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Glia-mediated gut-brain cytokine signaling couples sleep to intestinal inflammation

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Abstract

Sickness-induced sleep is a behavior conserved across species that promotes recovery from illness, yet the underlying mechanisms are poorly understood. Here, we show that interleukin-6-like cytokine signaling from the *Drosophila* gut to brain glial cells regulates sleep. Under healthy conditions, this pathway promotes wakefulness. However, elevated gut cytokine signaling in response to oxidative stress – triggered by immune and inflammatory responses in the intestine – induces sleep. The cytokines Unpaired 2 and -3 are upregulated by oxidative stress in enteroendocrine cells and activate JAK-STAT signaling in glial cells, including those of the blood-brain barrier (BBB). This activity maintains elevated sleep during oxidative-stress-induced intestinal disturbances, suggesting that the JAK-STAT pathway in glia inhibits wake-promoting signaling to facilitate sleep-dependent restoration under these conditions. We find that the enteric peptide Allatostatin A (AstA) enhances wakefulness, and during intestinal oxidative stress, gut-derived Unpaired 2/3 inhibits AstA receptor expression in BBB glia, thereby sustaining an elevated sleep state during gut inflammation or illness. Taken together, our work identifies a gut-to-glial communication pathway that couples sleep with intestinal homeostasis and disease, enhancing sleep during intestinal sickness, and contributes to our understanding of how sleep disturbances arise from gastrointestinal disturbances.

eLife assessment

This **important** work by Malita et al. describes a mechanism by which an intestinal infection causes an increase in daytime sleep through signaling from the gut to the blood-brain barrier. Their findings suggest that cytokines upd3 and upd2 produced by the intestine following infection act on the glia of the blood-brain barrier to regulate sleep by modulating Allatostatin A signaling. The evidence supporting the claims of the authors is **solid**. Further verification of certain critical tools, and addressing a few discrepancies from data previously published, would improve this work.

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Introduction

Sleep is a conserved behavior essential for physical health and mental well-being. This process maintains physiological balance and promotes recovery from illnesses and other stressors[1,2]. In healthy states, animals exhibit rhythmic periods of wakefulness and activity, but their sleep patterns significantly increase during illness. This adaptive behavior is related to the fundamental role of sleep in the recovery process, allowing the body to conserve energy and allocate resources towards eliminating pathogens and repairing tissue damage. Sleep patterns are generated by neural circuits within the brain. These circuits engage in complex interactions involving diverse brain regions, neurotransmitters, and signaling pathways to regulate the cycles of sleep and wakefulness[3,4]. For sleep to be effectively modulated during illness, there must be a dynamic interaction between the physiological states of the body's organs and these central sleep-regulatory circuits. However, the signals that mediate this communication and the mechanisms by which they modulate sleep during health and disease remain poorly defined.

Sickness-induced sleep, a behavior conserved across species including mammals and flies[5,6], is influenced by cytokines, which are key mediators of immune and inflammatory responses[1,2]. Cytokines such as Interleukin 1 (IL-1) and tumor necrosis factor alpha (TNFα) are expressed in the healthy mammalian brain in regions that are implicated in sleep regulation, and their circulating levels change during the normal sleep-wake cycle, peaking during the sleep phase. Furthermore, these factors' effects on sleep appear to be dose-dependent, as low levels of IL-1 can enhance sleep, whereas higher doses can inhibit sleep, indicating a dual functionality. Since immune responses alter the expression of these cytokines, they have been hypothesized to act as "somnogens" that promote sleep during times of infection or illness. However, the connection between sleep and immune function is bidirectional, since sleep deprivation in mammals has been linked to increased inflammatory response via IL-1 and TNFα[2]. In the fruit fly *Drosophila*, sleep deprivation also seems to influence TNFα in astrocyte-like cells to regulate homeostatic sleep responses that enable sleep rebound after deprivation[7]. However, the effect of these cytokines on sleep has mostly been linked to their central expression and function within the central nervous system (CNS), while the coupling of cytokines produced by peripheral tissues to sleep-regulatory systems within the brain remains poorly understood.

Disorders affecting the gastrointestinal tract can lead to sleep disturbances[8], which are also associated with virtually all mental illnesses[9–11]. Conditions including depression, anxiety, and disturbed sleep are frequently observed in individuals with gut inflammation, and the gut microbiome has also been linked to sleep quality and mental health[8,12–14]. These associations suggest a strong connection between gut health and sleep. For changes in gut status to bring about behavioral changes, the gut must sense its state of health, damage, or presence of pathogens and release signals that lead to altered cellular function within the brain. This gut-to-brain signaling is mediated in large part by hormonal factors released from specialized endocrine cells of the gut, the enteroendocrine cells (EECs)[15,16]. Like the mammalian intestine, the *Drosophila* gut produces numerous diverse hormones from specialized EECs[17–22]. Some of these gut hormones are released in response to nutritional intake, and they diet-dependently modulate sleep patterns and arousability through communication with neuroendocrine centers and brain circuits[23–25].

In the fly, enteric infection or damage leads to the production of reactive oxygen species (ROS) and the increased expression of the IL-6-like inflammatory cytokines Unpaired 2 and -3 (Upd2/3) in the absorptive enterocytes, a response required for local gut regeneration[26,27]. These cytokine factors signal through their receptor Domeless (Dome) to activate the JAK/STAT signaling pathway, which is important for both immune function and metabolism in flies, demonstrating a conserved function of cytokine action in this species. While the three related cytokines, Upd1, Upd2, and

Upd3, all signal through Dome, Upd2 and in particular Upd3 are IL-6-like cytokines mainly triggered by infection and are directly linked with cellular immune responses[28–30]. As in mammals, cytokines are also produced centrally within the *Drosophila* brain, and neuronal Upd1 acts in a leptin-like manner to regulate feeding[31], a behavior that is also linked with sleep[4]. Peripheral cytokine signaling has also been shown to modulate sleep in this species, where the fat tissue releases Upd2 to reflect adequate nutrition, and this signal modulates sleep[32]. Furthermore, Unpaired cytokines have been implicated in the modulation of feeding behavior through effects on glial cells[33]. Glial cells, including those making up the blood-brain barrier (BBB), have recently gained attention for their role in sleep regulation in both flies and mammals[34–37]. Neurons in the CNS are separated from the circulatory system by the BBB, a selectively semi-permeable cell layer[38], which presents a challenge for peripheral hormones to enter and signal to neurons within the brain. However, glial cells within the BBB are ideally positioned to receive and integrate systemic signals from peripheral organs and modulate neuronal function, thereby relaying peripheral information into the brain.

Here, we demonstrate Upd2 and Upd3 cytokine signaling from endocrine EECs in the intestine in *Drosophila*. Our findings show that Upd2/3 signaling from the EECs to BBB glial cells, plays a dual role in sleep regulation. Under normal, healthy conditions, EEC-derived Unpaired signaling sustains wakefulness, whereas in response to oxidative stress that leads to gut inflammation, elevated Unpaired signaling instead promotes sleep. Stress-induced EEC-derived Upd2/3 activates the JAK-STAT pathway in glial cells at the blood-brain interface and adjusts sleep through this activation based on intestinal homeostasis and levels of inflammatory signaling from the gut. Our results suggest that gut-derived Unpaired signaling influences sleep regulation through glial gating of wake-promoting AstA-mediated signals, thus linking intestinal health with CNS-dependent behaviors. These results identify a gut-brain connection by which gut disease impacts sleep regulation.

Results

Gut-derived Unpaired cytokine signaling regulates sleep

To investigate whether cytokine signaling from the gut regulates sleep, we silenced the expression of *upd2* and *upd3* in the EECs, which are a principal endocrine cell type in the gut that releases signals with systemic effects and constitutes the functional basis of gut-brain signaling. Using *voilà-GAL4* (a driver that targets all EECs) to drive RNAi in EECs in conjunction with *Tub-GAL80^{ts}* (hereafter referred to as *voilà>*) for temperature-induced RNAi induction exclusively in the adult stage to prevent developmental effects, we observed significant knockdown of the main IL-6 cytokine, *upd3*[30], in dissected adult female midguts (**Fig. 1a**), demonstrating that *upd3* is expressed in EECs under normal homeostatic conditions. To eliminate potential neuron-derived phenotypes, we employed *R57C10-GAL80*, a form of *nSyb-GAL80* that effectively inhibits neuronal GAL4 activity, in combination with *Tub-GAL80^{ts}* and *voilà-GAL4*[25,39]. To evaluate the effectiveness of this temperature-sensitive EEC-specific driver (referred to as *EEC>* hereafter) upon adult-restricted induction, we examined midgut *upd2* and *upd3* transcript levels and observed significant knockdown of both cytokines, which was reproduced with a second independent RNAi line targeting *upd3* (**Fig. 1b**). Additionally, *GFP* expressed under the control of an *upd3-GAL4* driver containing *upd3* enhancer sequences, was apparent in midgut EECs, marked by staining against the EEC fate determinant Prospero (**Fig. 1c**). These results show that EECs of the adult female midgut are a source of Upd2 and Upd3 under normal conditions.

We then explored whether these EEC-derived cytokines govern sleep under such normal conditions, given the known role of cytokines in both healthy and inflammatory states. We found that the knockdown of the two main immune-related cytokines, *upd2* or *upd3* using *voilà>* increased the amount of time animals spent asleep (defined as a period of inactivity lasting at least

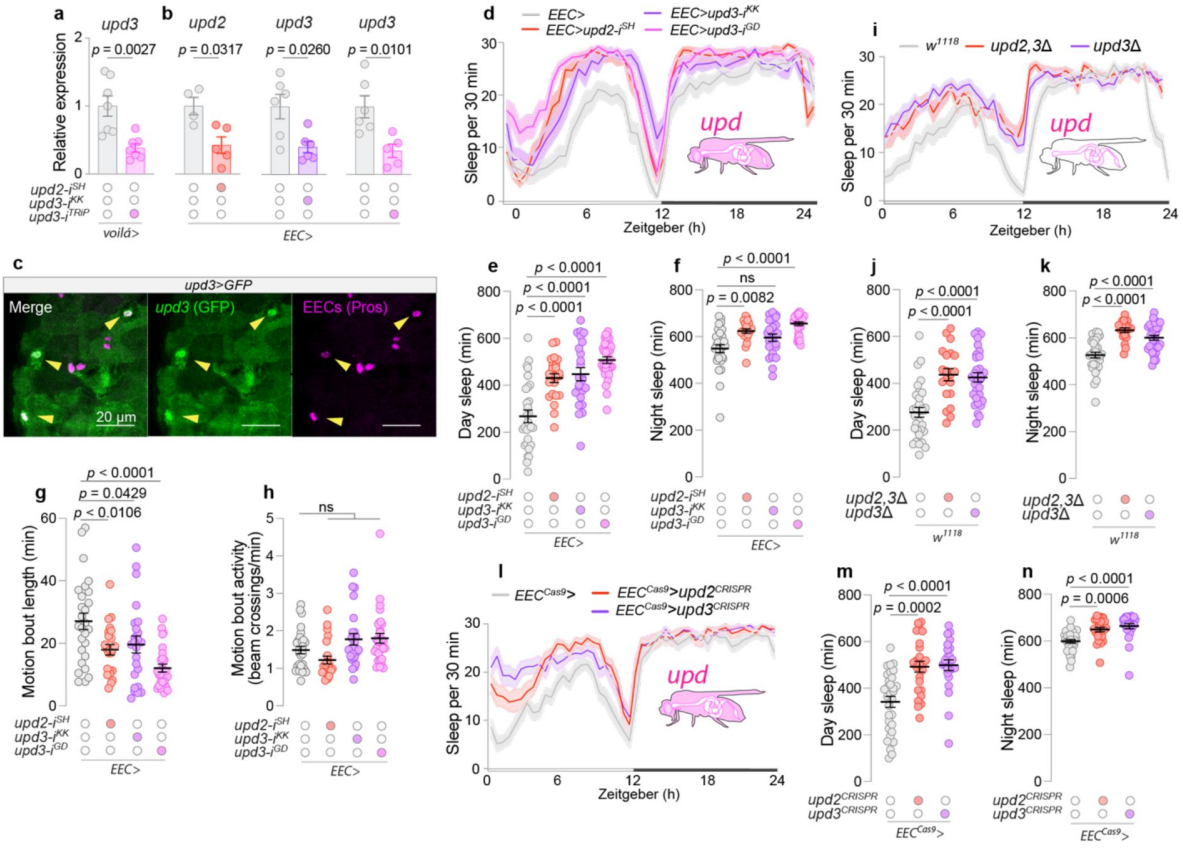

Figure 1.

EEC-derived Unpaired signaling regulates sleep.

a, *upd3* expression levels in midguts RNAi-mediated knockdown of *upd3* in EECs using *voilá-GAL4* in combination with *Tubulin-GAL80ts* (*voilá>*) (N=7). **b**, *upd2* and *upd3* expression levels in midguts in animals with EEC knockdown of *upd2* or *upd3* using *voilá-GAL4* in combination with *Tubulin-GAL80ts* and *R57C10-GAL80* (*EEC>*) (N=4-6). **c**, Confocal imaging of *upd3-GAL4* driven *UAS-GFP* expression in EECs, co-stained with Prospero (Pros) as an EEC marker (Scale bar, 20 µm). **d**, Twenty-four-hour sleep profiles in animals with EEC-specific *upd2* and *upd3* knockdown (N=29-34). **e**, Total daytime sleep and **f**, nighttime sleep durations in animals with EEC-specific *upd2* and *upd3* knockdown flies (N=29-34). **g**, Motion bout length for control and EEC-specific *upd2* and *upd3* knockdown flies (N=23-27). **h**, Motion bout activity across experimental groups (N=23-30). **i**, Twenty-four-hour sleep profiles for global *upd2/3* mutants (N=19-32). **j**, Daytime and **k**, nighttime sleep durations in global *upd2/3* mutants (N=19-32). **l**, Sleep profiles following EEC-specific CRISPR-mediated *upd2* and *upd3* knockout (N=24-31). **m**, Daytime and **n**, night sleep durations in animals with EEC-specific CRISPR-mediated *upd2* and *upd3* activation (N=25-31). Statistical analyses performed using Mann-Whitney tests for panels a and b; Ordinary one-way ANOVA with Dunnett's multiple comparisons test for panels e, g, j, and k; Kruskal-Wallis One-Way ANOVA with Dunn's multiple comparison test for panels f, h, m, and n. Data are presented as mean ± SEM. ns, non-significant (p>0.05).

five minutes), especially during the day (**Fig. S1a-c**). This phenotype was reproducible using the more restricted *EEC*> driver that includes *R57C10-GAL80*, suggesting that the EEC-specific loss of these cytokines promotes sleep with pronounced effects during daytimes when females are typically active (**Fig. 1d-f**). This outcome was not attributable to off-target effects, as two independent RNAi lines targeting the main IL-6-like cytokine, *upd3*, produced a similar phenotype or effects of the RNAi transgenes themselves (**Fig. 1d-f** and **S1d-f**). Animals lacking EEC-derived Unpaired signaling also exhibited shorter motion bouts (periods of activity; **Fig. 1g** and **S1g**). However, the EEC-specific knockdown of *upd2* or *upd3* did not reduce motion-bout activity (the intensity of activity during wake periods), implying that the lack of gut cytokine signaling did not reduce general activity when the animals were awake (**Fig. 1h** and **S1h**), and these effects do not arise from RNAi transgene insertions themselves (**Fig. S1i,j**). These observations suggest a direct influence of gut-derived Upd2 and Upd3 on sleep rather than a broader impact on general activity levels. Given that sleep and feeding are mutually exclusive behaviors, we measured feeding. We did not detect significant alterations in feeding behavior as a consequence of *upd2* or *upd3* knockdown in EECs over a 24-hour period using the automated FLIC system (**Fig. S1k**), nor did we observe an effect of EEC-specific *upd3* overexpression on food consumption via a dye assay (**Fig. S1l**). Furthermore, EEC-specific *upd3* knockdown did not affect the animals' metabolic state as reflected in their levels of stored triacylglyceride (TAG) (**Fig. S1m**). Therefore, the sleep phenotype exhibited by animals with EEC-specific *upd2/3* knockdown is not associated with changes in metabolism, appetite or feeding behavior.

