Gap Junctions and NCA Cation Channels Are Critical for Developmentally Timed Sleep and Arousal in *Caenorhabditis elegans*

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ABSTRACT An essential characteristic of sleep is heightened arousal threshold, with decreased behavioral response to external stimuli. The molecular and cellular mechanisms underlying arousal threshold changes during sleep are not fully understood. We report that loss of UNC-7 or UNC-9 innexin function dramatically reduced sleep and decreased arousal threshold during developmentally timed sleep in *Caenorhabditis elegans*. UNC-7 function was required in premotor interneurons and UNC-9 function was required in motor neurons in this paradigm. Simultaneous transient overexpression of UNC-7 and UNC-9 was sufficient to induce anachronistic sleep in adult animals. Moreover, loss of UNC-7 or UNC-9 suppressed the increased sleep of EGL-4 gain-of-function animals, which have increased cyclic-GMP–dependent protein kinase activity. These results suggest *C. elegans* gap junctions may act downstream of previously identified sleep regulators. In other paradigms, the NCA cation channels act upstream of gap junctions. Consistent with this, diminished NCA channel activity in *C. elegans* robustly increased arousal thresholds during sleep bouts in L4-to-adult developmentally timed sleep. Total time in sleep bouts was only modestly increased in animals lacking NCA channel auxiliary subunit UNC-79, whereas increased channel activity dramatically decreased sleep. Loss of EGL-4 or innexin proteins suppressed UNC-79 loss-of-function sleep and arousal defects. In *Drosophila*, the ion channel narrow abdomen, an ortholog of the *C. elegans* NCA channels, drive the pigment dispersing factor (PDF) neuropeptide release, regulating circadian behavior. However, in *C. elegans*, we found that loss of the PDF receptor PDFR-1 did not suppress gain-of-function sleep defects, suggesting an alternative downstream pathway. This study emphasizes the conservation and importance of neuronal activity modulation during sleep, and unequivocally demonstrates that gap junction function is critical for normal sleep.

KEYWORDS *Caenorhabditis elegans* sleep; gap junction; cGMP-dependent kinase; NCA channel

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DURING sleep, animals have heightened arousal thresholds due to decreased response and desensitization of specific neurons (Campbell and Tobler 1984; Schwarz *et al.* 2011; Nichols *et al.* 2017). How does this transient and profound state change occur at a molecular level? Previous work has revealed multiple players, including neurotransmitters, neuropeptides, receptors, and kinases (Cirelli 2009; Allada *et al.* 2017). However, genes regulating sleep act in diverse pathways, suggesting that additional critical genes are not yet known and that understanding the relationship between these genes will be critical. Conserved genes regulating sleep can be identified in many model organisms, including *Caenorhabditis elegans* (Trojanowski and Raizen 2016).

C. elegans sleeps spontaneously at specific times or under specific conditions (Van Buskirk and Sternberg 2007; Ghosh and Emmons 2008; Raizen *et al.* 2008; Hill *et al.* 2014). The best characterized sleep states are developmentally timed sleep and stress-induced sleep. Molecular and genetic pathways required for sleep in these paradigms do not completely overlap, although commonalities have been identified (Trojanowski *et al.* 2015). Here, we focus almost exclusively on developmentally timed sleep, which occurs during a 2.5-hr period, called lethargus, coincident with cuticle molting during diapause. During lethargus, *C. elegans* exhibits interspersed bouts of sleep and motion that vary in duration, lasting from seconds to minutes. During sleep bouts, animals spontaneously cease locomotion, assume a specific posture, and show diminished response to external stimuli. During motion bouts, animals resume sinusoidal locomotion and respond robustly to external stimuli (Raizen *et al.* 2008; Singh *et al.* 2011; Iwanir *et al.* 2013). The mechanisms underlying this state change remain elusive.

Three major components of sleep behavior can be defined: circadian regulation, sleep homeostasis, and the state of sleep itself (Campbell and Tobler 1984). At this point, circadian regulation is well described, but the other two are less understood. The molecular pathways that regulate these three components of sleep are likely interlinked and complex. Using an evolutionary and ethological strategy, it may be possible to disentangle these behaviors by studying species that have fewer circadian behaviors. While the state of sleep and sleep homeostasis are conserved in *C. elegans*, many aspects of circadian biology are not conserved. There is no evidence for an endogenous, genetically encoded 24-hr internal clock. *C. elegans* lacks classical proteins involved in light entrainment, like Cryptochrome. However, proteins intimately involved in circadian rhythms in other species, like Period and timeless, have *C. elegans* orthologs that play essential roles in the developmental timing of diapause and lethargus, but not circadian behavior (Banerjee *et al.* 2005; Monsalve *et al.* 2011). Thus far, endogenous *C. elegans* circadian behaviors have not been described, although cyclic gene expression changes can be artificially entrained (van der Linden *et al.* 2010). Other components of sleep are well conserved and a decade of *C. elegans* sleep research has established the utility of this model organism in the field. There is deep conservation of genes and pathways regulating sleep and sleep homeostasis between *C. elegans* and other species (Singh *et al.* 2014; Trojanowski and Raizen 2016). Studies in *C. elegans* have contributed to a growing list of genes affecting sleep, including *egl-4*/cyclic-GMP (cGMP)–dependent kinase, *lin-12/glp-1*/Notch receptors, *aptf-1*/AP2-family transcription factor, and *gpb-2*/G_β protein (Raizen *et al.* 2008; Singh *et al.* 2011; Huang *et al.* 2017b).

Here, we synthesize previous work in the sleep field with the results of an unbiased forward genetic screen for sleep genes in *C. elegans*, revealing a hierarchy of genes involved in sleep and arousal. In a classic genetic screen, we identify a new role for UNC-7 and UNC-9 innexins in sleep and find that these gap junction proteins are likely required downstream of the *C. elegans* cGMP-dependent protein kinase G (PKG), EGL-4. We also find that the NCA cation channels, identified in a mouse genetic screen for sleep genes (Funato *et al.* 2016), regulate arousal and sleep in *C. elegans*. Finally, we determine that EGL-4 PKG, but not *C. elegans* pigment dispersing factor (PDF) signaling, is required downstream of NCA channels to regulate sleep.

Materials and Methods

C. elegans culture and strain information

C. elegans were cultured on standard nematode growth media (NGM) seeded with OP50 *E. coli*. Animals were grown at 25° and assayed at 22° unless noted otherwise. Strains used are listed in Supplemental Material, Table S1.

Microfluidic chamber-based assessment of L4/A sleep

The microfluidic, chamber-based sleep assay was adapted from previous studies (Singh *et al.* 2011). Briefly, kanamycin-treated OP50 was resuspended as described in NGM (without agar) as a food source for the animals in the chambers. Mid-L4 stage animals, L4 substages L4.3–L4.4 (Mok *et al.* 2015), were loaded into each chamber and covered with a glass coverslip, which was sealed to the chip-containing chambers using molten 2% agar. Images were recorded every 10 sec for 12 hr and analyzed using a MATLAB (MathWorks, Natick, MA) script for image subtraction (Singh *et al.* 2011) and a custom Python script for calculating total time in sleep bouts (Huang *et al.* 2017a).

Arousal threshold assessment (touch and light)

Arousal threshold assays with blue light stimulation were conducted as previously described (Huang *et al.* 2017b). Briefly, L4/A lethargus animals were picked to freshly made assay plates with food and allowed to recover for 15 min. During the assay, the plates remained unperturbed with the lids off. A blue light laser pointer was used to stimulate the animal over the head region. Response latency for animals during both sleep bouts and motion bouts was recorded. Examination of *unc-7(e5)* animals suggested possible defects in their response to blue light stimulation during motion bouts. Therefore, arousal thresholds determinations for *unc-7* and *unc-9* studies tested latency to respond to posterior body gentle touch (Singh *et al.* 2011) in both sleep bouts and motion bouts. For all arousal determinations, at least eight animals per genotype and per condition (asleep or in motion) were tested in each trial. At least three independent trials were conducted. The experimenter was blinded to genotype.

PLM imaging

PLM GCaMP6 imaging in roaming animals was done with a custom-made system published earlier (Venkatachalam *et al.* 2016). One L4 animal, either before lethargus or during

lethargus, was picked onto each assay plate (NGM in 100 mm plate seeded with 10 μl OP50). Animals were covered with a 48 × 65 mm #1 coverslip and left unperturbed for between 10 and 20 min before imaging. When imaging, the agar plate was fit into a groove and secured with a custom-made metal plate screwed down on the plate. An automated tapping device will exert controlled tapping when triggered. To avoid desensitization, the interval between tapping for an individual animal was at least 10 min.

