Title: Sleep Circuits and Physiology in Non-Mammalian Systems

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Abstract:

Research over the last 20 years has firmly established the existence of sleep states across the animal kingdom. Work in non-mammalian animal models such as nematodes, fruit flies, and zebrafish has now uncovered many evolutionarily conserved aspects of sleep physiology and regulation, including shared circuit architecture, homeostatic and circadian control elements, and principles linking sleep physiology to function. Non-mammalian sleep research is now shedding light on fundamental aspects of the genetic and neuronal circuit regulation of sleep, with direct implications for the understanding of how sleep is regulated in mammals.

Introduction:

Sleep is an evolutionarily ancient behavior observed in every animal species that has been extensively studied [1], even those that lack a centralized nervous system, such as jellyfish [2]. In these diverse taxa, sleep is defined as a rapidly reversible period of quiescence that is characterized by a decreased sensitivity to sensory stimuli. Other core features of sleep that are usually observed across species include species-specific

sleep postures and locations, the regulation of sleep timing by the 24-hour circadian clock, and the homeostatic modulation of sleep depth and amount as a function of the prior time spent awake (Table 1). The use of behavioral criteria to define sleep has allowed for the expansion of sleep research over the past 20 years to include tractable genetic model systems, such as *Caenorhabditis elegans*, *Drosophila melanogaster*, and zebrafish (*Danio rerio*) (Box 1). Insights from these models have led to a greater appreciation for the ancient evolutionary conservation of sleep regulation and function, especially at the genetic level. For example, Shaker potassium channels regulate sleep amount from flies to mammals [3,4], and Salt-inducible kinase 3 (Sik3) modulates the phosphorylation of synaptic proteins to affect sleep homeostasis from nematode worms to mice [5]. Although some aspects of mammalian sleep physiology, such as the interaction between thermoregulation and sleep [6], are unlikely to be broadly conserved, those elements of sleep physiology that constitute and regulate the core functions of sleep will be found across phylogeny.

Despite these advances, one area of sleep research in which non-mammalian models have somewhat lagged behind is the characterization of the neural underpinnings of sleep. In mammals, circuit-level understanding of sleep has rapidly progressed on two main fronts. First, a combination of electrophysiological recordings, lesion studies, and optogenetic or chemogenetic manipulations have identified numerous populations of sleep-active, sleep-driving neurons, including the GABAergic neurons of ventrolateral preoptic area (VLPO) [7,8], the parafacial zone [9], the zona incerta [10], the ventral tegmental area [11], and the nucleus accumbens [12], as well as glutamatergic, neurotensin-expressing neurons of the amygdala, midbrain, and

brainstem [13-15]. Second, electroencephalographic (EEG) recordings have long identified sleep features of brain activity, such as the slow-wave oscillations observed during non-REM sleep, and rapid progress has been made on understanding both the neuronal contributions to and the functions of these circuit oscillations [16-18]. However, recent work, particularly in the Drosophila and zebrafish models, has begun to identify cellular and circuit level mechanisms that are crucial for converting circadian and homeostatic sleep need into sleep behavior. Consistent with the ancient evolutionary conservation of sleep, new experiments show that at least some of these sleep-regulatory processes are also present in mammals.

This review summarizes the insights into sleep physiology and function gleaned over the past few years from these sleep models. We focus on two particular aspects of sleep physiology: 1) conserved circuits of circadian and homeostatic sleep regulation, and 2) advances in the characterization of neural activity and brain-states during sleep in non-mammalian systems.

Conserved Schematics of Sleep-Regulatory Circuits

Drosophila

Numerous cell populations have been identified in the Drosophila brain that are capable of regulating sleep and wake states, including neurons of the mushroom body, peptidergic cells of the pars intercerebralis, and neurons the project to the dorsal fan-shaped and ellipsoid bodies. Most of these have been well-reviewed elsewhere [19], so

we focus here on likely conserved mechanisms by which circuits regulate the homeostatic and circadian control of sleep.

ExFl2 neurons of the dorsal fan-shaped body

Neurons known as ExFl2 project to the dorsal fan-shaped body (dFB) and are strongly sleep-promoting when activated [20]. This and other features of the dFB neurons, including the expression of the arthropod-specific inhibitory neuropeptide, allatostatin, which signals through the Drosophila ortholog of the vertebrate Galanin receptor [21], sensitivity to the anesthetic isoflurane [22,23], and direct inhibition by a wake-promoting circuit [24], have prompted the suggestion that dFB neurons are analogous to the mammalian sleep-regulatory neurons of the VLPO. Moreover, because manipulations that prevent dFB neurons from switching into an electrically active state leads to insomnia and defects in sleep homeostasis, the dFB neurons have been suggested to represent the output arm of the Drosophila sleep homeostat [25], a role also recently suggested for zebrafish [26] and mammalian [27] Galanin preoptic neurons.

Electrophysiological measurements of dFB neuronal excitability has revealed that these cells switch between an electrically silent state (OFF) during waking and an excitable state during sleep (ON). The excitability of dFB neurons also tracks changes in homeostatic sleep pressure, showing increased excitability after sleep deprivation [25]. These observations, combined with behavioral and genetic manipulations, have allowed for rapid progress in identifying key components of the ON-OFF (sleep-wake) and OFF-ON (wake-sleep) transitions of these sleep-driving dFB neurons.

The wake promoting neurotransmitter, dopamine, which is produced by two sets of dFB-projecting neurons of the PPL1 and PPM3 clusters [24,28], initiates the switch of the sleep-promoting dFB neurons from the electrically active, ON state to the silent OFF state [29]. This inhibition is via the direct activation of either Dop1R1 [24] or Dop1R2 [29] receptors on dFB neurons, which signals a long-lasting switch in membrane excitability caused by both a reduction in the potassium current conducted by the voltage-gated potassium channels Shaker and Shab and an increase in potassium leak currents generated by a novel two-pore domain potassium channel called Sandman. Thus, when dFB neurons are in the ON (sleep-promoting) state, the Shaker/Shab generated A-type currents predominate. In response to dopamine, the Sandman leak channel current predominates, rapidly switching the dFB neurons into the OFF state of wakefulness [29].

