Mitochondrial origins of the pressure to sleep

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Summary

The neural control of sleep requires that sleep need is sensed during waking and discharged during sleep. To obtain a comprehensive, unbiased view of molecular changes in the brain that may underpin these processes, we have characterized the transcriptomes of single cells isolated from rested and sleep-deprived flies. Transcripts upregulated after sleep deprivation, in sleep-control neurons projecting to the dorsal fan-shaped body (dFBNs) but not ubiquitously in the brain, encode almost exclusively proteins with roles in mitochondrial respiration and ATP synthesis. These gene expression changes are accompanied by mitochondrial fragmentation, enhanced mitophagy, and an increase in the number of contacts between mitochondria and the endoplasmic reticulum, creating conduits for the replenishment of peroxidized lipids. The morphological changes are reversible after recovery sleep and blunted by the installation of an electron overflow in the respiratory chain. Inducing or preventing mitochondrial fission or fusion in dFBNs alters sleep and the electrical properties of sleep-control cells in opposite directions: hyperfused mitochondria increase, whereas fragmented mitochondria decrease, neuronal excitability and sleep. ATP levels in dFBNs rise after enforced waking because of diminished ATP consumption during the arousal-mediated inhibition of these neurons, which predisposes them to heightened oxidative stress. Consistent with this view, uncoupling electron flux from ATP synthesis relieves the pressure to sleep, while exacerbating mismatches between electron supply and ATP demand (by powering ATP synthesis with a light-driven proton pump) promotes sleep. Sleep, like ageing, may be an inescapable consequence of aerobic metabolism.

Introduction

Sleep pressure, the process variable in sleep homeostasis, currently lacks a physical interpretation. Although prolonged waking is associated with numerous changes in the brain—of neuronal firing patterns[1,2], the strengths of synaptic connections[3], metabolite concentrations[4,5], and metabolic and gene expression programs[6-8]—, it remains generally indeterminable whether these changes are causes or consequences of a growing need for sleep. Perhaps the only realistic opportunity for separating causation from correlation exists in specialist neurons with active roles in the induction and maintenance of sleep[9]; in these cells, sleep's proximate (and maybe also its ultimate) causes must interlock directly with the processes that regulate spiking. To delineate the molecular determinants of these processes in as complete and unbiased a manner as possible, we collected single-cell transcriptomes[10] of the brains of rested and sleep-deprived flies (Supplementary Fig. 1a). An encodable fluorescent marker allowed us to identify and enrich for two dozen sleep-inducing neurons with projections to the dorsal fan-shaped body of the central complex[11] (dFBNs) and compare their transcriptomic response to sleep loss with that of other identifiable cell types. A companion paper[12] verifies the sleep-promoting role of dFBNs with genetic tools refined on the basis of new transcriptomic information.

Results

Transcriptomic traces of sleep loss in mitochondria and synapses

Drosophila brains were dissociated into single-cell suspensions, and dFBNs expressing GFP under the control of *R23E10-GAL4*[13] were isolated by flow cytometry. We performed single-cell RNA-sequencing (10X Chromium) on cells contained in the GFP-positive and GFP-negative fractions; among the 13,173 high-quality cells retrieved (Supplementary Fig. 1b, c), neurons nominated by *R23E10-GAL4* were identified by the expression of *GFP*, the neuronal markers *embryonic lethal abnormal vision* (*elav*) and *neuronal synaptobrevin* (*nSyb*), and *Allatostatin A* (*AstA*) *receptor 1* (*AstA-R1*), whose transcriptional enhancer provides the genomic fragment driving expression in the *R23E10-GAL4* line. Most neurons within the *R23E10-GAL4* domain contained machinery for the synthesis and release of either glutamate or GABA (Supplementary Fig. 1d); some also transcribed the gene encoding the vesicular acetylcholine transporter VAChT (Supplementary Fig. 1d)—a common occurrence in non-cholinergic neurons, which rely on a microRNA to suppress its translation[14].

In the *Drosophila* CNS, glutamate and GABA both act on ligand-gated chloride channels, consistent with the known inhibitory effect of dFBNs on their postsynaptic partners[12,15]. Members of the *R23E10-GAL4* pattern that expressed one or the other of these inhibitory transmitters were anatomically segregated[12]: a pair of GABAergic cells targeted the suboesophageal ganglion, while a larger glutamatergic population innervated the dFB. This latter group of *bona fide* dFBNs formed a distinct gene expression cluster of 323 cells among all glutamatergic neurons in the brain (Fig. 1a, Supplementary Fig. 1e, f). The cluster was defined by genes controlled by known dFBN enhancers (Supplementary Fig. 1g) and encoding known dFBN markers, such as the Rho GTPase-activating protein crossveinless-c[13], the dopamine receptor Dop1R2[16], the serotonin receptor 5-HT2B[17], and the neuropeptide AstA[15] (Fig. 1b), but the distributions of some markers suggested further subdivisions. For example, AstA was confined to a subset of dFBNs (Fig. 1b), consistent with the comparatively mild sleep phenotype following RNA-mediated interference (RNAi) with its expression in the entire dFBN population[15].

Gene ontology analyses of the 122 transcripts whose levels in dFBNs changed after 12 h of overnight sleep deprivation pointed to only two sleep need-dependent biological processes: mitochondrial energy metabolism and synaptic transmission (Fig. 1c, d, Supplementary Fig.

2a–d). Sleep loss led to the selective upregulation of transcripts encoding components of electron transport complexes I–IV, ATP synthase (complex V), the ATP/ADP carrier sesB, and enzymes of the tricarboxylic acid cycle, whereas gene products involved in synapse assembly, synaptic vesicle release, and presynaptic homeostatic plasticity[18] were selectively downregulated (Fig. 1c). Within the practical limits of our analysis, which cannot extend to every conceivable neuron type, this transcriptomic signature of sleep loss appeared unique to dFBNs: it was absent from two cell populations with comparable numerical representation, namely projection neurons of the antennal lobe (PNs, 317 cells, Supplementary Fig. 3a–d) and Kenyon cells of the mushroom body (KCs, 603 cells, Supplementary Fig. 3e–h), and it was also undetectable in a combined analysis of all 12,850 non-dFBN cells (Supplementary Fig. 3i, j). There are, however, subtle hints from an independent transcriptomic study[8] that sleep history may alter levels of mitochondrial components not only in dFBNs but also in R5 neurons of the ellipsoid body, another element of the sleep homeostat[19].

The remainder of this paper examines the causes and consequences of the differential expression of genes encoding mitochondrial proteins in dFBNs; a companion study[12] investigates the role of presynaptic plasticity in the wider context of sleep need-dependent dFBN dynamics.

A mitochondrial electron surplus induces sleep

The prominence of mitochondrial components in the transcriptional response of dFBNs to sleep deprivation (Fig. 1c, d, Supplementary Fig. 2) offers unbiased support for the hypothesis that sleep and aerobic metabolism are fundamentally connected. This hypothesis gained a firm mechanistic footing with the discovery that dFBNs contain machinery that gears their sleep-inducing spike discharge to mitochondrial respiration[20]. The centrepiece of this mechanism is Hyperkinetic, the β-subunit of the voltage-gated potassium channel Shaker, which regulates the electrical activity of dFBNs[16,20]. Hyperkinetic is an unusual aldo-keto reductase[21] with a stably bound nicotinamide adenine dinucleotide phosphate cofactor whose oxidation state (NADPH or $NADP^+$) reflects the fate of electrons entering the respiratory chain[20]. When the demands of ATP synthesis are high, the vast majority of electrons reach O_2 in an enzymatic reaction catalyzed by cytochrome *c* oxidase (complex IV); only a small minority leak prematurely from the upstream mobile carrier coenzyme Q (CoQ), producing superoxide and other reactive oxygen species[22,23] (ROS) (Fig. 2a). The probability of these non-enzymatic single-electron reductions of O_2 increases sharply under conditions that

overfill the CoQ pool as a consequence of increased supply (high $NADH/NAD^+$ ratio) or reduced demand (large protonmotive force and high ATP/ADP ratio)[22,23] (Fig. 2a). The mitochondria of dFBNs appear prone to this mode of operation during waking[20], when caloric intake is high but the neurons' electrical activity is reduced, leaving their ATP reserves full. Indeed, measurements with the genetically encoded ATP sensors iATPSnFR and ATeam revealed ~15-fold higher ATP concentrations in dFBNs, but not PNs, after a night of sleep deprivation than at rest (Fig. 2b, c, Supplementary Fig. 4a). ATP levels rose acutely when dFBNs were inhibited by an arousing heat stimulus, which releases dopamine onto their dendrites[12,16] (Fig. 2a, d), and fell below baseline when dFBNs themselves were stimulated, mimicking sleep[12] (Fig. 2a, e).

Even if the chance of an individual electron spilling from the CoQ pool is low, however, metabolically highly active cells, such as neurons, will by the sheer number of electrons passing through their respiratory chains generate significant amounts of ROS[22-24]. Their anti-cyclical relationship between energy availability (which peaks during waking) and energy consumption (which for sleep-active neurons peaks during sleep) may thus predispose dFBNs to an exaggerated form of the electron leak experienced by many neurons in the awake state, making them an effective early warning system against widespread damage. As polyunsaturated membrane lipids appear especially at risk[21], dFBNs estimate the size of the mitochondrial electron leak indirectly, by counting reductions of lipid peroxidation-derived carbonyls at Hyperkinetic's active site[21].

Several lines of evidence point to a mismatch between the number of high-energy electrons entering the mitochondrial transport chain and the number needed to fuel ATP production as a root cause of sleep. First, opening an exit route for surplus electrons from the CoQ pool (by equipping the mitochondria of dFBNs with the alternative oxidase[25] (AOX) of *Ciona intestinalis*, which produces water in a controlled four-electron reduction of O_2) relieved not only the basal pressure to sleep[20] but also remedied the excessive sleep need of flies whose ability to remove breakdown products of peroxidized lipids was impaired[21]. Second, increasing the demand of dFBNs for electrons (by overexpressing the uncoupling proteins Ucp4A or Ucp4C, which short-circuit the proton electrochemical gradient across the inner mitochondrial membrane[26], Fig. 2a) decreased sleep (Fig. 2f). And third, powering ATP synthesis with photons rather than electrons (by illuminating a mitochondrially targeted

version of the light-driven archaeal proton pump delta-rhodopsin, Fig. 2a) made NADH-derived electrons in dFBNs redundant and precipitated sleep (Fig. 2g, h).

Sleep alters mitochondrial dynamics

Given this wealth of mechanistic evidence, it is not surprising that mitochondria would emerge as one of two pivots in the reorganization of dFBN gene expression after sleep deprivation (Fig. 1c, d, Supplementary Fig. 2). However, it remains ambiguous whether the upregulation of transcripts encoding mitochondrial proteins signals a net increase in mitochondrial mass or a compensatory response to organelle damage. To disambiguate these scenarios, we labelled the mitochondria of dFBNs with matrix-localized GFP (mito-GFP) and performed automated morphometry on deconvolved image stacks of the neurons' dendritic fields. Sleep loss left the total number of mitochondria unaltered but reduced their size, elongation, and branching (Fig. 3a, b), and it led to the recruitment of dynamin-related protein 1 (Drp1), the key fission dynamin of the outer membrane[27-29], from the cytosol to the mitochondrial surface (Fig. 3c, d). The mitochondria of antennal lobe PNs, in contrast, bore no vestiges of sleep history (Supplementary Fig. 4b).

The presence of AOX protected dFBN mitochondria against sleep loss-induced fragmentation (Fig. 3a, b), underlining that ROS generation during waking[20] is the initial spark that triggers fission[23,30,31]. In the same vein, depolarization of dFBNs during mechanical sleep deprivation, which increases ATP consumption by the Na^+/K^+ pump and thereby reduces the diversion of high-energy electrons into ROS (Fig. 2a), preserved the morphology of their mitochondria (Fig. 3a, b).

Mitochondrial fission is a prelude to the proliferation of mitochondria at contact sites with the endoplasmic reticulum[32] (ER) and/or the shedding and clearance of dysfunctional organelle fragments by (trans)mitophagy[30,33-35] (Fig. 3c). Sleep deprivation stimulated both processes: the reconstitution of fluorescence from GFP fragments anchored in the outer mitochondrial and ER membranes ($SPLICS_{short}$) revealed a higher contact site count in dFBNs of sleep-deprived flies (Fig. 3e), as electron microscopy did in cortical pyramidal neurons of sleep-deprived mice[36], whereas mito-QC, a ratiometric sensor detecting the entry of mitochondria into acidic autophagolysosomes, reported enhanced mitophagy (Fig. 3f). Mitochondria–ER contacts (MERCS) concentrate the fission and fusion machineries whose dynamic equilibrium determines the steady-state morphology of the mitochondrial network[32] and

support mitochondrial biogenesis by allowing the passage of phospholipids from the ER[37,38]. The abundance of MERCS in sleep-deprived dFBNs (Fig. 3e) may thus not only echo a recent wave of mitochondrial fission (Fig. 3a, b) but also prefigure, as we suspect the abundance of transcripts for mitochondrial proteins does (Fig. 1c), the proliferation and fusion of mitochondria during subsequent recovery sleep, which caused their count, volume, shape, and branch length to rebound above baseline values (Fig. 3a, b).