To further explore the role of Upd2 and Upd3 in sleep regulation, we made use of deletion mutations that disrupt either both *upd2* and *upd3* (*upd2,311*) or *upd3* alone (*upd311*). Both mutants exhibited a pronounced increase in sleep (**Fig. 1i-k**), with strong effects on daytime sleep phenocopying the RNAi-mediated knockdown in the EECs. We additionally disrupted the *upd2* or *upd3* genes specifically in the EECs using somatic tissue-specific CRISPR-mediated deletion. EEC-specific CRISPR-mediated knockout of *upd2* or *upd3*, induced by UAS-controlled gRNA pairs designed to excise portions of each gene's coding sequence, led to significantly elevated sleep (**Fig. 1l-n**), further reinforcing these cytokines' role in sleep modulation. Collectively, these findings demonstrate that Upd2 and Upd3 expressed by EECs are important modulators of diurnal sleep patterns in *Drosophila* under normal homeostatic conditions.

Glial cytokine JAK-STAT signaling regulates sleep

To identify the CNS targets of EEC-derived Upd2/3 cytokine signaling by which they regulate sleep, we examined the effects of targeted knockdown of the Upd2/3 JAK-STAT-linked receptor *dome* in neurons or glia, the two main cell types in the CNS. Pan-neuronal *dome* knockdown using the driver *R57C10-GAL4*[25] did not significantly alter daytime sleep in adult females (**Fig. 2a**), contrary to the sleep increase observed upon loss of *upd2* or *upd3* in EECs (**Fig. 1**). This suggests that neurons are not the targets by which gut Unpaired cytokine signaling regulates sleep. In contrast, knockdown of *dome* in glial cells using the pan-glial driver *repo-GAL4* (*repo>*) resulted in a pronounced increase in daytime sleep (**Fig. 2a**), similar to the phenotype observed upon EEC-specific loss of Unpaired cytokines. To substantiate this observation further, we silenced *dome* expression in glia using three independent RNAi lines, which all increased sleep with strong induction of sleep during the day, effectively ruling out any off-target or transgene-background effects (**Fig. 2b-d** and **S2a-e**). Additionally, animals with glial *dome* knockdown displayed shorter daytime motion-bout lengths, suggesting reduced periods of wakefulness (**Fig. 2e** and **S2f**), without exhibiting decreased activity during these bouts (**Fig. 2f** and **S2g**), phenocopying the effects of *upd2/3* knockdown in EECs (**Fig. 1g,h**). We also assessed whether glial-specific *dome* knockdown might affect feeding and energy storage, but we observed no reduction in food intake (**Fig. S2h**), and no changes in TAG levels were detected (**Fig. S2i**). These findings collectively argue that augmented sleep resulting from impaired JAK-STAT signaling in glia is not due to a general decline in activity but rather represents a specific regulation of sleep itself.

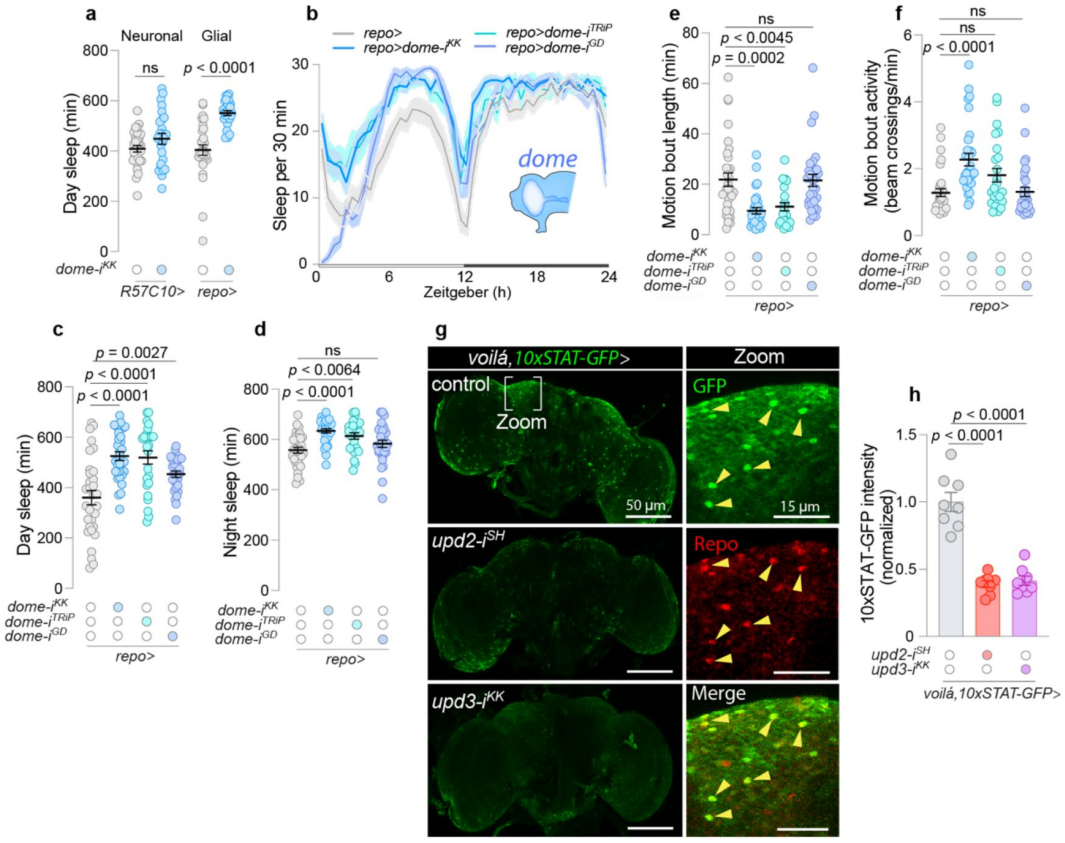

Figure 2.

EEC-derived Unpaired signaling regulates glial JAK-STAT activity that modulates sleep.

a, Day sleep measurements for flies with knockdown of IL-6 related Unpaired cytokine receptor *domeless* (*dome*), which activates JAK-STAT, in neurons (*R57C10-GAL4, R57C10*>) and glial cells (*repo-GAL4, repo*>) (N=25-39). **b**, Twenty-four-hour sleep profiles for controls and animals with glial-specific *dome* knockdown (N=25-32). **c**, Total day sleep duration and **d**, total night sleep duration for animals with glial-specific *dome* knockdown and control flies (N=25-32). **e**, Motion bout length and **f**, motion bout activity in animals with glial-specific *dome* knockdown and controls (N=25-32). **g**, Images of brain from controls and animals with EEC knockdown *upd2* or *upd3* using *voilá>10xSTAT-GFP*. STAT-GFP expression (green) with Repo labeling (red) indicates glial cells (Scale bar, 50 µm). Insets show zoomed views of STAT-GFP^{+} and Repo^{+} glial cells (Scale bar, 15 µm). **h**, Quantitative analysis of GFP intensity in the layer of glial cells located at the surface of the brain in animals with EEC knockdown of *upd2* or *upd3* and controls (N=8). Statistical analyses were conducted using parametric t-tests for panel a; Kruskal-Wallis One-Way ANOVA with Dunn's multiple comparison test for panels c, d, e and f; and Ordinary one-way ANOVA with Dunnett's multiple comparisons test for panel h. Data are presented as mean ± SEM. ns, non-significant (p>0.05).

To directly assess the functional Unpaired-mediated communication between the gut and glial cells, we manipulated *upd2* and *upd3* in EECs of animals carrying a ubiquitous transgenic JAK-STAT reporter (*10xSTAT-GFP*)[40]. Knockdown of either *upd2* or *upd3* in the EECs led to a marked decrease in JAK-STAT reporter activity within Repo-positive glial cells under normal conditions (**Fig. 2g,h**), suggesting that Upd2/3-mediated signaling from the EECs to the brain's glial cells activates JAK-STAT signaling. Taken together this suggests that gut-to-glia communication via Upd2 and Upd3 modulates diurnal sleep patterns through glial JAK-STAT activation and that these cytokines are required for the maintenance of wakefulness during the day under healthy conditions.

Oxidative stress modulates sleep through gut-derived cytokine signaling

Having established the significance of gut-derived Unpaired cytokines in maintaining wakefulness under normal conditions, we next explored their role in sleep-regulation during gut disturbances that trigger immune and inflammatory responses. Infection or inflammation in the gut leads to increased levels of ROS, causing local intestinal oxidative stress[26,27]. To probe the effects of enteric ROS on sleep and cytokine signaling, we induced oxidative stress in the gut by feeding animals food containing 1% hydrogen peroxide (H_2O_2) for 20 hours. We asked whether intestinal oxidative stress might elevate the levels of Upd2 and Upd3 in the gut and found substantial upregulation of *upd3* expression in ROS-challenged dissected midguts of adult females (**Fig. 3a**). This effect mirrored the upregulation observed with EEC-specific overexpression of *upd3*, indicating that it reflects physiologically relevant production of Upd3 by the gut in response to oxidative stress. Although oxidative stress also promoted *upd2* expression, albeit to a lesser extent, this effect was not modulated by simultaneous EEC-specific *upd3* overexpression.

We next investigated whether sleep is modulated by intestinal oxidative stress and if Unpaired signaling from EEC is required for this response. We induced intestinal oxidative stress by exposing animals to a diet supplemented with H_2O_2 at Zeitgeber Time 0 (ZT0), the onset of the light phase in a 12-hour light/dark cycle. Exposure to a lower H_2O_2 concentration (0.1%) incrementally increased daytime sleep amount over successive days (**Fig. 3b**). In contrast, a higher H_2O_2 concentration (1%) triggered an immediate augmentation of daytime sleep (**Fig. 3c**). These observations suggest dose-dependent modulation of sleep by intestinal oxidative stress. Since 1% H_2O_2 feeding induced robust responses both in *upd3* expression and in sleep behavior, we asked whether gut-derived Unpaired signaling might be essential for the observed ROS-induced sleep modulation. Indeed, EEC-specific RNAi targeting *upd2* or *upd3* abolished the sleep response to 1% H_2O_2 feeding. These animals with EEC-specific knockdown of *upd2* or *upd3* did not exhibit increased daytime sleep in response to the induction of oxidative stress in the intestine, even over two consecutive days exposed to 1% H_2O_2-containing diet (**Fig. 3d,e**). The specificity of this response was corroborated by three independent RNAi lines targeting *upd3*, negating the possibility of RNAi off-target effects (**Fig. 3d,e**), and the loss of response to ROS was also not attributable to the transgenes themselves (**Fig. S3a-d**). Intriguingly, animals lacking *upd3* in the EECs not only did not increase their sleep under oxidative stress but indeed appear to lose nighttime sleep in response to enteric stress.

We next tested whether this sleep phenotype might be associated with general physiological processes rendering animals lacking EEC Unpaired signaling more susceptible to ROS-induced damage. However, when we assessed survival on 1% H_2O_2-containing food, animals with *upd2* or *upd3* knockdown in EECs displayed no additional sensitivity to oxidative stress, compared to controls (**Fig. 3f**). This lack of effect indicates that the loss of EEC-derived Unpaired signaling specifically leads to an impaired behavioral sleep response to intestinal oxidative stress, rather than to compromised physiological processes that would make the animals more vulnerable to oxidative stress insults. We also examined whether animals lacking EEC-derived Unpaired signaling exhibit normal behavioral responses to other conditions that modulate sleep, which

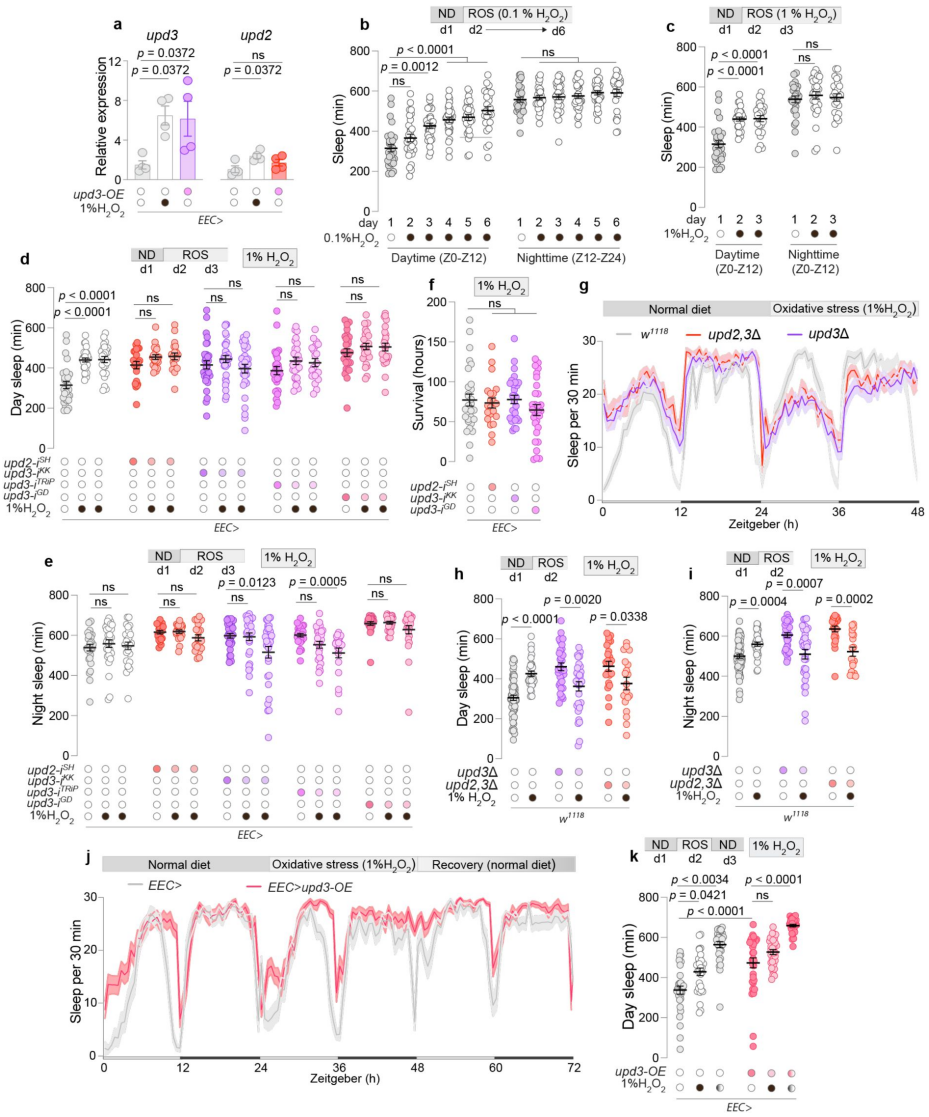

Figure 3.