More recently, a surprising mechanism for the dFB switch from the silent OFF state to the electrically excitable ON state was discovered (Figure 1). This OFF-ON switch is also regulated through modulation of the Shaker potassium conductance of dFB neurons, through a redox-sensing $NADP^+$/NADPH binding site located in Shaker's beta subunit, Hyperkinetic [30]. When dFB neurons are in the silent OFF state during wakefulness, the low demand for ATP causes the leak of electrons from the mitochondrial transport chain and the generation of reactive oxygen species (ROS). This change in the redox state of dFB neurons leads to a long-lasting oxidation of the NADPH bound to Hyperkinetic, which will ultimately augment the firing rate of dFB neurons by slowing the inactivation of the Shaker-dependent currents once the inhibitory Sandman leak current is released. The signal responsible for Sandman's

release is not yet known [30], although the removal of Sandman from the plasma membrane is likely accomplished via the activity of the Rho-GTPase Crossveinless-C [25].

What emerges from these observations is a sleep homeostasis model in which the redox state of Hyperkinetic-bound NADPH acts as a “memory” of ROS generated during wake by mitochondria of OFF dFB neurons. Upon switching to the electrically active ON state, this redox memory would then dissipate until a dopaminergic cue arrives to revert dFB neurons back into the OFF state. Since the redox state of silent OFF-state dFB neurons will correlate with total waking time, in principle, dFB neurons may intrinsically contain many, if not all, of the components necessary to act as a homeostatically-regulated sleep switch. Changes to the amount of homeostatic sleep need, which in Drosophila can be modulated by starvation [31], courtship [32], and neuronal manipulations [33], could be achieved by alterations to the reaction rates along this cascade within dFB neurons, including mitochondrial ROS production, NADPH-$NADP^+$ exchange on Hyperkinetic, Sandman localization to and from the plasma membrane, or the sculpting of the strength of Shaker currents.

R5 neurons of the ellipsoid body

Another neuronal population in the Drosophila brain involved in sleep homeostasis are the R5 (previously called R2, see [34]) neurons of the Drosophila ellipsoid body, a part of the fly brain’s central complex that is involved in computing position and orientation within space [35,36]. When stimulated, these neurons do not acutely drive sleep; instead, rebound sleep is observed after the cessation of forced R5 neuronal activation [37]. During prolonged wakefulness, the activity of these R5 neurons

increases, and changes in synaptic strength via increased NMDA receptor and Bruchpilot expression causes a switch into burst firing mode following sleep deprivation. These features of the R5 neurons suggest that their electrical activity and synaptic properties represent a homeostatic integrator circuit component that encodes sleep pressure.

Both dFB and R5 neurons participate in a recurrent, reciprocal circuit, although the interaction between these populations is not a direct connection (Figure 2). Via signaling through the allatostatin receptor R1 (an ortholog of vertebrate Galanin receptors), the dFB neurons inhibit a population of neurons called Helicon cells [21], which coordinate visual responses to locomotor output. Helicon cells also directly activate the sleep homeostatic R5 neurons, providing a potential circuit mechanism by which dFB neurons, when active during sleep, may act to dissipate the build-up of sleep pressure encoded in R5 neurons by inhibiting R5 activity. Conversely, R5 neurons indirectly activate dFB neurons and require an intact dFB to drive rebound sleep. Thus, the continuous activity of R5 neurons during sleep deprivation may convey an animal's sleep pressure state to dFB neurons, which drive the animal to sleep and in turn silence R5 activity through Helicon cell modulation. In this way, as proposed in [21], dFB and R5 neurons may participate in a relaxation oscillator circuit, in which the continuous buildup of sleep pressure is converted into a binary read-out (e.g. dFB neuron ON during sleep and OFF during wakefulness). How this circuit-level regulation may interact with the dFB-intrinsic redox-Hyperkinetic sleep pressure mechanism remains unclear.

Circadian input onto sleep homeostasis circuits

How do these fly sleep regulatory circuits receive circadian signals to sculpt sleep timing across the 24-hour day? One dorsal set of circadian clock neurons, the DN1s, is a mixed population of clock output neurons with several sleep/wake regulatory roles. For example, DN1 cells feed back onto other clock neurons to shape the timing and duration of the Drosophila mid-day siesta [38]. A subset of DN1 neurons also project to the pars intercerebralis to modulate Diuretic Hormone 44 producing neurons and drive wakefulness via a spike pattern temporal code [39,40]. A second set of molecularly distinct anteriorly projecting DN1 neurons, also called Anterior-Projecting Dorsal Neurons (APDNs), send both excitatory and inhibitory signals onto subsets of TuBu neurons, which are then sufficient to induce sleep via indirect and direct synaptic connectivity with the sleep homeostatic R5 neurons of the ellipsoid body [41,42]. Thus, direct circuitry from APDN to R5 neurons conveys circadian cues onto sleep-homeostasis centers in the fly brain (Figure 2).

Other sleep regulatory signals

Finally, in addition to circadian and homeostatic cues, the dFB/ellipsoid body sleep circuits may also serve as substrates upon which other sleep regulatory cues act. For example, the antimicrobial peptide NEUMURI drives increased sleep and may participate in changes in sleep in response to immune modulation [43]. Although NEUMURI does not activate nor require dFB neurons to drive changes in sleep, it does accumulate in the fan-shaped body and thus may be acting on downstream circuits involved in this pathway. The neurotransmitter serotonin is also linked to sleep/wake regulation in Drosophila, although, as in other species, this regulation is complex. For example, enhancement of serotonin signaling increases sleep, while disruption of its

synthesis decreases sleep and disrupts sleep homeostasis [44,45]. Sleep promoting serotonin (5-HT) signaling has been linked to the mushroom body via 5HT1A receptors [45] and to dFB neurons via 5HT2b receptors [44]. However, another serotonin signaling mechanism acts through 5HT7 receptors in the ellipsoid body (albeit not on the R5 homeostatic neurons) to promote the fragmentation of sleep without affecting overall sleep duration [46].