Plasmids and transgenic strains

Transgenic strains were generated by microinjecting plasmids into nematodes using standard methods (Evans 2006). Plasmids used and transgene concentrations are listed in Table S2. Phenotypic rescue studies showed dose dependence. For *unc-7* transgenes, high concentrations caused paralysis. For results herein, only transgene concentrations that did not cause paralysis were used.

Transient overexpression using heat shock

Transgenic animals with transgenes under the control of heat shock promoter were picked onto fresh plates at the young adult stage to induce transgene expression. Plates were sealed with Parafilm (Bemis Company) and heat shocked for 75 min in a 33.5° water bath, agar side down. Parafilm was removed and animals allowed to recover in a 20° incubator for 60 min. Then, animals were scored in the next 20-min interval. To avoid disturbing animals, plate lids were kept closed and plates were placed on the dissection scope agar side up (to avoid condensation on the inner surface of the plate lid when scoring). Animals were scored as Ans (in anachronistic sleep) if they did not move spontaneously or pump for 5 sec; either pharyngeal pumping or spontaneous locomotion was sufficient to score an animal as nonAns (not in anachronistic sleep).

To score locomotion and pharyngeal pumping separately, animals were treated as described above. Animals were scored as not moving if they did not move spontaneously for 5 sec, independent of the pumping status. Animals were scored as not pumping if they did not pump for 5 sec, regardless of locomotion. For all heat shock studies, at least 10 animals per genotype were tested for each trial and at least three independent trials were conducted. The experimenter was blinded to genotype.

Statistics

Statistical analyses used Student's *t*-test for most of the studies. Mann–Whitney–Wilcoxon tests were performed for analyses of arousal thresholds with blue light because of the non-normally distributed data.

Data and reagent availability

Strains and plasmids are available upon request, unless otherwise stated. Full data tables are available upon request. Supplemental material available at Figshare: https://doi.org/10.25386/genetics.7187531.

Results

Loss of UNC-7 or UNC-9 gap junction proteins dramatically decreases sleep bouts during lethargus

To identify conserved genes required for *C. elegans* developmentally timed sleep during the transition to adulthood (L4/A lethargus), we previously undertook a forward genetic screen (Huang *et al.* 2017b). Subsequently, we determined that the *unc-7(rt212)* mutant allele, a premature stop mutation (W365Opal, isoform a), caused decreased total time in sleep bouts during L4/A lethargus (total sleep time, Figure S1A). The *unc-7* gene encodes a *C. elegans* gap junction/innexin protein with at least 10 alternative splice forms, differing at the N terminus. The *unc-7(rt212)* missense allele is predicted to prematurely truncate all *unc-7* splice forms. *unc-7(rt212)* decreased endogenous L4/A lethargus sleep defects (Figure S1A) and suppressed adult anachronistic sleep induced by Notch coligand OSM-11 overexpression (Figure S1B).

UNC-7 normally functions with UNC-9 to form gap junctions between cells. Loss of either UNC-7 or UNC-9 causes the animals to have uncoordinated locomotion with inappropriate posture predominantly during forward locomotion, but activity levels of adult animals are not decreased (Starich *et al.* 1993, 1996, 2009). We found that *unc-7(e5)* loss-of-function or *unc-9(e101)* loss-of-function animals had dramatically reduced L4/A lethargus total sleep (Figure 1A) and decreased lethargus sleep bout duration (Figure S1C). Introduction of a transgene containing the *unc-9* promoter driving *unc-9* complementary DNA (cDNA) almost completely restored total sleep quantity in *unc-9(e101)* animals (Figure S1D). To corroborate the sleep defects observed based on motion detection, we also examined another important aspect of sleep: altered arousal thresholds. During *C. elegans* L4/A lethargus sleep bouts, response to a variety of external stimuli is diminished (Raizen *et al.* 2008; Singh *et al.* 2011; Huang *et al.* 2017b); animals carrying mutations that perturb sleep quantity usually also have perturbed arousal thresholds during sleep bouts (Singh *et al.* 2014). Consistent with this general rubric, we found arousal defects in *unc-7(lf)* and *unc-9(lf)* animals. While animals lacking *unc-7* or *unc-9* always responded to gentle touch outside of lethargus or during lethargus motion bouts, loss of either *unc-7* or *unc-9* decreased arousal thresholds specifically during sleep bouts (Figure 1B, response to gentle touch). Additionally, we undertook a small pilot study and found that changes in stimulus-evoked, intracellular calcium signals in mechanosensory neurons that are characteristic of lethargus (Schwarz and Bringmann 2013; Cho and Sternberg 2014; Nichols *et al.* 2017) were not seen in *unc-9(lf)* animals (Figure S1E). Consistent with reduced time in sleep bouts, decreased arousal thresholds suggests a poor quality of sleep together with loss of UNC-7 or UNC-9 function. Double mutant *unc-9(lf) unc-7(lf)* animals did not have additive defects (Figure 1, A and B), suggesting that UNC-7 and UNC-9 likely function together. Combined, these results demonstrate that UNC-7

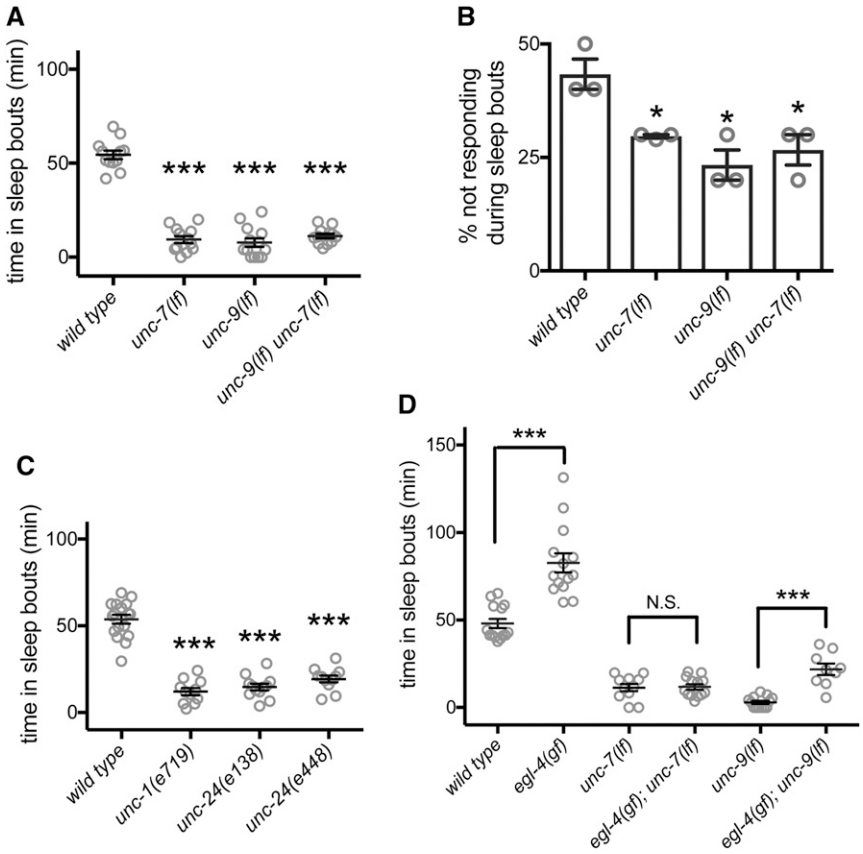

Figure 1 UNC-7 and UNC-9 gap junction proteins are required for normal L4/A developmentally timed sleep and arousal and are downstream of EGL-4 PKG. (A) Total time in sleep bouts for wild-type, *unc-7(e5lf)*, *unc-9(e101lf)*, and *unc-9(e101lf) unc-7(e5lf)* animals during L4 to adult lethargus. (B) Percent of wild-type, *unc-7(e5lf)*, *unc-9(e101lf)*, and *unc-9(e101lf) unc-7(e5lf)* animals not responding to gentle touch during L4 to adult sleep bouts. Because of the response defect of *unc-7(e5lf)* and *unc-9(e101lf)* animals to blue light stimuli, the arousal threshold was measured by gentle touch instead. Three independent trials were tested. $n = 10$ per genotype for each trial. (C) Total time in sleep bouts for wild-type, *unc-1(e719lf)*, *unc-24(e138lf)*, and *unc-24(e138lf)* animals during L4 to adult lethargus. (D) Total time in sleep bouts for *egl-4(ad450gf)*, *unc-7(e5lf)*, *egl-4(ad450gf)*; *unc-7(e5lf)*, *unc-9(e101lf)*, and *egl-4(ad450gf)*; *unc-9(e101lf)* animals during L4 to adult lethargus. Error bars show the SEM. * $P < 0.05$, *** $P < 0.001$. All error bars represent standard error of the mean.

and UNC-9 function is important for modulating sensory neuron response, setting arousal thresholds during sleep bouts, and normal sleep quantity during lethargus.

