prepare Drosophila *for optogenetic (see Steps 14–19) or thermogenetic (see Steps 20 and 21) experiments.*

Optogenetic Experiments

14. Using a CO_2 diffusion setup (including CO_2 tank, regulator, tubing, and Flypad), anesthetize flies at room temperature. Under a stereomicroscope, use a paintbrush to select *Drosophila* of appropriate parental genotypes that can be mated together to generate the desired fly lines to test.

    There are many options available at this point, but the experimental goal is to create flies that will express a red-light-activated optogenetic sensor under the control of a driver line that expresses within neurons of interest. Although using the GAL4/UAS binary system is most common, there are available tools that use other systems such as LexA/LexAOp and QF2/QUAS as well. The lines that we have used the most are (1) w^-;;UAS-CsChrimson attP2 (III) (BL 55136), (2) GAL4 line of interest, backcrossed onto the w^-;; line, and (3) w^-;;.

15. Cross parental flies together in bottles with ATR^- or ATR^+ food using male flies for strains on the left and virgin females for strains on the right. The total number of flies to use depends on the desired number of offspring (we typically aim for 30 females), but regardless it is recommended to use a ratio that slightly favors females. Leave flies together for ~4–7 d before transferring them to a new bottle or discarding them.

    - Cross 1: GAL4 × w^-;;UAS-CsChrimson
    - Cross 2: GAL4 × w^-;;
    - Cross 3: w^-;; × w^-;;UAS-CsChrimson

      Sexes could be reversed, but in our experience, it is beneficial to use virgin females of the UAS-CsChrimson line because they are healthy and the transgene is homozygous, so all virgins are useful to use as parents.

16. Determine the lighting conditions.

    - If using ATR^+ food throughout development, raise all crosses in total darkness to avoid unwanted activation of the targeted neurons throughout development and bleaching of ATR.

      This ensures that offspring have a functional optogenetic sensor (in this case, Chrimson) as soon as they are collected from the crosses.

    - If crosses are raised on ATR^- food, use normal 12-h–12-h light–dark conditions and then transfer collected progeny to ATR^+ food in total darkness for at least 3 d before experimentation.

17. Once the crosses are set up, flip the parental flies into new bottles every 3–5 d.

    At a temperature of 25°C, progeny will begin to eclose ~10–12 d after a cross is added to a bottle. To ensure that flies from the F1 generation are used for the experiment, do not collect adults from bottles that had parents added to them more than 20 d prior.

18. Collect young (0- to 3-d-old) experimental flies.

    i. As in Step 14, anesthetize the flies with CO_2 and then place them onto a CO_2 pad.

    ii. Using a microscope, select flies with the desired genotypes based on visible markers. Other than eye color, none of the lines described here have visible markers. If the driver line being used has markers, select against them in the offspring. For example, if using a GAL4 line over the classically used CyO balancer, flies with curly wings would be selected against.

iii. Add males and females into separate vials containing ATR+ food. Add some males (genotype does not matter) to the female vial to ensure that all of them are mated.

While collecting flies, keep all vials in a dark area.

19. Place the vials into an incubator with constant dark conditions until it is time to load the experiment (typically 3–4 d).

If initial collections did not yield the desired number of flies for each group, a second collection may be necessary.

Proceed to Step 22.

Thermogenetic Experiments

20. Using a CO_2 diffusion setup (including CO_2 tank, regulator, tubing, and Flypad), anesthetize flies at room temperature. Under a stereomicroscope, use a paintbrush to select *Drosophila* of appropriate parental genotypes that can be mated together to generate the desired fly lines to express temperature-gated neuronal activators or silencers in neurons using UAS-GAL4, LexA/LexAop, or QF2/QUAS systems. Cross parental flies together in bottles, using male flies for strains on the left and virgin female flies for strains on the right.

- Cross 1: GAL4 × w^-;;UAS-dTRPA1 (BL 26263)/UAS-Shi^{ts1} (BL 66600)
- Cross 2: GAL4 × w^- (BL 5905);
- Cross 3: w^-;; × w^-; UAS-dTRPA1 or w^-;;UAS-Shi^{ts1}

This is a list of fly stocks, crosses, and genotypic controls commonly used in our laboratory.

For large-scale screening of GAL4 lines in a common genetic background and genomic insertion site (e.g., attP2), enhancer-less empty GAL4 (BL 68384) crossed to effectors can be used as an additional control.