Zebrafish

At least three sleep-homeostasis regulating neuronal populations have been recently discovered in the zebrafish—Galanin-expressing neurons [26], neurons that produce the RF-amide Neuropeptide VF (NPVF) [47], and the serotonergic neurons of the raphe [48]. The full extent to which these populations interact with zebrafish wake-promoting circuits of Hypocretin (Hcrt) [49], norepinephrine neurons of the locus coeruleus [50], the arousing neuropeptide Neuromedin U (Nmu) [51], and Insulin-like Growth Factor [52], or with other sleep-promoting signals, such as the circadian sleep-output signal melatonin [53], the locus coeruleus-inhibiting Neuropeptide Y [54], or even each other is not yet clear and has been reviewed elsewhere [55]. Additionally, the discovery of sleep regulatory circuits in mammals continues in tandem with elucidation of their conservation in zebrafish. One example is the recent characterization of neurotensinergic sleep regulatory neurons in mouse thalamo-amygdalar, midbrain and brainstem circuits [13-15], which foreshadowed the discovery of neurotensin's sleep regulatory role in zebrafish [56]. Nonetheless, interesting parallels exist between zebrafish sleep homeostasis circuitry those in both Drosophila and mammalian species (Figure 3).

Galanin neurons

Galanin is an inhibitory neuropeptide expressed in a cluster of neurons in the zebrafish preoptic area and a scattered set of cells in the hypothalamus. In response to increased brain activity, generated by either acute administration and wash-out of wake-promoting drugs such as caffeine or forced prolonged wakefulness, Galanin neurons become more active and *galanin* expression is induced [26]. Galanin induction strongly correlates with both the magnitude of brain activity during prior wakefulness as well as with the duration of subsequent rebound sleep, suggesting that *galanin* is sensitive to sleep pressure and increases sleep in response. Indeed, Galanin itself is critical for the homeostatic sleep rebound response, as mutants that lack *galanin* fail to increase sleep in response to increased sleep pressure. Interestingly, under baseline light:dark conditions, *galanin* mutants sleep only modestly less than siblings, suggesting that the mechanisms that govern baseline sleep and rebound sleep after deprivation may not be identical. Galanin neurons are also required for other sleep behavioral phenomena, including the light-dependent induction of sleep by the neuropeptide Prokineticin 2 [57]. Thus, Galanin neurons may represent a hub of sleep behaviors mediated by both homeostatic and other cues (Figure 3).

The requirement for Galanin neurons in the homeostatic regulation of sleep has also been recently substantiated in mice. Conditional ablation of Galanin neurons in the rodent median preoptic area had a minimal effect on total sleep but strongly blocked the induction of both rebound sleep as well as homeostatic increases in slow-wave delta

power after sleep deprivation [27]. Thus, experimental evidence in zebrafish predicted the subsequent discovery of a mammalian sleep phenomenon. Furthermore, these findings provide insight into the potential mechanisms underlying the hypothesized role of Galanin neurons in human sleep regulation [58].

Serotonergic raphe

Another set of neurons in the zebrafish brain that have been implicated in sleep are the serotonergic neurons of the raphe. Genetic deletion of tryptophan hydroxylase 2 (Tph2), the enzyme that exclusively produces serotonin in the raphe, leads to reductions in sleep and weaker responses to sleep deprivation [48]. Consistent with a sleep-promoting role, ablation of these neurons also reduced sleep, while optogenetic activation of these neurons increased sleep. Curiously, however, electrophysiological recordings revealed these neurons are also most active during the day, when zebrafish are predominantly awake. It has therefore been proposed that the activity of the serotonergic raphe tracks wake time and homeostatic sleep need. The zebrafish serotonergic raphe might therefore be analogous to the R5 neurons of the Drosophila ellipsoid body, whose activity also tracks homeostatic sleep need. If so, examination of the signaling and functional relationships between the serotonergic raphe and Galanin neurons, which are analogous to the dFB sleep homeostat output neurons, is warranted (Figure 3).

The wake-active, sleep-inducing properties of serotonergic raphe neurons are also conserved in mammals. The role of serotonin in mammalian sleep had been

controversial, because these neurons are predominantly wake active. Revisiting this topic with GCaMP imaging revealed that mouse raphe neurons are indeed wake active; however, selective ablation of these neurons without affecting the thermoregulatory medullary raphe (a likely confound of previous experiments) both increased wakefulness and impaired the homeostatic response to sleep deprivation [48]. Consistently, optogenetic induction of tonic firing led to increased sleep, although induction of burst firing led to increased transitions to wakefulness. Thus, regulation of sleep by the serotonergic raphe is conserved in mice and zebrafish.

RFamides, and EGFR signalling

A class of neuropeptides called RFamides (which contain a C-terminal Arginine-Phenylalanine motif) has been implicated in regulating sleep in both *C. elegans* and Drosophila [59,60]. Two RFamides, QRFP [61] and NPVF [47] have also been shown to drive sleep in zebrafish. NPVF is expressed in a small cluster of glutamatergic neurons of the dorsomedial hypothalamus. Optogenetic activation of these neurons induces sleep, while their ablation increases wake [47]. NPVF neurons in zebrafish are required for the full induction of sleep by Epidermal Growth Factor Receptor (EGFR) signaling, as mutants that lack NPVF have weakened responses to EGFR activation [62]. Similarly, EGFR signaling in both *C. elegans* and Drosophila induces sleep in an RFamide dependent manner [59,60]. In Drosophila, EGF-induced sleep is mediated by the pars intercerebralis [63], suggesting similarities between this Drosophila circuit and the zebrafish EGFR-NPVF pathway.

Prompted by findings in zebrafish, variants in EGFR signaling components were found to affect sleep structure in human genome-wide association studies [62],

providing yet another example of zebrafish results having a direct impact on insights into human and mammalian sleep.