Gap junction accessory proteins are also required for normal lethargus sleep bouts

The stomatin-like proteins UNC-1 and UNC-24 are thought to regulate UNC-7/UNC-9 gap junction function via an unknown mechanism (Chen *et al.* 2007); *unc-24(lf)* and *unc-1(lf)* animals have severe locomotion defects that are almost identical to *unc-7(lf)* and *unc-9(lf)* animals. Both of the *unc-24(lf)* alleles examined resulted in decreased total sleep time during L4/A lethargus (Figure 1C). Additionally, we tested the loss-of-function allele *unc-1(e719)* and the dominant gain-of-function allele *unc-1(e1598)* (Park and Horvitz 1986). *unc-1* loss of function reduced total sleep time and *unc-1* gain of function increased total sleep time (Figure 1C and Figure S1F). Combined, these results confirm that reduced gap junction accessory protein function results in decreased developmentally timed sleep.

Gap junction proteins likely act downstream of EGL-4 PKG

Orthologs of the *C. elegans* EGL-4 PKG, regulate sleep in vertebrates and invertebrates (Raizen *et al.* 2008; Langmesser *et al.* 2009). In *C. elegans*, loss of EGL-4 function decreases sleep quantity and lowers arousal thresholds during developmentally timed sleep. Further, increased EGL-4 activity increases both sleep quantity and arousal thresholds (Raizen *et al.* 2008). We confirmed these changes in sleep quantity and arousal thresholds for *egl-4(n479)* and *egl-4(ad450)*, which are loss- and gain-of-function alleles, respectively. To our knowledge, no downstream targets of EGL-4 PKG pertinent to sleep have been identified in any species. To determine if *C. elegans* gap junction proteins might act downstream, we examined double mutant animals, as the downstream gene usually suppresses in this context (Huang and Sternberg 1995). We found that the increased sleep of *egl-4(ad450)* gain-of-function animals was completely suppressed by loss of *unc-7* and that *egl-4(ad450)* increased sleep was almost completely suppressed by loss of *unc-9* (Figure 1D). This suggests that EGL-4 PKG acts upstream in a genetic pathway and that EGL-4 PKG requires UNC-7 gap junction function, and is heavily dependent on UNC-9 function, to increase sleep.

UNC-7 and UNC-9 act in interneurons and motor neurons, respectively

UNC-7 and UNC-9 are broadly expressed in *C. elegans neurons* and body wall muscles (Starich *et al.* 2009). To determine where these genes are required for sleep, we drove the expression of *unc-7* or *unc-9* cDNAs in mutant animals using tissue-specific promoters. Expression of UNC-7 or UNC-9 in all neurons, rather than body wall muscle, ameliorated sleep defects in *unc-7(lf)* or *unc-9(lf)* animals, respectively (Figure 2, A and B). These and rescue studies presented below did

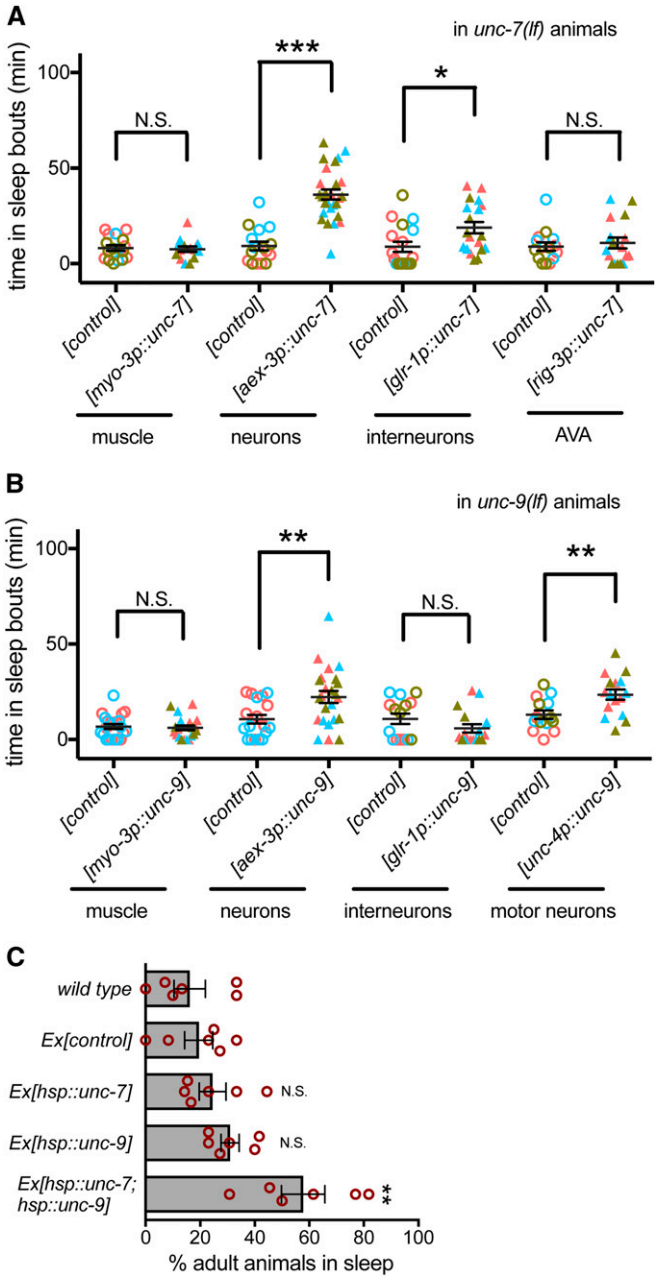

Figure 2 UNC-7 and UNC-9 is partially required in premotor interneurons and motor neurons, respectively, during sleep. (A) Total time in sleep bouts for *unc-7(e5lf)* animals in L4 to adult lethargus rescued with *myo-3* promoter, *aex-3* promoter, *glr-1* promoter, and *rig-3* promoter driven *unc-7* and their corresponding promoter driven GFP as controls. Colors represent three independent extrachromosomal arrays tested for each condition (strain information in Table S2). (B) Total time in sleep bouts for *unc-9(e101lf)* animals in L4 to adult lethargus rescued with *myo-3* promoter, *aex-3* promoter, *glr-1* promoter, and *unc-4* promoter driven *unc-9* and their corresponding promoter driven GFP as controls. Colors represent two or three independent extrachromosomal arrays tested for each condition (strain information in Table S2). (C) Percent of animals with anachronistic sleep in wild-type animals, animals overexpressing *hsp*::*gfp* ([control]), *hsp*::*unc-7*, *hsp*::*unc-9*, and both *hsp*::*unc-7* and *hsp*::*unc-9*. Data from three independent extrachromosomal arrays were pooled for each genotype. At least three independent trials were tested. $n = 10$ per genotype for each trial. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. All error bars represent standard error of the mean.

not restore wild-type level sleep; this may be a consequence of chimeric extrachromosomal array expression.

UNC-7 function is required in premotor AVA interneurons for coordinated locomotion (Kawano *et al.* 2011). To determine if UNC-7 function is also required in AVA for normal sleep, we expressed UNC-7 using the *glr-1* promoter, which drives expression in 17 classes of interneurons, including AVA interneurons (Hart *et al.* 1995). This partially rescued sleep defects (Figure 2A). Expression of UNC-7 in AVA neurons alone using the *rig-3* promoter did not rescue sleep. Combined, these results suggest that UNC-7 function is required in neurons, and that UNC-7 function in a subset of *glr-1*-expressing neurons plays a role in developmentally timed sleep.

UNC-9 function is required in A-class motor neurons for coordinated locomotion (Kawano *et al.* 2011). Consistent with this, expression of UNC-9 cDNA using the *glr-1* promoter did not restore sleep (Figure 2B). However, expression of UNC-9 in cholinergic neurons, which includes the A-class motor neurons, partially restored sleep. These results suggest that UNC-7/UNC-9 gap junctions may function between premotor interneurons and motor neurons to promote developmentally timed sleep, but their function in other neurons is likely also important. UNC-7 and UNC-9 act, at least in part, in interneurons and motor neurons to promote lethargus sleep bouts. The partial rescue of lethargus sleep bouts observed here may suggest that these gap junction proteins act at other sites to facilitate activity changes associated with sleep.