Sexes could be reversed, but in our experience, it is beneficial to use virgin females of the UAS-dTRPA1 (II) and UAS- Shi^{ts1} (III) lines because they are healthy and the transgene is homozygous, so all virgins are useful to use as parents for the crosses.

21. Collect and raise experimental animals.

i. Maintain crosses at 21°C to ensure that the channels are not activated during development.

ii. Flip the parental flies into new bottles every 3–5 d.

At this temperature, progeny will typically begin to eclose ~12–15 d after a cross is added to a bottle. To ensure that flies from the F1 generation are used for the experiment, do not collect adults from bottles that had parents added to them more than 24 d prior.

iii. As in Steps 18.i and 18.ii, collect young (0- to 3-d-old) experimental flies.

iv. Place the vials into an incubator maintained at 21°C until it is time to load the experiment (typically 3–4 d).

If initial collections did not yield the desired number of flies for each group, a second collection may be necessary.

Activity-Monitoring Experiments

Making Tubes for Activity-Monitoring Experiments

In addition to Bacto agar, some laboratories use 5% sucrose and 1% agarose or regular fly food in these tubes. There has not been a systematic study of medium composition on baseline sleep or at different temperatures, so researchers should test out different formulations to appropriately address their research question.

22. Add 5 g of Bacto agar (final concentration, 2% [w/v]) and 12.5 g of sucrose (final concentration, 5% [w/v]) to an Erlenmeyer flask. Add Milli-Q-purified H_2O to a final volume of 250 mL to the flask. Microwave the flask, removing and swirling the flask to disperse the bubbles that form. Continue to microwave until the agar and sucrose dissolve (the sucrose will take longer).

The solution will bubble over if neglected.

A volume of 250 mL will make enough sleep tubes for 20+ DAMs. The recipe can be scaled as needed.

23. Remove the flask from the microwave and place it on the counter to cool.
24. (Optional) If running an optogenetic experiment, once the flask is only warm to the touch, add 2.5 mL of 100 mM ATR to achieve a final ATR concentration of 1 mM and swirl the flask to mix.

    Wear gloves when handling ATR.
25. For every 250 mL of food being made, set out 10 open Petri dishes on a counter and use rubber bands to create 10 bundles of at least 32 uncapped polycarbonate tubes each (see Steps 40–49 for how to clean these reusable tubes and their black caps).
26. Pipette 20 mL of the food into each Petri dish and then transfer a bundle of tubes into the food. Repeat for all 10 Petri dishes.

    Make sure that the tubes are all level and touching the bottom of the dish.
27. Place Petri lids or paper towels over the top of the tubes and store in a dark environment (i.e., a cabinet) as the food cools.
28. Once the food has solidified, either proceed to Step 29 or place the dishes into a zip-lock bag and store for up to 2 wk at 4°C until needed. If the food contains ATR, store in the dark to prevent ATR from degrading.

    Tubes must be capped before they can be loaded into DAMs.
29. Carefully remove the tubes from the Petri dishes and place them on a paper towel. Clean excess food off each tube with a Kimwipe and add black caps to the end of the tube containing the plug of food. Make sure that the plug of food is not shifted down the length of the tube during this process. Either proceed to Step 30 or put the tubes back in the zip-lock bag and store at 4°C.

Loading DAMs and Connecting to the Incubator

30. Remove the needed number of sleep tubes from the refrigerator and allow them to warm to room temperature. If they contain ATR, protect them from light exposure during this time as much as possible. Using scissors, cut several strips of Parafilm.

    These will later be used to seal the open end of the tube.
31. During the course of the experiment, appropriately store the vials containing the experimental flies.
    - For optogenetic experiments, store vials at 25°C in a dark area (i.e., a drawer or cabinet) and be sure to limit light exposure to the flies as much as possible throughout the loading process. Flies should continue to be kept in the dark after they have emerged and been collected.
    - For thermogenetic experiments, store vials at 21°C on regular fly food, typically in a standard light–dark cycle. After collection, experimental flies should be maintained on the same light–dark cycle that will be used in the experimental procedure.
32. Anesthetize one vial of flies with CO_2 and tap them onto the Flypad.
33. Using a small paintbrush, sweep a single fly into the open end of a sleep tube and slide the tube into one of the openings of the DAM. Use Parafilm to then seal off the open end.

    Only a small amount of Parafilm is required to do this, so one strip can be used for multiple tubes.
34. Repeat until the monitor is full or no more flies are left to load, making sure that each tube is added to the monitor in the same direction. For optogenetics, place this monitor in a dark area before loading the next monitor. Each monitor has 32 flies and takes 10–15 min to load so presorting helps in keeping the time flies are exposed to anesthesia consistent between genotypes and monitors.