Neurophysiology of Sleep: Brain States and Neural Activity in Non-Mammalian Systems

Given the centrality of the brain and nervous system to sleep processes, and of sleep to neural and cognitive function, it is clear that neural activity is at the core of the generation and functional importance of sleep. Thus, features of neural activity that are readily identifiable in electrophysiological recordings of mammalian and avian species have occupied a predominant position in thinking about sleep states and behaviours. However, this focus on electrophysiological recording of sleep-related neural activity has been an impediment to efforts to exploit alternative animal models of sleep, as the diversity of brain structural organization across taxa results in little apparent correspondence between EEG or extracellular field recordings in mammals and other species. Indeed, until relatively recently recordings of sleep-linked neural activity in non-mammalian species were limited, although some exciting progress has been made in the last few years.

Early experiments in Drosophila used extracellular recordings to identify a broadband decrease in local field potential power in the central brain during sleep compared to wake [64,65]. They also found electrophysiological signatures corresponding to specific sleep periods, such as a prominent 7-10 Hz oscillation early within sleep bouts [66]. However, extracellular recording in non-mammalian models like

Drosophila is severely limited because the activity of neurons that are either small in number, form specific sub-populations within larger structures, or are not spatially arranged in an open field conformation, is largely invisible to extracellular field recordings [67]. To overcome these limitations, a recent, innovative study took advantage of advances in voltage imaging tools to optically record the activity of the R5 neurons during sleep and wake states [68]. This imaging revealed slow-wave like oscillations that arise from co-ordinated UP-DOWN phases in individual R5 neurons. In line with sleep pressure, both the magnitude of the membrane potential oscillations within individual cells and the interneuronal synchrony of these UP-DOWN oscillations were altered to generate prominent compound population oscillations in the <4 Hz range. Furthermore, this synchronous oscillation was essential for the maintenance of elevated sensory thresholds during sleep [68]. This unveiling of slow-wave activity at the level of single neuronal and population activity in the sleeping insect brain suggests that such sleep-related oscillatory activity might be fundamental to sleep regulation and its functions.

The power of these optical imaging approaches can be used to maximum advantage in sleep models that are optically transparent, such as the nematode *C. elegans*, where *in vivo* imaging approaches have allowed for comprehensive recording of neuronal activity during sleep and waking behaviour [69,70]. For example, whole-animal calcium imaging in *C. elegans* during developmental sleep found a broad suppression of neuronal activity during sleep bouts [70]. This study was also able to systematically map individual GABAergic or peptidergic sleep-active neurons, including

several already ascribed a sleep function through genetic experiments [71,72]. As this whole-brain recording was performed at single-cell resolution, computational analysis could describe whole-animal neuronal population dynamics associated with progression through and transitions between behavioural states [70].

The larval zebrafish is another model whose optical transparency has been leveraged to perform whole-brain calcium imaging approaches during a variety of behavioural tasks, including hunting [73,74], optomotor responses [75], and spontaneous alternations between exploration and exploitation behaviours [76]. To date, few zebrafish imaging studies have investigated sleep. One recent study employed light-sheet imaging to simultaneously record brain and spinal cord neuronal activity, muscle activity, eye movements, and heart rate to replicate mammalian polysomnography techniques, an approach that was dubbed fluorescence polysomnography [77]. This approach was able to detect and characterise altered behavioural- and brain-states following extremely long-duration (3 days) continuous physical sleep deprivation, pharmacological perturbations, and genetic manipulations of melanin-concentrating hormone (MCH) signalling. While the large seizure-like propagating waves they observed would in principle be detectable by *in vivo* electrophysiological field potential recordings [78], the role of periventricular, non-neuronal cells in initiating these waves (which was also reported in other zebrafish models of seizures [79]), would be difficult to identify with electrophysiological methods. An important next step will be to use similar imaging methods to characterize physiological sleep states and natural wake-sleep transitions.

Functional imaging also has the power to elucidate the nature of sleep states in mammalian brains, which will allow for a greater understanding of neuronal dynamics beyond that attainable with electrophysiology alone. A good example is the use of *in vivo* calcium imaging in mice to reveal that neocortical activity during REM sleep is globally suppressed relative to either waking or slow-wave sleep [80], a finding that contradicts previous conclusions derived from electrophysiological studies that neocortical cells generally fire action potentials at a higher rate during REM [81]. As our ability to comprehensively and simultaneously record more neurons at cellular resolution grows, conclusions and assumptions based on older, more limited tools will likely need systematic re-evaluation.

To summarise, non-mammalian species are particularly suited to *in vivo* imaging approaches that facilitate the recording of the neuronal activity that underlies the electrophysiological measurements traditionally used to characterise brain state, sleep stages, and sleep-related neural activity. These approaches furthermore allow for the identification of phenomena related to sleep physiology, sleep function, and sleep regulation that escape investigation by electrophysiological means, either because they arise from cell types (e.g. non-neuronal cells [82,83]) or cell populations (e.g. neurons that contribute little to the extracellular field [67]) that do not produce a clear electrophysiological signal. Imaging also facilitates the examination of other sleep-related phenomena, such as changes in properties of the extracellular space [84] or the sleep-linked augmentation of neuronal chromatin dynamics that is associated with

nuclear maintenance and DNA repair [85]. The potential of *in vivo* imaging will be augmented in tandem with the advent of both novel imaging modalities and enhanced imaging technologies, such as multiphoton light-sheet imaging [86,87], luminescent voltage indicators, [88], sensors of oxidative stress [30], or indicators of neuropeptidergic transmission [89]. Applying these techniques to sleep in non-mammalian systems promises to uncover both global and local changes in neuronal and non-neuronal dynamics that underpin evolutionarily conserved physiology and functions of sleep.

Declaration of Interest

The authors declare they have no conflict of interest.

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Figure Legends

Figure 1. A redox sensor that links metabolism to sleep homeostasis. A) In Drosophila, during waking, the dFB neurons are electrically silent, which is reinforced by a potassium leak channel, Sandman. Sandman is translocated to the plasma membrane in response to a wake-promoting dopamine (DA) signal. In this silent state,

free electrons from the mitochondria generate reactive oxygen species (ROS). This leads to an exchange of NADPH to $NADP^+$ bound to the Hyperkinetic (beta subunit) of Shaker potassium channels, which can act as a “memory” of sleep pressure accumulated during wakefulness. B) In response to an unknown signal, Sandman is removed from the plasma membrane, and the dFB neurons switch into an electrically excitable state. Fewer ROS will be generated, allowing for the Hyperkinetic-bound $NADP^+$ to exchange for NADPH, thereby dissipating the “memory” of sleep pressure.