Mitochondrial dynamics alter sleep

If shifts in the balance between mitochondrial fission and fusion are part of a feedback mechanism[23,30,31] which corrects mismatches between NADH supply and ATP demand that cause sleep pressure to rise or fall, then the experimental induction of these homeostatic responses in dFBNs should move the set points for sleep: mitochondrial fragmentation is predicted to decrease, and mitochondrial fusion is predicted to increase, sleep duration and depth. To test these predictions, we took experimental control of the three GTPases with central regulatory roles in mitochondrial dynamics[23,30,31] (Fig. 4a): the fission dynamin Drp1[27-29], and integral proteins of the inner and outer mitochondrial membranes, termed optic atrophy 1 (Opa1) and mitofusin or mitochondrial assembly regulatory factor (Marf), respectively, whose oligomerization in *cis* and *trans* fuses the corresponding membranes[28,39-41].

As predicted, fragmenting dFBN mitochondria through the overexpression of Drp1 or the RNAi-mediated depletion of Opa1—and, to a lesser extent, of Marf—decreased sleep (Fig. 4b, Extended Data 5a–c), abolished the homeostatic response to sleep deprivation (Fig. 4c), and reduced ATP levels in dFBNs regardless of sleep history (Supplementary Fig. 6). Tipping the equilibrium toward mitochondrial fusion had the opposite effect: *R23E10-GAL4*-restricted knockdown of Drp1 or the overexpression of Opa1 plus Marf —or of Opa1 alone, but not of Marf alone (Fig. 4b, Supplementary Fig. 5d)—increased baseline as well as rebound sleep (Fig. 4b, c) and elevated the arousal threshold (Supplementary Fig. 7a, b) without causing overexpression artefacts or overt developmental defects (Supplementary Fig. 7c, d). None of these interventions altered sleep when targeted to PNs or KCs (Supplementary Fig. 7e, f).

In dFBNs, the large and opposite behavioral consequences of promoting mitochondrial fission or fusion (Fig. 4b, c) went hand-in-hand with established biophysical signatures of

low or high sleep pressure[13]. dFBNs in short sleepers overexpressing Drp1 had shallower current–spike frequency functions than neurons in control animals, whereas the converse was true in somnolent overexpressers of Opa1 and Marf (Fig. 4d–f), whose dFBNs generated an elevated number of somnogenic bursts[12] as part of their enhanced responses (Fig. 4d, g).

A striking feature of sleep-deprived brains is the depletion of phosphatidic acid[21] (PA), a glycerophosopholipid whose lack of a bulky head group reduces the mechanical strain in fusion intermediates with large negative membrane curvature (Fig. 4a). Mitochondrial PA is a cleavage product of cardiolipin, generated by a local phospholipase D (mitoPLD)[31,42] (Fig. 4a). Consistent with the importance of PA for the fusion reaction[31,42], and of mitochondrial fusion for the regulation of sleep, *R23E10-GAL4*-driven interference with the expression of the mitoPLD zucchini or the outer membrane protein Mitoguardin (Miga), which stabilizes catalytically active mitoPLD[43] and/or transfers phospholipids (including PA) from other cellular membranes to mitochondria[44], recapitulated the sleep losses seen when the protein-based fusion machinery of these neurons was targeted by RNAi or antagonized by the overexpression of Drp1 (Fig. 4b, Supplementary Fig. 5e).

Discussion

Aerobic metabolism was the innovation that, following two large increases in atmospheric O_2 levels 2.4 billion and 550 million years ago, enabled the evolution of eukaryotes and subsequently the Cambrian explosion of multicellular life[45], during which complex nervous systems appeared[46,47]—and with them, apparently, the need for sleep[48]. Although sleep is likely since to have acquired additional functions, such as synaptic homeostasis or memory consolidation[3], an empirical power law[49] that relates daily sleep amount to mass-specific O_2 consumption[50] suggests that sleep serves an ancient metabolic purpose also in mammals. The allometric exponent in this power law is a multiple of $\frac{1}{4}$ rather than the $\frac{1}{3}$ expected from Euclidean geometric scaling—a clear sign that the distribution of resources by centralized networks, such as the vascular and respiratory systems, is responsible[49,51,52]. Thanks to higher terminal branch densities, these networks allocate more O_2 to each cell in small animals, allowing their metabolism to run 'hotter' than that of large mammals, whose cells are supply-limited[52]. The price to pay is a shorter life, a greater fraction of which is spent asleep.

If sleep indeed evolved to fulfill a metabolic need, it is not surprising that neurons controlling sleep and energy balance would be regulated by similar mechanisms. In the mammalian hypothalamus, the mitochondria of orexigenic neurons expressing agouti-related protein (AgRP) and of anorexigenic neurons expressing pro-opiomelanocortin undergo antiphasic cycles of fission and fusion[53]. These cycles are coupled to changes in the energy balance of mice[53], just as cycles of mitochondrial fission and fusion in dFBNs are coupled to changes in the sleep balance of flies. The electrical output of AgRP neurons increases after mitochondrial fusion to promote weight gain and fat deposition[53], just as the electrical output of dFBNs increases after mitochondrial fusion to promote sleep. Deletions of mitofusins from AgRP neurons impair the consumption of food[53], just as interference with mitochondrial fusion in dFBNs impairs the induction of sleep. These parallels suggest that sleep pressure and hunger both have mitochondrial origins, and that electrons flow through the respiratory chains of the respective feedback control neurons like sand in the hourglass that determines when balance must be restored.

Methods

***Drosophila* strains and culture.** Flies were grown on media of cornmeal (62.5 g l^{-1}), inactive yeast powder (25 g l^{-1}), agar (6.75 g l^{-1}), molasses (37.5 ml l^{-1}), propionic acid (4.2 ml l^{-1}), tegosept (1.4 g l^{-1}), and ethanol (7 ml l^{-1}) under a 12 h light:12 h dark cycle at 25 °C in ~60% relative humidity, unless stated otherwise. To prevent the undesired activation of optogenetic actuators or the photoconversion of all-*trans* retinal by ambient light, flies expressing CsChrimson or delta-rhodopsin and their controls were reared and housed in constant darkness and transferred to food supplemented with 2 mM *all-trans* retinal (Molekula) in DMSO, or to DMSO vehicle only, 2 days before the optical stimulation experiments, at an age of 1–2 days post eclosion. Flies expressing TrpA1 were cultured and maintained at 21 °C and shifted to 29 °C for 12 h.

Driver lines *R23E10-GAL4*[54], *GH146-GAL4*[55], and *OK107-GAL4*[56] were used to target dFBNs, PNs, or KCs, respectively. Effector transgenes encoded fluorescent markers for flow cytometry (*UAS-6xEGFP*[57]), visually guided patch-clamp recordings (*UAS-mCD8::GFP*[58]), mitochondrial morphometry (*UAS-mito-GFP*[59,60]), labeling of the outer mitochondrial membrane (*UAS-OMM-mCherry*[61]), or ratiometric imaging (*UAS-RFP* or *UAS-tdTomato*); the ATP sensors iATPSnFR[1.062,63] or ATeam1.03NL[64,65]; the mitochondrial alternative oxidase AOX from *Ciona intestinalis*[66]; delta-rhodopsin from *Haloterrigena turkmenica* with an inner mitochondrial membrane-targeting sequence[67,68] (mito-dR); the opto- or thermogenetic actuators[69] CsChrimson[70] or TrpA1[71]; the mitochondria–ER contact site or mitophagy reporters $SPLICS_{short}$[72,73] or mito-QC[74,75]; overexpression constructs encoding Ucp4A or Ucp4C[76,77], Drp1 (3 independent transgenes[78-80]), Opa1[79], or Marf (2 independent transgenes[79,81]); or RNAi transgenes for interference with the expression of *Drp1*[81], *Opa1* (7 independent transgenes[78,82,83]), *Marf* (5 independent transgenes[78,82,83]), *zucchini*[83], or *Mitoguardin* (2 independent transgenes[82]). A recombinant strain carrying the *R23E10-GAL4* and *UAS-Marf* transgenes on the third chromosome was generated to enable the co-expression of Opa1 and Marf. Endogenous Drp1 was colocalized with mitochondria in *Drp1::FLAG-FlAsH-HA* flies[84].

Sleep measurements, sleep deprivation, and sleep induction by photoenergized mitochondria. In standard sleep assays, females aged 2–4 days were individually inserted

into 65-mm glass tubes containing food reservoirs, loaded into the Trikinetics *Drosophila* Activity Monitor system, and housed under 12 h light:12 h dark conditions at 25 °C in 60% relative humidity. Flies were allowed to habituate for one day before sleep was averaged over two consecutive recording days. Periods of inactivity lasting >5 minutes were classified as sleep[85,86] (Sleep and Circadian Analysis MATLAB Program[87]). Immobile flies (< 2 beam breaks per 24 h) were manually excluded.

To deprive flies of sleep before single-cell transcriptomic studies, snapshot measurements of ATP concentrations, or quantification of mitochondrial morphology, mitophagy, mitochondria-ER contacts, or Drp1 localization, a spring-loaded platform stacked with Trikinetics monitors was slowly tilted by an electric motor, released, and allowed to snap back to its original position[88]. The mechanical cycles lasted 10 s and were repeated continuously for 12 h, beginning at zeitgeber time 12. Sleep deprivation of flies expressing TrpA1 was performed at 29 ˚C.

Rebound sleep was quantified after sleep deprivation between zeitgeber times 12 and 24. An Ohaus Vortex Mixer stacked with Trikinetics monitors produced horizontal circular motion stimuli with a radius of ~1 cm at 25 Hz for 2 s; stimulation periods were randomly normally distributed within 20-s bins. A cumulative sleep loss plot was calculated for each individual by comparing the percentage of sleep lost during overnight sleep deprivation to the immediately preceding unperturbed night. Individual sleep rebound was quantified by normalizing the difference in sleep amount between the rebound and baseline days to baseline sleep. Only flies losing >95% of baseline sleep were included in the analysis.

Arousal thresholds were determined by applying horizontal circular motion stimuli with a radius of ~1 cm at 8 Hz, generated by a Talboys Multi-Tube Vortexer. Stimuli lasting 0.5 to 20 s were delivered once every hour between zeitgeber times 0 and 24, and the percentages of sleeping flies (if any) awakened within 1 minute of each stimulation episode were quantified.

For experiments with photoenergized mitochondria[67,68], females aged 3–5 days that expressed *R23E10-GAL4*-driven mito-dR and their parental controls were reared for 2 days on standard food supplemented with all-*trans* retinal (or DMSO vehicle only, as indicated) and individually transferred into the wells of a flat-bottom 96-well plate. Each well contained 150 µl sucrose food (5% sucrose, 1% agar) with or without 2 mM all-*trans* retinal. The plate was

sealed with a perforated transparent lid, inserted into a Zantiks MWP Z2S unit operated at 25 °C, and illuminated from below by infrared LEDs while a camera captured 31.25 frames s^{-1} from above. Zantiks software extracted time series data of individual movements, which were converted to sleep measurements with the help of a custom MATLAB script that detected continuous stretches of zero-speed bins lasting >5 minutes. After flies had been allowed to habituate for at least one day in the absence of stimulation light, high-power LEDs running on an 80% duty cycle at 2 Hz (PWM ZK-PP2K) delivered ~7 mW cm^{-2} of 530-nm light for 1 h. Movement was monitored for 24 h, including the initial hour of optogenetic stimulation. Immobile flies (>98% zero-speed bins during ≥2 consecutive hours until the end of the recording) were excluded from the analysis, beginning with the hour preceding the onset of immobility.

Brain dissociation and cell collection for single-cell RNA-sequencing. On each experimental day, averages of 186 rested and 144 sleep-deprived female flies were retrieved alive from Trikinetics monitors (Extended Data Fig, 1a) and dissected in parallel in ice-cold Ca^{2+}- and Mg^{2+}-free Dulbecco's PBS (Thermo Fisher) supplemented with 50 µM D(-)-2-amino-5-phosphonovaleric acid, 20 µM 6,7-dinitroquinoxaline-2,3-dione, and 100 nM tetrodotoxin (tDPBS) to block excitatory glutamate receptors and voltage-gated sodium channels. The lower number of sleep-deprived flies recovered reflects excess mortality associated with sleep deprivation. Brains were transferred to Protein LoBind microcentrifuge tubes containing ice-cold Schneider's medium supplemented with the same toxins (tSM), washed once with 1 ml tSM, incubated in tSM containing 1.11 mg ml^{-1} papain and 1.11 mg ml^{-1} collagenase I for 30 minutes at room temperature, washed again with tSM, and subsequently triturated with flame-rounded 200 µl pipette tips. Dissociated brain cells were collected by low-speed centrifugation (2,000 rpm, 3 minutes), resuspended in 1 ml tDPBS, and filtered through a 20 µm CellTrics strainer. For the isolation of cells by flow cytometry (FACS), dead cells were excluded with the help a DAPI viability dye (1 µg ml^{-1}, BD Pharmingen). Single cells were gated for based on forward and side scatter parameters, followed by subsequent gating for EGFP-negative cells, using FACSDiva software (Becton Dickenson). Both the EGFP-positive fraction and the EGFP-negative flow-through were collected for sequencing. Samples were partitioned into single cells and barcoded using droplet microfluidics[10] (10X Chromium v3 and v3.1) and multiplexed during Illumina NovaSeq6000 sequencing. Brains and dissociated cells were kept on ice or at ice-cold temperatures from dissection to sample submission, including during the FACS sorting

procedure, but not during the enzymatic and mechanical dissociation steps, which took place at room temperature.

scRNA-seq data processing and alignment. Raw transcriptomic data were pre-processed with a custom command line script[10,89,90], which extracted cell barcodes and aligned associated reads to a combination of the *Drosophila melanogaster* genome release BDGP6.22 and the reference sequences of the *GAL4* and *EGFP-p10*$^{3'UTR}$ transgenes, using STAR with default settings. Flybase version FB2018_03 gene names were used for annotation. The cumulative fraction of reads as a function of cell barcodes, arranged in descending order of the number of reads, was inspected, and only cells with a high number of reads, up to a clearly visible shoulder, were retained[10]; beads with few reads, potentially ambient RNA, were discarded. All subsequent analyses were performed in R, using the Seurat v4.1 package[91].

