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A sleep state during *C. elegans* development

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Abstract

Caenorhabditis elegans is the simplest animal shown to sleep. It sleeps during lethargus, a larval transition stage. Behavior during lethargus has the sleep properties of a specific quiescent posture and elevated arousal threshold that are reversible to strong stimulation and of increased sleep drive following sleep deprivation. Genetic similarities between sleep regulation during *C. elegans* lethargus and sleep regulation in other animals point to a sleep state that was an evolutionarily ancestor to sleep both in *C. elegans* and other animals. Recent publications have shed light on key questions in sleep biology: (1) How is sleep regulated? (2) How is sensory information gated during sleep? (3) How is sleep homeostasis mediated? (4) What is the core function of sleep?

Our detailed understanding of the mechanism by which organisms keep the time of day derives to a large extent from work in genetically-tractable organisms such as *Neurospora* and *Drosophila* [1,2]. But in contrast to circadian rhythms, which can be explained largely by cell autonomous molecular interactions, sleep behavior is an emergent property of cellular communications [3]. The importance of understanding sleep in context of neural circuits has been appreciated for nearly a century, when discrete neuronal groups regulating human sleep and wake were described [4]. As such, *Caenorhabditis elegans* provides a powerful model system for the study of sleep, given its compact, well-described nervous system, consisting of 302 neurons [5]. A simple and defined neural network coupled with powerful molecular genetic tools endows this system with unique advantages for determining the molecular processes driving sleep with single-cell resolution.

C. elegans sleep shows similarities to sleep in other animals

The gold standard for assessing sleep in mammals is measurement of cerebral cortex electrical activity using an electroencephalogram (EEG) [6]. Since EEG signals are a function of the structure of the vertebrate brain, they do not translate to invertebrates. Instead, the following behavioral criteria are used to define sleep [7]: 1) there is behavioral quiescence, an absence of both locomotive and feeding movements, with the animal assuming a specific posture; 2) there is a heightened arousal threshold. Both the behavioral quiescence and the elevated arousal threshold are reversible by strong stimulation; 3) depriving the animal of sleep results in an increased sleep drive (homeostasis) [8]; and 4) the quiescent episodes are episodic and are regulated by a conserved molecular clock. These criteria are satisfied by behavior during *C. elegans* lethargus, a stage that occurs before each

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of the four molts during the animal's larval development (Fig 1). Recent publications have shed light on the mechanisms of each of these phenomena.

Specific posture

With the geometry of a flexible rod, there are a limited number of postures *C. elegans* can assume. Nonetheless, differences exist between bouts of lethargus quiescence and active locomotion. During locomotion, the animal typically assumes a sinusoidal posture, whereas during lethargus the average curvature of the worm is reduced as the animal straightens its body [9]. This reduction in curvature is specific to lethargus quiescence and not simply a function of reduced locomotion, as during bouts of reduced locomotion outside of lethargus animals do not show this property [9]. Moreover, whereas outside of lethargus the bending of the body is distributed throughout the animal, during lethargus quiescence bouts the bends are concentrated closer to the anterior end of the animal [9]. Outside lethargus, the animals show frequent feeding movements and move their noses nearly constantly, presumably in order to sample the environment; During lethargus, feeding stops and the foraging nose movements essentially cease [10].

A key feature of lethargus that distinguishes it from sleep in other animals is its association with the molt, the shedding of an old cuticle and the secretion of a new one [11]. One trivial explanation for the lethargus-specific quiescent posture is that the molting process restricts movement and bending of the animal. Several lines of evidence argue that this is not the case. First, while the animal spends a significant fraction of the time during lethargus quiescent, it has brief bouts of locomotion, where it assumes the sinusoidal posture of an awake animal [9,12]. Second, using genetically-encoded calcium indicators (GECI), Schwarz and colleagues demonstrated that muscle calcium levels are reduced during lethargus [10], providing a physiological correlate to the behavioral quiescence. Finally, Iwanir and colleagues showed using GECI that a key interneuron regulating backwards locomotion has reduced activity during lethargus quiescent bouts, just as it does during forward locomotion bouts during wakefulness [9]. These measurements of physiological activity show that quiescent behavior during lethargus is a consequence of active regulation by the nervous system, much like mammalian sleep.

Behavioral quiescence during lethargus

The molecular mechanisms regulating lethargus quiescence have only begun to be identified. Raizen and colleagues identified a role for EGL-4, a cyclic guanine monophosphate (cGMP)-dependent protein kinase, during the regulation of lethargus quiescence [12]. *egl-4* mutant animals display a reduction of quiescence, suggesting that increases in cGMP second messenger levels support sleep, a mechanism conserved in *Drosophila* [12,13]. Another phylogenetically conserved mechanism is the wake promoting effects of increased signaling by cyclic adenosine monophosphate (cAMP) dependent-protein kinase (PKA) [14]. Increased PKA activity, which results from loss-of-function of the cAMP phosphodiesterase PDE-4 [12], gain-of-function of the adenylyl cyclase ACY-1 [12], or loss-of-function of the PKA regulatory subunit KIN-2 [58], causes strong reductions in quiescence during lethargus.