EEC-derived Unpaired signaling modulates sleep in response to intestinal oxidative stress.

a, Measurement of *upd2* and *upd3* expression in the midguts upon 20-hour treatment with 1% H_2O_2-laced food or overexpression of *upd3* (*upd3-OE*) using *EEC*> (N=4). **b** and **c** Assessment of sleep duration over consecutive days during daytime (ZT0-ZT12) and nighttime (ZT12-ZT24) in animals exposed to food containing (b) 0.1% H_2O_2 (N=26-31) or (c) 1% H_2O_2 (N=23-29). **d**, Daily sleep and **e**, nightly sleep amounts measured over one day under standard food conditions followed by two consecutive days on 1% H_2O_2-containing food in animals with EEC-specific *upd2* or *upd3* knockdown and controls (N=23-30). Experiments measuring sleep levels in controls and animals lacking EEC-derived *upd2* or *upd3* were performed concurrently and share the control but are presented in separate figures (b, c, d, and e) for clarity. **f**, Survival rates under a 1% H_2O_2-induced oxidative stress diet in controls and animals with EEC-specific *upd2* or *upd3* knockdown (N=23-30). **g**, A 48-hour sleep profile comparison between global *upd2/3* mutants and w^{1118} controls under one day of standard food conditions followed by one day of 1% H_2O_2-induced stress (N=18-63). **h** and **i**, Quantification of daytime and nighttime sleep durations in *upd2/3* mutants versus w^{1118} controls under normal food conditions and the following day exposed to food containing 1% H_2O_2 (N=18-63). **j**, Observation of sleep patterns and **k**, measurements of daytime sleep across a three-day period, encompassing a day on standard diet, subsequent day on 1% H_2O_2-laced food to induce oxidative stress, and a final day back on standard diet to monitor recovery, in flies with EEC-specific overexpression of *upd3* (*upd3-OE*) compared to control (N=29-32). Statistical analyses were performed using Kruskal-Wallis One-Way ANOVA with Dunn's multiple comparison test for panels a, b, c, d, e, f, and k; Mann-Whitney test for panels h and i. Data are presented as mean ± SEM. ns, non-significant (p>0.05).

would suggest a specific requirement for this signaling in responding to intestinal oxidative stress. Animals typically suppress their sleep in response to nutritional deprivation, a behavior conserved across species that is believed to facilitate food-seeking activities and that is also influenced by EEC-mediated hormone signaling[25,41]. Animals lacking EEC-derived *upd3* suppressed their sleep similar to controls in response to starvation, indicating a normal sleep response to nutritional stress (**Fig. S3e-g**).

Although we observed behavioral phenotypes with manipulations of either *upd2* or *upd3*, suggesting that both are required for normal function, Upd2 and Upd3 likely function at least partially redundantly or additively in their regulation of sleep, as is the case for other processes[42]. Moreover, RNAi effects do not result in a complete loss of function. Therefore, we speculated that a stronger disruption and combined knockout of both *upd2* and *upd3* might lead to even more pronounced phenotypes. We therefore tested the *upd3Δ* deletions and the double *upd2,3Δ* double deletion mutants. Whereas *upd3Δ* and *upd2,3Δ* mutants exhibited increased baseline sleep under homeostatic conditions, these animals not only failed to increase their sleep in response to oxidative stress but indeed showed a strong reduction in daytime and nighttime sleep under oxidative-stress conditions (**Fig. 3g-i**). These results suggest that contrary to its role in promoting wakefulness during normal homeostatic conditions, the enhanced ROS-induced Unpaired signaling from EECs helps sustain a higher sleep level during periods of oxidative stress. This indicates a dual functionality of Unpaired cytokine signaling, in which low Unpaired signaling promotes wakefulness under normal conditions, whereas higher ROS-induced Unpaired signaling facilitates a shift to restorative sleep during intestinal stress.

We therefore investigated whether higher levels of Unpaired signaling from the gut, comparable to the level produced during oxidative stress, could enhance sleep in the absence of exogenous stressors. We analyzed the effect of *upd3* overexpression in EECs, which drives expression of midgut *upd3* to levels similar to those induced by 1% H_2O_2 feeding (**Fig. 3a**). Consistent with a model in which high levels of Upd3, which would occur during high levels of intestinal oxidative stress, promotes daytime sleep, animals overexpressing *upd3* in the EECs exhibited increased sleep during the day, even in the absence of H_2O_2-induced oxidative stress (**Fig. 3j,k**). These animals further increased their sleep in response to H_2O_2-induced enteric oxidative stress, unlike those lacking gut-derived *upd3* (**Fig. 3d,e**), suggesting they are able to mount an additional ROS-induced Unpaired signaling response on top of the overexpression-induced levels. After animals were switched back to normal food after one day of oxidative stress, both control animals and those with EEC-specific *upd3* overexpression exhibited even more sleep than during the previous day under oxidative-stress conditions (**Fig. 3j,k**). This suggests a robust recovery sleep response following the insult, likely mediated by Unpaired signaling, since the effect is more pronounced with upd3 overexpression. Taken together our results show that control animals increase their sleep during oxidative stress, likely as an adaptive recovery response. In contrast, animals with EEC-specific knockdown of *unpaired* cytokines do not exhibit this ROS-induced sleep response; instead, they experience sleep loss during such stress conditions. This suggests that while keeping the animal awake during normal healthy conditions, ROS-induced gut Unpaired signaling temporarily suppress these wake-promoting effects, leading to more sleep.

EEC-derived Unpaired cytokine signaling activates glial JAK-STAT under oxidative stress

To investigate whether oxidative stress enhances glial JAK-STAT signaling and if this enhancement is mediated by gut-derived Upd2 and Upd3, we assessed glial JAK-STAT reporter activity using the dual-color TransTimer system, which provides temporal information about JAK-STAT signaling[43]. In this system, active STAT promotes the expression of a construct encoding a short-lived destabilized GFP (dGFP, half-life ~2 hours) and a long-lived RFP (half-life ~20 hours) separated by a 2A peptide (*6xSTAT-dGFP:2A::RFP*); a higher ratio of GFP to RFP in a given cell reflects more recent JAK-STAT signaling. We explored whether JAK-STAT signaling responds

dynamically to intestinal oxidative stress and assessed two daily time points. We observed no circadian changes between ZT0 (lights on) and ZT12 (lights off) in the superficial layer of cells surrounding the brain (**Fig. 4a,b**), which is composed of glia[44]. However, we observed a significant increase in GFP signal at ZT0 in animals fed for 20 hours with 1% H_2O_2-containing food, indicating recent JAK-STAT activity in the surface glia. Next, we investigated whether gut-derived Unpaired signaling is responsible for this upregulation by combining the *10xSTAT-GFP* reporter with knockdown of *upd2* or *upd3* in the EECs. Whereas glial JAK-STAT reporter activity was upregulated by oxidative stress (20 hours 1%-H_2O_2 feeding) in control animals, this response was abolished in animals with EEC-specific knockdown of *upd2* or *upd3*, indicating that this response is dependent on these EEC-derived cytokines (**Fig. 4c,d**). Since in this case we used *voilá>* without pan-neuronal *R57C10-GAL80* to drive knockdown in EECs, we measured the expression of *upd2* and *upd3* in heads to check for any unintended neuronal effects that might contribute to the observed effect on glial JAK-STAT activity. We detected no changes in the expression of these genes in the heads, confirming that the observed JAK-STAT activation in glial cells is attributable to cytokines derived from EECs (**Fig. S4a,b**). To test the ability of gut-derived Upd3 to drive events in the brain in another way, we made use of cells' homeostatic response to changes in signaling input. Receptor expression is often upregulated in response to low levels of a ligand as a compensatory mechanism to enhance cellular sensitivity[45]. We observed an upregulation of *dome* transcript levels in the heads of animals with EEC-specific knockdown of *upd3*, exposed to oxidative stress induced by 15 hours of feeding with food laced with 1% H_2O_2 (**Fig. 4e**). Increased *dome* expression suggests reduced Unpaired ligand availability as a result of the loss of EEC-derived Upd3. Together, our results demonstrate that EEC-derived Unpaired cytokine signaling is required for activating glial JAK-STAT under oxidative stress.

Glia JAK-STAT modulates sleep in response to oxidative stress

Since EEC-derived Unpaired signaling promotes oxidative stress-induced sleep and glial JAK/STAT activity, we investigated whether the observed glial JAK-STAT signaling is involved in the modulation of sleep in response to intestinal oxidative stress. Knockdown of *dome* in all glial cells using the *repo>* driver completely abolished the ROS-induced daytime sleep response when animals were fed 1% H_2O_2 supplemented diet. The specificity of this effect was confirmed using three distinct RNAi lines, and with transgenic RNAi controls (**Fig. 5a** and **S5a-c**). Similar to the effects observed with *upd2,3* mutants (**Fig. 3g-i**), glia-specific *dome* knockdown ($p < 0.00001$ for *dome-i*^{KK} and $p = 0.0556$ for *dome-i*^{TRiP}) resulted in progressive and substantial sleep loss over two consecutive days on 1% H_2O_2-containing food. To rule out developmental effects, we restricted knockdown of *dome* in glial cells to the adult stage using the *repo>* driver in combination with *Tub-GAL80*^{ts} (*repo*^{TS}*>*) and observed similar effects (**Fig. 5b**).

We then examined the dynamics of glial sleep-regulatory JAK-STAT signaling by inducing oxidative stress for one day and then transferring the animals to normal food to observe the recovery response. The results showed that, in response to 1% H_2O_2-containing food, animals lacking glial *dome* expression displayed a sleep response opposite from that of controls, with a reduction in sleep duration rather than an increase, confirmed using independent RNAi lines (**Fig. 5c**). This phenotype is similar to that seen in the *upd2,3Δ* double mutants (**Fig. 3h-j**). During the recovery phase, after the animals had been switched back to normal food, the sleep level of controls increased even further, but the sleep duration exhibited by animals with glia-specific *dome* knockdown immediately reverted to pre-stress levels. This pattern indicates that animals with inhibited glial JAK-STAT signaling display an aberrant dynamic sleep response to oxidative stress that is not a consequence of a physiological breakdown but rather arises from altered inhibitory sleep-regulating mechanisms. In line with this and similarly to the loss of *upd2* or *upd3* in the EECs, *dome* knockdown in glial cells did not decrease survival on H_2O_2-containing food (**Fig. 5d**). This supports the notion that physiological resistance to oxidative stress remains unaltered by gut-glial Unpaired signaling, which in turn indicates that the signaling modulation leads to a specific sleep phenotype. We further assessed whether loss of glial *dome* affected sleep responses induced by

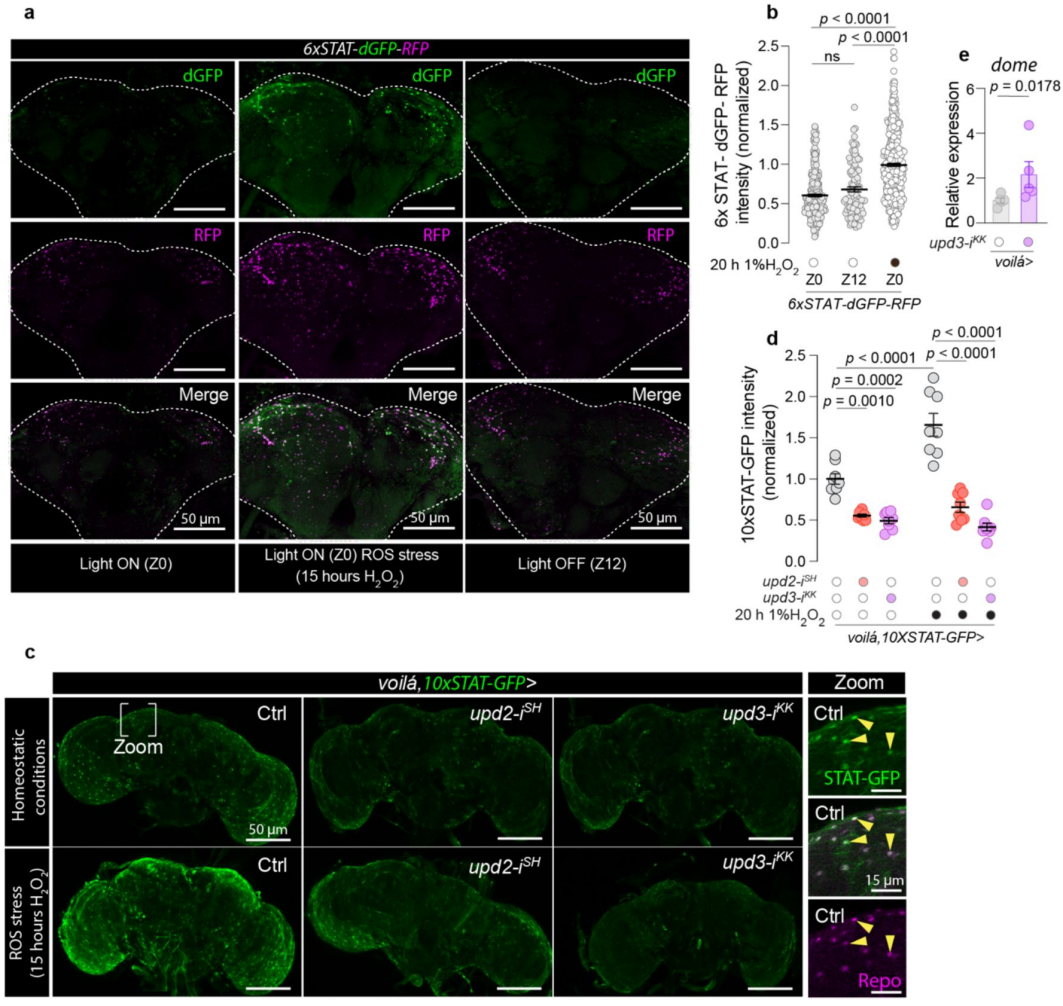

Figure 4.

Activation of glial JAK-STAT by EEC-derived Unpaired cytokines in response to oxidative stress.

a, Representative images of brains from flies expressing *STAT::dGFP:2A::RFP* reporter, with green representing destabilized GFP (dGFP) and purple indicating stable RFP. Left panels show brains under normal light conditions (ZT0), middle panels show brains with lights off (ZT12), and right panels depict brains after 20 hours of oxidative stress induced by 1% H_2O_2-containing food, imaged at light-on time ZT0. White dotted lines outline the brain perimeter. Scale bar, 50 µm. **b**, Ratio of dGFP to RFP fluorescence intensity under normal light conditions at ZT0, at ZT12, and after oxidative stress (ZT0), as depicted in panel a, to show dynamic changes in JAK-STAT activity (N=108-461, indicating the number of cells counted). **c**, Representative images of brains displaying *10xSTAT::GFP* expression under homeostatic conditions and after oxidative stress in control flies (Ctrl) and flies with EEC-specific *upd2* or upd3 knockdown. Scale bar, 50 µm. Inset 'Zoom' panels provide magnified views of glial cells labeled by Repo. Scale bar, 15 µm. **d**, Quantification of 10xSTAT::GFP intensity in glial cells under homeostatic and oxidative stress conditions, demonstrating the impact of EEC-specific cytokine knockdown (N=8, indicating the number of brains). **e**, qPCR analysis of *dome* expression in the brains of flies with EEC-specific *upd3* knockdown in comparison to *voilà>* controls (N=5). Statistical analyses were conducted using Kruskal-Wallis One-Way ANOVA with Dunn's multiple comparison test for panel b; ordinary one-way ANOVA with Tukey's multiple comparison test for panel d; and t-tests for panel e. Data are presented as mean ± SEM. ns, non-significant (p>0.05).