35. Once all the DAMs have been loaded, use a pin to poke a hole through the Parafilm on each tube to allow air to enter the tube. On the other side of the monitor, weave a rubber band around the black caps of each tube in one row and repeat for every row to stabilize the tubes.

36. Once every row has been banded, gently push the tubes so that half of the empty space within each tube is on each side of the monitor. Repeat for each monitor.

Rubber banding keeps the tubes from shifting during the experiment.

37. Connect each DAM to a CAB2 cable and then place them directly over or under the LED light grid, so that each one will be exposed to the same amount of light (Fig. 1A–C). Ensure that the environmental monitor is in an appropriate position to detect the lighting conditions and temperature before closing the incubator.

38. Set up the light and temperature conditions.

- For optogenetic experiments, control red-light stimulation under the "Lights" control tab in the DAMSystem3 software.

This system is quite flexible for generating single or repeated pulses. For example, single pulses ranging in duration from <1 sec up to several hours can be delivered. In this protocol, we show an experimental design (Fig. 1D) with 1 h of stimulation with red light at circadian time (CT)7 on the second day of the experiment. In this study examining the effects of activating neurons expressing short neuropeptide F (sNPF), the daily sleep data (Fig. 2A) show a rapid increase in sleep during red-light stimulation that only occurs in experimental animals but not in controls. Subtracting the baseline from the experimental day shows that this increase in sleep persisted for multiple hours after red-light stimulation ceased (Fig. 2B).

We typically collect data using one copy of the DAMSystem3 software set to collect data every 1 min and then use a second copy of the same software to control lights, with the monitoring cycle set to 10 sec to allow for shorter pulses to be delivered. Repeated pulses can also be generated using this