Van Buskirk and Sternberg have described a role in quiescence regulation for epidermal growth factor (EGF) signaling, which has been implicated in sleep in mammals and in *Drosophila* [15–17]. Elevated expression of EGF induces quiescence by signaling through the EGF receptor, phospholipase-C (PLC), and diacylglycerol (DAG). The response to EGF occurs in a single neuron called ALA. The mechanism by which ALA in turn induces quiescence of locomotion and feeding is unknown. However, it may involve the release of neuropeptides, as mutations in genes required for peptidergic neurotransmission block the

quiescence-inducing effects of EGF [18] and ALA has anatomical features of a peptidergic neuron [5]. Since removal of ALA has only small effects on lethargus quiescence [18], there are likely to be additional sleep-inducing neurons that have not yet been identified (Fig 1).

Singh and colleagues have identified a quiescence-regulating role for Notch signaling. Elevated expression of a Notch co-ligand OSM-11 causes anachronistic behavioral quiescence and reduction in Notch signaling decreases quiescence during lethargus [19]. OSM-11 is expressed in the epidermis of the animal yet the Notch receptors appear to function in neurons, pointing to an interaction between neural and non-neural tissues in the regulation of quiescence.

Other types of quiescence

While during typical cultivation conditions, quiescence of feeding or locomotion is observed only during lethargus, adult quiescence has been described following specific environmental manipulations. These include conditions of satiety (which can be induced by a cycle of starvation and then feeding) [20], heat stress [21], exposure to chemical toxicants [21] or general anesthetics [22], infection [23] and extended duration without food while housed in liquid [24]. The regulation of satiety quiescence has both common and distinct features with the regulation of lethargus quiescence [20]. Infection-induced quiescence is regulated by neural signaling [23], though whether this signaling has molecular similarities with the regulation of lethargus quiescence remains to be determined.

Elevated sensory arousal threshold

Reduced responsiveness is a key property of sleep that distinguishes it from quiet wakefulness. In mammals, both anatomical and physiological mechanisms play a role in the gating of sensory information. For example, eyelid closure attenuates the intensity of light reaching the retina and reduced activity of the thalamus places a gate on sensory information that can reach the cortex [25]. In *C. elegans* too there are both anatomical and physiological mechanisms to sensory gating.

During lethargus, an extracellular material forms a plug at the opening to the buccal cavity [11]. The plug prevents food and other material from entering the pharynx and thus minimizes sensory stimulation of pharyngeal neurons. Likely more important is a neuronal process of sensory gating. Multiple lines of evidence suggest that one key site of regulation is in the sensory neurons themselves.

First, *egl-4* mutants show increased responsiveness to sensory stimulation during lethargus suggesting that EGL-4 gates sensation during lethargus by dampening signaling in primary sensory neurons. In support of this notion are studies showing a role for EGL-4 in sensory neurons during non-lethargus periods in the adaptation to prolonged exposure to odorants [26]. The defect in sensory gating during lethargus is rescued in *egl-4* mutants by restoring expression of the gene in sensory neurons [12]. Second, Notch signaling, which is required for sensory gating during lethargus, functions in ciliated sensory neurons through an EGL-4-dependent mechanism [19].

Third, evidence that dampening occurs in sensory neurons was shown by Schwarz and colleagues, who observed a reduction in calcium levels in mechanosensory neurons during lethargus [27]. In addition, calcium increases in mechanosensory neurons in response to mechanical stimuli are attenuated during the quiescent lethargus stage [27]. It will be of future interest to test whether these physiological changes in sensory neurons are perturbed in *egl-4*, Notch, and PKA signaling mutants.

Homeostasis

The phenomenon of homeostasis, which refers to the maintenance of sleep amount or depth following sleep deprivation, indicates that sleep serves a fundamental function that cannot be bypassed [28]. In rats and in *Drosophila*, prolonged sleep deprivation is lethal [29,30], providing evidence for an essential function of sleep. Driver and colleagues showed that lethargus quiescence too is essential, since *C. elegans* animals forced to move continuously during lethargus die [31].