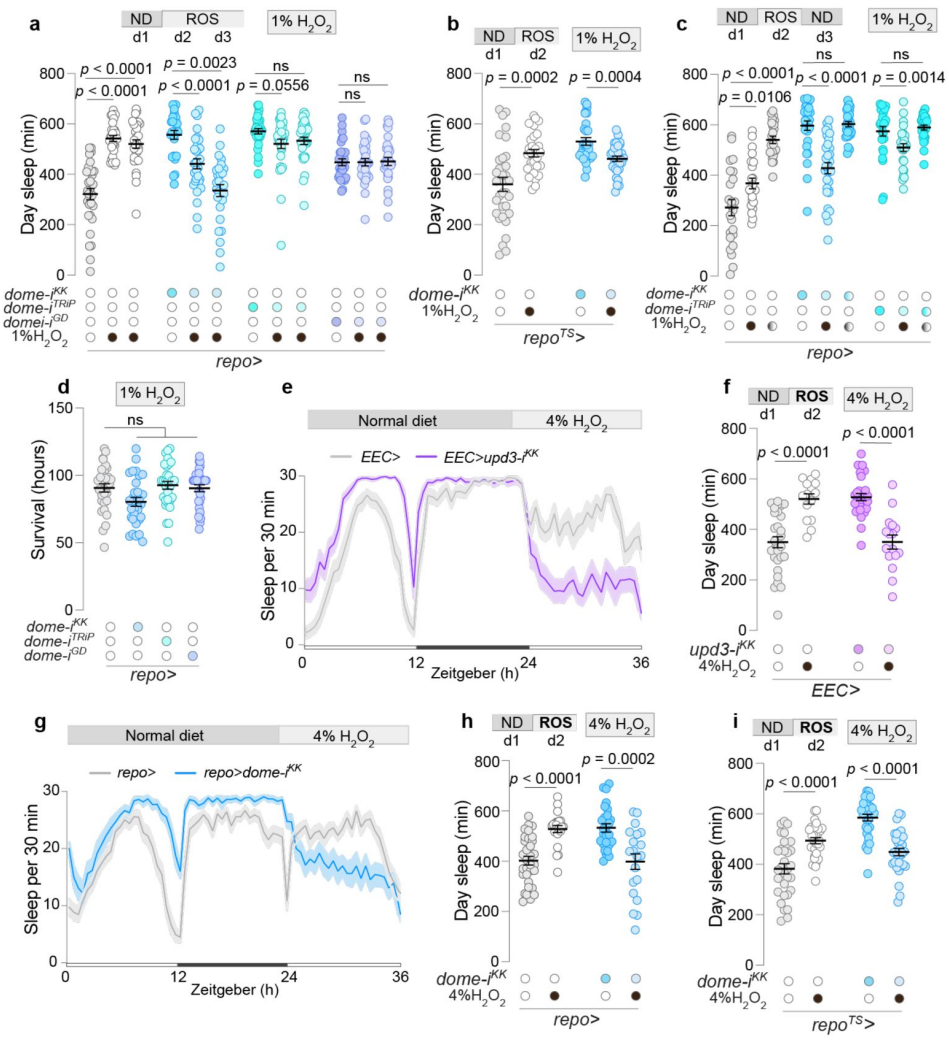

Figure 5.

EEC-derived Unpaired and glial Domeless signaling modulate sleep during intestinal oxidative stress.

a, Daytime sleep duration in flies with glial-specific *dome* knockdown under standard and oxidative stress conditions induced by 1% H_2O_2 in food (N=25-32). **b**, Daytime sleep duration in flies with *repo*-driven *dome* knockdown restricted to the adult stage using *Tub-GAL80^{ts}*(*repoTS*>) under normal conditions and during exposure to 1% H_2O_2-containing food (N=29-32). **c**, Daytime sleep during a three-day period, encompassing a day on standard diet, subsequent day on 1% H_2O_2-laced food to induce oxidative stress, and a final day back on standard diet to monitor recovery, in controls and animals with glial-specific *dome* knockdown (N=24-32). **d**, Survival rates of controls and flies with glial-specific *dome* knockdown after exposure to oxidative stress by 1% H_2O_2-laced food (N=31). **e**, Sleep profiles and **f**, daytime sleep duration for animals with EEC-specific *upd3* knockdown compared to control flies across a 36-hour period encompassing 24 hours on standard diet followed by 12 hours on oxidative stress conditions, induced by 4% H_2O_2-containing food (N=15-30). **g**, Sleep profiles and **h**, daytime sleep duration for animals with glial-specific *dome* knockdown compared to control flies across a 36-hour period encompassing 24 hours on standard diet followed by 12 hours on oxidative stress conditions, induced by 4% H_2O_2-containing food (N=20-32). **i**, Nighttime sleep durations for animals under 4% H_2O_2 oxidative stress with adult restricted knockdown of *dome* in glial cells and control (N=31-32). Statistical tests: Kruskal-Wallis One-Way ANOVA with Dunn's multiple comparison test for panels a, c and d; Unpaired t-tests for panels b, f, and h; and Mann-Whitney test for panel i. Data are presented as mean ± SEM. ns, non-significant (p>0.05).

sleep deprivation by evaluating the animals' ability to recover sleep after deprivation occurring during the second half of the night (ZT18-ZT24). Like controls, animals with glial-specific *dome* knockdown exhibited increased sleep (rebound sleep) in the morning hours (ZT0-ZT2) following sleep deprivation (**Fig. S5d,e**). This indicates that they exhibit normal rebound sleep responses to deprivation and retain the capability to further increase their sleep. Collectively, these data suggest that Dome-mediated JAK-STAT signaling in the glial cells specifically regulates ROS-induced sleep responses.

Furthermore, we investigated whether increased intestinal oxidative stress would exacerbate the phenotypes associated with the loss of *upd3* in EECs or *dome* in glial cells by exposing the animals to food containing 4% H_2O_2 and observing sleep-architecture changes. Oxidative stress resulted in increased sleep in control animals as anticipated (**Fig. 5e-h**). However, in animals with EEC-specific *upd3* knockdown, or glia-specific *dome* RNAi, exposure to 4% H_2O_2-containing food led to a pronounced loss of sleep during the daytime. For the EEC-specific *upd3* knockdown, the RNAi effect was induced at the adult stage (**Fig. 5e,f**). We therefore also confirmed that adult-restricted knockdown of *dome* in glial cells resulted in similar phenotypes (**Fig. 5i**). Thus, under conditions of intensified intestinal stress induced by 4% H_2O_2 in the food, the loss of *upd3* in EECs phenocopies the glial knockdown of *dome*, leading to reduced sleep and increased wakefulness.

BBB glial JAK-STAT pathway activation drives sleep in response to intestinal oxidative stress

To determine the subset of glial cells responsible for mediating Unpaired-driven sleep regulation, we focused on the perineurial and subperineurial glial cells that form the BBB. These BBB glial cells serve as the interface between the CNS and the periphery, including its organs[44], and are ideally situated to receive circulating signals from the intestine. Using the *10xSTAT-GFP* reporter, we assessed whether Upd3 from the gut activates JAK-STAT signaling within BBB glial cells. Knockdown of *upd3* in the EECs using *voilá>* (without *R57C10-GAL80*) which drives specific knockdown in the gut without any neuronal effects (**Fig. S4a,b**), resulted in decreased GFP intensity in the outermost glial cell layer of the central brain facing the periphery after 15 hours of exposure to 1% H_2O_2-containing food, indicating reduced JAK-STAT activity in these cells (**Fig. 6a,b**). Interestingly, this knockdown did not affect JAK-STAT activity in the outer glial layer of the ventral nerve cord (VNC), suggesting that Upd3 acts specifically on brain BBB glia in response to intestinal oxidative stress.

We further examined BBB-specific JAK-STAT signaling effects on sleep by driving expression in subperineurial glial cells – those that form the permeability barrier – with *moody-GAL4* (*moody>*). As observed with other manipulations, knockdown of *dome* in these BBB glial cells led to increased sleep during normal homeostatic conditions (**Fig. 6e,f**). Whereas controls exhibited the expected sleep increase both during 1%-H_2O_2 exposure and during the recovery period, loss of *dome* in the subperineurial BBB glia blocked these effects. When oxidative stress levels were elevated further using 4% H_2O_2-containing food, *dome-RNAi* in BBB glial cells led to sleep loss in response to oxidative stress, with sleep levels rebounding to pre-stress levels on the subsequent recovery day when animals were returned to a normal diet (**Fig. 6e,f**). These results suggest that disrupting *dome* specifically in BBB glial cells recapitulates the phenotypes observed with pan-glial knockdown or EEC-specific *upd3* knockdown, indicating that Dome-mediated JAK-STAT activation in subperineurial BBB cells is required for maintaining an increased sleep state during intestinal oxidative stress.

To assess the sufficiency of subperineurial JAK-STAT signaling in inducing sleep, we activated the pathway in these cells by expressing a hyperactivated variant of the *Drosophila* JAK ortholog *hopscotch* (hop^{Tum}). Activation of JAK in BBB glia increased sleep under normal conditions without oxidative stress, consistent with a sleep-promoting effect of high JAK-STAT signaling (**Fig. 6c-f**). Moreover, animals with overactivate JAK signaling in BBB glia exhibited further enhanced sleep

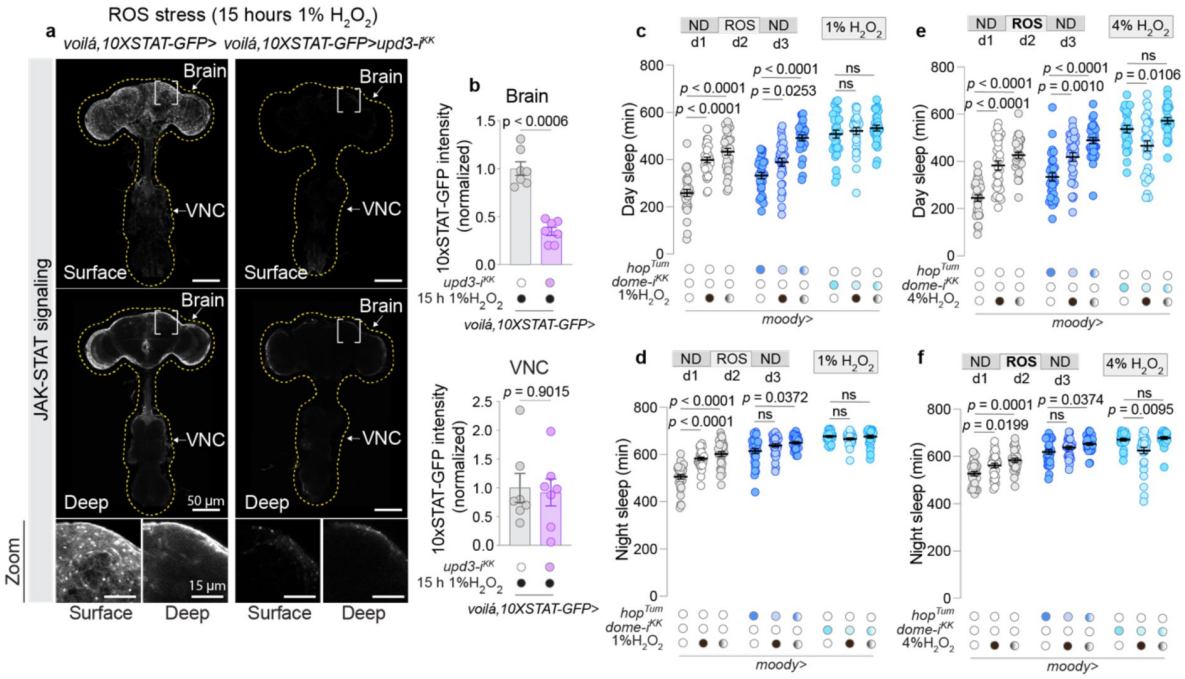

Figure 6.

BBB glia drives Domeless-mediated sleep responses to intestinal oxidative stress.

a, Representative images showing GFP expression driven by *10xSTAT::GFP* in controls and animals with *voilà-GAL4* (*voilà>*) driven *upd3* knockdown in EECs under intestinal ROS induced by 20 hours exposure to 1% H_2O_2-containing food. The top panels depict overall brain and ventral nerve cord (VNC) structure with views of surface or deeper layers; the bottom panels provide zoomed-in views, highlighting the BBB glia at the interface between the brain and external environment. Dotted lines indicate brain and VNC perimeters. Scale bars, 50 µm (top) and 15 µm (bottom). **b**, Quantification of GFP intensity in the brain and VNC in BBB glial controls and animals with EEC knockdown of *upd3* under ROS stress, induced by exposure to 1% H_2O_2-laced food (N=7). **c**, Daytime and **d**, nighttime sleep durations in flies with BBB glial-specific knockdown of *dome* or overexpression of hop^{Tum} under normal conditions, during exposure to 1% H_2O_2-containing food, and subsequent recovery on normal diet (N=23-32). **e**, Daytime and **f**, nighttime sleep durations in flies with BBB glial-specific knockdown of *dome* or overexpression of hop^{Tum} under normal conditions, during exposure to 4% H_2O_2-containing food, and subsequent recovery on normal diet (N=23-32). Statistical tests used: Unpaired t-tests for panel b; Kruskal-Wallis One-Way ANOVA with Dunn's multiple comparison test for panels c, d and f; Ordinary one-way ANOVA with Dunnett's multiple comparisons test for panel e. Data are presented as mean ± SEM. ns, non-significant (p>0.05).

both during oxidative stress and in the subsequent recovery phase, as opposed to those with *dome* knockdown. This suggests that the combined activation of JAK-STAT induced by intestinal ROS and expression of hop^{Tum} leads to additive increases in sleep. Collectively, our data indicate that JAK-STAT signaling specifically in the subperineurial glial of the BBB links sleep responses to intestinal oxidative stress.