Three biological replicates, collected on different days from independent genetic crosses, were merged, variation driven by individual batches was regressed out, and the data were normalized by dividing by the number of unique molecular identifiers (UMIs) per cell and multiplying by 10,000. Applying standard criteria for fly neurons[8,90,92,93], we rejected genes detected in fewer than 3 cells and retained only cells associated with 800–10,000 UMIs and 200–5,000 transcripts.

Principal component analysis was used to compress the expression data from an initial dimensionality of 10,000 (the number of variable features) to 50 (the number of principal components we chose to consider); the scores along these 50 dimensions were then visualized in a two-dimensional uniform manifold approximation and projection (UMAP) embedding. Clusters were identified by constructing a shared nearest neighbour graph and applying the Louvain algorithm with resolution 0.2. Clusters were manually annotated according to the presence of established markers[90,92]: Cholinergic, glutamatergic, and GABAergic neurons expressed the neuronal markers *embryonic lethal abnormal vision* (*elav*) and *neuronal synaptobrevin* (*nSyb*) and, respectively, genes encoding the vesicular acetylcholine (VAChT) or glutamate transporters (VGlut) or glutamic acid decarboxylase 1 (Gad1) at levels >2; KCs were identified by the expression of *eyeless* and *Dop1R2* and partitioned into αβ, α'β', and γ divisions according to the distributions of sNPF, fasciclin 2, and trio; monoaminergic neurons were identified by the presence of the vesicular monoamine transporter Vmat and divided

into dopaminergic, serotonergic, and octopaminergic/tyraminergic neurons by the coexpression of genes for biosynthetic enzymes (Dopa decarboxylase, tyrosine hydroxylase, tyrosine decarboxylase 2 (Tdc2), tryptophan hydroxylase, and tyramine β-hydroxylase) and vesicular transporters for dopamine or serotonin (DAT and SerT); PNs were defined and classified by the expression of the transcription factors cut and abnormal chemosensory jump 6 (acj6), with or without Lim1; glia lacked elav and nSyb but expressed the Na^+/K^+ ATPase encoded by the *nervana 2* gene, while astrocytes also contained the astrocytic leucine-rich repeat molecule (alrm); cells of the fat body were recognized by the expression of *Secreted protein, acidic, cysteine-rich* (*SPARC*), *Metallothionein A* (*MtnA*), *I'm not dead yet* (*Indy*), and *pudgy*; *R23E10* neurons expressed *elav* and *nSyb* plus *AstAR-1* and *EGFP* at a level >2. Expression level cutoffs for *VAChT*, *VGlut*, *Gad1*, and *EGFP* were chosen to bisect bimodal distributions (Supplementary Fig. 1d). For re-clustering neurons expressing specific fast-acting neurotransmitters, dFBNs, KCs, or PNs, 150 genes were used as variable features.

Differential gene expression and gene ontology analyses. Differentially expressed genes were identified in Seurat via the 'FindMarkers' function, using the 'RNA assay' counts of the two comparison groups, and restricted to genes detected in ≥1% of cells in either of the two groups with, on average, a ≥0.01-fold (log scale) expression level difference between the rested and sleep-deprived states. Expression levels were compared by Bonferroni-corrected Wilcoxon rank-sum test.

Gene ontology terms enriched in the set of differentially expressed nuclear genes were identified using PANTHER v17 or the ViSEAGO 1.4.0 and topGO 2.42.0 packages. PANTHER compared the list of differentially expressed genes to the *Drosophila melanogaster* reference list and the GO annotation database (doi: 10.5281/zenodo.7942786, version 2023-05-10). In ViSEAGO and topGO, differentially expressed genes were compared to a reference set of all variable genes used in Seurat and annotated in Ensembl; only GO terms with more than 40 attached genes were considered, and enriched terms with unadjusted $P < 0.001$ were clustered hierarchically according to Wang's distance[94].

Two-photon imaging. Females aged 3–4 days were head-fixed to a custom mount with eicosane (Sigma) and imaged on a Movable Objective Microscope with resonant scanners (MOM, Sutter Instruments) controlled through ScanImage software (Vidrio Technologies). Cuticle, adipose tissue, and trachea were removed to create an optical window, and the brain

was superfused with carbogenated extracellular solution (95% O_2 – 5% CO_2, pH 7.3, 275 mOsm) containing (in mM) 103 NaCl, 3 KCl, 5 TES, 8 trehalose, 10 glucose, 7 sucrose, 26 $NaHCO_3$, 1 NaH_2PO_4, 1.5 $CaCl_2$, 4 $MgCl_2$.

To excite iATPSnFR and co-expressed tdTomato, or the FRET donor (CFP) of ATeam, a Mai Tai DeepSee Ti:sapphire laser (Spectra Physics model eHP DS) produced excitation light pulses with centre wavelengths of 930 and 840 nm, respectively, whose power was modulated by a Pockels cell (302RM, Conoptics). Emitted photons were collected by a 20×/1.0 NA water immersion objective (W-Plan-Apochromat, Zeiss), split into two channels by dichromatic mirrors (green and red: Chroma 565dcxr; cyan and yellow: Semrock BrightLine FF509-Fdi01), and detected by GaAsP photomultiplier tubes (H10770PA-40 SEL, Hamamatsu Photonics). The emission paths contained bandpass filters for iATPSnFR (Chroma ET525/70m-2p) and tdTomato (Chroma ET605/70m) or CFP (Semrock BrightLine FF01-482/25) and YFP (Semrock Avant AF01-539/27), respectively. Photocurrents were passed through high-speed amplifiers (HCA-4M-500K-C, Laser Components) and custom-designed integrator circuits to maximize the signal-to-noise ratio. Images of 256 × 256 pixels were acquired at a rate of 29.13 Hz.

For optogenetic stimulation, a 625-nm LED (M625L3, ThorLabs) controlled by a dimmable LED driver (ThorLabs) delivered 0.5–25 mW cm^{-2} of optical power through a bandpass filter (Semrock BrightLine FF01-647/57-25) to the head of the fly. Stimulus trains lasted for 2 minutes and consisted of ten 25-ms light pulses in 500-ms bursts recurring once per s. To apply arousing heat, an 808-nm laser diode (Thorlabs L808P500MM) was mounted on a temperature-controlled heat sink (ThorLabs TCDLM9 with ThorLabs TED200C controller) and aimed at the abdomen of the fly. The diode was restricted to a maximal output of 50 mW by a ThorLabs LDC210C laser diode controller, and 2-s pulses were delivered every 30 s for 2 minutes. The voltage steps controlling the LED or laser diode were recorded in a separate imaging channel for post-hoc alignment.

Time series of average fluorescence in manually selected dendritic regions of interest (ROIs) were analysed in MATLAB, following the subtraction of a time-varying background. ΔF/F curves were calculated separately for each trial as $\Delta F_t/F_0 = (F_t - F_0)/F_0$, where F_0 is the mean fluorescence intensity during 170 s before stimulation onset and F_t is the fluorescence intensity in frame t; $\Delta R_t/R_0$ represents the iATPSnFR/tdTomato (green-red) or YFP/CFP

(yellow-cyan) intensity ratio, as indicated. The stimulus-aligned $\Delta R_t/R_0$ signals were averaged across two trials in the case of optogenetic stimulation and then across flies; the statistical units are flies. For display purposes, traces were smoothed with a 15-s moving-average filter and down-sampled by a factor of 100.

Confocal imaging. Single-housed females aged 6 days post eclosion were dissected at zeitgeber time 0, following *ad libitum* sleep or 12 h of sleep deprivation. Experimental and control samples were processed in parallel. Brains were fixed for 20 min in 0.3% (v/v) Triton X-100 in PBS (PBST) with 4% (w/v) paraformaldehyde, washed five times with PBST, incubated with primary and secondary antibodies where indicated, mounted in Vectashield, and imaged. Only anatomically intact specimens from live flies (at the point of dissection) were analysed, blind to sleep history, using existing, adapted, or newly developed (semi-)automated routines in Fiji. The specific acquisition and analysis parameters for different experiments were as follows:

For mitochondrial morphometry[95], z-stacks of structures expressing mito-GFP were collected on a Leica TCS SP5 confocal microscope with an HCX IRAPO L 25×/0.95 NA water immersion objective at the Nyquist limit, with identical acquisition parameters across all conditions. Point-spread functions were created with the PSF Generator plugin[96] in Fiji and used to deconvolve the images with DeconvolutionLab2 software[97,98] employing the Richardson-Lucy TV algorithm with regularization set to 0.0001 and a maximum of two iterations[95]. Functions of the Mitochondria Analyser plugin in Fiji were applied in an automated fashion to the deconvolved images to remove background noise ('subtract background' with a radius of 1.25 µm), reduce noise and smooth objects while preserving edges ('sigma filter plus'), enhance dim areas while minimizing noise amplification ('enhance local contrast' with slope 1.5), and optimize the use of image bits ('gamma correction' with a value of 0.90) before thresholding ('weighted mean' with block size 1.25 µm and a C value of 5 for dFBNs and 12 for PNs, determined empirically to minimize background noise[95]). The resulting binary images were examined via Batch 3D analysis on a 'per-cell' basis, to extract morphological metrics. The full dendritic fields of dFBNs were analysed, but the high packing density of PN dendrites in the glomeruli of the antennal lobe forced us to select 20 substacks per glomerulus, each with an axial depth of 5.8 µm, using a Fiji random number generator function, to make computations practical. Morphometric parameters were then averaged across all substacks per glomerulus.

For ratiometric snapshot imaging of the ATP sensors iATPSnFR (normalized to co-expressed RFP) and ATeam and the mitophagy sensor mito-QC, fluorescence was quantified on summed z-stacks of the relevant channels, following the subtraction of average background in manually defined areas close to the structures of interest. Brains expressing ATeam were imaged on a Zeiss LSM980 with Airyscan2 microscope with a Plan-Apochromat 40×/1.3 NA oil immersion objective, an excitation wavelength of 445 nm for the FRET donor mCFP, and emission bands of 454–507 nm for CFP and 516–693 nm for the YFP variant mVenus[65]. Images of iATPSnFR plus RFP and mito-QC were acquired on a Leica TCS SP5 confocal microscope with HCX IRAPO L 25×/0.95 NA water and HCX PL APO 40×/1.30 NA oil immersion objectives, respectively. The excitation and emission wavelengths were 488 nm and 498–544 nm for iATPSnFR, 555 nm and 565–663 nm for RFP, and 488 nm and 503–538 nm, and 587 nm and 605–650 nm, for the GFP and mCherry moieties of mito-QC, respectively. To convert the emission ratios of ATeam1.03NL into approximate ATP concentrations, the 2-channel fluorescence of the sensor was linearly unmixed into that of its constituent FRET moieties, recombined at the donor and acceptor peak wavelengths for comparison with the published emission spectra of the sensor at different ATP concentrations[65], and integrated in the emission bands used for imaging while accounting for the spectral response of the photodetectors. ATP concentration estimates were validated by plugging the ATeam estimates into the dose-response curve[62] of iATPSnFR$^{1.0}$ and comparing the predicted iATPSnFR fluorescence ratios in sleep-deprived vs. rested flies (1.55 ± 0.24) with the experimentally measured values (1.44 ± 0.12; means ± s.e.m.; P=0.6840, t test).

SPLICS puncta[72,99] were imaged on a Leica TCS SP5 confocal microscope with an HCX PL APO 40×/1.30 NA oil immersion objective, excitation and emission wavelengths of 488 nm and 500–540 nm, respectively, and analysed in Fiji with a custom macro based on the existing 'Quantification 1 and 2' plugins[99]. Only puncta with a minimal volume of 0.026 μm^3 (10 voxels) were quantified.

For localizing Drp1, we labelled the mitochondria of dFBNs with mitoGFP in *Drp1::FLAG-FlAsH-HA* flies[84], whose genomic *Drp1* coding sequence is fused in frame with a FLAG-FlAsH-HA tag. Fixed brains were incubated sequentially at 4 °C in blocking solution (10% goat serum in 0.3% PBST) overnight, with mouse monoclonal anti-FLAG antibody (anti-DDK; 1:1,000, OriGene) in blocking solution for 2–3 days, and with goat anti-Mouse Alexa

Fluor 633 (1:500, ThermoFisher) in blocking solution for two days. The samples were washed five times with blocking solution before and after the addition of secondary antibodies, mounted, and imaged on a Leica TCS SP5 confocal microscope with an HCX PL APO 40×/1.30 NA oil immersion objective. The excitation and emission wavelengths were 488 nm and 500–540 nm for mito-GFP and 631 nm and 642–690 nm for Alexa Fluor 633, respectively. dFBN somata were identified manually in the green channel, which was then thresholded, despeckled, and binarized by an automated custom macro in Fiji. The mitochondria-associated fraction of endogenous FLAG-tagged Drp1 in dFBNs was quantified in summed z-stacks as the proportion of red-fluorescent pixels within the somatic volume that colocalized with mitochondrial objects.