The mechanisms of sleep homeostasis remain poorly understood but genetic experiments suggest that baseline sleep and the response to sleep deprivation are partially under different regulation. *Drosophila* and mice strains have been described that have a normal duration of baseline sleep yet a defective response to sleep deprivation [32,33]. Based on the observation that mammalian sleep deprivation is stressful [32], Driver and colleagues [31] investigated the role in sleep homeostasis of the gene *daf-16*, which encodes a FOXO transcription factor that is activated in response to stressful stimuli by movement from the cytoplasm into the nucleus [35]. They found that deprivation of lethargus quiescence causes DAF-16 translocation into the nucleus and that *daf-16* mutants are defective in the behavioral response to deprivation. Surprisingly, the *daf-16* sleep homeostasis defect was corrected by restoring *daf-16* function in muscle cells and not neurons. This finding suggests that sleep regulation is not entirely in the nervous system, as is commonly believed [36].

Iwanir and colleagues observed a positive correlation between the duration of individual quiescent bouts during lethargus and the duration of prior motion bouts, suggesting that sleep is regulated homeostatically on an episode-by-episode basis [9]; that it shows micro homeostasis. This positive correlation was lost in mutants with a gain-of-function mutation of an adenylate cyclase [9], implicating PKA signaling in micro homeostasis. Where PKA is acting to regulate quiescence and homeostasis is not yet known.

Regulation by a conserved molecular clock

The timing of sleep and wake in most animals is controlled by an internal circadian clock, which can be reset by cues external to the animal such as light or temperature [37]. The circadian clock functions as a cell-autonomous transcriptional-translational feedback loop. A core component of this loop is the protein PERIOD, whose function depends on its cyclical mRNA and protein expression [37]. The *C. elegans* PERIOD-like protein LIN-42 was identified by Jeon et al, who were studying the timing of developmental programs during larval development [38,39]. LIN-42, which is homologous to PERIOD both in the N-terminal PAS domain as well as in a C-terminal nuclear-localization domain [38,39], is required for the proper timing of multiple developmental events including cell migration and division. In *lin-42* mutants, these events occur precociously [39,40]. In addition to the amino acid sequence homology, *lin-42* shows homology in expression dynamics. Like PERIOD, it shows cyclical expression of its mRNA and protein, however, this is relative to the larval molting/lethargus cycle and not to the circadian cycle.

Like these developmental timing events, the timing of lethargus and the molt is also abnormal in *lin-42* mutants. Monsalve and colleagues found that inactivation of *lin-42* results in arrhythmic timing of the molts [41]. Forced expression of LIN-42 results in anachronistic molts and lethargus behavior at inappropriate times [41]. The quiescence-inducing effect of LIN-42 mis-expression was partially abrogated by a mutation that disrupts ALA function as well as by an *egl-4* mutation, implicating both the ALA neuron and EGL-4 as acting down-stream of the quiescence timer (Fig 1). It is unknown whether EGF is an output of the clock and from which cells EGF acts to activate ALA. An important future goal is to identify connections between the clock and sleep regulators.

In addition to PERIOD, several additional conserved components of the circadian clock have been identified in *Drosophila* and in mammals [37]. Genes encoding orthologous proteins are encoded in the *C. elegans* genome [42], though a function in the regulation of lethargus behavior or the molt has not yet been explored.

After completion of larval development, *C. elegans* adults display weak circadian rhythms of behavior [43], metabolism [44], mRNA [45], and oxidation [46]. These rhythms appear to be independent of *lin-42* function [45]. Whether sleep behavior too is under circadian control in adults remains to be determined.

Can *C. elegans* inform our understanding of sleep function?

Experimental prevention of sleep results in death in rats [29], *Drosophila* [30], and *C. elegans* [31]. During sleep, animals are vulnerable to predation and are apparently non-productive. Hence, the evolution of sleep and its persistence across phylogeny is a mystery. A comparison between sleep in diverse species can allow for distillation of core features of sleep that may speak to its function. Among the current leading theories are (1) sleep is a consequence of changes in neural activity required for carrying out certain metabolic functions [47], for example macromolecular biosynthesis [48], and (2) sleep is required for nervous system changes [49].

Events that occur during lethargus inform this debate. The most obvious association with lethargus is the molt, a major biosynthetic event during which a complete new cuticle is synthesized and the old one is shed [11] in a process known as ecdysis (Fig 1). In addition, the DNA content of intestinal nuclei is doubled during lethargus [50], again suggestive of biosynthetic cellular metabolism. Molecular evidence of anabolic activity was provided by analysis of DAF-16/FOXO sub cellular localization during larval development. DAF-16/FOXO, which is negatively regulated by the energetically-responsive kinase TOR [51] and insulin-like growth factors [52], was more cytoplasmic (possibly representing increased anabolic signaling) during lethargus than outside lethargus [31]. This molecular marker of anabolic signaling requires sleep behavior, since its disruption results in movement of DAF-16 into the nucleus [31].