AstA signaling promotes wakefulness and mediates ROS-induced sleep regulation in BBB glia

Our results indicate that the effect of gut-to-glial Unpaired cytokine signaling is both dose-dependent and context-dependent. During intestinal oxidative stress, ROS-induced EEC Unpaired signaling leads to high JAK-STAT activity in subperineurial glial cells. Given that animals lacking this gut cytokine-to-glial signaling fail to maintain a high sleep state during oxidative stress, instead exhibiting increased wakefulness, this pathway appears to suppress wake-promoting signals under such conditions. The role of such wake-suppressive effects is likely to enhance sleep, aiding the process of recovery from intestinal damage. To identify potential wake-promoting signals that might be gated by JAK-STAT signaling, we examined a published dataset of genes whose expression in glia is positively or negatively correlated with these cells' JAK-STAT activity following enteric infection[33]. The receptors for Allatostatin A (AstA), *AstA-R1* and *AstA-R2*, both ranked among the top 4% of genes most strongly downregulated by JAK/STAT signaling (with *AstA-R1* expression reduced by ~80% and *AstA-R2* by ~90%). Notably, these were the only peptide-hormone G-protein-coupled receptors (GPCRs) downregulated in the JAK-STAT-activate glial cells. This suggests that upon intestinal infection, the JAK-STAT pathway is activated in glial cells, which suppresses AstA signaling by reducing the expression of the AstA receptors. Considering the central role of neuronal AstA in sleep-regulatory circuits[46,47], we investigated whether AstA might constitute a wake-promoting signal that is inhibited in glial cells by gut-derived Unpaired signaling. To evaluate the expression pattern of *AstA-R1* and *AstA-R2* within glial populations, we employed *AstA-R1-GAL4* and *AstA-R2-GAL4* knock-in constructs to drive the expression of nuclear-localized *RFP*. We co-stained the brain with antibodies against the glial transcription factor Repo, which marks the nuclei of glial cells. We observed that the outer layer of glial cells at the barrier between the brain and the periphery – constituting the BBB – express both *AstA-R1* and *AstA-R2* (**Fig. 7a**). These findings are in line with previously reported data showing expression of *AstA-R1* and *AstA-R2* in glial cells referred to above[33]. To functionally characterize the role of AstA signaling in these cells, we knocked down *AstA-R2* in BBB glial using *moody>*. This led to a significant reduction in *AstA-R2* transcript levels in dissected brains, indicating that BBB glia is a significant source of *AstA-R2* expression (**Fig. S6a**). In support of an inhibitory role of Unpaired signaling, we observed that *AstA-R1* and *AstA-R2* expression was upregulated in the heads of animals with EEC-specific *upd3* knockdown, following gut-oxidative stress induced by feeding with 1% H_2O_2-laced food for 20 hours (**Fig. 7b**). To demonstrate that this is caused by a failure to suppress AstA receptors in glial cells, we examined AstA receptor expression in brains following glia-specific *dome* knockdown in animals fed 1% H_2O_2-containing food for 20 hours. Indeed, glial-specific *dome* knockdown led to strong upregulation of both *AstA-R1* and *AstA-R2*, indicating that ROS-induced glia-mediated Unpaired signaling is inhibiting AstA receptor expression (**Fig. 7c**).

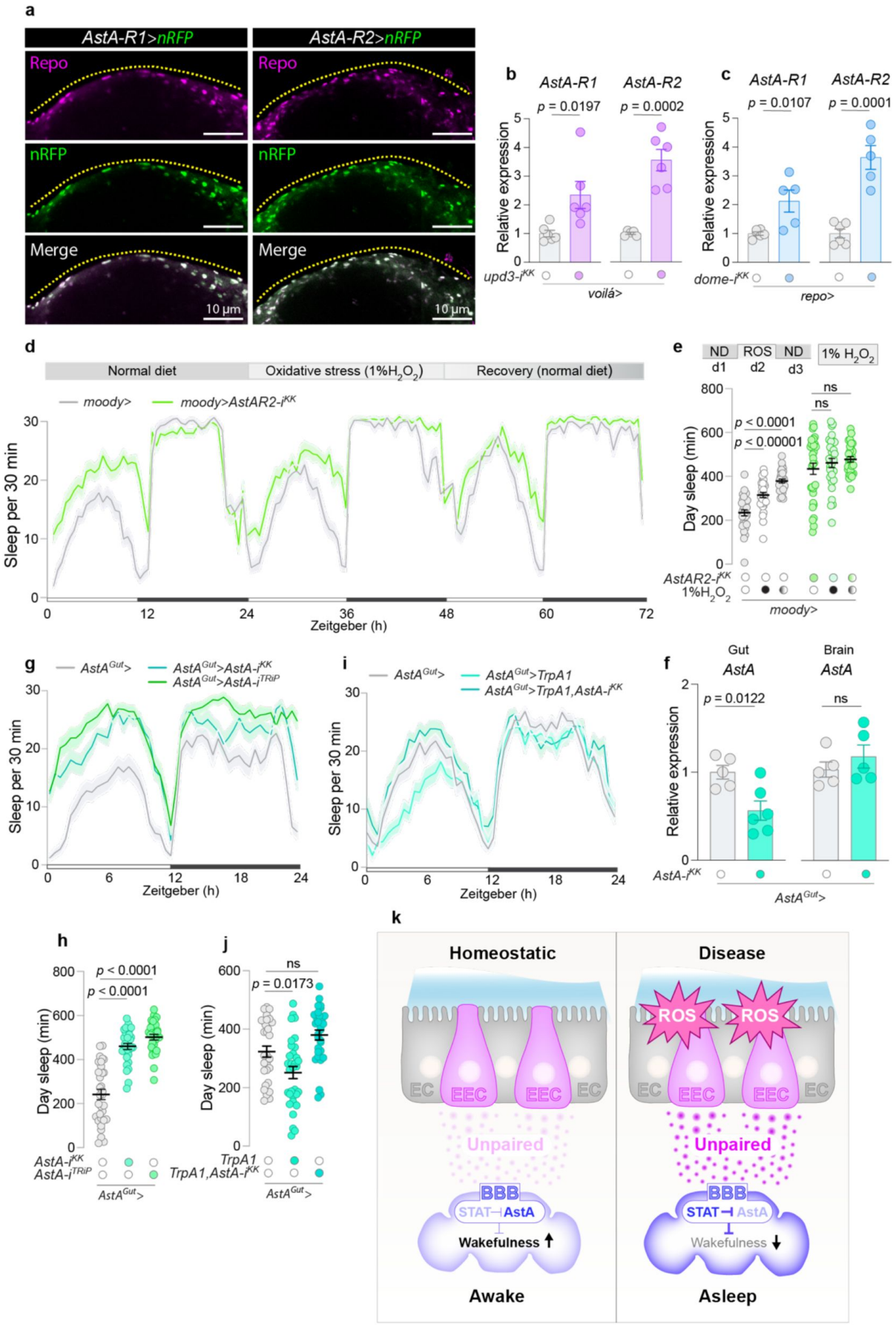
a
AstA-R1>nRFP
AstA-R2>nRFP
Repo
nRFP
Merge
10 µm
b
AstA-R1
AstA-R2
p = 0.0197
p = 0.0002
Relative expression
upd3-i^KK
voilá>
c
AstA-R1
AstA-R2
p = 0.0107
p = 0.0001
Relative expression
dome-i^KK
repo>
d
Normal diet
Oxidative stress (1%H2O2)
Recovery (normal diet)
moody>
moody>AstAR2-i^KK
Sleep per 30 min
Zeitgeber (h)
e
ND d1
ROS d2
ND d3
1% H2O2
p < 0.0001
p < 0.00001
ns
ns
Day sleep (min)
AstAR2-i^KK
1%H2O2
moody>
g
AstA^Gut>
AstA^Gut>AstA-i^KK
AstA^Gut>AstA-i^TRiP
Sleep per 30 min
Zeitgeber (h)
i
AstA^Gut>
AstA^Gut>TrpA1
AstA^Gut>TrpA1,AstA-i^KK
Sleep per 30 min
Zeitgeber (h)
f
Gut
AstA
Brain
AstA
p = 0.0122
ns
Relative expression
AstA-i^KK
AstA^Gut>
h
p < 0.0001
p < 0.0001
Day sleep (min)
AstA-i^KK
AstA-i^TRiP
AstA^Gut>
j
ns
p = 0.0173
Day sleep (min)
TrpA1
TrpA1,AstA-i^KK
AstA^Gut>
k
Homeostatic
Disease
ROS
EC
EEC
Unpaired
BBB
STAT⊣AstA
Wakefulness ↑
Wakefulness ↓
Awake
Asleep

Figure 7.

Gut Unpaired cytokine signaling inhibits wake-promoting AstA signaling.

a, Images of brains from animals with *AstA-R1-GAL4* and *AstA-R2-GAL4* driving nuclear *dsRed* (nRFP, magenta) and co-stained with anti-Repo antibodies (green) show glial cells. The yellow dashed line indicates the interface between the brain and the external space, with the area below housing the (BBB glial cells (Scale bar, 10 μm). The yellow demarcation accentuates the separation between the cerebral interior and the external milieu, identifying the location of BBB glial cells underneath this partition (Scale bar is 10 μm). **b**, Relative expression of *AstA-R1* and *AstA-R2* in heads from animals with *upd3* knockdown in EECs driven by *voilà-GAL4* (*voilà>*) compared to the control group after 20 hours on 1% H_2O_2-laced food to induce oxidative stress (N=5-6). **c**, Relative expression of *AstA-R1* and *AstA-R2* in brains from animals with *dome* knockdown in glial cells driven by *repo-GAL4* (*repo>*) compared to the control group after 20 hours on 1% H_2O_2-laced food to induce oxidative stress (N=6).**d** Sleep patterns and **e**, daytime sleep across a three-day period, encompassing a day on a standard diet, a subsequent day on 1% H_2O_2-laced food to induce oxidative stress, and a final day back on the standard diet to monitor recovery, in flies with BBB glial-specific knockdown of *AstA-R2* compared to control (N=28-32). **f,** *AstA* transcript levels in brains and midguts from controls and animals with knockdown of *AstA* in $AstA^+$ EECs using *AstA-GAL4* (*AstA>*) in combination with *R57C10-GAL80* to suppress neuronal GAL4 activity, referred to as $AstA^{GUT}$> (N=5-6). **g** Sleep profiles and **h**, daytime sleep on standard food of animals with *AstA* knockdown in $AstA^+$ EECs using $AstA^{GUT}$> and controls (N=30-32). **i** Sleep profiles, **j** daytime sleep on standard food of controls and animals with TrpA1-mediated activation of $AstA^+$ EECs, and animals with TrpA1-mediated activation of $AstA^+$ EECs with simultaneous knockdown of *AstA* (N=30-32). **k,** Diagram illustrating the role of enteroendocrine cells (EECs) in regulating wakefulness and sleep through Unpaired cytokine signaling under homeostatic and disease conditions. Left: Under homeostatic conditions, EECs release baseline levels of Unpaired, which interacts with the blood-brain barrier (BBB) to maintain normal JAK-STAT signaling, promoting healthy wakefulness. Right: In response to disease, reactive oxygen species (ROS) increase in EECs, leading to elevated release of Unpaired. This surge in Unpaired upregulates JAK-STAT signaling in BBB glia, which inhibits wake-promoting AstA signaling by suppressing AstA receptors, thus resulting in increased sleep, a state termed 'sickness-sleep,' to promote recovery. The diagrams depict the gut lining with EECs highlighted, the interface with the BBB, and the resulting systemic effects on the organism's sleep-wake states. EC; enterocyte. Statistical tests used: Unpaired t-tests for panel b, c and f; Ordinary one-way ANOVA with Dunnett's multiple comparisons test for panel e, h and j. Data are presented as mean ± SEM. ns, non-significant (p>0.05).

Next, we investigated whether AstA receptors are involved in mediating the glial-regulated sleep response to intestinal oxidative stress. Like the Unpaired cytokines, AstA is released from EECs in response to oxidative stress[48]. It may therefore act as a context-dependent wake-promoting signal, that under certain conditions, is inhibited by Unpaired signaling in BBB glial to promote sleep. We thus hypothesized that during intestinal disturbances characterized by oxidative stress, gut-derived Unpaired signaling via JAK-STAT activation either sustains or consolidates sleep through a mechanism that involves the downregulation of wake-promoting AstA receptor signaling in BBB glial cells. In this model, EEC-derived Unpaired signaling normally suppresses AstA signaling in BBB glial cells under oxidative stress. Consequently, knocking down *dome* in these cells or *unpaired* in the EECs leads to a failure to downregulate AstA receptors, causing the animals to wake up under these conditions. Thus, inhibition of glial AstA receptors would impair the animals' ability to respond to these wake-promoting signals altogether, leaving them unresponsive to intestinal ROS in terms of sleep. Consistent with this notion, we found that knocking down *AstA-R1* or *AstA-R2* in BBB glia attenuated the ROS-induced sleep response (**Fig. S6b,c**). Knockdown of *AstA-R2* with second, independent RNAi line resulted in a more pronounced phenotype, with an almost completely blunted sleep response to intestinal ROS, showing no significant sleep increase during oxidative stress or the following day of recovery (**Fig. 7d,e**). AstA-R2 was also the most highly upregulated in response to loss of *upd*3 in the EEC or *dome* in glia (**Fig. 7b,c**), and it was the more strongly downregulated of the two AstA receptors in response to glial JAK-STAT activation, together suggesting that AstA-R2 is a primary receptor

mediating these effects. Furthermore, knockdown of *AstA* receptors in BBB glia increased daytime sleep under normal homeostatic conditions, consistent with a wake-promoting role of AstA signaling in BBB glial (**Fig. 7d,e** and **S6b,c**).

AstA is produced by two cell types, neurons and the EECs in the gut[46]. Since BBB glial cells are well-positioned to receive hormonal signals from the periphery, they likely are regulated by gut-derived AstA. We thus examined whether gut-derived AstA acts as a wake-promoting signal, by conducting *AstA* knockdown in AstA-positive EECs using an *AstA:2A::GAL4* knock-in in combination with *R57C10-GAL80* to suppress GAL4 activity in the AstA-positive neuronal population. We confirmed that this driver efficiently reduces the expression of *AstA* in midguts without affecting neuronal *AstA* transcripts levels (**Fig. 7f**). Knockdown of *AstA* in AstA-positive EECs with either of two independent RNAi constructs led to increased sleep without any contribution of the transgenic insertion backgrounds (**Fig. 7g,h** and **S6d,e**), indicating that gut-derived AstA is indeed a wake-promoting factor. To assess whether EEC-derived AstA is sufficient to promote arousal, we employed the thermosensitive cation channel Transient Receptor Potential A1 (TrpA1)[49] to induce hormonal release from AstA-positive EECs. Activation of these EECs suppressed sleep, an effect that was abolished by simultaneous *AstA* knockdown, supporting that the wake-promoting role of EEC-derived AstA (**Fig. 7i,j**). Taken together, our findings suggest that enteric oxidative stress induces the release of Unpaired cytokines, which activate the JAK-STAT pathway in subperineurial glia of the BBB. This activation leads to the glial downregulation of receptors for AstA, which is a wake-promoting factor also released by EECs. Gut-derived Upd signaling thereby gates the effect of AstA at the BBB and permits increased sleep during periods of intestinal stress. This process may aid in recovery and maintain overall organismal homeostasis.

Discussion

Intestinal inflammation and microbial imbalance are strongly associated with sleep disturbances and mental disorders such as anxiety and depression[8,12–14]. The influence of gut health on CNS-dependent behaviors is thought to be mediated by the gut-brain axis, comprised of diverse signals secreted by the gut that act on the brain to induce behavioral responses[50]. Whereas the regulation of feeding behavior by this axis has been extensively studied, leading to revolutionary approaches to medical weight loss and diabetes control, the role of gut-brain signaling in regulating sleep – a behavior affected by nearly all illnesses[51,52] – remains poorly defined. Sickness induces a state of sleepiness, which is believed to be a conserved adaptive response that promotes recovery by supporting energy conservation and efficient immune activity[5,6]. However, the exact mechanisms driving sickness-induced sleep remain largely elusive. We have demonstrated here that intestinal illness, through the release of interleukin 6-like Unpaired cytokines from endocrine cells of the *Drosophila* gut, regulates sleep via a glia-mediated pathway. This gut-to-glia communication promotes sleep during intestinal illness, presumably to facilitate the restorative sleep essential for both physical and mental health. Our findings provide mechanistic insight into how perturbations of gut health can influence sleep, potentially contributing to understanding the link between gastrointestinal disorders, sleep disturbances, and mental illnesses.