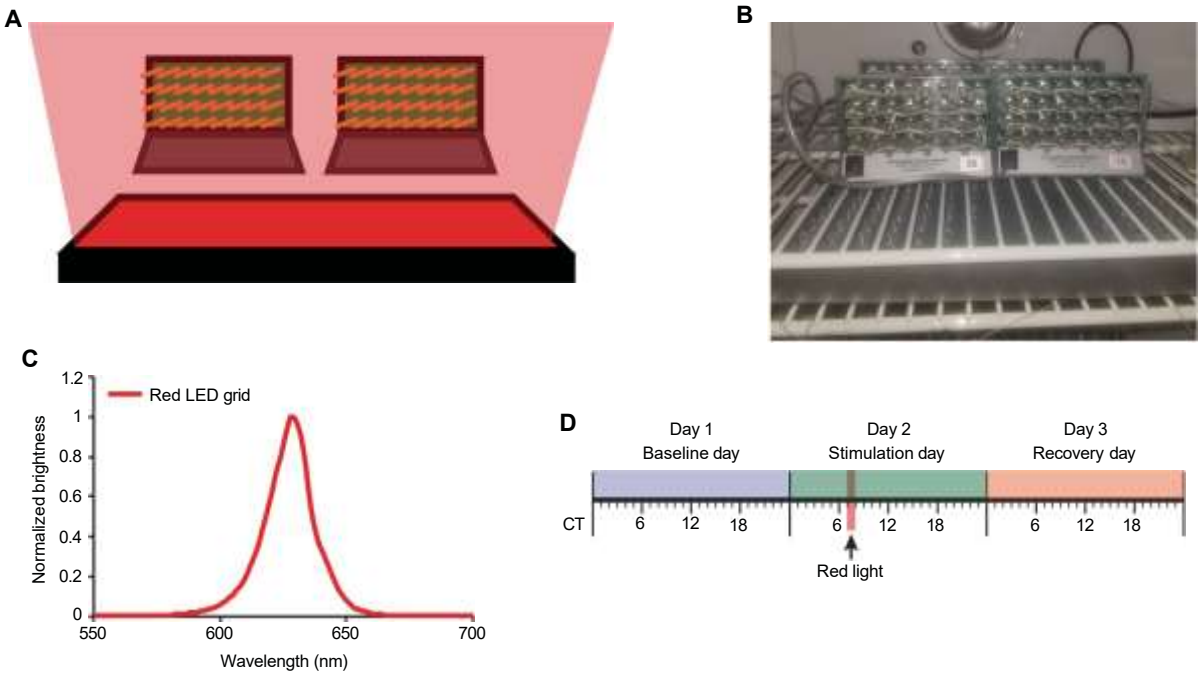

FIGURE 1. Methods for optogenetic stimulation during sleep studies in *Drosophila melanogaster*. (*A*) Schematic of an optogenetic stimulation experimental setup. In this case, a red LED grid was used to expose flies to red light to activate the CsChrimson sensor. (*B*) Image showing a typical experimental setup in an incubator. *Drosophila* activity monitors (DAMs) are placed directly above the LED grid with CAB2 cables plugged in and pulled to the side to prevent any light interference. (*C*) Emission spectrum from this red LED grid, demonstrating a single peak at 630 nm. (*D*) An example time line for a 3-d optogenetic stimulation experiment that consists of a baseline day, a stimulation day, and a recovery day. The arrow and red bar indicate the time and duration of light stimulation during day 2; in this case, flies were exposed to red light for 1 h from CT7 to CT8. *A*, *C*, and *D* were adapted from Juneau et al. (2019) with permission.

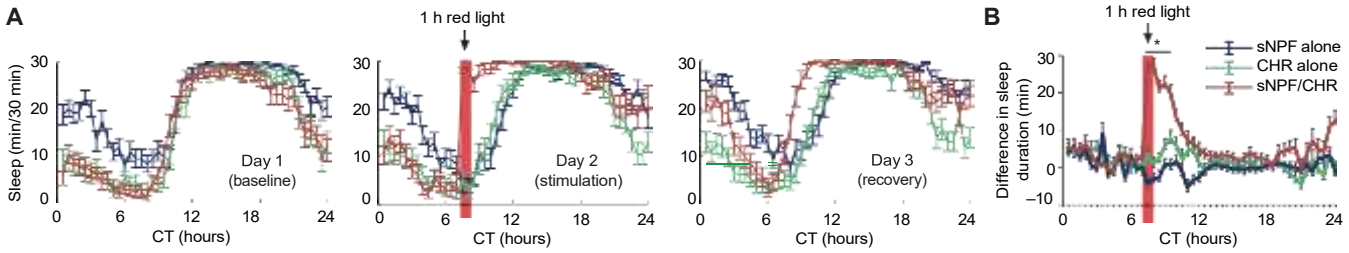

FIGURE 2. Assessment of sleep in *Drosophila* that have undergone optogenetic activation of neurons expressing short neuropeptide F (sNPF). Mated female flies containing sNPF-GAL4 alone, UAS-CsChrimson (CHR) alone, or sNPF-GAL4 and UAS-CsChrimson were placed in DAM2s just above a red LED illuminator grid and received red-light stimulation for 1 h at CT7 on day 2 as shown in Figure 1. (*A*) Sleep patterns across the three experimental days, with plots generated by SCAMP of the number of minutes of sleep per 30-min bin. (*B*) To determine how much sleep was altered by red-light stimulation, subtraction plots were created in SCAMP by subtracting the amount of sleep during each 30-min bin during the baseline day from the amount of sleep during the 30-min bin from the same CT of day during the subtraction day. Stimulation resulted in significantly increased sleep that took hours to decay back to baseline. Error bars represent the standard error of the mean, and the asterisk represents a post-hoc comparison with $P < 0.05$. The numbers of experimental animals were 28, 29, and 27 for sNPF alone, CHR alone, and sNPF/CHR groups, respectively. Figure and legend were adapted from Juneau et al. (2019) with permission.

software (see the TriKinetics website for more detailed documentation about DAMSystem3 software operation).

- For dTRPA1- and Shi^{ts1}-based thermogenetic manipulations, implement temperature changes using standard *Drosophila* incubators.

  These manipulations do not require ATR as a cofactor. Typically, temperatures of 21°C are used to monitor baseline (no activation or inhibition) and temperatures of 28°C–31°C are used for stimulation/inhibition. Depending on the research question, temperature changes can be applied specifically during daytime (zeitgeber time [ZT] 0–12), nighttime (ZT 12–24), or specific blocks. Here we show a typical 3-d experiment with dTRPA1 (Fig. 3A–D): day 1 (21°C), baseline/preactivation; day 2 (29°C), activation; and day 3 (21°C), recovery/postactivation.

39. Process data from the DAM system using the free TriKinetics software DAMFileScan, followed by any of several available software programs (see Introduction: Activity Monitoring for Analysis of Sleep in *Drosophila melanogaster* [Sitaraman et al. 2024]).