Nervous system changes also occur during lethargus. During the first lethargus stage, GABAergic neurons remove synapses from ventral muscles and form synapses on dorsal muscles [53], and GABAergic synaptic transmission is functionally reduced during lethargus [54]. However, the changes in GABAergic transmission do not depend on sleep behavior but rather, appear coupled to the developmental clock regulating lethargus [54]. Thus, while neural changes do occur in association with lethargus, sleep behavior is not required for these changes. In both *Drosophila* [55,56] and zebrafish [57], synaptic changes are a function of the clock as well as behavioral state with variable contribution of each. Hence, it is possible that the metabolic milieu, which is controlled by a clock and which is conducive to sleep, is also conducive to nervous system plasticity.

Conclusions

These are early days in the study of *C. elegans* sleep. While a few of the signaling pathways and relevant cells regulating lethargus behavior have been identified (Fig 1), much remains to be done. A major push will be to identify the full circuit of neurons regulating sleep and wake behavior. Finding additional sleep/wake genetic modulators will clarify how these cells communicate. For example, stability of behavioral state requires that sleep-inducing neurons inhibit wake-inducing neurons, and vice-versa [3]. Neuropeptides may be involved since the sleep-promoting ALA neuron appears to be peptidergic. Acceleration of progress will require technical advances, for example in the ability to simultaneously record behavior

of several animals [19,58,59], additional analyses of the micro-architecture of lethargus [9], and increased use of optogenetic methods for controlling neural activity. Insight into the function of lethargus behavior and sleep can be gained by examining the consequences of disrupting the behavior.

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Highlights

- *C. elegans* sleep occurs during lethargus, a larval stage transition period.
- Sensory gating during *C. elegans* sleep occurs in sensory neurons.
- *C. elegans* sleep is regulated by both neural and non-neural mechanisms.
- Genetic regulation of *C. elegans* sleep shares features with that of other species.
- Future work will identify cellular interactions regulating *C. elegans* sleep.

Figure 1.

A proposed model for the regulation of sleep during lethargus. Sleep-like quiescence occurs at larval transitions and is followed by ecdysis, the escape from the former stage cuticle. LIN-42/PERIOD sets the timing of lethargus quiescence by activating ALA and other sleep-promoting cells and by inhibiting wake-promoting cells. ALA and other sleep-promoting cells signal to sensory neurons leading to sensory gating which involves an increase in PKG and Notch activity, and decreased calcium. Hatched lines denote direct or indirect connections. Arrows denote activating interactions while perpendicular bars denote inhibitory interactions. The cellular site in which PKA promotes wake is not yet known. The source of EGF that regulates ALA is also unknown.

Table 1

Sleep-like properties of lethargus quiescence

Posture and Quiescence	<ul style="list-style-type: none"> • <u>Posture</u>: Body curvature is reduced; curvature is greater towards the anterior [9]. • Inhibition of feeding, foraging, and locomotion [11] • Brief bouts of movement where the animal takes on the active sinusoidal posture [9,12]. • Reduced calcium levels in muscle [10] and in neurons controlling backward locomotion [9]. • <u>Quiescence</u>: Conserved molecular regulators of lethargus quiescence <ul style="list-style-type: none"> - The cyclic GMP-dependent protein kinase PKG-1 is required for proper quiescence [12]. - Decreased cyclic AMP-dependent protein kinase activity is required for lethargus quiescence [12,53]. - Epidermal growth factor (EGF) over-expression induces behavioral quiescence [18]. <p>Signaling occurs through the EGF receptor LET-23, phospholipase C (PLC), and diacylglycerol (DAG) in a single interneuron, ALA [18].</p> <ul style="list-style-type: none"> - EGF signaling in ALA is required for normal lethargus quiescence [18]. - Over-expression of the Notch ligand OSM-11 induces quiescence anachronistically [19]. - Notch signaling is required for normal lethargus quiescence [19].
Sensory Gating	<ul style="list-style-type: none"> • <u>Structural</u>: Formation of a buccal plug, which likely blocks sensation to the mouth. • <u>Gating in sensory neurons</u>: <ul style="list-style-type: none"> - PKG is required for gating in sensory neurons [12]. - Notch is required in ciliated sensory neurons for sensory gating [19]. - Reduced calcium levels in sensory neurons during lethargus [26].
Homeostasis	<ul style="list-style-type: none"> • Arousal threshold is further increased following sleep deprivation by forced locomotion during lethargus [12,30]. • DAF-16/FOXO is required in muscle for a proper homeostatic response to sleep deprivation [30]. • Duration of individual quiescence bouts is partially predicted by the duration of the preceding motion bouts [9].
Clock	<ul style="list-style-type: none"> • Timing of lethargus quiescence is regulated by LIN-42/PERIOD: <ul style="list-style-type: none"> - mRNA and protein levels of LIN-42 cycle relative to lethargus and the molt [37,38]. - LIN-42 is required for synchronization of lethargus quiescence [40]. - Over-expression of <i>lin-42</i> can induce behavioral quiescence that is ALA and PKG-dependent [40].