Electrophysiology. For whole-cell patch-clamp recordings *in vivo*, female flies aged 2–4 days post eclosion were prepared as for functional imaging, but the perineural sheath was also removed for electrode access. The GFP-labelled somata of dFBNs were visually targeted with borosilicate glass electrodes (8–10 MΩ) filled with internal solution (pH 7.3, 265 mOsM) containing (in mM): 10 HEPES, 140 potassium aspartate, 1 KCl, 4 MgATP, 0.5 Na_3GTP, 1 EGTA, and 10 biocytin. Signals were acquired at room temperature (23 °C) in current-clamp mode with a MultiClamp 700B amplifier (Molecular Devices), lowpass-filtered at 5 kHz, and sampled at 10 kHz using an Axon Digidata 1550B digitizer controlled through pCLAMP 11.2 (Molecular Devices). Series resistances were monitored but not compensated. Data were analysed using the NeuroMatic package (http://neuromatic.thinkrandom.com) in Igor Pro (WaveMetrics). Current–spike frequency functions were determined from voltage responses to a series of current steps (5-pA increments from –20 to 105 pA, 1 s duration) from a pre-pulse potential of –60 ± 5 mV. Spikes were detected by finding minima in the second derivative of the membrane potential trace. Spike frequencies were normalized to membrane resistances, which were calculated from linear fits of the steady-state voltage changes elicited by hyperpolarizing current steps. Only dFBNs firing more than one action potential in response to depolarizing current injections, with resting potentials <–30 mV and series resistances <50 MΩ were characterized further. Spike bursts were defined as sets of spikes with an average intra-burst inter-spike interval (ISI) <50 ms and an inter-burst ISI >100 ms. These ISI thresholds were set after visual inspection of voltage traces of all recorded neurons. Cells were scored as bursting if they generated at least one action potential burst during the series of depolarizing current steps.

Quantification and statistical analysis. Gene expression levels were compared by Bonferroni-corrected Wilcoxon rank-sum test. Gene ontology enrichment was quantified using Fisher's exact test in PANTHER (with a false discovery rate-adjusted significance level of $P < 0.05$) or ViSEAGO (with an unadjusted significance level of $P < 0.001$).

Behavioral, imaging, and electrophysiological data were analysed in Prism 10 (GraphPad) and SPSS Statistics 29 (IBM). All null hypothesis tests were two-sided. To control type I errors, *P*-values were adjusted to achieve a joint α of 0.05 at each level in a hypothesis hierarchy; multiplicity-adjusted *P*-values are reported in cases of multiple comparisons at one level. Group means were compared by *t* test, one-way ANOVA, two-way repeated-measures ANOVA, or mixed-effects models, as stated, followed by planned pairwise analyses with Holm-Šidák's multiple comparisons test where indicated. Repeated-measures ANOVA and mixed-effect models used the Geisser-Greenhouse correction. Where the assumption of normality was violated (as indicated by D'Agostino-Pearson test), group means were compared by Mann-Whitney test or Kruskal-Wallis ANOVA, followed by planned pairwise analyses using Dunn's multiple comparisons test, as indicated. Frequency distributions were analysed by χ^2 test, and categories responsible for pairwise differences were detected[100] by locating cells with standardized residuals ≥ 2.

The investigators were blind to sleep history and/or genotype in imaging experiments but not otherwise. Sample sizes in behavioral experiments (typically n=32 flies per genotype) were chosen to detect 2-h differences in daily sleep with a power of 0.9. All behavioral experiments were run at least three times, on different days and with different batches of flies. The figures show pooled data from all replicates.

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Author contributions

R.S. performed and analysed all transcriptomic, imaging, and behavioral experiments and C.D.V. all electrophysiological recordings; N.M. assisted with imaging, genetics, and behavior; A. K. conducted initial studies with Ucp4. R.S. and G.M. designed the study, interpreted the results, and prepared the manuscript. G.M. devised and directed the research and wrote the final version of the paper.

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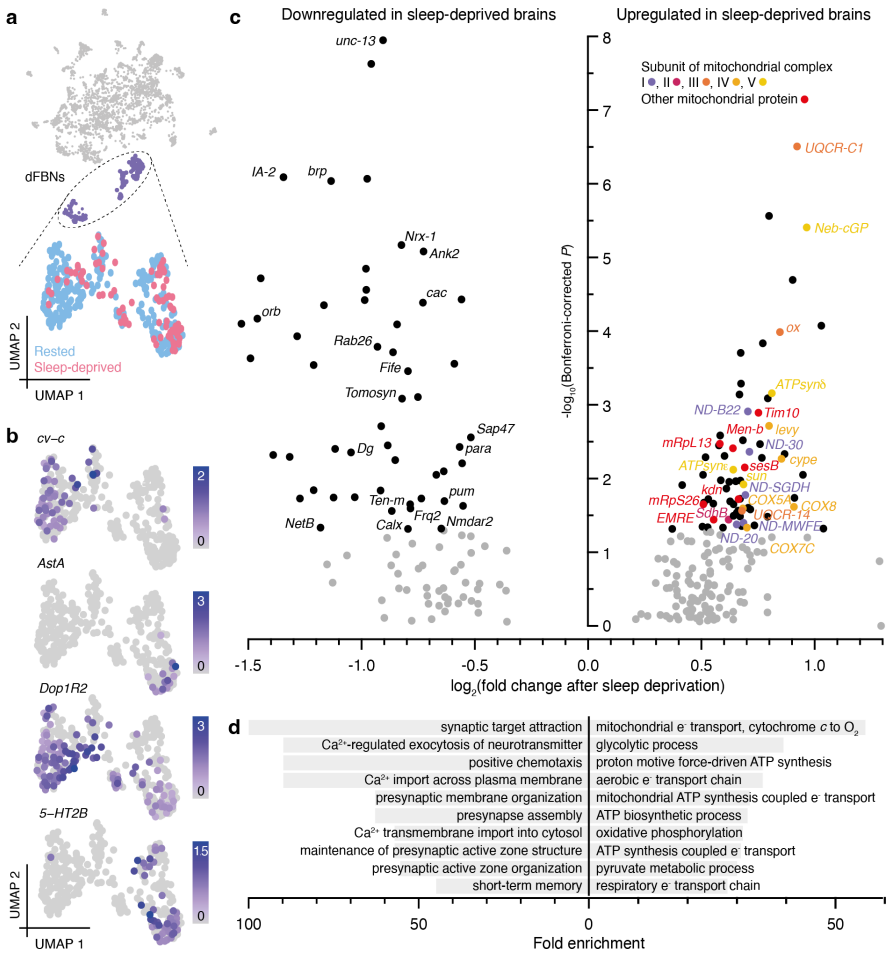

Fig. 1 | The transcriptional response of dFBNs to sleep deprivation.

a, Uniform manifold approximation and projection (UMAP) representation of glutamatergic neurons (gray) according to their gene expression profiles. dFBNs (top, purple) form a distinct cluster containing cells from rested (blue, *n*=237 cells) and sleep-deprived (red, *n*=86 cells) brains (bottom). **b**, Log-normalized expression levels of dFBN markers. **c**, Volcano plot of sleep history-dependent gene expression changes in dFBNs. Signals with Bonferroni-corrected *P*<0.05 are indicated in black; labels identify protein products localized to synapses or mitochondria; colors denote subunits of mitochondrial respiratory complexes. The *P*-value of *unc-13* exceeds the y-axis limit and is plotted at the top of the graph. **d**, Enrichment of the top 10 downregulated (left) and upregulated (right) 'biological process' gene ontology terms in the set of differentially expressed dFBN genes.

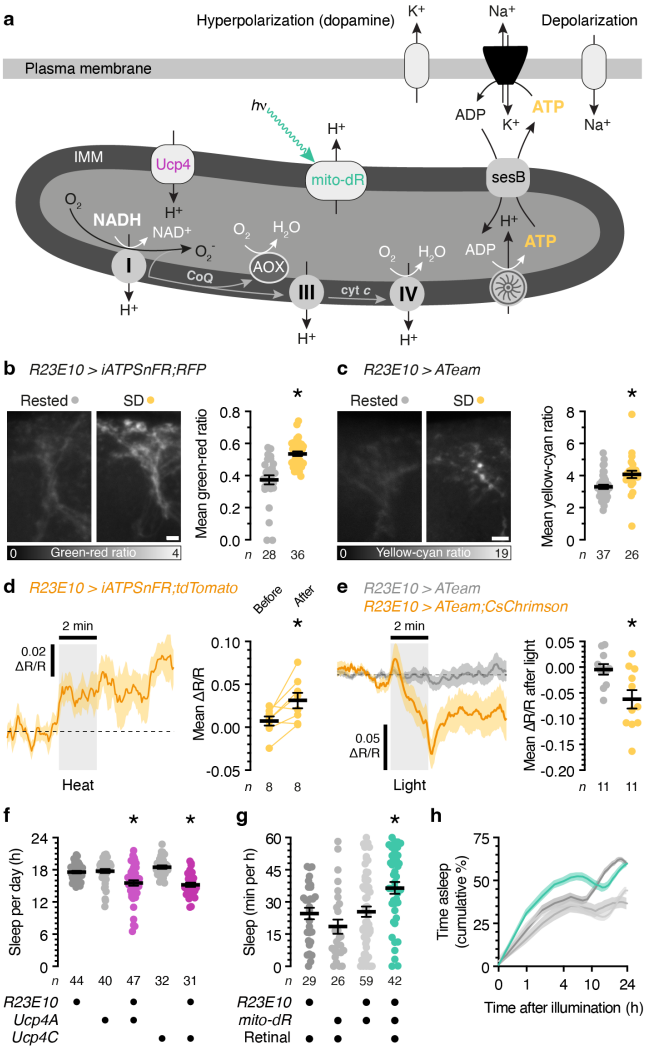

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Fig. 2 | A mitochondrial electron surplus relative to ATP demand induces sleep.

a, Proton-pumping complexes I, III, and IV convert the energy of electron transfers from NADH via coenzyme Q (CoQ) and cytochrome *c* (cyt *c*) to O_2 into a proton electrochemical gradient across the inner mitochondrial membrane (IMM). Uncoupling protein 4 (Ucp4) discharges, while illumination of mito-dR charges, the IMM. The return of extruded protons to the matrix spins the blades of the ATP synthase and produces ATP, which leaves the matrix via sesB in exchange for cytoplasmic ADP. Neuronal ATP consumption is activity-dependent and dominated by the plasma membrane Na^+/K^+ ATPase, which restores ion gradients diminished by action and excitatory synaptic currents. An oversupply (relative to ATP demand) of electrons to CoQ increases the risk of single-electron reductions of O_2 to O_2^- at complexes I and III. AOX mitigates this risk. **b**, **c**, Summed-intensity projections of dFBN dendrites expressing iATPSnFR plus tdTomato (**b**) or ATeam (**c**), in rested and sleep-deprived (SD) flies. Scale bars, 5 μm. Emission ratios are intensity-coded according to the keys below and increase after sleep deprivation (**b**, $P<0.0001$; **c**, $P=0.0002$; Mann-Whitney test). **d**, Arousing heat acutely elevates ATP in dFBNs expressing iATPSnFR plus RFP. Mean fluorescence was quantified in 20-s windows immediately before and after stimulation ($P=0.0152$, paired *t* test). **e**, Optogenetic stimulation acutely dissipates ATP in dFBNs expressing ATeam and CsChrimson, but

not in dFBNs lacking CsChrimson. Mean fluorescence was quantified in a 2-s window immediately after the optical stimulus (P=0.0103, t test). **f**, Sleep in flies expressing *R23E10-GAL4*-driven Ucp4A or Ucp4C and parental controls ($P \leq 0.0139$ for all planned pairwise comparisons after ANOVA). **g**, **h**, Sleep during the first 60 min after illumination (**g**, $P \leq 0.0279$ for all planned pairwise comparisons after ANOVA) and cumulative sleep percentages (**h**, time × proton pump interaction: $P<0.0001$, mixed-effects model) in flies expressing *R23E10-GAL4*-driven mito-dR, with or without retinal, and parental controls. Asterisks indicate significant differences ($P<0.05$) from both parental controls or in planned pairwise comparisons. Data are means ± s.e.m.; n, number of dendritic regions (**b**, **c**) or flies (**d**–**h**). For statistical details see Supplementary Table 1.