Simultaneous expression of both UNC-7 and UNC-9 induces anachronistic sleep in adult animals

UNC-7 and UNC-9 are required for developmentally timed sleep, but is their activation sufficient to induce sleep? We tested this by inducing expression of *unc-7*, *unc-9*, or both in adult animals using the heat-inducible *hsp-16.2* promoter to drive cDNA expression. After heat shock, *C. elegans* enter stress-induced quiescence, but in our hands, most wild-type animals resume locomotion and pharyngeal pumping 1 hr after heat shock ends. However, when UNC-7 and UNC-9 were simultaneously expressed, anachronistic sleep bouts were observed in adult animals 1 hr after heat shock (Figure 2C). Expression of either UNC-7 or UNC-9 was not sufficient, demonstrating that increasing UNC-7 and UNC-9 gap junctions is sufficient to induce anachronistic sleep in adult *C. elegans* and that both are required. UNC-7 and UNC-9 can function as heterotypic gap junction channels or as hemichannels; homomeric channels have been observed in ectopic expression systems (Starich *et al.* 2009; Bouhours *et al.* 2011; Meng *et al.* 2016). Heterotypic channels may be critical for the anachronistic sleep observed here.

The relationship between lethargus sleep and anachronistic sleep observed in adult animals after heat shock induced expression of *unc-7* and *unc-9* may be complicated by contribution of stress-induced sleep, ectopic overexpression, and uncoupling of this behavior from the developmental context

of diapause. Nevertheless, we were able to examine the consequences of *egl-4* PKG loss of function in anachronistic sleep caused by UNC-7 and UNC-9 ectopic overexpression. Loss of *egl-4* did not significantly alter the fraction of animals not pumping after UNC-7/UNC-9 ectopic overexpression (Figure S2A), suggesting that EGL-4 is irrelevant or acts upstream in this paradigm. However, *egl-4* loss did suppress the locomotion changes caused by UNC-7/UNC-9 ectopic overexpression (Figure S2B), suggesting that EGL-4 may act downstream in this anachronistic paradigm. We are hesitant to draw conclusions about developmentally timed sleep from anachronistic sleep, but *unc-7* and *unc-9* may act downstream of *egl-4* for cessation of pumping in anachronistic sleep.

Normal NCA channel activity is required during C. elegans sleep bouts

In *C. elegans*, UNC-7 and UNC-9 act downstream of NCA cation channels to modulate response to anesthetics (Sedensky and Meneely 1987). The NCA ion channels (NALCN channels in mammals) are conserved cation channels that are a member of the voltage-gated sodium and calcium channel family (Lee *et al.* 1999; Ren 2011; Liebeskind *et al.* 2012). The NALCN/NCA channels used to be considered sodium leak channels, although more functions have been proposed for these channels. (Senatore *et al.* 2013; Senatore and Spafford 2013; Boone *et al.* 2014) The NALCN/NCA channels have been implicated in various aspects of sleep in flies and mice (Lear *et al.* 2005; Funato *et al.* 2016). In flies, antiphase cycles in resting potassium and sodium conductance in clock neurons drive daily rhythms of activity, arousal, and sleep. This rhythmic sodium conductance is driven by sodium leak channel activity, which regulates circadian pacemaker neuron activity (Flourakis *et al.* 2015). We hypothesized that, in *C. elegans*, the NCA cation channels might also regulate arousal and sleep. The *C. elegans* NCA channel comprises the functionally redundant α1 subunits NCA-1 and NCA-2, which are encoded by *nca-1* and *nca-2* genes, hence the name NCA channels. NCA-1 and NCA-2 act redundantly to regulate resting membrane potential (Gao *et al.* 2015). Increased NCA channel function causes exaggerated body bends during locomotion, while NCA channel loss causes short transient pauses in *C. elegans* locomotion (Yeh *et al.* 2008). To assess the effect of NCA channels on *C. elegans* sleep during L4/A lethargus, we first examined the gain-of-function allele of *nca-1(e625)*: sleep bouts during L4/A lethargus were almost completely eliminated in these animals (Figure 3A), suggesting that heightened NCA channel activity disturbs sleep.

Animals lacking both *nca-1* and *nca-2* are referred to as *nca(lf)* animals in the literature (Yeh *et al.* 2008) and we adopt that convention here. We found that *nca(lf)* animals had normal total time in sleep bouts during L4/A lethargus (Figure 3A). Other aspects of sleep/motion bout timing and duration were also normal when compared to wild-type animals (Figure S3, A and B). This discordance led us to more closely examine the role of NCA channels and associated proteins in *C. elegans* sleep.

Functional NCA channels require two conserved auxiliary subunits, called UNC-79 and UNC-80 in *C. elegans*. These two genes do not function redundantly, as loss of either *unc-79* or *unc-80* perturbs NCA channel function with defects equal to *nca(lf)* animals (Yeh *et al.* 2008). When we examined L4/A lethargus sleep in *unc-79(lf)* animals, we observed a small increase in total sleep time for two different mutant allele strains (Figure 3A and Figure S3C). However, total sleep time and other measures of L4/A sleep were normal in *unc-80(lf)* animals (Figure 3A and Figure S3, A–C). Given the dramatic effect of *nca(gf)* on sleep, we considered the possibility that *nca(lf)* altered other aspects of L4/A lethargus sleep.

NCA loss-of-function animals had normal total sleep time, allowing us to assess arousal thresholds during sleep bouts. We previously established that blue light can be used to wake *C. elegans* from sleep bouts and that time to respond (response latency) is an effective measure of arousal threshold (Huang *et al.* 2017b). Loss-of-function alleles of NCA channel genes, including *nca(lf)*, *unc-79(lf)*, and *unc-80(lf)*, caused increased latency to respond during sleep bouts (Figure 3B, left panel). To rule out the possibility that NCA channel function is required for response to blue light, we also examined latency to respond during L4/A motion bouts. Time to respond to blue light was normal in *nca(lf)*, *unc-79(lf)*, and *unc-80(lf)* animals (Figure 3B, right panel). Assessing arousal thresholds in *nca-1(gf)* animals was more challenging. Sleep bouts are rare and the average sleep bout duration in these animals was roughly 50% shorter (Figure S3B). With these caveats, we found that arousal thresholds in *nca-1(gf)* animals were not different from wild-type animals (Figure 3C). Combined, these results suggest that appropriate *C. elegans* NCA activity is critical for normal sleep.

We also examined sleep and arousal in animals defective in *nlf-1* function; NLF-1 is an endoplasmic reticulum-associated protein that promotes, but is not absolutely required for, appropriate *C. elegans* NCA channel localization and function (Xie *et al.* 2013). The locomotion defects in adult *nlf-1(lf)* animals were not as severe as those of *unc-79(lf)*, *unc-80(lf)*, or *nca(lf)* animals (Xie *et al.* 2013). We did not observe changes in L4/A lethargus sleep quantity or in arousal thresholds for either sleep or motion bouts (Figure S3, D and E). Combined, all of these results confirm the conserved role of NCA channels in sleep-associated behaviors extends to *C. elegans*. Loss of NCA function leads to aberrantly increased arousal thresholds only during sleep bouts and increased NCA function prevents sleep bouts. Regulation of NCA channel activity might be critical for entry into or maintenance of the sleep state with appropriately increased arousal thresholds.

We undertook rescue experiments to pinpoint the site of action for NCA channels by expressing the channel pan-neuronally, in premotor-interneurons, and in different classes of motor neurons in *nca(lf)* animals. We found that expressing