Cytokines, key mediators of immune and inflammatory responses, are thought to modulate sleep/wake cycles under disease conditions[53]. Interleukins and TNFα, cytokines induced during illness in mammals, have been suggested to promote sleep to aid recovery from disease. However, most of these effects have been attributed to the actions of cytokines produced within the CNS, leaving open the question of how diseases affecting other parts of the body can drive sleep responses. In *Drosophila*, sleep induced by immune responses is known to be influenced by the NFκB ortholog Relish in fat tissue[54], and the neuronally expressed gene *nemuri* drives sleep and connects immune function with sleep regulation[6]. However, inter-organ signaling mechanisms

by which intestinal disease or stress regulate sleep have not yet been described in either flies or mammals. Intestinal infections or inflammation lead to elevated levels of ROS in the gut, and our findings demonstrate that enteric oxidative stress in the gut triggers the production of Upd2 and Upd3 cytokines by hormone-secreting EECs. These gut-derived cytokines signal the state of the intestine to brain glial cells, including those of the BBB, and modulate sleep. This glia-mediated gut-to-brain signaling promotes wakefulness in healthy animals under normal conditions, while inducing sleep in response to oxidative stress in the intestine. This indicates a dual functionality, with low levels of gut Unpaired signaling promoting wakefulness, while higher disease-induced levels act oppositely to enhance sleep (**Fig. 7k**). A similar dose-dependent effect has previously been observed for interleukins in rats, in which injection of IL-1 into the CNS stimulates or inhibits sleep depending on the dose[55].

Our results indicate that Unpaired signaling in subperineurial glial cells – those forming the BBB – activates the JAK-STAT pathway, and they suggest that this effect inhibits wake-promoting AstA signaling by downregulating *AstA* receptor expression. AstA and its receptors, which are orthologous with the mammalian Galanin signaling system, have been linked to the regulation of sleep, feeding, and metabolism[46,56,57]. Mammalian glia also express receptors for Galanin[58], which also regulates sleep[59,60], further underscoring a conserved role in sleep modulation across species. AstA-producing neurons induce sleep by releasing glutamate onto sleep-regulatory neuronal circuits, although recent findings also suggest a wake-promoting role for AstA signaling[47]. Irrespective of neuronal AstA, our experiments clearly show that AstA released from EECs of the gut acts as a wake-promoting signal and that activation of AstA receptor signaling in BBB glial cells induces wakefulness. This highlights the potential of peptide hormones to elicit different effects depending on their source tissue and thus their accessible target cells – whether they are produced by the gut outside the BBB or by the CNS inside the barrier. A similar phenomenon has been demonstrated for neuropeptide F[39,61].

Drosophila exhibit conserved behaviors such as sleep, arousal/wakefulness, and anxiety-like responses[4,62–67], and the EECs of the fly gut produce diverse hormones similar to those of mammals[17–22], potentially influenced by diet, microbiota, and inflammatory responses. This makes *Drosophila* an excellent model for studying behaviors influenced by gut conditions through gut-brain signaling. Our findings suggest that the oxidative-stress level within gut tissues, which is modulated by intestinal bacteria and immune activity[26,27], regulates sleep via EEC-derived Unpaired signaling, potentially explaining the observed links between gut microbiota and sleep disturbances in both flies and humans[13,68]. Furthermore, in mammals, conditions such as inflammatory bowel disease that are linked with oxidative stress[69] are often associated with sleep and mental-health disturbances[8,12,14]. Our results imply that cytokines, including interleukin signaling from an inflamed or diseased gut, might be a mechanism by which intestinal illnesses affect sleep and mental health. Our findings raise the possibility that these cytokines may act on glial cells that integrate and relay these gut signals to brain sleep-regulatory circuits.

The neurons of the CNS are isolated from the circulatory system by the BBB[70] that restricts the transmission of some hormonal and cytokine signals from the periphery to neurons within the brain. Our work suggests that the BBB receives AstA and Unpaired signaling from the periphery. Other reports indicate that Unpaired cytokines from tumors and from enterocytes also can activate JAK-STAT signaling in BBB glia cells in *Drosophila*[33,71]. One effect of glial JAK-STAT activity seems to be the alteration of BBB permeability[71], raising the possibility that EEC-derived Unpaired signaling in BBB glia, directly or through AstA signaling, modulates sleep via regulation of BBB permeability, which has been linked to homeostatic sleep regulation[36]. Furthermore, the endocytotic activity of BBB glia, important for cellular transport and barrier function, has also been associated with sleep regulation[37], and thus JAK-STAT-induced changes could regulate sleep through alterations in BBB trafficking. Another possibility is that JAK-STAT activity might regulate glial metabolic support for neuronal activity and in this way affect sleep patterns. In any case, our findings highlight the involvement of BBB glial cells in transmitting signals from the gut

to the brain, adding another layer to our understanding of body-to-brain communication, which suggests that the BBB does more than protect the brain; it also responds to peripheral signals to modulate brain function, presenting an intriguing area for future research into gut-brain signaling.

Methods

Drosophila stocks and husbandry

Flies were cultured using a standard cornmeal-based formulation (82 g/L cornmeal, 60 g/L sucrose, 34 g/L yeast, 8 g/L agar, 4.8 mL/L propionic acid, and 1.6 g/L methyl-4-hydroxybenzoate) maintained at 25 °C with 60% relative humidity under a 12-hour light/dark cycle. Post-eclosion, flies were transitioned to an adult-specific, cornmeal-free diet (comprising 90 g/L sucrose, 80 g/L yeast, 10 g/L agar, 5 mL/L propionic acid, and 15 mL/L of a 10% methyl-4-hydroxybenzoate solution in ethanol)[72] for 4-7 days prior to experiments. Adult mated females were used for all experiments. Flies were separated by sex one day prior to experimental procedures. Strains harboring the temperature-sensitive *Tubulin-GAL80^{ts}* transgene were initially reared at 18 °C on cornmeal food and then switched to the adult diet for 3-4 days post-eclosion, still at 18 °C. Subsequently, they were incubated at 29 °C for five to seven days to activate RNAi expression in advance of the experiments. To ensure optimal conditions, the flies were provided with fresh food every three days. The following lines used in this study were sourced from the Bloomington *Drosophila* Stock Center (BDSC) at the University of Indiana: *R57C10-GAL4* (#39171); *UAS-upd3-RNAi^{TRiP}* (*#32859*); *UAS-dome-RNAi^{TRiP}* (*#53890*); *AstA::2A::GAL4* (#84593); *AstA-R1::2A::GAL4* (#84709); *AstA-R2::2A::GAL4* (#84594); *UAS-AstA-RNAi^{TRiP}* (#25866); *UAS-AstA-R2-RNAi^{TRiP}* (#67864); *UAS-mCD8::GFP* (#5137); *Tub-GAL80^{ts}* (#7108); *repo-GAL4* (*#7415*); *moody-GAL4* (#90883); *UAS-TrpA1* (#26263); and *UAS-dsRed* was extracted from (#8546). Additional fly lines were acquired from the Vienna *Drosophila* Resource Center (VDRC): control line *w^{1118}* (#60000, which is isogenic with the VDRC RNAi lines); *UAS-upd2-RNAi^{SH}* (#330691); *UAS-upd3-RNAi^{KK}* (#106869); *UAS-upd3-RNAi^{GD}* (#27136); *UAS-dome-RNAi^{KK}* (*#106071*); *UAS-dome-RNAi^{GD}* (*#36356*); *UAS-AstA-RNAi^{KK}* (#103215); *UAS-AstA-R1-RNAi^{KK}* (#101395); *UAS-AstA-R2-RNAi^{KK}*(#108648). The *upd3Δ* and *upd2,3Δ* deletion mutants were kindly provided by Bruno Lemaitre. *UAS-upd3* and *UAS-hop^{Tum}* lines were gifts from David Bilder. The *STAT-dGFP:2A::RFP* line was generously supplied by Norbert Perrimon. The *voilà-GAL4* strain was graciously provided by Alessandro Scopelliti. The *R57C10-GAL80* transgene, situated on the X chromosome, was kindly donated by Ryusuke Niwa. The *10xSTAT::GFP* line was a gift from Julien Colombani. To ensure uniformity in genetic background and to create control groups with an appropriate genetic background, all *GAL4* and *GAL80* lines used in this study were backcrossed to a *w^{1118}* line for multiple generations before being outcrossed with the genetic background specific to the RNAi, CRISPR, or overexpression lines to serve as controls in the experiments[25].

Generation of tissue-specific CRISPR lines

To facilitate tissue-specific CRISPR-based disruption of the *upd2* and *upd3* loci, constructs were prepared containing two gRNA target sequences, flanked by efficiency-enhancing tRNA sequences. One construct was prepared for *upd2*, and two transgenes, targeting different genomic sites, were made for *upd3*. The *upd2* construct was designed to delete the region encoding the secreted Upd2 protein. One *upd3* construct should delete the initiator ATG codon, and the other – the one used in this work – deletes the second exon, which contains a significant portion of the coding sequence. Target-sequence cassettes were assembled by first cloning the tRNA insert from plasmid pCFD6 (Addgene #73915) between long oligos containing the gRNA target sequences using Q5 polymerase (New England Biolabs, #M0491S). The vector and the PCR products were then integrated using Gibson assembly (NEBuilder HiFi DNA Assembly Master Mix, New England Biolabs, #E2621S). Clones were sequenced to verify accuracy, and correct constructs were integrated into the fly

genome at the attP2 site (chromosome 3L) by BestGene (Chino Hills, CA). The sequences used for cloning the *upd2* and *upd3* CRISPR constructs, with gRNA sequences indicated in bold, are shown in **Supplementary Table 1**.

Sleep, activity, and survival assays

The *Drosophila* Activity Monitoring System (TriKinetics, Waltham, MA) was employed to track sleep and activity patterns. Single flies aged six to eight days after eclosion were placed into glass tubes using light CO_2 anesthesia. On one end, the tubes were sealed with a foam plug; on the other was placed a detachable 250-µL PCR tube containing 90 µL of feeding medium: either 5% sucrose in 1% agar/water, 5% sucrose mixed with various concentrations of H_2O_2 in 1% agar/water, or plain 1% agar/water for starvation conditions. All food media contained 0.5% propionic acid and 0.15% methyl-4-hydroxybenzoate to prevent microbial growth, with H_2O_2 being supplemented once the food had cooled to below 40 °C. Monitoring of the flies' locomotor activity and sleep began at the beginning of the light cycle, after the animals had spent their first day in the tubes acclimating. Following an additional 24 hours on the standard 5%-sucrose diet, the PCR tubes were replaced with fresh ones containing H_2O_2 or starvation media at the lights-on transition when animals were awake, to avoid unnecessary disturbances to the animals. For recovery experiments, animals were switched back to a 5%-sucrose diet after 24 hours on H_2O_2-laced food. Periods of inactivity lasting 5 minutes or longer were recorded as "sleep." In the sleep deprivation studies, the flies were placed in DAM monitors and subjected to mechanical stimulation, which was produced by attaching the monitors to a vortexer mounting plate (TriKinetics) and vibrating them for 2 seconds at the start of each minute throughout the 6-hour interval leading up to the lights-on time. Recovery sleep was assessed in flies that experienced a reduction of more than 60% in their typical sleep during the deprivation period, using their sleep patterns from the 24-hour period before the onset of sleep deprivation as a baseline. The occurrence of recovery sleep was specifically evaluated during the first two hours immediately following the sleep deprivation phase. For survival assays, flies were loaded into tubes filled with either plain 1% agar/water for starvation or 1% H_2O_2 in 1% agar/water to test oxidative stress resistance. The time of death was recorded upon the complete cessation of movement.

Feeding Assays

Short-term food consumption was quantified using a spectrophotometric dye-feeding assay[73,74]. All food intake experiments were conducted during the time of the normal morning meal, one hour after lights-on in a 12:12-hour dark/light cycle. Flies were transferred without anesthesia to food (90 g/L sucrose, 80 g/L yeast, 10 g/L agar, 5 mL/L propionic acid, and 15 mL/L of a 10% methyl-4-hydroxybenzoate solution in ethanol) supplemented with 0.5% erioglaucine dye (brilliant blue R, FD&C Blue No.1, Sigma-Aldrich, #861146) and allowed to feed for 1 hour. A control group of flies was provided with undyed food to establish the baseline absorbance levels of fly lysates. For each genotype, 1-2 flies per sample were homogenized in 100 µL phosphate buffer (pH 7.5) using a TissueLyser LT (Qiagen) with 5-mm stainless-steel beads. Homogenates were centrifuged at 16,000 g for 5 minutes, and 50 µL of the cleared supernatant was transferred to a 384-well plate. Absorbance was measured at 629 nm for erioglaucine using an Ensight multi-mode plate reader (PerkinElmer). Standard curves for dye were employed to correlate absorbance readings with the amounts of food consumed.

To assess feeding behavior, interactions with food were monitored over a 20-24 hour period using the Fly Liquid-Food Interaction Counter (FLIC) apparatus[75]. *Drosophila* Feeding Monitors (DFMs; Sable Systems) were placed in an incubator set to 25 °C (or 29 °C for strains carrying $GAL80^{ts}$), maintaining 70% humidity under a 12:12-hour light/dark cycle. Each of the 12 DFM chambers was filled with a 10% sucrose solution, and individual flies were introduced in the afternoon following the morning meal. After several hours of acclimation, evening feeding activity was recorded. The following morning, at lights-on, the DFMs were refilled with fresh sugar solution, and data from

the morning meal were collected. The feeding behavior was recorded using the manufacturer's software and analyzed using R Studio with the provided package (*https://github.com/PletcherLab/FLIC_R_Code*).