Figure 2. Drosophila sleep pressure circuits. The wake-promoting dopamine (DA) inhibits dFB neurons, while circadian clock sleep information is relayed to the R5 neurons of the ellipsoid body. The dFB and R5 neurons participate in a recurrent feedback network to regulate sleep amount and responses to homeostatic sleep pressure.

Figure 3. Zebrafish sleep pressure circuits. While it remains unclear how classic wake-promoting Norepinephrine (NA), Hypocretin (Hcrt), and Neuromedin U (Nmu) signals relay information to sleep regulatory neurons, Galanin neurons are required for light-dependent induction of sleep by Prok2 (negative masking). Whether the sleep homeostatic Serotonin (5-HT), Galanin, and NPVF neurons participate in recurrent feedback loops as in Drosophila remains speculative. Also unclear is how circadian sleep cues converge on this network, although melatonin synthesis is required for rhythmic sleep in zebrafish larvae.

Table 1. Evolutionary conservation of behavioral and physiological aspects of sleep.

a) Criteria used for the experimental definition of sleep. The behavioral criteria apply universally. Although present in other species, electrophysiological correlates of sleep are currently primarily used only in mammals and birds to define sleep.

* Although *C. elegans* lack sleep circadian rhythms, the timing of developmental sleep is regulated by an ortholog of the clock protein, Period.

b) Sleep regulatory mechanisms that have been found through research in non-mammalian models and subsequently found to be conserved in other taxa.

c) Currently understood characteristics of sleep-related neural activity that exist in both mammalian and non-mammalian species.

Box 1. Quantifying sleep in non-mammalian model organisms

Sleep Behavior

Accurately distinguishing sleep from wake depends on assessment of two of the criteria that define sleep, namely behavioral quiescence and the level of responsiveness to sensory stimuli. In mammals, experimental assessment of sleep-wake state is commonly based on measurement of electrophysiological correlates of behavioral states. However, in organisms where characterisation of brain electrophysiology has been unavailable, sleep-wake state has been assessed by measuring visible behaviours (primarily locomotion), with periods of immobility longer than a specified duration being considered as sleep bouts. In a given species, this threshold duration is defined based on the minimal period of immobility that is associated with a significant decrease in animals' responses to sensory stimuli (e.g. Drosophila: [90,91]; Zebrafish:

[49]; *C. elegans*: [92]).These criteria are necessarily probabilistic and their accuracy and precision will depend on the specifics of the behavioural tracking system used (e.g. [93]). Although progress is being made in the identification of the neurophysiological correlates of sleep behaviours in non-mammalian models (See “Neurophysiology of Sleep: Brain States and Neural Activity in Non-Mammalian Systems” section below), behavioral tracking will continue to underpin powerful, high-throughput approaches for monitoring sleep-wake states in non-mammalian systems. Indeed, the advent of sophisticated, high-resolution analysis of behaviour from video tracking [94-97] suggests that such approaches, potentially benchmarked to electrophysiological recordings, may also become more widely used in mammalian sleep research.

Sleep Homeostasis

Sleep homeostasis is the observed phenomenon that deficits in sleep are followed by an increase in the duration and intensity of sleep. In mammals, specific electrophysiological correlates (primarily slow-wave activity, approximately <4Hz) of this process have been identified in the neocortex [98]. These correlates are commonly used to track sleep pressure within periods of wake and subsequent sleep.

However, such electrophysiological signatures of homeostatic sleep drive have proven difficult to identify in non-mammalian species (but see [68,99,100] and “Neurophysiology of Sleep: Brain States and Neural Activity in Non-Mammalian Systems” below). Therefore, the study of sleep homeostasis and homeostatic sleep drive in non-mammalian model systems has largely depended on studying changes in sleep duration, consolidation, and depth subsequent to sleep deprivation. This sleep rebound has been demonstrated in Drosophila [101], zebrafish [26,102,103] and *C.*

elegans, [92], whereby sleep deprivation is followed by an increase in total sleep duration and sleep intensity (the proportion of a defined post-deprivation period spent asleep), and in a decreased sensitivity to sensory stimuli.

Interpretation of sleep pressure and sleep homeostasis subsequent to sleep deprivation (achieved by extending wake time) can often be complicated by the interaction of the induced homeostatic sleep drive with circadian influences, which can affect both sleep behaviour and neurophysiological correlates of sleep pressure [104]. Recently however, an approach was developed in the zebrafish larva model that allows for decoupling of elevated sleep drive from total waking time [26]. Acute, reversible, and time-limited (approximately 1 hour) increases in neural activity are induced pharmacologically, resulting in dramatic increases in sleep drive and rebound sleep behaviour. By acutely generating sleep pressure, this approach avoids the need to extend the waking period to deprive animals of sleep, and so potentially allows for the disentangling of the effects of the homeostatic and circadian mechanisms of sleep regulation.

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Papers of Special Interest:

25. Donlea, J.M., Pimentel, D., Talbot, C.B., Kempf, A., Omoto, J.J., Hartenstein, V., and Miesenbock, G. (2018). Recurrent Circuitry for Balancing Sleep Need and Sleep. Neuron 97, 378-389 e374.

[*] This study identified a neuronal circuit in Drosophila that may coordinate the homeostatic control, sensory, and motor aspects of sleep. dFB neurons, previously shown to operate in a homeostatic sleep switch, inhibit ‘helicon’ cells via inhibitory allatostatin signaling. This induces the reduction of visually-evoked locomotion that is associated with sleep, and also inhibits helicon cells’ excitation of sleep homeostasis neurons.