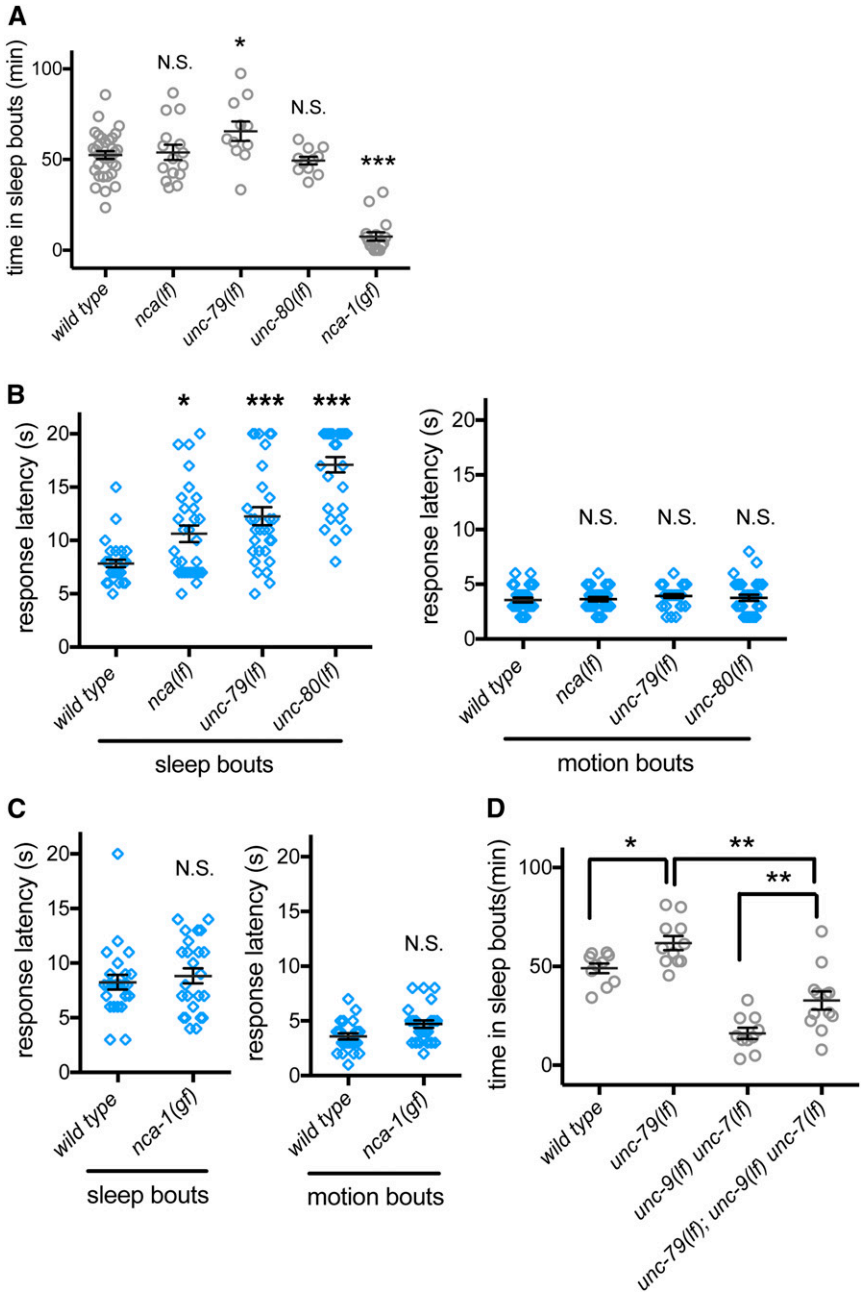

Figure 3 Normal NCA cation channel activity is required for proper response latency during sleep bouts. (A) Total time in sleep bouts for wild-type, *nca-1(gk5lf)*; *nca-1(gk9lf)* [simplified as *nca(lf)*], *unc-79(ec1lf)*, *unc-80(e1272lf)*, and *nca-1(e625gf)* animals during L4 to adult lethargus. (B) Response latency of wild-type, *nca(lf)*, *unc-79(ec1lf)*, and *unc-80(e1272lf)* animals to blue light stimulation during sleep bouts (left panel) and motion bouts (right panel) in L4 to adult lethargus. (C) Response latency of *nca-1(e625gf)* animals to blue light stimulation during sleep bouts (left panel) and motion bouts (right panel) in L4 to adult lethargus. (D) Total time in sleep bouts for wild-type, *unc-79-(ec1lf)*, *unc-9(e101lf) unc-7(e5lf)*, and *unc-79(ec1lf)*; *unc-9(e101lf) unc-7(e5lf)* animals during L4 to adult lethargus. All sleep bouts and motions bouts were assessed during L4 to adult lethargus. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. All error bars represent standard error of the mean.

NCA in any of these neuron populations rescued the arousal defects during lethargus (Figure S4C). These results are reminiscent of the distributed sites of action for *C. elegans egl-4* in sleep bout arousal (Raizen *et al.* 2008) and consistent with the hypothesis that arousing factors can act at various locations to awaken animals.

Gap junction protein function contributes to NCA channel modulation of sleep

Either NCA channel gain of function or gap junction loss of function results in decreased sleep. We examined the possibility that these genes act in the same pathway for sleep, as observed in other contexts (Sedensky and Meneely 1987; Bouhours *et al.* 2011). For this analysis, we used *unc-79(lf)* animals, which have increased sleep, and introduced loss-of-function alleles for *unc-7* and *unc-9*. Loss of UNC-7, UNC-9, or simultaneous loss of both gap junction proteins suppressed *unc-79(lf)* defects; total sleep time in triple mutant animals was not as low as sleep time in *unc-9(lf) unc-7(lf)* animals (Figure 3D), but was dramatically lower than the quantity of sleep observed in wild-type animals. We found that loss of only *unc-7* or *unc-9* also decreased sleep in *unc-79(lf)* animals (Figure S3F), to levels comparable to the triple mutant in Figure 3D. The incomplete suppression means either that these genes function in independent pathways or that gap junctions act genetically downstream of NCA channels in a pathway critical for lethargus sleep, along with other effectors.

C. elegans NCA channel loss of function leads to "fainting bouts," which cause transient cessation of locomotion in adult animals (Yeh *et al.* 2008). We examined the consequences of *nca-1(gf)* in anachronistic sleep caused by UNC-7 and UNC-9 ectopic overexpression in adult animals. *nca-1(gf)* did not suppress pumping or locomotion changes observed in animals ectopically overexpressing UNC-7 and UNC-9 (Figure S2, A and B), suggesting that NCA channels either act upstream of gap junctions or are irrelevant in this paradigm.

EGL-4 PKG may act downstream of NCA channels in C. elegans sleep

We also examined the relationship between EGL-4 and NCA channels during *C. elegans* L4/A lethargus, focusing initially on sleep quantity. We found that the increased sleep of *unc-79(ec1)* loss-of-function animals was completely eliminated by loss of *egl-4*; total time in sleep bouts of animals lacking both *unc-79* and *egl-4* was indistinguishable from animals with loss of *egl-4* function alone (Figure 4A). Therefore, *egl-4* likely acts downstream of *unc-79* in a genetic pathway that regulates sleep quantity. Next, we examined the arousal thresholds in the double mutant animals. Again, *unc-79(ec1)* arousal changes during sleep bouts were completely suppressed by loss of *egl-4* (Figure 4B). Combined, these results suggest that EGL-4 likely acts downstream of NCA channels for both sleep bouts and arousal threshold changes in a single pathway.

Sometimes, additional information can be gained by examining gain-of-function alleles in epistasis studies. Therefore, we constructed double mutant *unc-79(lf)*; *egl-4(gf)* animals. There was no additivity in arousal changes between *unc-79(lf)* and *egl-4(gf)* animals (Figure 4C), consistent with EGL-4 and NCA acting in the same pathway for arousal threshold changes during L4/A sleep. However, we noted that *unc-79(lf)*; *egl-4(gf)* double animals had increased sleep compared with *unc-79(lf)* and *egl-4(gf)* single mutant animals (Figure 4A). Because the increased sleep in double mutant animals is more than additive, interpretation is difficult, but not inconsistent with these two genes acting in the same pathway. Loss of NCA channel function may increase downstream EGL-4 kinase activity. Alternatively, NCA channels may act through EGL-4, as well as other mechanisms. Combined, these genetic epistasis studies suggest that NCA channels likely act through the EGL-4 PKG to regulate sleep and arousal.

NCA channels act downstream or in parallel to PDF signaling

Previous studies in *Drosophila* have suggested that NCA channels and PDF receptors act coordinately in $DN1_p$ circadian neurons to regulate circadian behavior (Zhang *et al.* 2010). In *C. elegans*, there are two PDF neuropeptide-encoding genes (*pdf-1 and pdf-2*) and one gene encoding a receptor (*pdfr-1*). Previous work in *C. elegans* has shown in animals lacking neuropeptide Y signaling, loss of PDF-1 or the PDFR-1 receptor restores L4/A lethargus sleep quantity (Choi *et al.* 2013). PDF-1 or PDFR-1 loss also decreases motility in adult *C. elegans* (Meelkop *et al.* 2012; Choi *et al.* 2013). However, a requirement for PDF signaling in L4/A lethargus sleep and arousal has not been reported previously.