Immunohistochemistry and confocal imaging

Adult midguts, brains, and VNCs were dissected in cold PBS and fixed for 1 hour at room temperature in 4% paraformaldehyde/PBS with gentle shaking. After a quick rinse with PBST (PBS with 0.1% Triton X-100, Merck #12298), the tissues were washed three times for 15 minutes each in PBST. The tissues were then blocked for 30 minutes at room temperature in PBST containing 5% normal goat serum (Sigma) and subsequently incubated overnight (or two days for CNS samples) at 4 °C with primary antibodies diluted in the blocking solution with gentle agitation. After removing the primary antibody solution, tissues were rinsed once and washed three times for 20 minutes each in PBST. Secondary antibodies diluted in PBST were applied, and tissues were incubated overnight at 4 °C, followed by three PBST washes and one PBS wash. The samples were then mounted on poly-L-lysine-coated slides (Sigma, #P8920) in Fluoroshield mounting medium with DAPI (Nordic Biosite, #GTX30920), and imaged on a Zeiss LSM-900 confocal microscope using a 20× air or 40× oil objective with Zen software. Image stitching was performed using the Stitching function of Zeiss Zen Blue 3.1, and analysis was conducted using the open-source FIJI/ImageJ software package[76]. All samples within each figure panel were dissected, stained, and imaged simultaneously using identical settings and reagents. To quantify STAT-dGFP:2A::RFP staining, dissected brains were fixed for 10 minutes in 4% paraformaldehyde at room temperature with agitation, rinsed once with PBST, mounted, and imaged immediately without antibody staining. For 10xSTAT::GFP quantification, brain and VNC samples were stained as described above, Z-stacks were projected in FIJI using the "sum" method, and Repo-positive cells at the surface were manually segmented to measure the raw integrated density with local background subtraction. For measuring 10xSTAT::GFP across the BBB, linear regions of interest were drawn through the glial layer perpendicular to the brain surface at the plane showing maximum brain size using FIJI's line tool; GFP intensity was then quantified along these lines, and the peak was recorded for each transect. Antibodies used included 1:50 mouse anti-Repo (University of Iowa Developmental Studies Hybridoma Bank, #8D12), 1:20 mouse anti-Prospero (University of Iowa Developmental Studies Hybridoma Bank, #MR1A), 1:500 mouse anti-GFP (ThermoFisher, #A11120), 1:500 chicken anti-GFP (ThermoFisher, #A10262), 1:1000 rat anti-mCherry (used against Ds Red, ThermoFisher, #M11217), 1:500 Alexa Fluor 488-conjugated goat anti-mouse (ThermoFisher, #A11001), 1:500 Alexa Fluor 555-conjugated goat anti-mouse (ThermoFisher, #A21422), 1:500 Alexa Fluor 647-conjugated goat anti-mouse (ThermoFisher, #A-32728), 555-conjugated goat anti-rat (ThermoFisher, #A21434), and 488-conjugated goat anti-chicken (ThermoFisher, #A11039).

Triacylglyceride measurements

Triacylglyceride concentrations were determined following established methods[72,77] using the Randox Triglycerides (GPO-PAP) method (Randox, #TR210). For each sample, flies were homogenized in 50 µl PBS per fly (between 2-4 flies per sample) containing 0.1% Tween-20 (Sigma #1379) using a TissueLyser LT (Qiagen) with 5-mm stainless-steel beads, 50 oscillations/second for 30 seconds. Homogenates were heated at 70 °C for 10 minutes to inactivate endogenous enzymes and centrifuged at 11,000 g for 1 min. Aliquots of cleared and vortexed supernatants (4 µl) were added to 36 µl of triglyceride reagent (Randox, #TR210) in a 384-well plate, covered with ultra-high optical clarity film (ThermalSeal RT2RR^{TM}, Z722553, Excel Scientific). The plate was spun down 1,500 g 1 min and incubated for 10 minutes at room temperature. Absorbance for each sample was measured at 540 nm on an Ensight multimode plate reader (PerkinElmer). The readings were then converted to TAG concentrations using standard curves, prepared with triglycerides standard (Randox, 1352TR CAL Standard).

Measurement of transcript levels using qPCR

Several tissue samples containing 3 dissected guts, brains, or heads were collected for each condition or genotype. These samples were then homogenized in 2-ml Eppendorf tubes filled with lysis buffer containing 1% beta-mercaptoethanol, utilizing a TissueLyser LT bead mill (Qiagen) with 5-mm stainless steel beads (Qiagen #69989). RNA extraction was carried out with the NucleoSpin RNA kit (Macherey-Nagel, #740955) following the guidelines provided by the manufacturer. cDNA was synthesized using the High-Capacity cDNA Synthesis kit (Applied Biosystems, #4368814). Quantitative PCR was performed with RealQ Plus 2x Master Mix Green (Ampliqon, #A324402) using a QuantStudio 5 (Applied Biosystems) instrument. Gene expression results were normalized to the housekeeping gene *Rp49* using the delta-delta-Ct method. The specific oligonucleotides used are given in Supplementary Table 2.

Statistics

Statistical analyses were performed using the Prism software package (GraphPad, version 10). Data were tested for normality before assessments of significance. For data following a normal distribution, pairwise analyses were conducted using two-tailed unpaired Student's t-tests, and comparisons involving multiple samples used one-way ANOVA with subsequent post-hoc tests for multiple comparisons. Non-normally distributed data were analyzed using two-tailed unpaired Mann-Whitney U tests or one-way Kruskal-Wallis ANOVA, followed by tests for multiple comparisons. Additionally, interactions between genotype and diet were calculated using 2-way ANOVA. All plots represent the mean ± standard error of the mean (SEM).

Data Availability

Reasonable data requests will be fulfilled by the lead author.

Code Availability

The MatLAB scripts used for analyzing sleep are described[10,78].

Acknowledgements

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Author contributions

AM and KR conceived and designed the study. AM, OK, XC, ECC, NA, TK, and AK performed the experiments and analyzed the data. DSS, KVH, and MJT provided input and contributed to editing the manuscript. AM and KR wrote the manuscript.

Competing interests

Authors declare that no competing interests exist.

Supplementary Information

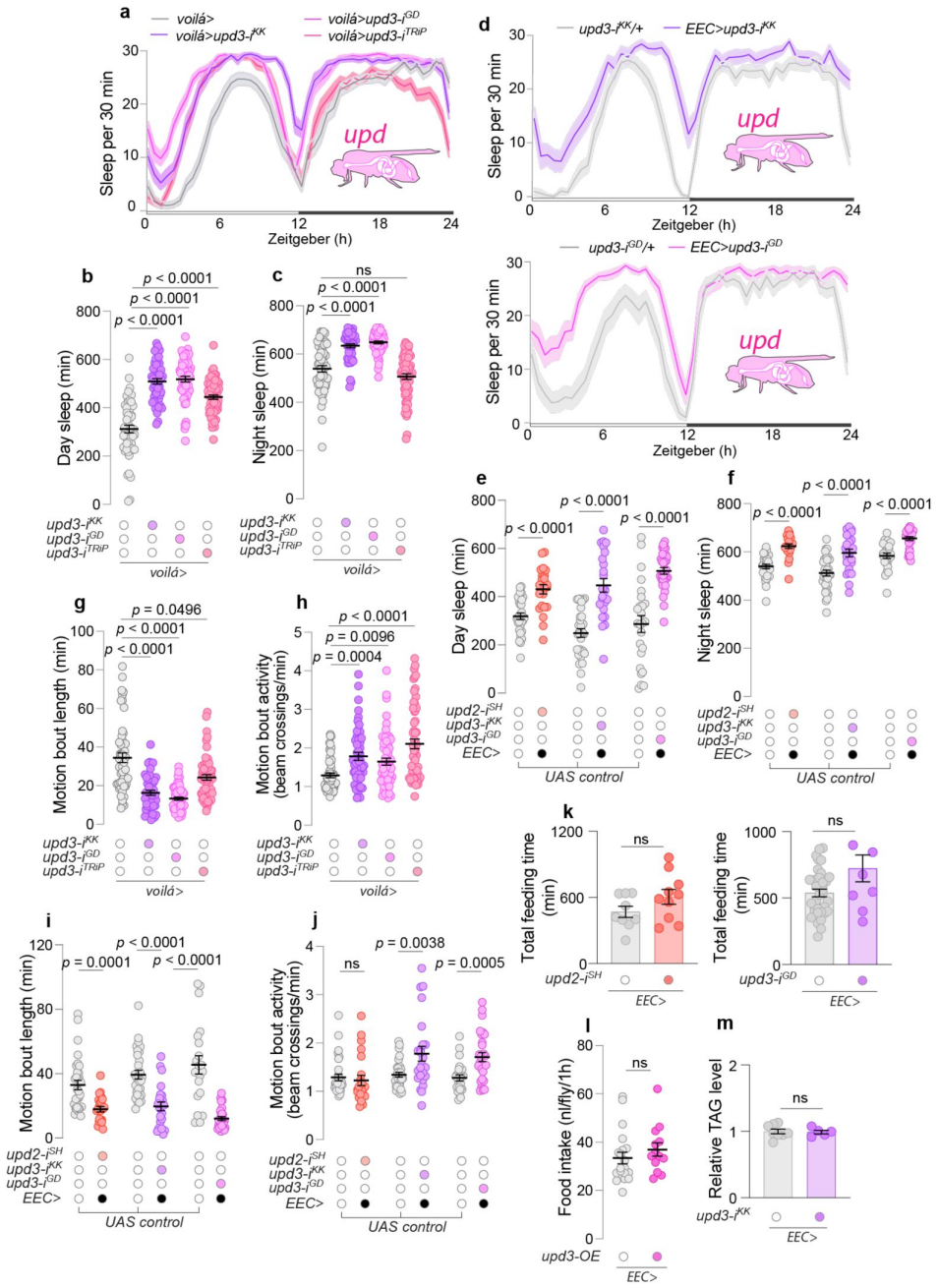


Figure S1.

Effects on sleep, feeding, and metabolic parameters of Unpaired cytokine manipulation in EECs.

a, Twenty-four-hour sleep profile for animals with EEC knockdown of *upd2* and *upd3* using *voilà-GAL4* (*voilà*>). **b**, Day and **c**, night sleep durations for EEC *upd2* and *upd3* knockdown and control groups (N=50-61). **d**, Sleep profiles for EEC-specific *upd3* knockdown using *R57C10-GAL80, Tub-GAL80^{ts}, voilà*> (*ECC*>) and *UAS-upd3-RNAi* controls (N=23-31). **e**, Day and **f**, night sleep durations for UAS-RNAi controls and EEC-specific *upd2* and *upd3* knockdown (N=23-31). **g**, Motion bout length and **h**, motion bout activity for EEC *upd2* and *upd3* knockdown using *voilà*> and control flies (N=49-60). **i**, Motion bout length and **j**, motion bout activity for EEC *upd2* and *upd3* knockdown and UAS-RNAi control flies (N=21-32). **k**, Total feeding time for EEC-specific *upd2* and *upd3* knockdown and control flies using FLIC (N=9-36). **l**, Food intake over 1 hour for animals with EEC-specific *upd3* overexpression (*upd3-OE*) and controls (N=13-20). **m**, Relative triacylglyceride (TAG) levels in EEC-specific *upd3* knockdown and control flies (N=5-8). Statistical analyses were performed using t-tests for panel e, f, and m; Mann Whitney tests for panels i,j, k, and l; Kruskal-Wallis One-Way ANOVA with Dunn's multiple comparison test for panels b, c, g, and h. Data are presented as mean ± SEM. ns, non-significant (p>0.05).

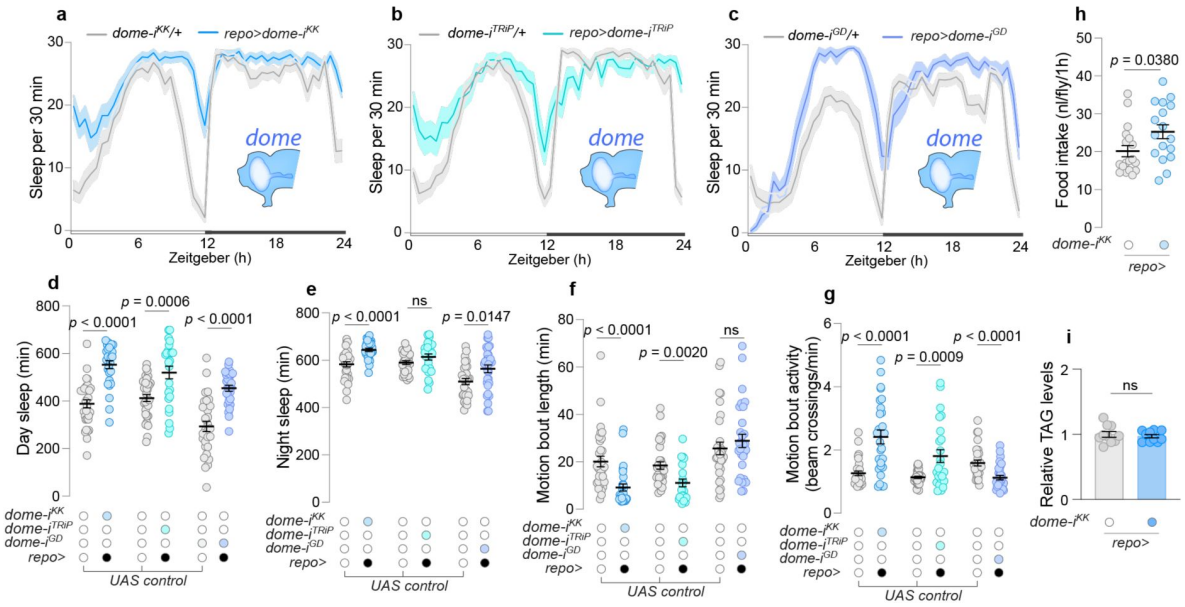


Figure S2.

Characterization of sleep patterns, activity, and metabolic impact of *dome* knockdown in glial cells.

a-c, Sleep profiles over a 24-hour period for animals with glial-specific *dome* knockdown and UAS-RNAi controls (N=25-32). **d**, Total daytime sleep and **e**, nighttime sleep for UAS-RNAi controls and animals with glial-specific *dome* knockdown (N=25-32). **f**, Duration of motion bouts and **g**, motion bout activity for the same groups as in a-d (N=25-32). **h**, Food intake measured over a 1-hour period for animals with *dome* knockdown in glial cells and UAS-RNAi control flies (N=23-31). **i**, Triacylglyceride (TAG) levels relative to controls in glial-specific *dome* knockdown flies. Statistical analyses were performed using Mann Whitney tests for panels d,e, f, g, h, and i, except t-tests, were performed for *dome-i*^{TRiP} versus control in panel d and g, and for *dome-i*^{GD} versus control in panel d. Data are presented as mean ± SEM. ns, non-significant (p>0.05).

Figure S3.

Sleep duration influenced by EEC-specific Unpaired cytokine disruption and dietary changes.

a-**d**, Daytime sleep duration in flies with *upd2* or *upd3* knockdown in EECs and UAS-RNAi controls measured over one day under standard food conditions followed by two consecutive days on 1% H_2O_2-containing food (N=21-32). **e**, Sleep profiles over a 48-hour period for flies with EEC-specific *upd3* knockdown under a transition from standard to 1% agar starvation diet compared to control flies (N=27-32). **f** and **g**, Daytime and nighttime sleep durations for flies with EEC-specific *upd3* knockdown under standard diet and 1% agar starvation diet conditions (N=27-32). Statistical tests: Kruskal-Wallis One-Way ANOVA with Dunn's multiple comparison test was used for panels a, b, c, d; two-way ANOVA with Sidak's multiple comparisons test was used for panels f and g. Data are presented as mean ± SEM. ns, non-significant (p>0.05).