26. Reichert, S., Pavon Arocas, O., and Rihel, J. (2019). The Neuropeptide Galanin Is Required for Homeostatic Rebound Sleep following Increased Neuronal Activity. Neuron 104, 370-384 e375.

[**] This study in larval zebrafish found that pharmacological manipulations of brain activity increased homeostatic sleep pressure independently of prior wake time. Genetic manipulations revealed a critical role of the neuropeptide Galanin in converting homeostatic sleep pressure into sleep behavior.

30. Kempf, A., Song, S.M., Talbot, C.B., and Miesenbock, G. (2019). A potassium channel beta-subunit couples mitochondrial electron transport to sleep. Nature 568, 230-234.

[**] This paper describes the effect of redox state on the firing properties of sleep-regulating dFB neurons. Via several innovative manipulations of intra-neuronal reactive oxygen species, this study builds a model in which changes to the internal redox state of dFB neurons during wake are sensed by NADPH/$NADP^+$ co-factor binding to a K^+ channel subunit, thereby altering electrical excitability and the switch into a sleep-promoting state.

40. Tabuchi, M., Monaco, J.D., Duan, G., Bell, B., Liu, S., Liu, Q., Zhang, K., and Wu, M.N. (2018). Clock-Generated Temporal Codes Determine Synaptic Plasticity to Control Sleep. Cell 175, 1213-1227 e1218.

[**] This paper shows that the circadian modulation of clock neuron firing patterns generates a temporal code that influences downstream arousal neurons via input pattern-dependent synaptic plasticity, thereby controlling the consolidation of sleep behavior.

43. Toda, H., Williams, J.A., Gulledge, M., and Sehgal, A. (2019). A sleep-inducing gene, nemuri, links sleep and immune function in Drosophila. Science 363, 509-51

[**] This study identified an anti-microbial peptide, *nemuri*, as a promotor of Drosophila sleep. This represents a mechanism by which the response to infection and other stressors might be linked to increased sleep.

48. Oikonomou, G., Altermatt, M., Zhang, R.W., Coughlin, G.M., Montz, C., Gradinaru, V., and Prober, D.A. (2019). The Serotonergic Raphe Promote Sleep in Zebrafish and Mice. Neuron 103, 686-701 e688.

[**] Initially in zebrafish and then in mice, this paper showed that tonic serotonergic output from raphe neurons is sleep promoting but that burst firing can drive wakefulness. The paper suggests that raphe firing during waking may encode the buildup of homeostatic sleep pressure, thereby reconciling conflicting data of the serotonergic raphe's role in sleep.

70. Nichols, A.L.A., Eichler, T., Latham, R., and Zimmer, M. (2017). A global brain state underlies C. elegans sleep behavior. Science 356.

[**] This work used whole-brain, cellular-resolution calcium imaging in *C. elegans* to characterize the global dynamics of neuronal activity during sleep and wake behavioral states.

77. Leung, L.C., Wang, G.X., Madelaine, R., Skariah, G., Kawakami, K., Deisseroth, K., Urban, A.E., and Mourrain, P. (2019). Neural signatures of sleep in zebrafish. Nature 571, 198-204.

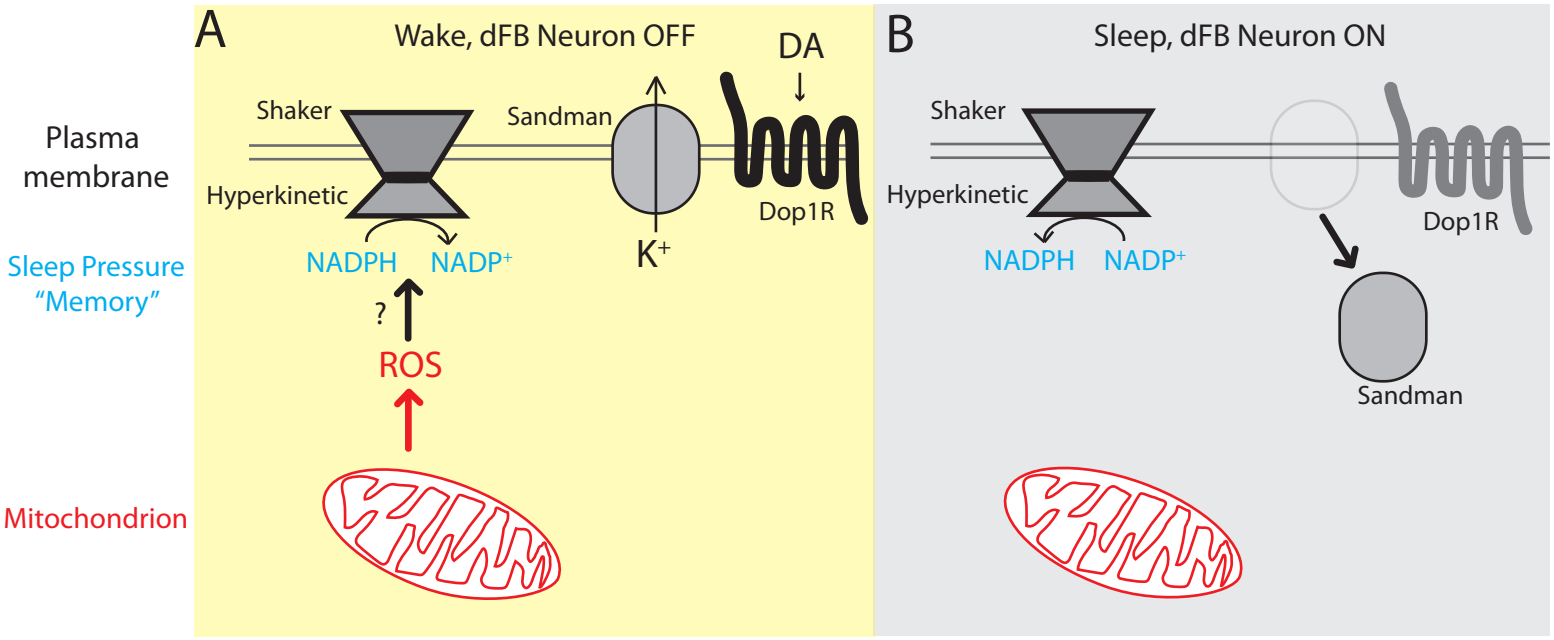
[*] This paper implemented imaging-based analogues of the electrophysiological measurements used in mammalian polysomnography in the larval zebrafish and characterized changes in neurophysiology after a several-days' long mechanical sleep deprivation.