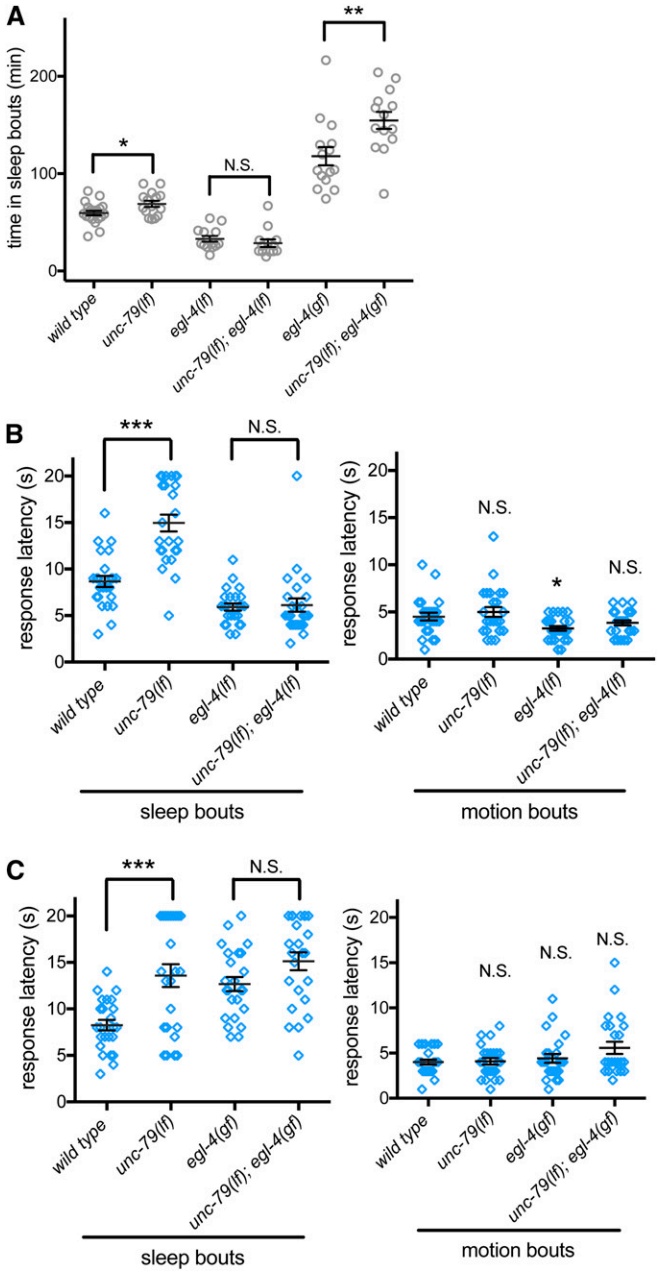

Figure 4 EGL-4 PKG is downstream of NCA channels during L4/A lethargus. (A) Total time in sleep bouts for wild-type, *unc-79(ec1lf)*, *egl-4(n479lf)*, *unc-79(ec1lf)*; *egl-4(n479lf)*, *egl-4(ad450gf)*, and *unc-79(ec1lf)*; *egl-4(ad450gf)*. (B) Response latency of wild-type, *unc-79(ec1lf)*, *egl-4(n479lf)*, and *unc-79(ec1lf)*; *egl-4(n479lf)* animals to blue light stimulation during sleep bouts (left panel) and motion bouts (right panel). (C) Response latency of wild-type, *unc-79(ec1lf)*, *egl-4(ad450gf)*, and *unc-79(ec1lf)*; *egl-4(ad450gf)* animals to blue light stimulation during sleep bouts (left panel) and motion bouts (right panel). All sleep bouts and motions bouts were assessed during L4 to adult lethargus. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. All error bars represent standard error of the mean.

To directly determine the role of PDF signaling in lethargus sleep, we measured the total time in sleep bouts in *pdfr-1(ok3425)*, *pdfr-1(lst34)*, *pdf-1(tm1996)*, or *pdf-2(tm4393)* loss-of-function animals and in *pdf-1(lf)*; *pdf-2(lf)* double mutant animals. All of these mutant strains had normal total sleep during L4/A lethargus (Figure 5A and Figure S4A). We also examined arousal thresholds during L4/A lethargus sleep and motion bouts. Both *pdfr-1(lf)* animals and *pdf-1(lf)*; *pdf-2(lf)* double mutant animals had slightly increased arousal thresholds only during sleep bouts (Figure 5B and Figure S4B). Response latency was normal during motion bouts for all genotypes. *pdf-1(lf)* and *pdf-2(lf)* may act redundantly in this scenario, through the *pdfr-1* receptor. While the effect of altered PDF signaling was less dramatic than that of NCA channel perturbations, these results suggest that PDF neuropeptide signaling contributes to arousal during lethargus sleep bouts. Next, we examined the relationship between PDF signaling and NCA channels based on genetic epistasis. We generated *pdfr-1(lf)*; *nca-1(gf)* double mutant animals. PDFR-1 loss did not ameliorate the sleep defects of *nca-1(gf)* animals (Figure 5C). Combined, these results suggest that PDF signaling is not required for NCA channel modulation of *C. elegans* lethargus sleep and that loss of PDF signaling has a modest effect on arousal threshold during lethargus sleep bouts.

In summary, we have unequivocally established a requirement for UNC-7 and UNC-9 gap junctions in L4/A lethargus sleep and arousal thresholds. We also find that NCA cation channels and EGL-4 PKG likely act upstream of UNC-7 and UNC-9 gap junction proteins in this behavior. NCA and PKG play critical roles in sleep and arousal across species, including *C. elegans*, *Drosophila*, and mice. We suggest that a pathway including these proteins and gap junctions may play a conserved role regulating sleep across all animal species (Figure 5D).

Discussion

Here, we report that loss of *C. elegans* UNC-7 or UNC-9 innexin gap junction proteins resulted in dramatically reduced time in sleep bouts during L4/A lethargus, decreased bout duration, and decreased arousal thresholds during these sleep bouts. UNC-7 function was partially required in premotor interneurons and UNC-9 function was partially required in motor neurons during sleep. Moreover, transient, simultaneous ectopic overexpression of UNC-7 and UNC-9 induced anachronistic sleep in adult animals. Based on genetic epistasis studies in lethargus sleep, *unc-7* and *unc-9* acted downstream of *egl-4*, which encodes the *C. elegans* PKG. Additionally, we found that loss of the *C. elegans* NCA channel increased arousal thresholds during L4/A lethargus sleep bouts and increased cation channel activity decreased sleep in this context. NCA channels acted upstream of *egl-4* and gap junctions in lethargus sleep. Loss of *C. elegans* PDF neuropeptides or receptors had no effect on sleep quantity during lethargus, but their loss slightly increased arousal thresholds during these sleep bouts. Our results suggest that in *C. elegans* lethargus sleep, PDF signaling likely does not act downstream of NCA channels. This unequivocally demonstrates a requirement for gap junction proteins in sleep and demonstrates that EGL-4 PKG regulation of sleep is dependent on gap junction function.

A recent forward genetic screen in mice for genes involved in sleep, identified a dominant, missense mutation in murine NALCN, which increases channel activity and leads to decreased rapid eye movement sleep (Funato *et al.* 2016). Murine *Nalcn* encodes the direct ortholog of *C. elegans nca-1* and *nca-2*. Our work in *C. elegans* finds that increased NCA channel activity decreases sleep bout quantity during lethargus, which is consistent with a requirement for NALCN channels in murine sleep (Funato *et al.* 2016). We speculate that if these channels act in wake-promoting neurons or sensory neurons, their loss might increase arousal thresholds during sleep. In *Drosophila*, PDF signaling and cation channels coordinately regulate *Drosophila* circadian behavior, based on activity and entrainment to light/dark cycles (Zhang *et al.* 2010). *Drosophila* PDF-expressing neurons play critical roles in activating neurons that express NCA channels, which in turn helps drive circadian behaviors. In *C. elegans*, PDF signaling regulates activity levels in adult animals and suppresses sleep quantity defects in animals lacking neuropeptide Y signaling (Meelkop *et al.* 2012; Choi *et al.* 2013). Our work confirms that loss of PDF signaling does not change *C. elegans* lethargus sleep quantity (Choi *et al.* 2013), but reveals that PDF signaling regulates arousal thresholds during sleep bouts, confirming a cross-species role for PDF signaling in arousal and sleep. We note that loss of *nlf-1*, which encodes an ER-localized protein required for NCA channel trafficking, did not perturb sleep or arousal thresholds (Figure S3, D and E). As the fainting locomotion defects in *nlf-1* loss-of-function animals are less severe than those seen in *nca* loss-of-function animals (Xie *et al.* 2013), NLF may also interact with or regulate additional, unidentified channels that affect both locomotion and sleep, antagonizing NCA channel function. Combined, these studies in disparate animal species highlight the conserved requirement for NACLN channels in sleep, although the molecular mechanisms underlying sleep regulation may require further study.

Invertebrate gap junctions comprise innexin proteins, while vertebrate gap junctions comprise connexin proteins. Despite their analogous function in electrically coupling adjacent cells, innexin and connexin protein amino acid sequences cannot be easily aligned, which makes drawing conclusions about orthology or orthologous function difficult (Abascal and Zardoya 2013). Vertebrates also have genes that encode innexin protein orthologs, called pannexins. Innexin and pannexin proteins share sequence similarity and both can function as hemichannels (Bruzzone *et al.* 2003; Bouhours *et al.* 2011), which are nonspecific transmembrane channels.