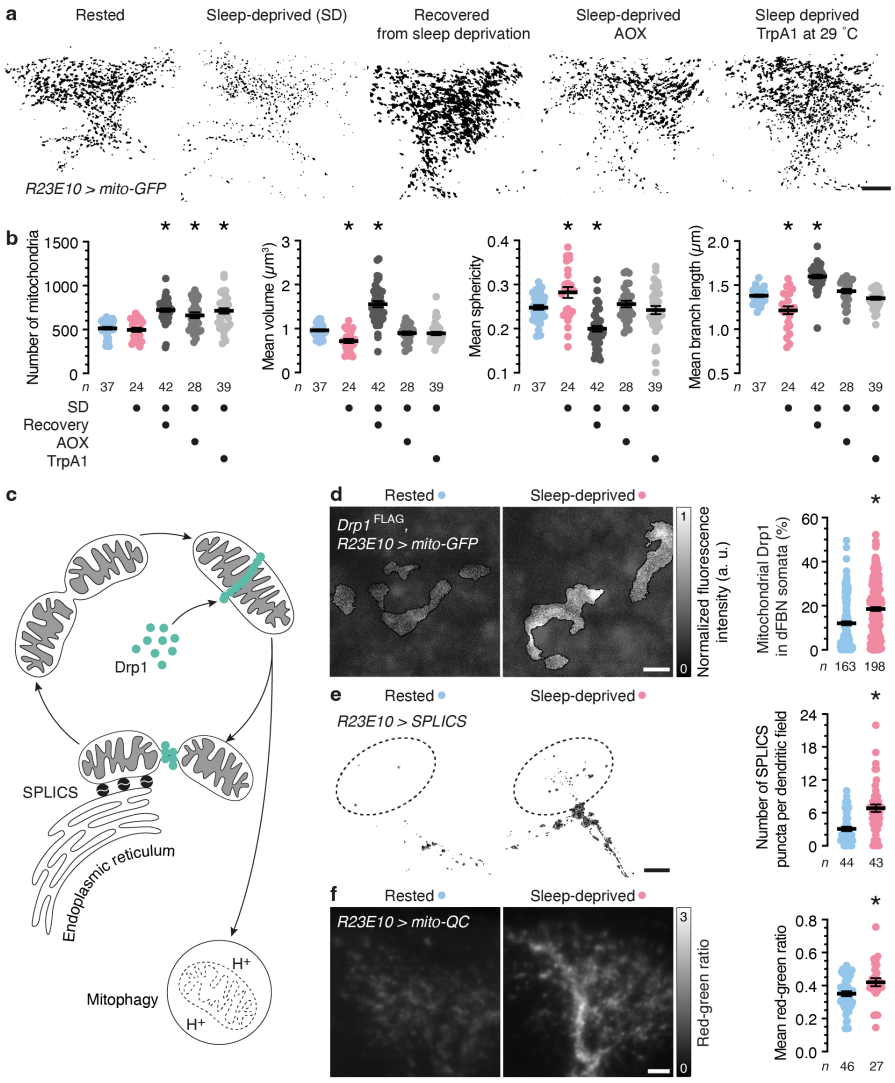


Fig. 3 | Sleep alters mitochondrial dynamics.

a, **b,** Maximum intensity projections of automatically detected mitochondria (**a,** black) and their morphometric parameters (**b**) in dFBN dendrites of rested flies, sleep-deprived flies, flies allowed to recover for 24 h after sleep deprivation, and sleep-deprived flies co-expressing *R23E10-GAL4*-driven AOX or TrpA1, which was activated by performing sleep deprivation at 29 ˚C. Scale bar, 10 μm. Sleep history-dependent changes in mean mitochondrial volume (P<0.0001, t test), sphericity (P=0.0059, t test), and branch length (P<0.0001, t test) are occluded by the co-expression of AOX or the simultaneous activation of TrpA1 (P≥0.1944, Dunn's or Holm-Šídák test after ANOVA) and overcorrected after recovery sleep (P<0.0001, Dunn's or Holm-Šídák test after ANOVA). The number of mitochondria is unchanged by sleep deprivation (P=0.5002, t test) but elevated after recovery sleep and in sleep-deprived flies co-expressing AOX or activated TrpA1 (P≤0.0008, Dunn's test after Kruskal-Wallis ANOVA). **c**, Visualization of events in mitochondrial fission: oligomerization of cytoplasmic Drp1 at the mitochondrial surface; reconstitution of fluorescence from split-GFP-based contact site sensors (SPLICS) with the endoplasmic reticulum (ER); mitophagy of daughter organelles. **d**, Drp1 recruitment. Single confocal image planes through dFBN somata of flies carrying

the *Drp1*^{FLAG} allele and expressing *R23E10-GAL4*-driven mito-GFP. Sleep deprivation increases anti-FLAG fluorescence (intensity-coded according to the key on the right) within automatically detected mitochondrial outlines (P<0.0001, Mann-Whitney test). Scale bar, 2 μm. **e**, Mitochondria–ER contacts. Isosurface renderings (voxel value 128) of SPLICS puncta in dFBN dendrites (dashed outlines), obtained by trilinear interpolation of thresholded and despeckled confocal image stacks. Sleep deprivation increases the number of SPLICS puncta per dendritic field (P<0.0001, Mann-Whitney test). Scale bar, 10 μm. **f**, Mitophagy. Summed-intensity projections of dFBN dendrites expressing mito-QC. Emission ratios are intensity-coded according to the key on the right and increase after sleep deprivation (P=0.0101, t test). Scale bar, 5 μm. Data are means ± s.e.m.; n, number of dendritic regions (**b**, **e**, **f**) or somata (**d**); asterisks, significant differences (P<0.05) in planned pairwise comparisons. For statistical details see Supplementary Table 1.

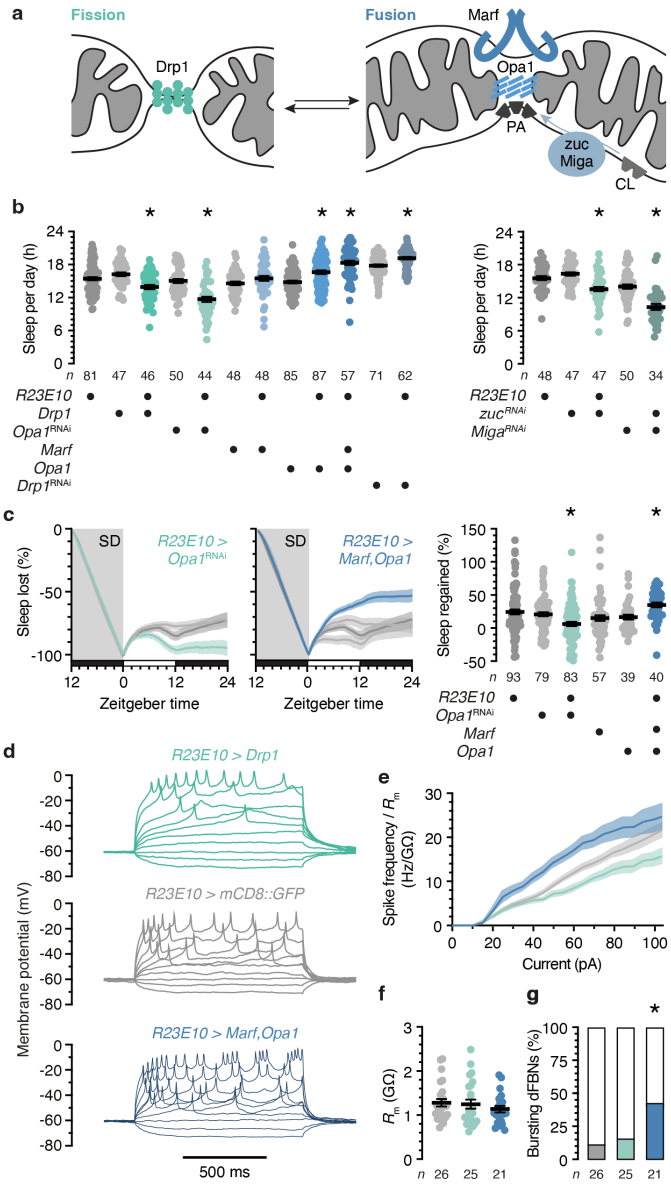
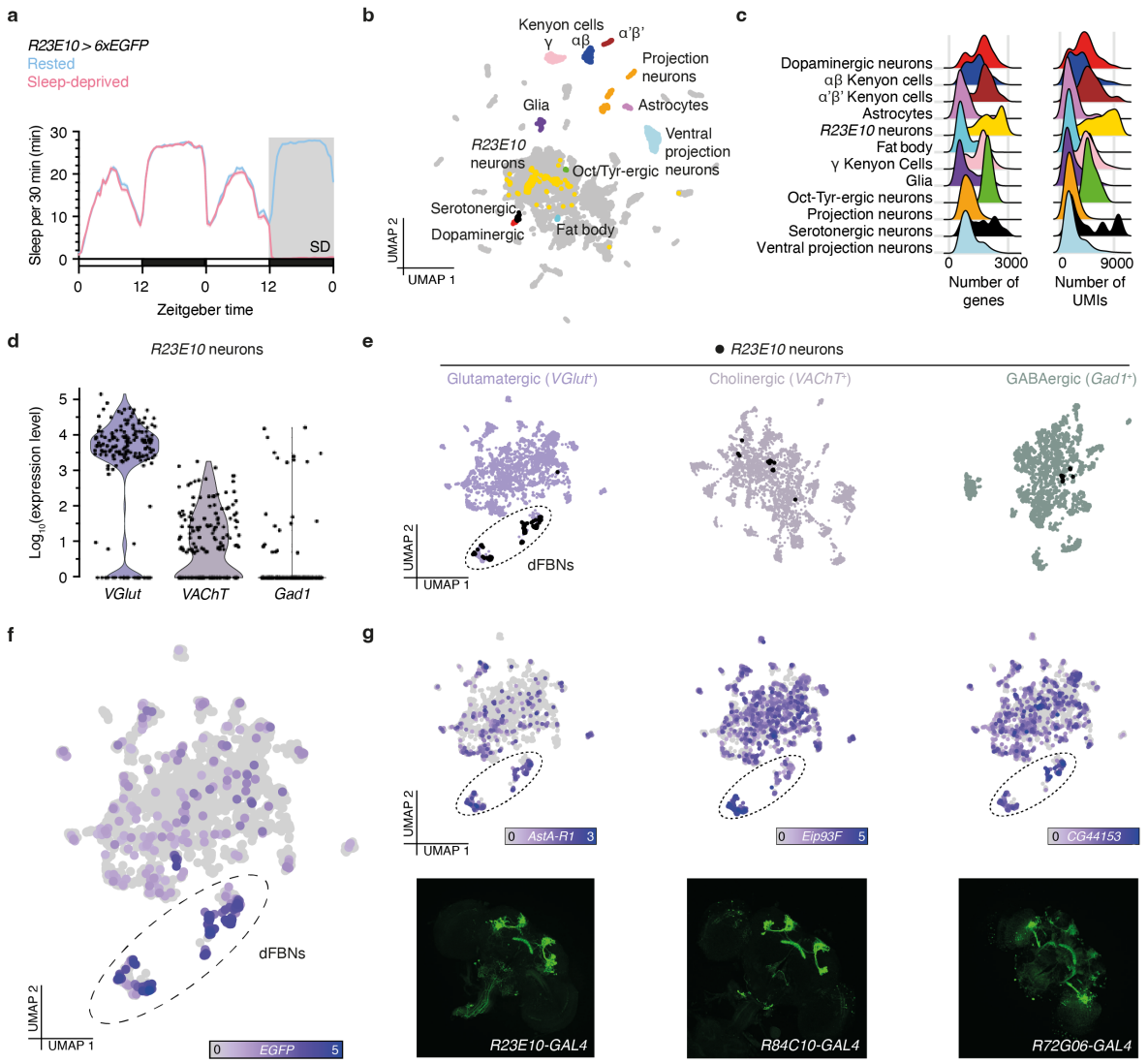


Fig. 4 | Mitochondrial dynamics alter sleep.

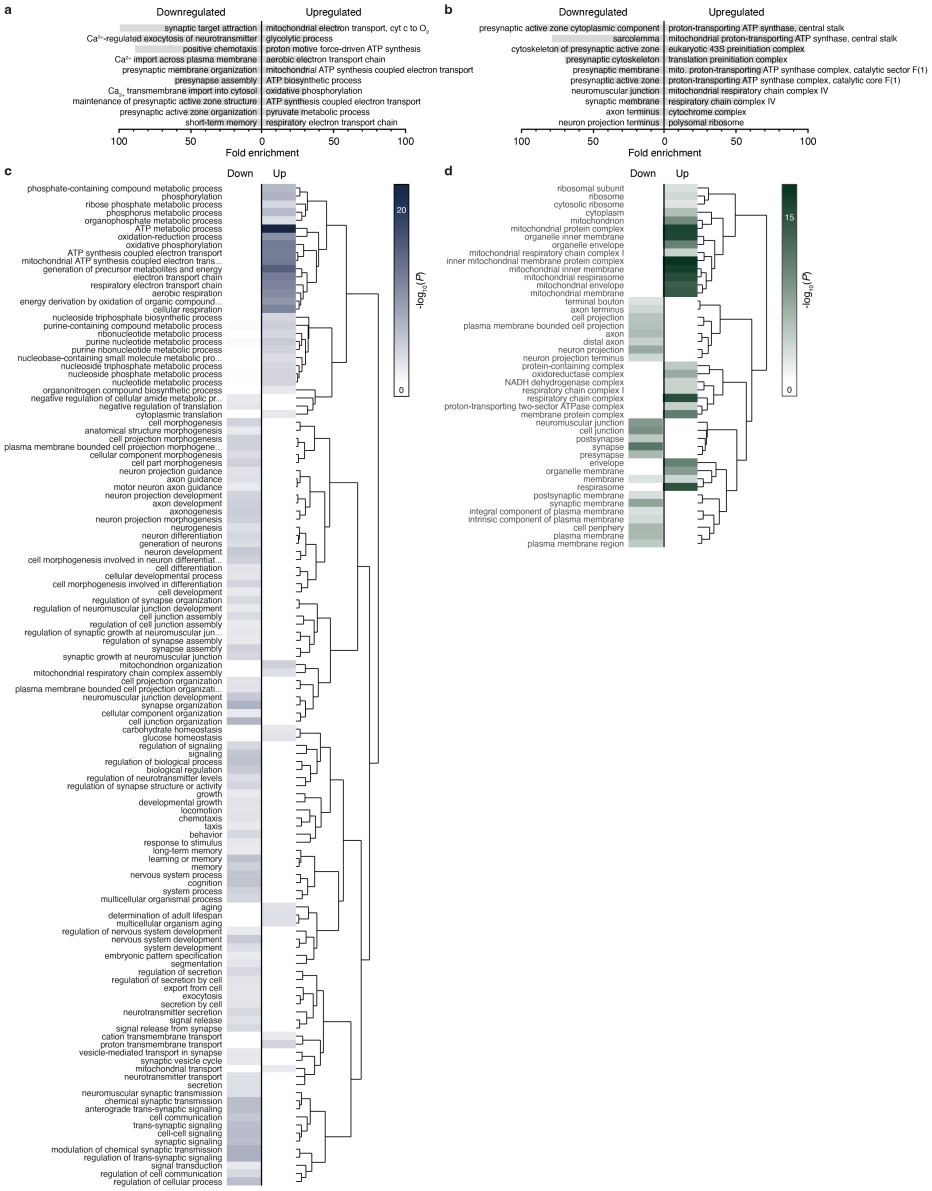
a, The mitochondrial fission (green) and fusion machineries (blue) comprise Drp1, the outer and inner membrane proteins Marf and Opa1, and the mitoPLD zuc, which releases phosphatidic acid (PA) from cardiolipin (CL). Miga stimulates zuc activity and/or directly supplies PA from other membranes. **b**, Sleep in flies expressing *R23E10-GAL4*-driven fission or fusion proteins, or RNAi transgenes directed against transcripts encoding these proteins (left) or proteins regulating PA levels (right), and their parental controls. With the exception of the overexpression of Marf alone ($P \geq 0.1622$, Holm-Šídák test after ANOVA), manipulations that increase fission (green) or fusion (blue) alter sleep in opposite directions (GTPases: $P \leq 0.0115$, PA regulators: $P \leq 0.0003$; Holm-Šídák test after ANOVA). **c**, Manipulations that increase fission (*R23E10-GAL4* > *Opa1*^{RNAi}, green) or fusion (*R23E10* > *Marf;Opa1*, blue) alter the time courses (left panels, time x genotype interactions: $P < 0.0001$, two-way ANOVA) and percentages of sleep rebound after deprivation (SD) in opposite directions (right panel, genotype effect: $P \leq 0.0186$, Dunn's test after ANOVA). **d**, Example voltage responses to current steps