85. Zada, D., Bronshtein, I., Lerer-Goldshtein, T., Garini, Y., and Appelbaum, L. (2019). Sleep increases chromosome dynamics to enable reduction of accumulating DNA damage in single neurons. Nat Commun 10, 895.

[**] Using *in vivo* imaging in larval zebrafish, this paper showed that sleep is associated with increases in neuronal chromosome dynamics and the reversal of DNA double-strand breaks that occur during waking activity.

Table 1

		Features	Characteristics	Mammals		Ray finned fish	Insects	Nematode worms	Jellyfish
				Humans	Rodents	Zebrafish	Drosophila melanogaster	C. elegans	Cassiopea
(a)	Sleep Definition	Immobility	During sleep animals display reduced movement, often in species-specific postures or locations	YES	YES	YES	YES	YES	YES [2]
		Elevated Sensory Threshold	Sleep is associated with reduced sensitivity to sensory stimuli	YES	YES	YES	YES	YES	YES [2]
		Rapid Reversibility	Sufficiently strong stimuli will rapidly wake an animal	YES	YES	YES	YES	YES	YES [2]
		Homeostatic Regulation	Sleep deprivation induces a rebound in sleep duration and intensity	YES	YES	YES	YES	YES	YES [2]
		Circadian Regulation	Sleep predominates at specific periods, with this timing regulated by circadian clock mechanisms	YES	YES	YES	YES	YES *	YES [2]
		Electrophysiological Signature	A specific set of measurements of neuronal or muscular activity is used to identify sleep	YES	YES	NO	NO	NO	NO
(b)	Sleep Regulatory Mechanisms	Sleep induction by EGFR signalling	RF-amide signalling mediates a major part of EGFR's role in sleep regulatio	YES [62]	?	YES [62]	YES [60,63]	YES [105,106]	?
		Homeostatic Regulation - Sleep induction via Galanin receptor homologs	Transmission from Galanin/allatostatin-A neurons is necessary for rebound sleep induction by increased homeostatic sleep pressure	?	YES [27]	YES [26]	YES [21]	?	?
		Homeostatic Regulation - Serotonin	Serotonergic neurons are predominantly wake-active, but serotonergic signalling promotes sleep and homeostatic sleep rebound	?	YES [48]	YES [48]	YES [44,45,46]	?	?
		Homeostatic Regulation - ROS mechanisms	Sleep-promoting neurons' activity is regulated by mitochondrial ROS production	?	?	?	YES [30]	?	?
(c)	Sleep Physiology and Functions	Unit neuronal activity	Sleep is characterized by an overall decrease in single-unit neuronal activity in the brain	YES	YES	?	YES [64,65]	YES [70]	?
		Sub-threshold population oscillatory activity	Slow subthreshold oscillations of neurons' membrane potential, which sum to generate population oscillations. Population oscillatory power is dependent on synchronisation of these neurons' oscillations and reflects sleep pressure.	YES	YES	?	YES [68]	?	?


A
Wake, dFB Neuron OFF
DA
Shaker
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Hyperkinetic
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B
Sleep, dFB Neuron ON
Shaker
Hyperkinetic
Dop1R
NADPH
NADP+
Sandman
Plasma membrane
Sleep Pressure "Memory"
Mitochondrion

Figure 1

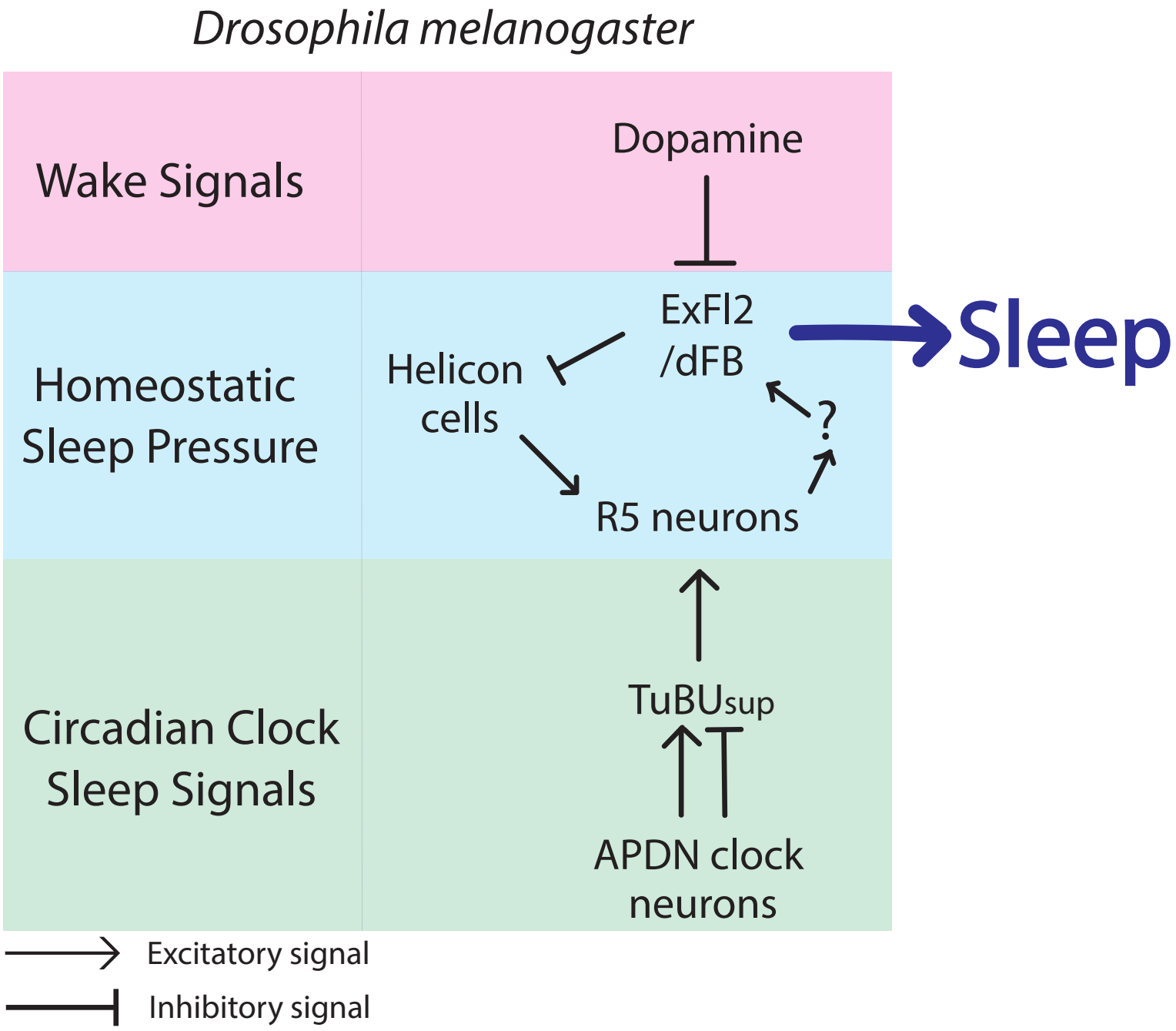
Drosophila melanogaster
Wake Signals
Dopamine
Homeostatic Sleep Pressure
ExFl2 /dFB
Sleep
Helicon cells
?
R5 neurons
Circadian Clock Sleep Signals
TuBUsup
APDN clock neurons
Excitatory signal
Inhibitory signal