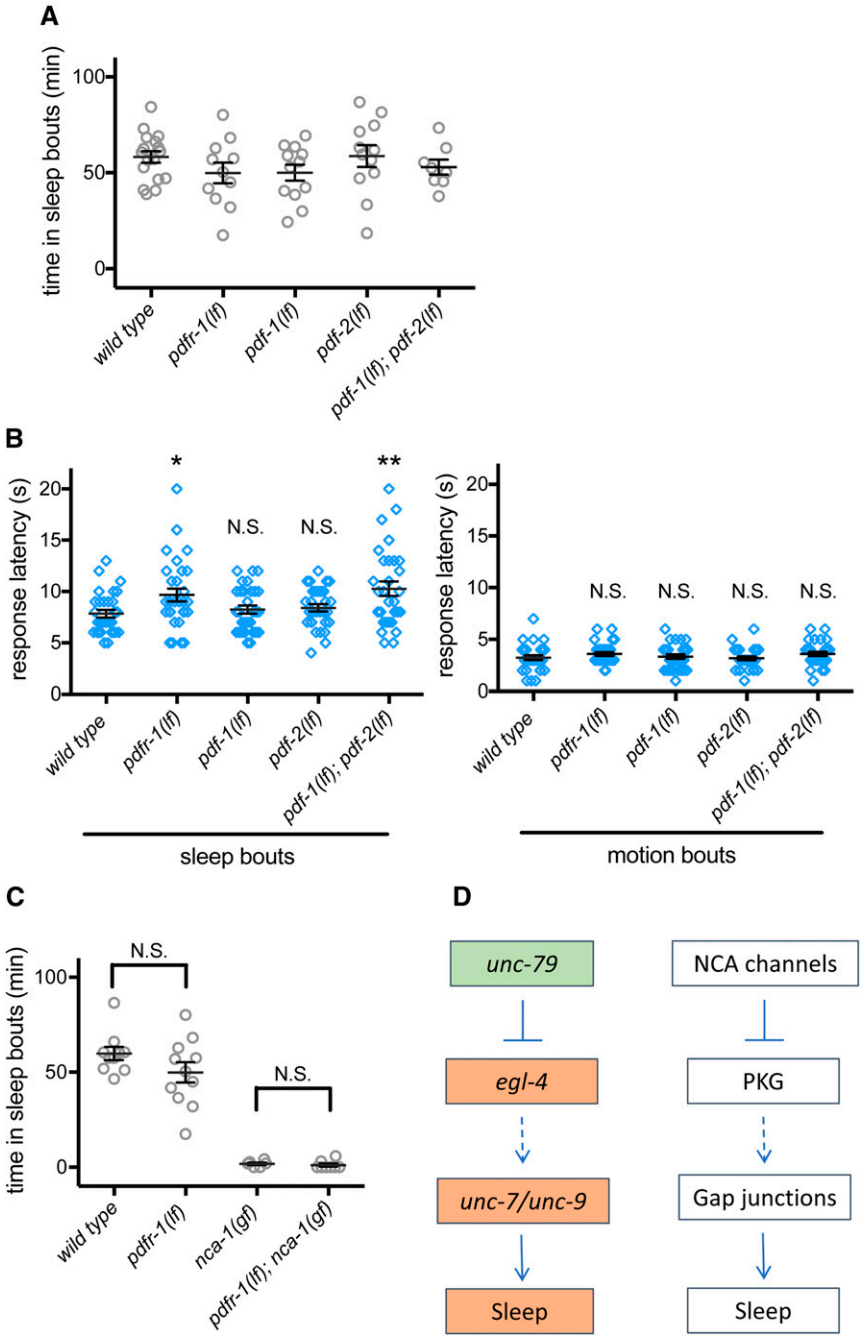

Figure 5 PDF signaling acts upstream or in parallel to NCA channels. (A) Total time in sleep bouts for wild-type, *pdfr-1(ok3425lf)*, *pdf-1(tm1996lf)*, *pdf-2-(tm4393lf)*, and *pdf-1tm1996(lf)*; *pdf-2(tm4393lf)* animals. (B) Response latency of wild-type, *pdfr-1(ok3425lf)*, *pdf-1(tm1996lf)*, *pdf-2(tm4393lf)*, and *pdf-1(tm1996lf)*; *pdf-2(tm4393lf)* animals to blue light stimulation during sleep bouts (left panel) and motion bouts (right panel). (C) Total time in sleep bouts for wild-type, *pdfr-1(ok3425lf)*, *nca-1(e625gf)*, and *pdfr-1(ok3425lf)*; *nca-1(e625gf)* animals. (D) Model for genetic pathways of the conserved cation channel and gap junction proteins regulating sleep across species. Dashed arrows represent possible parallel pathway. All sleep bouts and motions bouts were assessed during L4 to adult lethargus. * $P < 0.05$, ** $P < 0.01$. All error bars represent standard error of the mean.

Prior work is not inconsistent with a conserved role for gap junctions in sleep. Rapid eye movement sleep in rats is disrupted by quinine, a nonspecific blocker of gap junctions and various other channels and solute pumps (Franco-Pérez and Paz 2009; Connors 2012). Further, mice lacking Pannexin 1 function show increased wake and decreased slow-wave sleep, possibly due to the depleted extracellular adenosine (Kovalzon *et al.* 2017). Also, Connexin 43 knockout mice show excessive sleepiness and fragmented wakefulness, which may be attributed to connexin loss in astrocytes causing impaired lactate shuttling that impairs orexin neuron function (Clasadonte *et al.* 2017). Additionally, Connexin 36 knockout mice show dampened circadian activity rhythms that may be attributed to loss of synchronized spiking in the suprachiasmatic nucleus (Long *et al.* 2005). Finally, a recent study in *Drosophila* has demonstrated requirement for *innexin 6* activity in dorsal fan-shaped body neurons that regulate arousal during both sleep and awaking states (Troup *et al.* 2018). Our work here demonstrates that gap junction function is required for normal *C. elegans* lethargus sleep and that these gap junctions act genetically downstream of EGL-4 PKG. We suggest that increased gap junction activity may increase coupling between neurons and the consequent shunting may serve to decrease neuronal excitability

in circuits, allowing normal *C. elegans* sleep. Or, the synchronized neuronal activity characteristic of sleep in many species may require gap junction function. Additional studies are clearly required to understand how gap junction proteins act to regulate sleep and arousal across species.

Do *C. elegans* UNC-7 and UNC-9 proteins function as hemichannels or as gap junctions proteins in the context of *C. elegans* sleep? Previous work has established that *C. elegans* UNC-7 may function as a hemichannel in some contexts, but must function as a gap junction for normal locomotion (Bouhours *et al.* 2011). We do not directly test if UNC-7 acts as a hemichannel in the context of sleep. However, our overexpression data showed that only by overexpressing both UNC-7 and UNC-9 together were we able to induce sleep in adult animals. This suggests that either collaboration of UNC-7 and UNC-9 in heterotypic gap junctions is required for normal sleep, or that in the ectopic overexpression paradigm, high levels of both UNC-7 and UNC-9 hemichannels are required.

During lethargus, *C. elegans* stop feeding and pharyngeal pumping ceases. Why pumping normally ceases during lethargus is unclear. Previous work has demonstrated a requirement for UNC-7 in EGF-signaling induced pumping cessation during sleep bouts (Van Buskirk and Sternberg 2007). Further, a recent report found that optical silencing of body wall muscles can induce pumping inhibition in adult animals via UNC-7 gap junctions between the I1 neuron and RIP neurons, the only intra- and extrapharyngeal neural connection (Takahashi and Takagi 2017). However, even in animals lacking *unc-7* function, we observed that pharyngeal pumping ceases during lethargus, suggesting that other factors are at work. We note that lethargus-specific decreases in pharyngeal muscle excitability may play a major role in suppressing pharyngeal pumping and feeding during lethargus, as optical stimulation of pharyngeal muscles cannot drive contraction (Trojanowski *et al.* 2016). Gap junction proteins may contribute to cessation of feeding during lethargus, but other regulatory pathways are likely major players.

Results presented here suggest that gap junctions function downstream of other proteins known to regulate *C. elegans* sleep. Indeed, *C. elegans* gap junctions function downstream of the cGMP-dependent protein kinase EGL-4 in sleep, suggesting a model in which EGL-4 directly or indirectly regulates gap junction function. Gap junction activity could be regulated at multiple levels, including protein local expression, membrane protein endocytosis and exocytosis, and/or protein modifications that might alter gap junction permeability (Kjenseth *et al.* 2010; Axelsen *et al.* 2013; Pogoda *et al.* 2016). Phosphorylation and redox changes can regulate connexin activity and trafficking (Pogoda *et al.* 2016), but a connection between gap junction proteins and cGMP-dependent kinase activity has not been examined to our knowledge. The only exception is one *in vitro* study showing that nitric oxide can lead to PKG phosphorylation of connexins (Patel *et al.* 2006). Further work examining how gap junctions are regulated might provide novel insights into how cGMP-dependent protein kinases regulate sleep and arousal across species.