    Processing using DAMFileScan will depend on which software will be used afterward for analysis. Data in Figures 2 and 3 were processed using SCAMP software, instructions for which are detailed in Protocol: Analysis of Sleep and Circadian Rhythms from Drosophila *Activity-Monitoring Data using SCAMP (Vecsey et al. 2024) and in Supplemental Document (Vecsey SCAMP_v4 Instructions, https://figshare.com/s/75f69e5054b88ecf04c5).*

Recycling Activity-Monitoring Tubes

Cleaning Tubes

40. Remove the cap from a used tube and place the tube into a large beaker, with the plug of food facing toward the open end of the beaker. Once the beaker is filled with tubes, add tap H_2O to completely submerge the tubes. Cover the top of the beaker in aluminum foil.

41. Autoclave the tubes using a liquid setting (18 PSI for 30 min at 121°C). Once cool, dump the dirty H_2O from the beaker, transfer the tubes to a colander, and rinse the tubes with hot tap H_2O. Remove any remaining Parafilm and dead flies from the tubes.

42. Place the tubes back into the beaker and rinse them with Milli-Q-purified H_2O.

43. Make a 50% (v/v) solution of bleach in H_2O in a separate beaker and mix well.

44. Remove H_2O from the tubes and then add the 50% bleach solution. Cover the beaker with plastic wrap and leave overnight at room temperature.