Figure 2

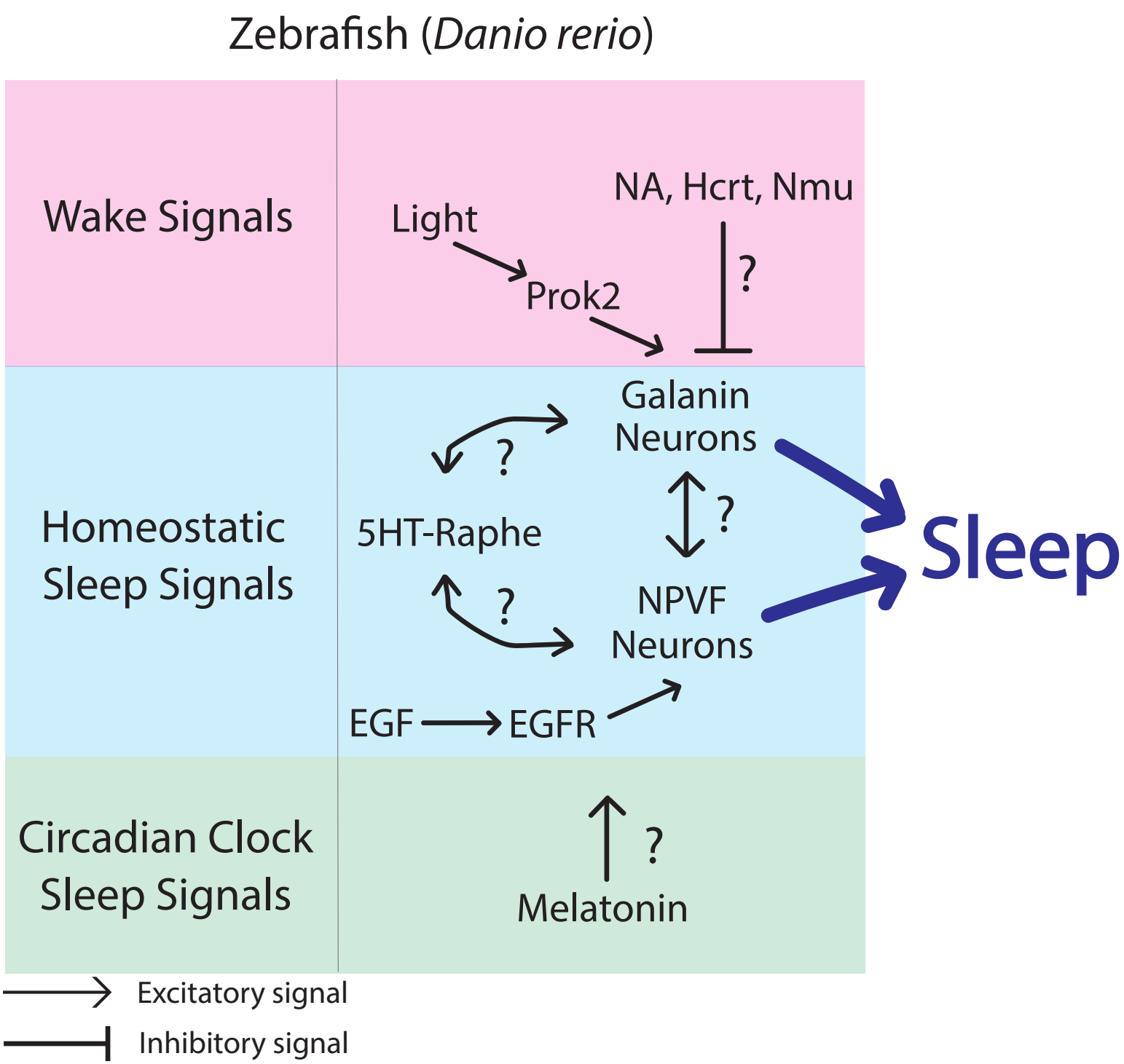
Zebrafish (*Danio rerio*)
Wake Signals
Light
Prok2
NA, Hcrt, Nmu
?
Homeostatic Sleep Signals
Galanin Neurons
?
?
5HT-Raphe
?
NPVF Neurons
Sleep
EGF
EGFR
Circadian Clock Sleep Signals
?
Melatonin
Excitatory signal
Inhibitory signal

Figure 3