of dFBNs expressing mCD8::GFP (gray) and Drp1 (green) or Marf plus Opa1 (blue). **e**, Manipulations that increase fission (green) or fusion (blue) alter the membrane resistance (R_m)-normalized spike frequency in opposite directions (time × genotype interaction: $P<0.0001$, mixed-effects model, sample sizes in **f**). **f**, Membrane resistances (genotype effect: $P=0.5099$, ANOVA). **g**, The overexpression of Marf plus Opa1 increases the percentage of dFBNs generating bursts of action potentials ($P=0.0241$, χ^2 test; standardized residuals +2.01). Data are means ± s.e.m.; *n*, number of flies (**b**, **c**) or cells (**f**, **g**); asterisks, significant differences ($P<0.05$) in planned pairwise comparisons. For statistical details see Supplementary Table 1.



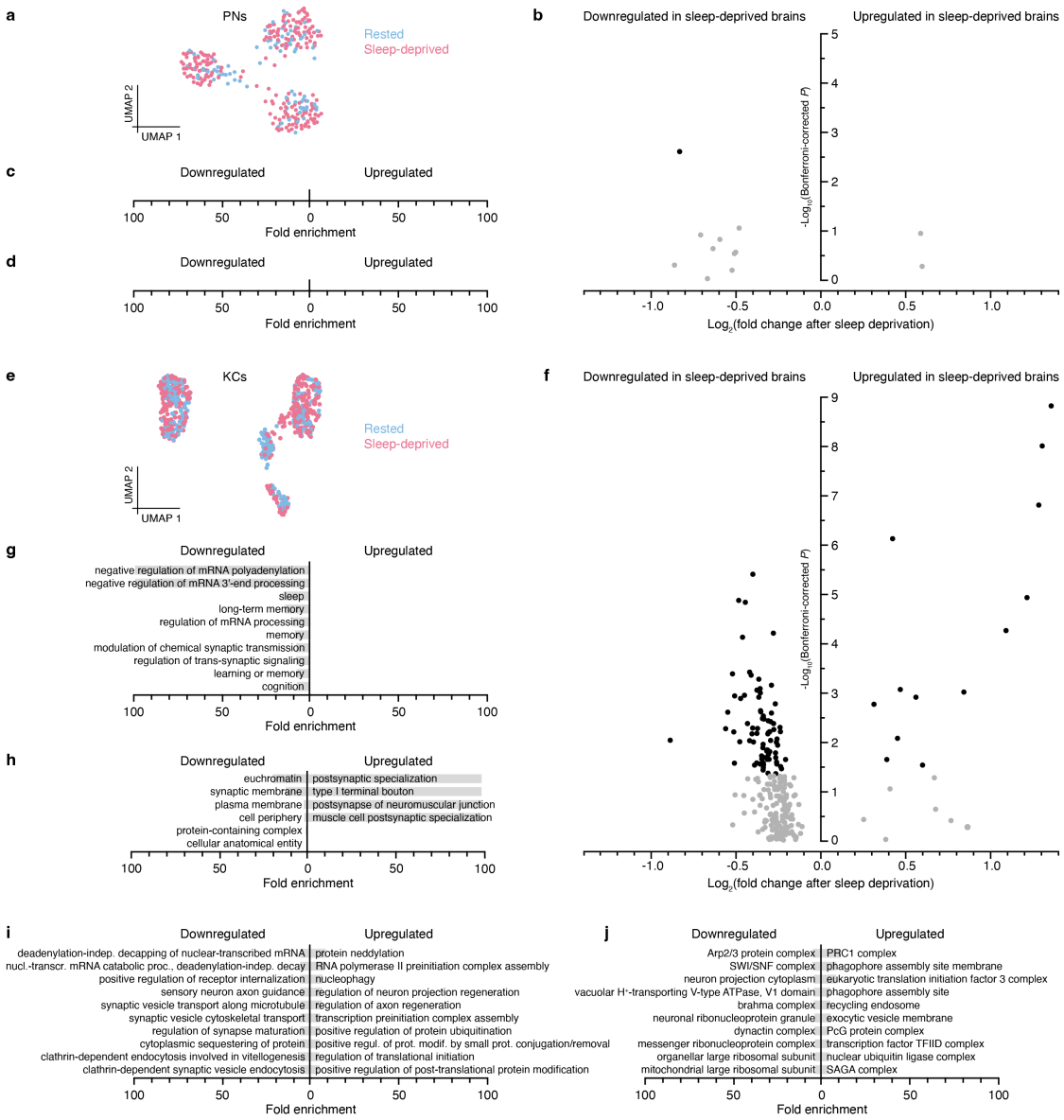
Supplementary Fig. 1 | Identification of dFBNs and their transcriptomic response to sleep deprivation by single-cell RNA-sequencing.

a, Sleep profiles of flies expressing *R23E10-GAL4*-driven 6xEGFP under control (blue, *n*=558) and sleep deprivation (SD) conditions (red, *n*=432) before single-cell RNA sequencing. **b**, Uniform manifold approximation and projection (UMAP) representation of cells in the fly brain. Highlighted cell types, including neurons nominated by *R23E10-GAL4* (yellow), were identified as detailed in Methods. **c**, Distribution of the number of unique molecular identifiers (UMIs) and genes per cell for each annotated cell type. **d**, Log-normalized distribution of the expression levels of markers for the fast-acting neurotransmitters glutamate, acetylcholine, and GABA in *R23E10-GAL4* neurons. **e**, *R23E10-GAL4* neurons (black) mapped onto re-clustered representations of cells expressing glutamatergic, cholinergic, and GABAergic markers. *Bona fide* dFBNs form a distinct glutamatergic cluster (dashed outline). **f**, **g**, Compared to the rest of the glutamatergic brain, dFBNs are enriched in EGFP (**f**) and transcripts of genes whose enhancer fragments label dFBNs in *GAL4* lines generated and imaged by the FlyLight project[54] (**g**). Log-normalized expression levels are color-coded according to the keys below each panel.



Supplementary Fig. 2 | Gene ontology analysis of sleep history-dependent gene expression in dFBNs.

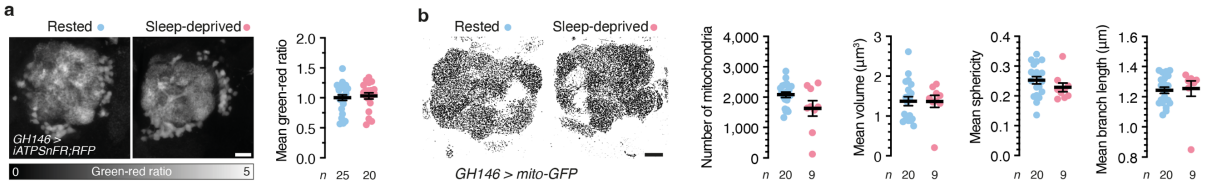
a–**d,** Enrichment of gene ontology (GO) 'biological process' (**a**, **c**) and 'cellular component' (**b**, **d**) terms in the set of genes whose expression in dFBNs varies with sleep history. Panels **a** and **b** plot the top 10 enriched terms by PANTHER Overrepresentation Test (fold enrichments >100 are truncated). Panels **c** and **d** show heatmaps (computed with ViSEAGO and topGO) of GO terms attached to downregulated (left) and upregulated (right) differentially expressed genes. Dendrograms represent semantic groupings among GO terms. *P*-values are color-coded according to the keys on the right.



Supplementary Fig. 3 | Sleep history-dependent gene expression in KCs, PNs, and non-dFBN cells.

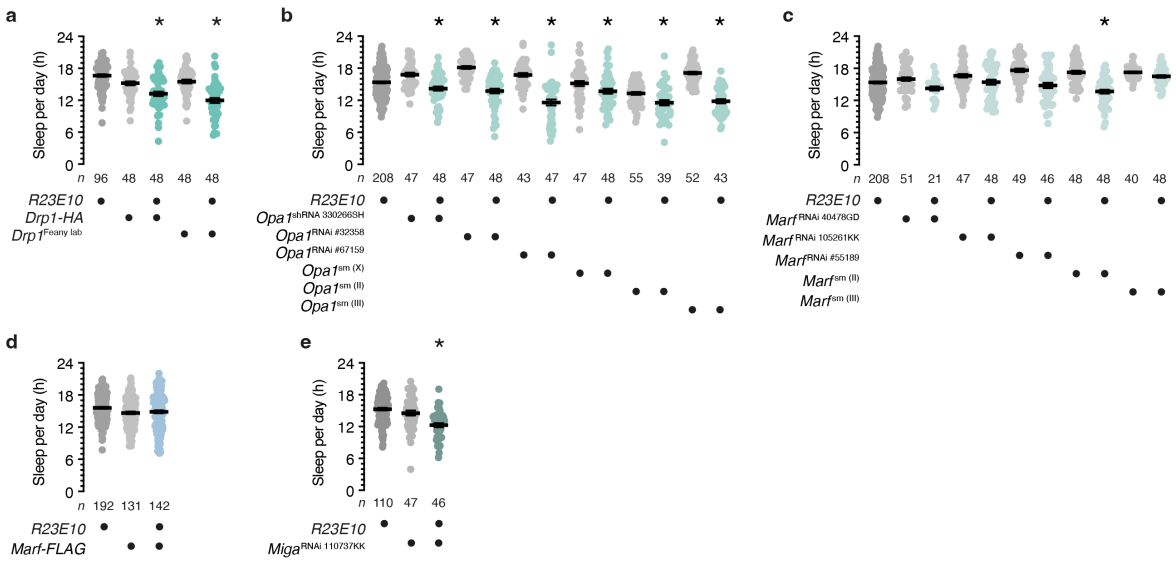
a, UMAP representation of PNs (*n*=317) from rested (blue) and sleep-deprived brains (red) according to their gene expression profiles. **b**, Volcano plot of sleep history-dependent gene expression changes in PNs. A single signal with Bonferroni-corrected *P*<0.05 is indicated in black. **c**, **d**, PANTHER Overrepresentation Test fails to detect enriched 'biological process' (**g**) and 'cellular component' (**h**) gene ontology terms in the set of differentially expressed PN genes. **e**, UMAP representation of KCs (*n*=603) from rested (blue) and sleep-deprived brains (red) according to their gene expression profiles. **f**, Volcano plot of sleep history-dependent gene expression changes in PNs. Signal with Bonferroni-corrected *P*<0.05 are indicated in black. **g**, **h**, Enrichment of the top 10 downregulated and upregulated 'biological process' (**g**) and 'cellular component' (**h**) gene ontology terms in the set of differentially expressed KC genes by PANTHER Overrepresentation Test (fold

enrichments >100 are truncated). **i**, **j**, Enrichment of the top 10 downregulated and upregulated 'biological process' (**i**) and 'cellular component' (**j**) gene ontology terms in the set of genes with differential expression in all non-dFBN cells by PANTHER Overrepresentation Test.