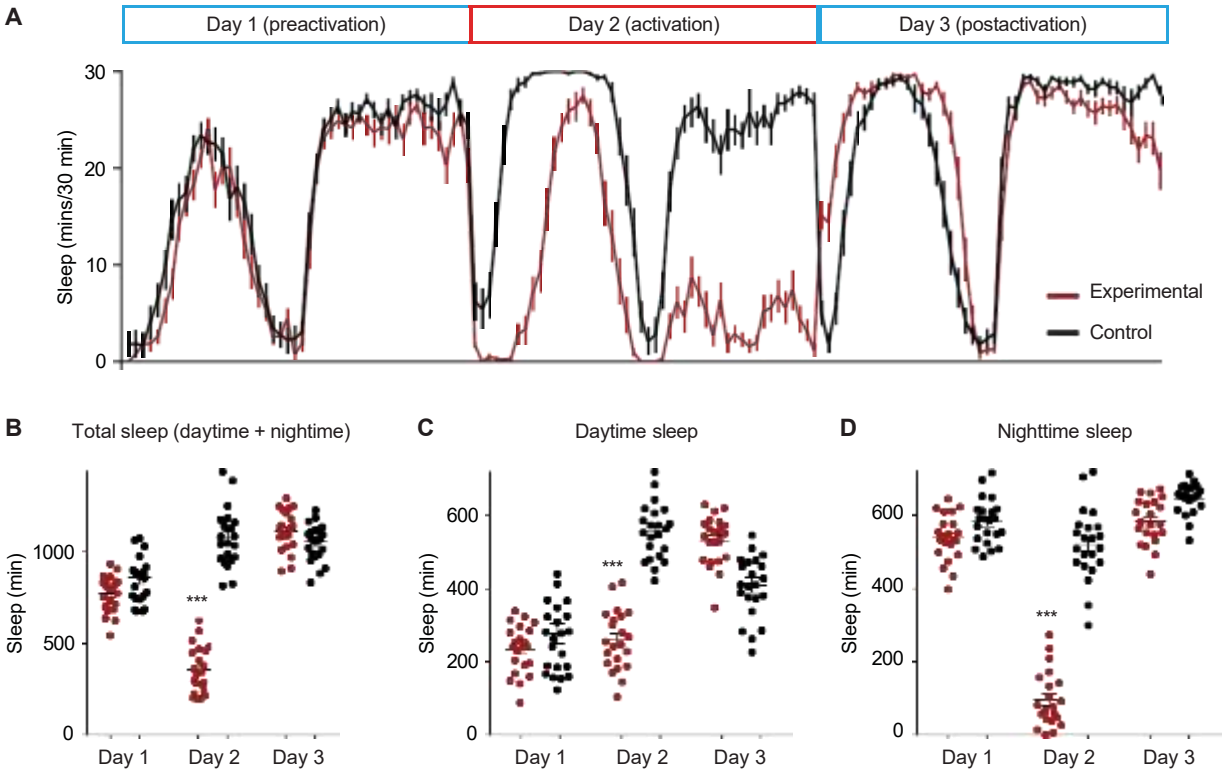

FIGURE 3. Assessment of sleep in *Drosophila* that have undergone thermogenetic activation of mushroom body output neurons. Mated male flies expressing the warmth-activated *Drosophila* transient receptor potential A1 (dTRPA1) in mushroom body output neuron γ5β′2 clusters (targeted by MB011B-GAL4) were placed in DAM2s in an incubator maintained at 21°C with a 12–12-h light–dark cycle. The flies were allowed to recover from anesthesia and acclimate for 2 d, and the subsequent days were used to record baseline, activation, and recovery. Temperature shifts were made at ZT 0 and monitored throughout the experiment. (*A*) Sleep patterns across three experimental days with plots showing sleep amount (min/30 min). (*B–D*) To determine how much sleep was altered by temperature elevation, we compared total sleep (daytime and nighttime, daytime and nighttime) on day 1, day 2, and day 3 between MB011B-GAL4/UAS-dTRPA1 (experimental; red) and pBDPGAL4/UAS-dTRPA1 (control; black). The negative control contains the vector backbone used to generate each GAL4 line but lacks any active enhancer motif to drive GAL4 or dTRPA1 expression. Error bars represent the standard error of the mean, and the asterisk represents a post-hoc comparison with $P < 0.05$ and three asterisks represent $P < 0.0001$. The numbers of experimental animals were 22 for both groups.

45. Thoroughly rinse out the bleach solution with tap H_2O until no bleach smell is detectable. Next, rinse with Milli-Q-purified H_2O. Place the tubes back into the beaker and submerge with Milli-Q-purified H_2O.
46. Autoclave the tubes again using the liquid setting as in Step 41.
47. After the tubes are autoclaved, use a colander to drain H_2O from the tubes. Use a rubber band to hold the tubes together and then place them upright on paper towels overnight to dry (and loosely cover with paper towels or foil). Once dry, separate the tubes into smaller bundles.

Cleaning Black Caps

48. Place the black caps removed from used tubes into a beaker. Once the beaker is about half-filled with caps, fill it 0.75 of the way with Milli-Q H_2O. Cover the beaker with aluminum foil and autoclave the caps using the liquid setting as in Step 41.
49. Pour the caps into a colander and shake them. Next, cover the caps with paper towels or foil and leave them to dry above a layer of paper towels. Once dry, store the caps for future use.

RECIPES

*All-*Trans*-Retinal (ATR, 100 mM)*

Dissolve all-*trans*-retinal (Toronto Research Chemicals R240000) in 100% ethanol (200-proof; Fisher Scientific) to 100 mM.

Store for up to 3 mo at 4°C in the dark.

Drosophila *Cornmeal–Yeast Food*

Reagent	Vendor	Amount
Distilled H_2O		4 L
Cornmeal (Quaker yellow)	Genesee Scientific	400 g
Agar (*Drosophila* agar, 100 mesh)	Genesee Scientific	24 g
Yeast (Red Star active dry yeast)	Genesee Scientific	72 g
Dextrose	Fisher Scientific	240 g
Sucrose	Fisher Scientific	120 g
Methyl paraben	Josh's Frogs	2 g
Mix of propionic acid (41.8%, v/v) and phosphoric acid (4.15%, v/v)	Fisher Scientific	40 mL

1. Combine 1 L of distilled H_2O with sucrose, dextrose, and yeast in a large pot on a hot plate and stir.
2. Slowly add the cornmeal and continue stirring.
3. While doing Steps 1 and 2, slowly add agar to 3 L of distilled H_2O in a large pot on another hot plate while stirring. Boil until solution is clear.
4. Pour the agar solution into the cornmeal solution while still on the hot plate. Solution should be steaming but not boiling.
5. Pour solution into three separate 2-L glass beakers, cover each with foil, place the beakers in a metal tray, and autoclave on liquid setting at 18 PSI for 30 min at 121°C.
6. While autoclaving, combine the acid mixture with methyl paraben.
7. After autoclaving, recombine the three beakers' contents into a single pot, add acid/methyl paraben mix, and stir well.
8. (Optional): Add All-*trans*-retinal (ATR, 100 mM) <R> to a final concentration of 1 mM.

   ATR+ food can also be prepared by melting a known volume of cornmeal food in the microwave, mixing in the ATR solution at 1:100, and then pouring the resulting 1 mM ATR food into bottles and/or vials as needed.
9. Dispense into containers as needed.
10. Store for up to 2 wk at 4°C.

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