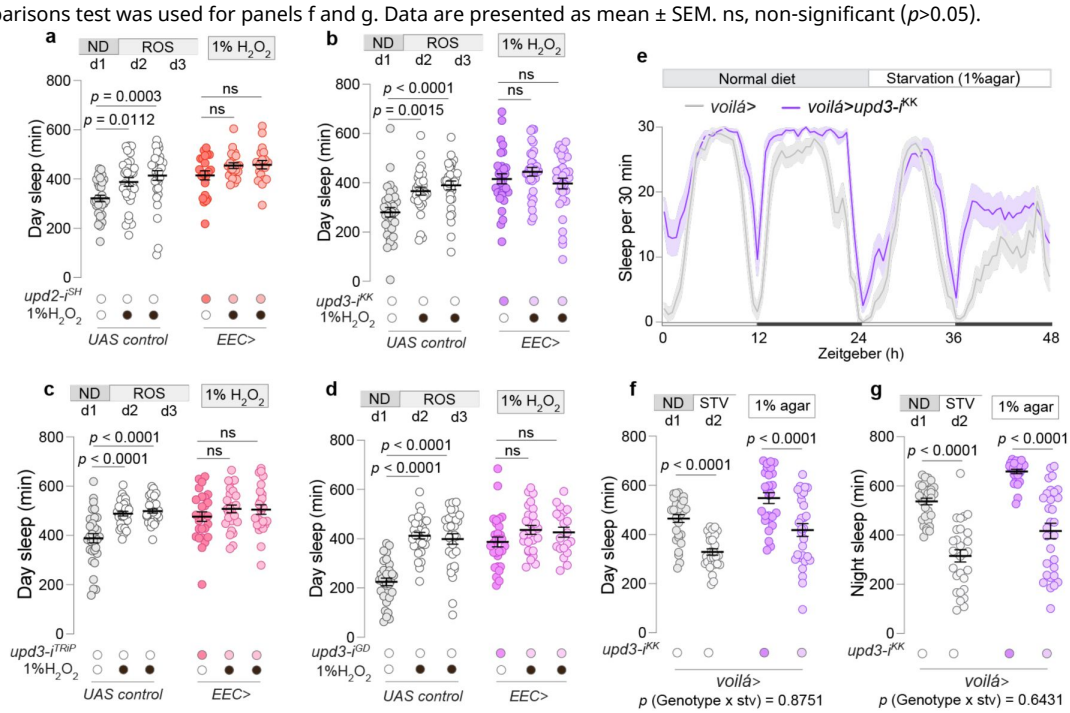
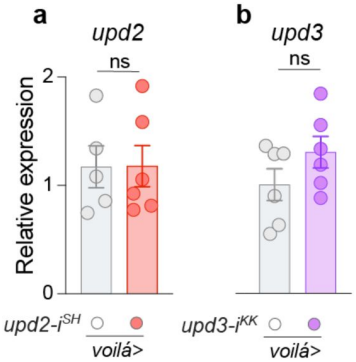


Figure S4.

Expression of upd2 and upd3.

a *upd2* and *upd3*, **b** expression levels in heads of animals with RNAi-mediated knockdown in EECs using *voilá-GAL4* in combination with *Tubulin-GAL80ts* (*voilá*>) (N=6). Statistical analyses were performed using t-tests for panel a and b. Data are presented as mean ± SEM.



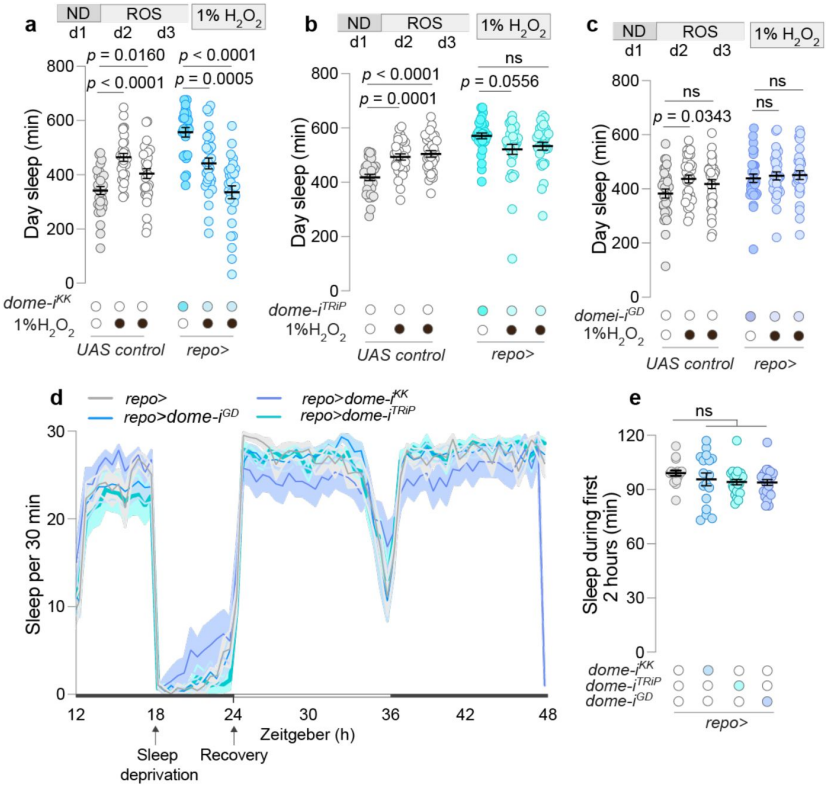


Figure S5.

Impact of glial-specific *dome* knockdown on sleep patterns post oxidative stress and after sleep deprivation.

a-**c**, Daytime sleep measured over three days, starting with a normal diet, followed by a day with 1% H_2O_2-supplemented food to induce oxidative stress, and concluding with a return to a normal diet to assess recovery, in flies with UAS-RNAi constructs without *dome* knockdown (UAS-RNAi controls) and those with glial-specific *dome* knockdown (N=25-31). **d**, Sleep profiles during a night that included a 6-hour period of sleep deprivation, followed by a recovery phase, in control flies and those with glial-specific *dome* knockdown (N=17-24). **e**, Sleep quantity measured during the first 2 hours of the recovery period following sleep deprivation in control flies and those with glial-specific *dome* knockdown (N=17-24). Statistical tests: Kruskal-Wallis One-Way ANOVA with Dunn's multiple comparison test for panels a, b, and e; Ordinary one-way ANOVA with Dunnett's multiple comparison test for panel c. Data are presented as mean ± SEM. ns, non-significant (p>0.05)

Figure S6.

AstA signaling from EECs promotes wakefullness.

a, *AstA-R2* expression in heads of flies with knockdown of *AstA-R2* in BBB glia using *moody-GAL4* (*moody>*) compared to the control group (N=5). **b**, Sleep profiles over two consecutive days, encompassing one day under standard diet conditions followed by one day on oxidative stress conditions, induced by 1% H_2O_2-containing food, in animals with BBB glial-specific knockdown of *AstA-R1* or *AstA-R2* and controls (N=30-32). **c**, Daytime sleep durations over two consecutive days, encompassing one day under standard diet conditions followed by one day on oxidative stress conditions, induced by 1% H_2O_2-containing food, in animals with BBB glial-specific knockdown of *AstA-R1* or *AstA-R2* and controls (N=30-32). **d**, Sleep profiles and **e**, daytime sleep on standard food in animals with *AstA* knockdown in $AstA^+$ EECs and UAS-RNAi controls (N=30-31). Statistical tests used: Unpaired t-tests for panel a; two-way ANOVA with Sidak's multiple comparisons test was used for panel b; Unpaired t-tests for panel d. Data are presented as mean ± SEM.

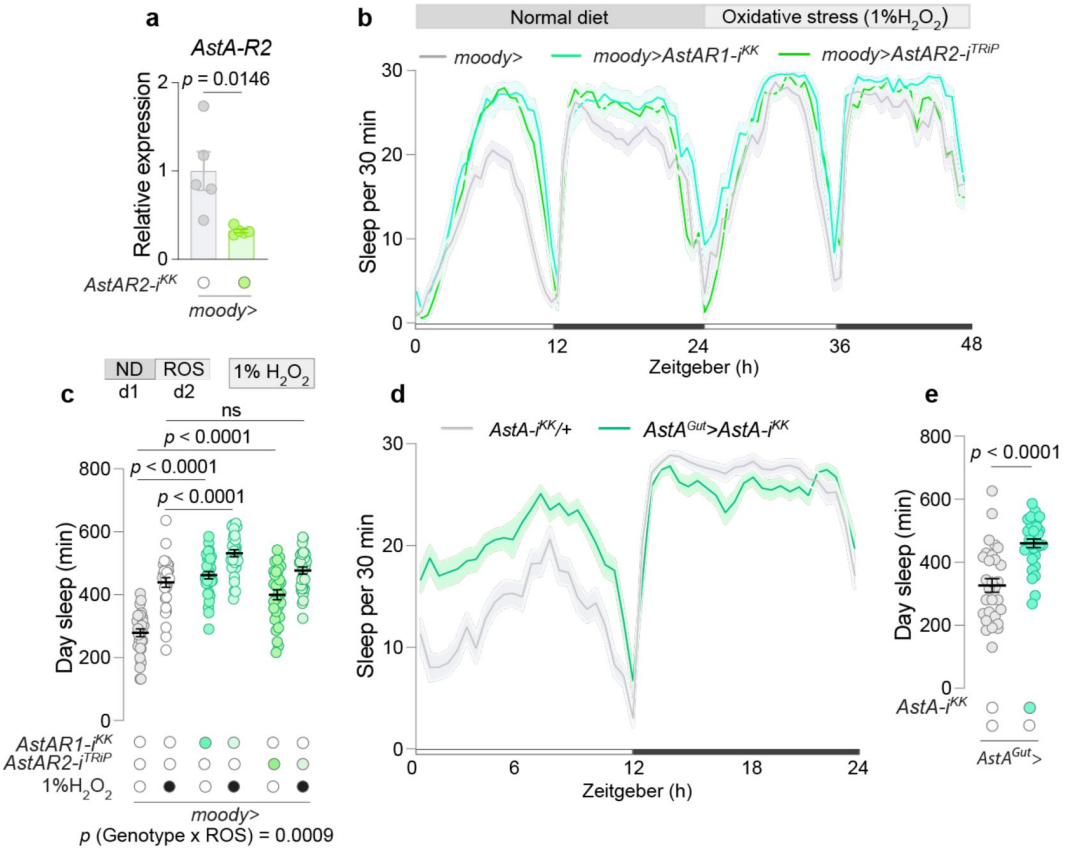


Table S1

Oligos used for cloning the *upd2* and *upd3* CRISPR constructs, with gRNA sequences indicated in bold.

Gene	Sequence
upd2fwd	CGGCCCGGGTTCGATTCCCGGCCGATGCA**TTCTCGCCCGCTCGATTGGT**GTTTCAGAGCTATGCTGGAAAC
upd2rev	ATTTTAACTTGCTATTTCTAGCTCTAAAAC**CATGCAACAGTCACTGACGA**TGCACCAGCCGGGAATCGAACC
upd3fwd	CGGCCCGGGTTCGATTCCCGGCCGATGCAGACAACTGAACTGAACCGACGTTTCAGAGCTATGCTGGAAAC
upd3rev	ATTTTAACTTGCTATTTCTAGCTCTAAAACTTTGGTTCTGTAGATTCTGCTGCACCAGCCGGGAATCGAACC

Gene	Primer	Sequence
upd2	Forward	CGGAACATCACGATGAGCGAA
upd2	Reverse	TCGGCAGGAACTTGTACTCG
upd3	Forward	TGTCGAGAAGAACAAGTGGCG
upd3	Reverse	CGTGGCGAAGGTTCAACTGT
dome	Forward	CTCACGTCTCGACTGGGAAC
dome	Reverse	AGAATGGTGCTTGTCAGGCA
AstAR1	Forward	GCCACTGGAAACGGTAGTATC
AstAR1	Reverse	CGTGTGTTCCGAGGTGAATG
AstAR2	Forward	CGCAGTGTCCAGTACCTGATT
AstAR2	Reverse	GAGCGAATGGGATGAACCAC
Rp49	Forward	AGTATCTGATGCCCAACATCG
Rp49	Reverse	CAATCTCCTTGCGCTTCTTG

Table S1

List of qPCR primers

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Joint Public Review

Summary:

The authors sought to elucidate the mechanism by which infections increase sleep in Drosophila. Their work is important because it further supports the idea that the blood-brain barrier is involved in brain-body communication, and because it advances the field of sleep research. Using knock-down and knock-out of cytokines and cytokine receptors specifically in the endocrine cells of the gut (cytokines) as well as in the glia forming the blood-brain barrier (BBB) (cytokines receptors), the authors show that cytokines, upd2 and upd3, secreted by entero-endocrine cells in response to infections increase sleep through the Dome receptor in the BBB. They also show that gut-derived Allatostatin (Alst) A promotes wakefulness by inhibiting Alst A signaling that is mediated by Alst receptors expressed in BBB glia. Their results suggest there may be additional mechanisms that promote elevated sleep during gut inflammation.
The authors suggest that upd3 is more critical than upd2, which is not sufficiently addressed or explained. In addition, the study uses the gut's response to reactive oxygen molecules as a proxy for infection, which is not sufficiently justified. Finally, further verification of some fundamental tools used in this paper would further solidify these findings making them more convincing.

Strengths:

(1) The work addresses an important topic and proposes an intriguing mechanism that involves several interconnected tissues. The authors place their research in the appropriate context and reference related work, such as literature about sickness-induced sleep, ROS, the effect of nutritional deprivation on sleep, sleep deprivation and sleep rebound, upregulated receptor expression as a compensatory mechanism in response to low levels of a ligand, and information about Alst A.

(2) The work is, in general, supported by well-performed experiments that use a variety of different tools, including multiple RNAi lines, CRISPR, and mutants, to dissect both signal-sending and receiving sides of the signaling pathway.

(3) The authors provide compelling evidence that shows that endocrine cells from the gut are the source of the upd cytokines that increase daytime sleep, that the glial cells of the BBB are the targets of these upds, and that upd action causes the downregulation of Alst receptors in the BBB via the Jak/Stat pathways.

Weaknesses:

(1) There is a limited characterization of cell types in the midgut which are classically associated with upd cytokine production.

(2) Some of the main tools used in this manuscript to manipulate the gut while not influencing the brain (e.g., Voilà and Voilà + R57C10-GAL80), are not directly shown to not affect gene expression in the brain. This is critical for a manuscript delving into intra-organ communication, as even limited expression in the brain may lead to wrong conclusions.

(3) The model of gut inflammation used by the authors is based on the increase in reactive oxygen species (ROS) obtained by feeding flies food containing 1% H2O2. The use of this model is supported by the authors rather weakly in two papers (refs. 26 and 27): The paper by Jiang et al. (ref. 26) shows that the infection by Pseudomonas entomophila induces cytokine responses upd2 and 3, which are also induced by the Jnk pathway. In addition, no mention of ROS could be found in Buchon et al. (ref 27); this is a review that refers to results showing that ROS are produced by the NADPH oxidase DUOX as part of the immune response to pathogens in the gut. Thus, there is no strong support for the use of this model.

(4) Likewise, there is no support for the use of ROS in the food instead a direct infection by pathogenic bacteria. Furthermore, it is known that ROS damages the gut epithelium, which in turn induces the expression of the cytokines studied. Thus the effects observed may not reflect the response to infection. In addition, Majcin Dorcikova et al. (2023). Circadian clock disruption promotes the degeneration of dopaminergic neurons in male Drosophila. Nat Commun. 2023 14(1):5908. doi: 10.1038/s41467-023-41540-y report that the feeding of adult flies with H2O2 results in neurodegeneration if associated with circadian clock defects. Thus, it would be important to discuss or present controls that show that the feeding of H2O2 does not cause neuronal damage.

(5) The novelty of the work is difficult to evaluate because of the numerous publications on sleep in Drosophila. Thus, it would be very helpful to read from the authors how this work is different and novel from other closely related works such as: Li et al. (2023) Gut AstA mediates sleep deprivation-induced energy wasting in Drosophila. Cell Discov. 23;9(1):49. doi: 10.1038/s41421-023-00541-3.

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