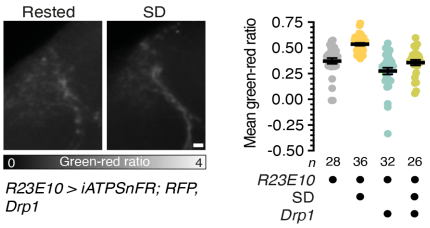
Supplementary Fig. 4 | ATP levels and mitochondrial morphology of PNs are insensitive to sleep history.

a, Summed-intensity projections of PN dendrites expressing iATPSnFR plus RFP, in rested and sleep-deprived (SD) flies. Emission ratios are intensity-coded according to the key below and unaltered by sleep deprivation (P=0.6616, t test) **b**, Maximum intensity projections of automatically detected mitochondria (black) and their morphometric parameters in PN dendrites of rested and sleep-deprived flies. Mitochondrial number (P=0.2534, Mann-Whitney test), mean volume (P= 0.7637, Mann-Whitney test), mean sphericity (P=0.1953, Mann-Whitney test), and mean branch length (P=0.5014, Mann-Whitney test) are unaffected by sleep deprivation. Scale bars, 20 μm. Data are means ± s.e.m.; n, number of antennal lobe glomeruli. For statistical details see Supplementary Table 2.



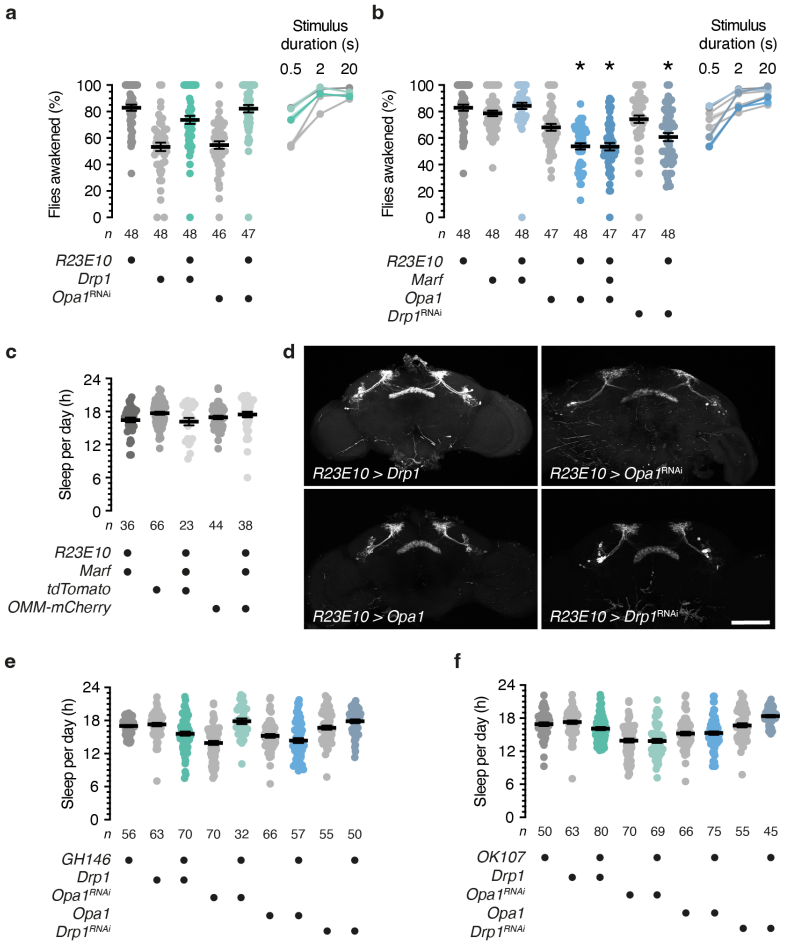
Supplementary Fig. 5 | Mitochondrial dynamics alter sleep.

a–**e**, Sleep in flies carrying *R23E10-GAL4*-driven overexpression constructs or RNAi transgenes directed against mitochondrial fission or fusion machinery that are not included in Fig. 4b: two independent constructs for Drp1 overexpression (**a**, $P \leq 0.0209$, Dunn's test after ANOVA); six independent RNAi transgenes directed against Opa1 (**b**, $P \leq 0.0199$, Holm-Šídák test after ANOVA); five independent RNAi transgenes directed against Marf (**c**, $P \geq 0.1017$ relative to ≥1 parental control with the exception of *R23E10* > *Marf*$^{sm(II)}$, Holm-Šídák test after ANOVA); one independent construct for Marf overexpression (**d**, $P \geq 0.1252$, Dunn's test after ANOVA); and one independent RNAi transgene directed against Miga (**e**, $P \leq 0.0001$, Holm-Šídák test after ANOVA). Data are means ± s.e.m.; *n*, number of flies; asterisks, significant differences ($P < 0.05$) from both parental controls. For statistical details see Supplementary Table 2.



Supplementary Fig. 6 | Mitochondrial fission reduces dFBN ATP levels in rested and sleep-deprived flies.

Summed-intensity projections of dFBN dendrites expressing Drp1 and iATPSnFR plus RFP, in rested and sleep-deprived (SD) flies. Emission ratios are intensity-coded according to the key below and reduced in dFBNs expressing Drp1, irrespective of sleep history (Drp1 effect: $P<0.0001$, sleep history effect: $P<0.0001$, Drp1 × sleep history interaction: $P=0.1112$; two-way ANOVA). Population data from Fig. 2b are reproduced for comparison. Scale bar, 5 μm. Data are means ± s.e.m.; *n*, number of dendritic fields. For statistical details see Supplementary Table 2.



Supplementary Fig. 7 | Manipulations of mitochondrial dynamics in dFBNs, but not KCs or PNs, alter sleep and arousal thresholds without causing overexpression artefacts or anatomical defects.

a, **b**, Percentages of flies awakened by mechanical stimuli lasting 0.5 s (left panels), 2 s, or 20 s (right panels). The average percentages awakened by 0.5-s stimuli in the left panels are reproduced on the right. Manipulations that increase fission fail to lower the arousal threshold, possibly because of a floor effect linked to the *R23E10-GAL4* strain (**a**, $P \geq 0.3354$ relative to ≥1 parental control, Dunn's test after ANOVA). With the exception of the overexpression of Marf alone ($P > 0.9999$), manipulations that increase fusion raise the arousal threshold (**b**, $P \leq 0.0371$, Dunn's test after ANOVA). **c**, Sleep in flies expressing *R23E10-GAL4*-driven Marf is insensitive to the coexpression of fluorescent proteins in the cytoplasm (tdTomato, $P \geq 0.2899$) or the outer mitochondrial membrane (OMM-mCherry, $P \geq 0.1106$, Dunn's test after ANOVA), in contrast to the synergistic effect of overexpressing Opa1 (Fig. 4b). **d**, Example morphologies of dFBNs in flies carrying *R23E10-GAL4*-driven overexpression constructs or RNAi transgenes directed against mitochondrial fission or fusion machinery. Scale bar, 100 μm. **e**, Sleep in flies carrying *GH146-GAL4*-driven overexpression constructs or RNAi transgenes directed against mitochondrial fission or fusion machinery in PNs ($P \geq 0.0842$ relative to ≥1 parental control, Holm-Šídák test after ANOVA). **f**, Sleep in flies carrying *OK107-GAL4*-driven overexpression constructs or RNAi transgenes directed against mitochondrial fission or fusion machinery in KCs

($P \geq 0.0660$ relative to ≥1 parental control, Holm-Šídák test after ANOVA). Data are means ± s.e.m.; *n*, number of flies; asterisks, significant differences ($P < 0.05$) from both parental controls. For statistical details see Supplementary Table 2.

Supplementary Table 1. Statistical analyses of Figures 2–4.

Figure	Statistical test	Pairwise comparison	Test statistic	*P*
2b	Mann-Whitney test	Rested vs. sleep-deprived	U = 141	<0.0001
2c	Mann-Whitney test	Rested vs. sleep-deprived	U = 218.5	0.0002
2d	Paired *t* test	Before vs. after heat	t_7 = 3.192	0.0152
2e	Unpaired *t* test	*R23E10>ATeam* vs. *R23E10>ATeam; CsChrimson*	t_{20} = 2.830	0.0103
2f	Kruskal-Wallis ANOVA			
	Genotype		H_5 = 42.82	<0.0001
	Dunn's test	*R23E10>Ucp4A* vs. *R23E10*	Z = 2.923	0.0139
	Dunn's test	*R23E10>Ucp4A* vs. *Ucp4A*	Z = 3.685	0.0009
	Dunn's test	*R23E10>Ucp4C* vs. *R23E10*	Z = 3.852	0.0005
	Dunn's test	*R23E10>Ucp4C* vs. *Ucp4C*	Z = 5.217	<0.0001
2g	Kruskal-Wallis ANOVA			
	Fly group		H_4 = 17.92	0.0005
	Dunn's test	*R23E10>mito-dR* (+ ret.) vs. *R23E10>mito-dR* (– ret.)	Z = 2.930	0.0102
	Dunn's test	*R23E10>mito-dR* (+ ret.) vs. *R23E10* (+ ret.)	Z = 2.600	0.0279
	Dunn's test	*R23E10>mito-dR* (+ ret.) vs. *mito-dR* (+ ret.)	Z = 4.002	0.0002
2h	Mixed-effects model			
	Time		$F_{3.346,502.9}$ = 267.9	<0.0001
	Genotype		$F_{3,152}$ = 13.79	<0.0001
	Time x genotype		$F_{69,3457}$ = 15.95	<0.0001
3b	Number of mitochondria			
	Unpaired *t* test			
	Effect of sleep deprivation	Rested vs. sleep-deprived	t_{59} = 0.6784	0.5002
	Kruskal-Wallis ANOVA			
	Effect of recovery, AOX, TrpA1		H_4 = 41.13	<0.0001
	Dunn's test	Rested vs. recovered	Z = 5.920	<0.0001
	Dunn's test	Rested vs. sleep-deprived + AOX	Z = 3.661	0.0008
	Dunn's test	Rested vs. sleep-deprived + TrpA1	Z = 5.187	<0.0001
	Mean volume			
	Unpaired *t* test			
	Effect of sleep deprivation	Rested vs. sleep-deprived	t_{59} = 4.896	<0.0001
	Kruskal-Wallis ANOVA			
	Effect of recovery, AOX, TrpA1		H_4 = 64.06	<0.0001
	Dunn's test	Rested vs. recovered	Z = 5.465	<0.0001
	Dunn's test	Rested vs. sleep-deprived + AOX	Z = 0.8956	>0.9999
	Dunn's test	Rested vs. sleep-deprived + TrpA1	Z = 1.625	0.3125
	Mean sphericity			
	Unpaired *t* test			
	Effect of sleep deprivation	Rested vs. sleep-deprived	t_{59} = 2.859	0.0059
	One-way ANOVA			
	Effect of recovery, AOX, TrpA1		$F_{3,142}$ = 12.62	<0.0001
	Holm-Šidák test	Rested vs. recovered	t_{142} = 4.902	<0.0001
	Holm-Šidák test	Rested vs. sleep-deprived + AOX	t_{142} = 0.6945	0.7384
	Holm-Šidák test	Rested vs. sleep-deprived + TrpA1	t_{142} = 0.5879	0.7384
	Mean branch length			
	Unpaired *t* test			
	Effect of sleep deprivation	Rested vs. sleep-deprived	t_{59} = 4.184	<0.0001
	Kruskal-Wallis ANOVA			
	Effect of recovery, AOX, TrpA1		H_4 = 67.30	<0.0001
	Dunn's test	Rested vs. recovered	Z = 6.663	<0.0001
	Dunn's test	Rested vs. sleep-deprived + AOX	Z = 1.847	0.1944
	Dunn's test	Rested vs. sleep-deprived + TrpA1	Z = 0.5999	>0.9999
3d	Mann-Whitney test	Rested vs. sleep-deprived	U = 11182	<0.0001
3e	Mann-Whitney test	Rested vs. sleep-deprived	U = 449.5	<0.0001
3f	Unpaired *t* test	Rested vs. sleep-deprived	t_{71} = 2.643	0.0101
4b, left	One-way ANOVA			
	Effect of GTPases		$F_{11,714}$ = 33.80	<0.0001

	Holm-Šidák test	*R23E10>Drp1* vs. *R23E10*	t_{714} = 3.170	0.0079
	Holm-Šidák test	*R23E10>Drp1* vs. *Drp1*	t_{714} = 4.332	0.0001
	Holm-Šidák test	*R23E10>Opa1*^{RNAi} vs. *R23E10*	t_{714} = 7.729	<0.0001
	Holm-Šidák test	*R23E10>Opa1*^{RNAi} vs.*Opa1*^{RNAi}	t_{714} = 6.219	<0.0001
	Holm-Šidák test	*R23E10>Marf* vs. *R23E10*	t_{714} = 0.1551	0.8768
	Holm-Šidák test	*R23E10>Marf* vs. *Marf*	t_{714} = 1.726	0.1622
	Holm-Šidák test	*R23E10>Opa1* vs. *R23E10*	t_{714} = 2.963	0.0115
	Holm-Šidák test	*R23E10>Opa1* vs. *Opa1*	t_{714} = 4.505	<0.0001
	Holm-Šidák test	*R23E10>Opa1,Marf* vs. *R23E10*	t_{714} = 6.398	<0.0001
	Holm-Šidák test	*R23E10>Opa1,Marf* vs. *Opa1*	t_{714} = 7.802	<0.0001
	Holm-Šidák test	*R23E10>Opa1,Marf* vs. *Marf*	t_{714} = 7.301	<0.0001
	Holm-Šidák test	*R23E10>Drp1*^{RNAi} vs. *R23E10*	t_{714} = 8.503	<0.0001
	Holm-Šidák test	*R23E10>Drp1*^{RNAi} vs. *Drp1*^{RNAi}	t_{714} = 2.990	0.0115
4b, right	One-way ANOVA			
	Effect of PA regulators		$F_{4,221}$ = 30.13	<0.0001
	Holm-Šidák test	*R23E10>zuc*^{RNAi} vs. R23E10	t_{221} = 3.714	0.0003
	Holm-Šidák test	*R23E10>zuc*^{RNAi} vs. *zuc*^{RNAi}	t_{221} = 5.097	<0.0001
	Holm-Šidák test	*R23E10>Miga*^{RNAi} vs. *R23E10*	t_{221} = 8.891	<0.0001
	Holm-Šidák test	*R23E10> Miga*^{RNAi} vs. *Miga*^{RNAi}	t_{221} = 6.320	<0.0001
4c, left	Two-way ANOVA			
	Time		$F_{1.371,345.6}$ = 450.1	<0.0001
	Genotype		$F_{2, 252}$ = 3.015	0.0508
	Time x genotype		$F_{142, 17892}$ = 7.224	<0.0001
4c, centre	Two-way ANOVA			
	Time		$F_{1.367, 307.5}$ = 357.9	<0.0001
	Genotype		$F_{3, 225}$ = 3.296	0.0213
	Time x genotype		$F_{213, 15975}$ = 6.160	<0.0001
4c, right	Kruskal-Wallis ANOVA			
	Genotype		H_6 = 37.30	<0.0001
	Dunn's test	*R23E10>Opa1*^{RNAi} *vs. R23E10*	Z = 3.545	0.0020
	Dunn's test	*R23E10>Opa1*^{RNAi} *vs. Opa1*^{RNAi}	Z = 3.272	0.0053
	Dunn's test	*R23E10>Opa1,Marf vs. R23E10*	Z = 2.900	0.0186
	Dunn's test	*R23E10>Opa1,Marf vs. Marf*	Z = 4.266	<0.0001
	Dunn's test	*R23E10>Opa1,Marf vs. Opa1*	Z = 3.484	0.0025
4e	Mixed-effects model			
	Time		$F_{1.856, 114.6}$ = 227.5	<0.0001
	Genotype		$F_{2, 69}$ = 15.56	<0.0001
	Time x genotype		$F_{42, 1297}$ = 5.822	<0.0001
4f	Kruskal-Wallis ANOVA			
	Genotype		H_3 = 1.465	0.4806
	Dunn's test	*R23E10>mCD8::GFP* vs. *R23E10>Drp1*	Z = 0.8508	>0.9999
	Dunn's test	*R23E10>mCD8::GFP* vs. *R23E10>Opa1;Marf*	Z = 1.159	0.7392
	Dunn's test	*R23E10>Drp1* vs. *R23E10>Opa1;Marf*	Z = 0.3438	>0.9999
4g	Contingency table			
	χ^2 test		χ^2 = 7.451	0.0241
	Standardized residuals	*R23E10>mCD8::GFP* non-bursting	+ 0.62	
		R23E10>mCD8::GFP bursting	- 1.16	
		R23E10>Drp1 non-bursting	+ 0.35	
		R23E10>Drp1 bursting	- 0.66	
		R23E10>Opa1;Marf non-bursting	- 1.07	
		R23E10>Opa1;Marf bursting	+ 2.01	

Supplementary Table 2. Statistical analyses of Extended Data Figures 4–7.

Figure (ED)	Statistical test	Pairwise comparison	Test statistic	*P*
4a	Unpaired *t* test	Rested vs. sleep-deprived	t_{43} = 0.4407	0.6616
4b	Mann-Whitney test			
	Number of mitochondria	Rested vs. sleep-deprived	U = 65	0.2534
	Mean volume	Rested vs. sleep-deprived	U = 83	0.7637
	Mean sphericity	Rested vs. sleep-deprived	U = 62	0.1953
	Mean branch length	Rested vs. sleep-deprived	U = 75	0.5014
5a	Kruskal-Wallis ANOVA			
	Genotype		H_5 = 78.08	<0.0001
	Dunn's test	*R23E10>Drp1-HA* vs. *R23E10*	Z = 6.175	<0.0001
	Dunn's test	*R23E10>Drp1-HA* vs. *Drp1-HA*	Z = 2.793	0.0209
	Dunn's test	*R23E10>Drp1*$^{\text{Feany lab}}$ vs. *R23E10*	Z = 7.861	<0.0001
	Dunn's test	*R23E10>Drp1*$^{\text{Feany lab}}$ vs. *Drp1*$^{\text{Feany lab}}$	Z = 4.875	<0.0001
5b	One-way ANOVA			
	Genotype		$F_{12,759}$ = 29.05	<0.0001
	Holm-Šidák test	*R23E10>Opa1*$^{\text{shRNA330266SH}}$ vs. *R23E10*	t_{759} = 2.583	0.0199
	Holm-Šidák test	*R23E10>Opa1*$^{\text{shRNA330266SH}}$ vs. *Opa1*$^{\text{shRNA330266SH}}$	t_{759} = 4.532	<0.0001
	Holm-Šidák test	*R23E10>Opa1*$^{\text{RNAi \#32358}}$ vs. *R23E10*	t_{759} = 3.592	0.0014
	Holm-Šidák test	*R23E10>Opa1*$^{\text{RNAi \#32358}}$ vs. *UAS-Opa1*$^{\text{RNAi \#32358}}$	t_{759} = 7.650	<0.0001
	Holm-Šidák test	*R23E10>Opa1*$^{\text{miRNA.CDS}}$ vs. *R23E10*	t_{759} = 8.277	<0.0001
	Holm-Šidák test	*R23E10>Opa1*$^{\text{miRNA.CDS}}$ vs. *UAS-Opa1*$^{\text{miRNA.CDS}}$	t_{759} = 8.663	<0.0001
	Holm-Šidák test	*R23E10>smOpa1 (X)* vs. *R23E10*	t_{759} = 3.679	0.0013
	Holm-Šidák test	*R23E10>smOpa1 (X)* vs. *smOpa1 (X)*	t_{759} = 2.478	0.0199
	Holm-Šidák test	*R23E10>smOpa1 (II)* vs. *R23E10*	t_{759} = 7.777	<0.0001
	Holm-Šidák test	*R23E10>smOpa1 (II)* vs. *UAS-smOpa1 (II)*	t_{759} = 2.947	0.0099
	Holm-Šidák test	*R23E10>smOpa1 (III)* vs. *R23E10*	t_{759} = 7.495	<0.0001
	Holm-Šidák test	*R23E10>smOpa1 (III)* vs. *UAS-smOpa1 (III)*	t_{759} = 9.094	<0.0001
5c	One-way ANOVA			
	Genotype		$F_{10,643}$ = 11.60	<0.0001
	Holm-Šidák test	*R23E10>Marf*$^{\text{RNAi 40478GD}}$ vs. *R23E10*	t_{643} = 1.901	0.2117
	Holm-Šidák test	*R23E10>Marf*$^{\text{RNAi 40478GD}}$ vs. *Marf*$^{\text{RNAi 40478GD}}$	t_{643} = 2.642	0.0496
	Holm-Šidák test	*R23E10>Marf*$^{\text{RNAi 105261KK}}$ vs. *R23E10*	t_{643} = 0.1334	0.8939
	Holm-Šidák test	*R23E10>Marf*$^{\text{RNAi 105261KK}}$ vs. *Marf*$^{\text{RNAi 105261KK}}$	t_{643} = 2.310	0.1017
	Holm-Šidák test	*R23E10>Marf*$^{\text{RNAi \#55189}}$ vs. *R23E10*	t_{643} = 1.276	0.4397
	Holm-Šidák test	*R23E10>Marf*$^{\text{RNAi \#55189}}$ vs. *Marf*$^{\text{RNAi \#55189}}$	t_{643} = 5.295	<0.0001
	Holm-Šidák test	*R23E10>smMarf (II)* vs. *R23E10*	t_{643} = 4.144	0.0003
	Holm-Šidák test	*R23E10>smMarf (II)* vs. *smMarf (II)*	t_{643} = 6.863	<0.0001
	Holm-Šidák test	*R23E10>smMarf (III)* vs. *R23E10*	t_{643} = 2.733	0.0443
	Holm-Šidák test	*R23E10>smMarf (III)* vs. *smMarf (III)*	t_{643} = 1.356	0.4397
5d	Kruskal-Wallis ANOVA			
	Genotype		H_3 = 7.932	0.0189
	Dunn's test	*R23E10>Marf-FLAG* vs. *R23E10*	Z = 1.862	0.1252
	Dunn's test	*R23E10>Marf-FLAG* vs. *Marf-FLAG*	Z = 0.8203	0.8241
5e	One-way ANOVA			
	Genotype		$F_{2,200}$= 18.77	<0.0001
	Holm-Šidák test	*R23E10>Miga*$^{\text{RNAi 110737KK}}$ vs. *R23E10*	t_{200}=6.117	<0.0001
	Holm-Šidák test	*R23E10>Miga*$^{\text{RNAi 110737KK}}$ vs. *Miga*$^{\text{RNAi 110737KK}}$	t_{200}=3.944	0.0001
6	Two-way ANOVA			
	Drp1		$F_{1,118}$ = 29.05	<0.0001
	Sleep history		$F_{1,118}$ = 22.82	<0.0001
	Drp1 x sleep history		$F_{1,118}$ = 2.576	0.1112
7a-b	Kruskal-Wallis ANOVA			
	Genotype		H_{12} = 193.4	<0.0001
	Dunn's test	*R23E10>Drp1* vs. *R23E10*	Z = 2.229	0.3354
	Dunn's test	*R23E10>Drp1* vs. *Drp1*	Z = 4.528	<0.0001
	Dunn's test	*R23E10>Opa1*^{RNAi} vs. *R23E10*	Z = 0.04653	>0.9999
	Dunn's test	*R23E10>Opa1*^{RNAi} vs. *Opa1*^{RNAi}	Z = 6.468	<0.0001
	Dunn's test	*R23E10>Marf* vs. *R23E10*	Z = 0.6387	>0.9999
	Dunn's test	*R23E10>Marf* vs. *Marf*	Z = 1.700	>0.9999
	Dunn's test	*R23E10>Opa1* vs. *R23E10*	Z = 6.977	<0.0001
	Dunn's test	*R23E10>Opa1* vs. *Opa1*	Z = 3.132	0.0226
	Dunn's test	*R23E10>Marf,Opa1* vs. *R23E10*	Z = 6.806	<0.0001
	Dunn's test	*R23E10>Marf,Opa1* vs. *Marf*	Z = 5.751	<0.0001
	Dunn's test	*R23E10>Marf,Opa1* vs. *Opa1*	Z = 2.983	0.0371
	Dunn's test	*R23E10>Drp1*^{RNAi} vs. *R23E10*	Z = 5.139	<0.0001
	Dunn's test	*R23E10>Drp1*^{RNAi} vs. *Drp1*^{RNAi}	Z = 3.213	0.0170
7c	Kruskal-Wallis ANOVA			
	Genotype		H_5 = 9.949	0.0413
	Dunn's test	*R23E10>Marf,tdTomato* vs. *R23E10>Marf*	Z = 0.08112	>0.9999
	Dunn's test	*R23E10>Marf,tdTomato* vs. *tdTomato*	Z = 1.796	0.2899

	Dunn's test	*R23E10>Marf,OMM-mCherry* vs. *R23E10>Marf*	Z = 2.202	0.1106
	Dunn's test	*R23E10>Marf,OMM-mCherry* vs. *OMM-mCherry*	Z = 1.898	0.2310
7e	One-way ANOVA			
	Genotype		$F_{8,510}$= 16.13	<0.0001
	Holm-Šidák test	*GH146>Drp1* vs. *GH146*	t_{510} = 2.385	0.0842
	Holm-Šidák test	*GH146>Drp1* vs. *Drp1*	t_{510} = 3.031	0.0153
	Holm-Šidák test	*GH146>Opa1*^{RNAi} vs. *GH146*	t_{510} = 1.393	0.2211
	Holm-Šidák test	*GH146>Opa1*^{RNAi} vs. *Opa1*^{RNAi}	t_{510} = 6.731	<0.0001
	Holm-Šidák test	*GH146>Opa1* vs. *GH146*	t_{510} = 5.143	<0.0001
	Holm-Šidák test	*GH146>Opa1* vs. *Opa1*	t_{510} = 1.755	0.2211
	Holm-Šidák test	*GH146>Drp1*^{RNAi} vs. *GH146*	t_{510} = 1.609	0.2211
	Holm-Šidák test	*GH146>Drp1*^{RNAi} vs. *Drp1*^{RNAi}	t_{510} = 2.162	0.1187
7f	One-way ANOVA			
	Genotype		$F_{8,564}$ = 19.13	<0.0001
	Holm-Šidák test	*OK107>Drp1* vs. *OK107*	t_{564} = 1.638	0.2757
	Holm-Šidák test	*OK107>Drp1* vs. *Drp1*	t_{564} = 2.632	0.0429
	Holm-Šidák test	*OK107>Opa1*^{RNAi} vs. *OK107*	t_{564} = 6.202	<0.0001
	Holm-Šidák test	*OK107>Opa1*^{RNAi} vs. *Opa1*^{RNAi}	t_{564} = 0.09364	0.9764
	Holm-Šidák test	*OK107>Opa1* vs. *OK107*	t_{564} = 3.307	0.0070
	Holm-Šidák test	*OK107>Opa1* vs. *Opa1*	t_{564} = 0.1937	0.9764
	Holm-Šidák test	*OK107>Drp1*^{RNAi} vs. *OK107*	t_{564} = 2.396	0.0660
	Holm-Šidák test	*OK107>Drp1*^{RNAi} vs. *Drp1*^{RNAi}	t_{564} = 2.797	0.0316