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Literature Cited

Abascal, F., and R. Zardoya, 2013 Evolutionary analyses of gap junction protein families. Biochim. Biophys. Acta 1828: 4–14. https://doi.org/10.1016/j.bbamem.2012.02.007

Allada, R., C. Cirelli, and A. Sehgal, 2017 Molecular mechanisms of sleep homeostasis in flies and mammals. Cold Spring Harb. Perspect. Biol. 9. https://doi.org/10.1101/cshperspect.a027730

Axelsen, L. N., K. Calloe, N. H. Holstein-Rathlou, and M. S. Nielsen, 2013 Managing the complexity of communication: regulation of gap junctions by post-translational modification. Front. Pharmacol. 4: 130. https://doi.org/10.3389/fphar.2013.00130

Banerjee, D., A. Kwok, S. Y. Lin, and F. J. Slack, 2005 Developmental timing in C. elegans is regulated by kin-20 and tim-1, homologs of core circadian clock genes. Dev. Cell 8: 287–295. https://doi.org/10.1016/j.devcel.2004.12.006

Boone, A. N., A. Senatore, J. Chemin, A. Monteil, and J. D. Spafford, 2014 Gd3+ and calcium sensitive, sodium leak currents are features of weak membrane-glass seals in patch clamp recordings. PLoS One 9: e98808. https://doi.org/10.1371/journal.pone.0098808

Bouhours, M., M. D. Po, S. Gao, W. Hung, H. Li *et al.*, 2011 A co-operative regulation of neuronal excitability by UNC-7 innexin and NCA/NALCN leak channel. Mol. Brain 4: 16. https://doi.org/10.1186/1756-6606-4-16

Bruzzone, R., S. G. Hormuzdi, M. T. Barbe, A. Herb, and H. Monyer, 2003 Pannexins, a family of gap junction proteins expressed in brain. Proc. Natl. Acad. Sci. USA 100: 13644–13649. https://doi.org/10.1073/pnas.2233464100

Campbell, S. S., and I. Tobler, 1984 Animal sleep: a review of sleep duration across phylogeny. Neurosci. Biobehav. Rev. 8: 269–300. https://doi.org/10.1016/0149-7634(84)90054-X

Chen, B., Q. Liu, Q. Ge, J. Xie, and Z. W. Wang, 2007 UNC-1 regulates gap junctions important to locomotion in *C. elegans*. Curr. Biol. 17: 1334–1339. https://doi.org/10.1016/j.cub.2007.06.060

Cho, J. Y., and P. W. Sternberg, 2014 Multilevel modulation of a sensory motor circuit during *C. elegans* sleep and arousal. Cell 156: 249–260. https://doi.org/10.1016/j.cell.2013.11.036

Choi, S., M. Chatzigeorgiou, K. P. Taylor, W. R. Schafer, and J. M. Kaplan, 2013 Analysis of NPR-1 reveals a circuit mechanism

for behavioral quiescence in *C. elegans*. Neuron 78: 869–880. https://doi.org/10.1016/j.neuron.2013.04.002
Cirelli, C., 2009 The genetic and molecular regulation of sleep: from fruit flies to humans. Nat. Rev. Neurosci. 10: 549–560. https://doi.org/10.1038/nrn2683
Clasadonte, J., E. Scemes, Z. Wang, D. Boison, and P. G. Haydon, 2017 Connexin 43-mediated astroglial metabolic networks contribute to the regulation of the sleep-wake cycle. Neuron 95: 1365–1380.e5. https://doi.org/10.1016/j.neuron.2017.08.022
Connors, B. W., 2012 Tales of a dirty drug: carbenoxolone, gap junctions, and seizures. Epilepsy Curr. 12: 66–68. https://doi.org/10.5698/1535-7511-12.2.66
Evans, T. C., 2006 Transformation and microinjection (April 6, 2006), *WormBook*, ed. The *C. elegans* Research Community, WormBook, doi/10.1895/wormbook.1.108.1, http://www.wormbook.org.
Flourakis, M., E. Kula-Eversole, A. L. Hutchison, T. H. Han, K. Aranda *et al.*, 2015 A conserved bicycle model for circadian clock control of membrane excitability. Cell 162: 836–848. https://doi.org/10.1016/j.cell.2015.07.036
Franco-Pérez, J., and C. Paz, 2009 Quinine, a selective gap junction blocker, decreases REM sleep in rats. Pharmacol. Biochem. Behav. 94: 250–254. https://doi.org/10.1016/j.pbb.2009.09.003
Funato, H., C. Miyoshi, T. Fujiyama, T. Kanda, M. Sato *et al.*, 2016 Forward-genetics analysis of sleep in randomly mutagenized mice. Nature 539: 378–383. https://doi.org/10.1038/nature20142
Gao, S., L. Xie, T. Kawano, M. D. Po, S. Guan *et al.*, 2015 The NCA sodium leak channel is required for persistent motor circuit activity that sustains locomotion. Nat. Commun. 6: 6323 (erratum: Nat. Commun. 6: 7191). https://doi.org/10.1038/ncomms7323
Ghosh, R., and S. W. Emmons, 2008 Episodic swimming behavior in the nematode *C. elegans*. J. Exp. Biol. 211: 3703–3711. https://doi.org/10.1242/jeb.023606
Hart, A. C., S. Sims, and J. M. Kaplan, 1995 Synaptic code for sensory modalities revealed by *C. elegans* GLR-1 glutamate receptor. Nature 378: 82–85. https://doi.org/10.1038/378082a0
Hill, A. J., R. Mansfield, J. Lopez, D. M. Raizen, and C. Van Buskirk, 2014 Cellular stress induces a protective sleep-like state in *C. elegans*. Curr. Biol. 24: 2399–2405. https://doi.org/10.1016/j.cub.2014.08.040
Huang, H., K. Singh, and A. C. Hart, 2017a Measuring *Caenorhabditis elegans* sleep during the transition to adulthood using a microfluidics-based system. Bio Protoc. 7: e2174. https://doi.org/10.21769/BioProtoc.2174
Huang, H., C. T. Zhu, L. L. Skuja, D. J. Hayden, and A. C. Hart, 2017b Genome-wide screen for genes involved in *Caenorhabditis elegans* developmentally timed sleep. G3 (Bethesda) 7: 2907–2917. https://doi.org/10.1534/g3.117.300071
Huang, L. S., and P. W. Sternberg, 1995 Genetic dissection of developmental pathways. Methods Cell Biol. 48: 97–122. https://doi.org/10.1016/S0091-679X(08)61385-0
Iwanir, S., N. Tramm, S. Nagy, C. Wright, D. Ish *et al.*, 2013 The microarchitecture of *C. elegans* behavior during lethargus: homeostatic bout dynamics, a typical body posture, and regulation by a central neuron. Sleep (Basel) 36: 385–395. https://doi.org/10.5665/sleep.2456
Kawano, T., M. D. Po, S. Gao, G. Leung, W. S. Ryu *et al.*, 2011 An imbalancing act: gap junctions reduce the backward motor circuit activity to bias *C. elegans* for forward locomotion. Neuron 72: 572–586. https://doi.org/10.1016/j.neuron.2011.09.005
Kjenseth, A., T. Fykerud, E. Rivedal, and E. Leithe, 2010 Regulation of gap junction intercellular communication by the ubiquitin system. Cell. Signal. 22: 1267–1273. https://doi.org/10.1016/j.cellsig.2010.03.005
Kovalzon, V. M., L. S. Moiseenko, A. V. Ambaryan, S. Kurtenbach, V. I. Shestopalov *et al.*, 2017 Sleep-wakefulness cycle and behavior in pannexin1 knockout mice. Behav. Brain Res. 318: 24–27. https://doi.org/10.1016/j.bbr.2016.10.015
Langmesser, S., P. Franken, S. Feil, Y. Emmenegger, U. Albrecht *et al.*, 2009 cGMP-dependent protein kinase type I is implicated in the regulation of the timing and quality of sleep and wakefulness. PLoS One 4: e4238. https://doi.org/10.1371/journal.pone.0004238
Lear, B. C., J. M. Lin, J. R. Keath, J. J. McGill, I. M. Raman *et al.*, 2005 The ion channel narrow abdomen is critical for neural output of the Drosophila circadian pacemaker. Neuron 48: 965–976. https://doi.org/10.1016/j.neuron.2005.10.030
Lee, J. H., L. L. Cribbs, and E. Perez-Reyes, 1999 Cloning of a novel four repeat protein related to voltage-gated sodium and calcium channels. FEBS Lett. 445: 231–236. https://doi.org/10.1016/S0014-5793(99)00082-4
Liebeskind, B. J., D. M. Hillis, and H. H. Zakon, 2012 Phylogeny unites animal sodium leak channels with fungal calcium channels in an ancient, voltage-insensitive clade. Mol. Biol. Evol. 29: 3613–3616. https://doi.org/10.1093/molbev/mss182
Long, M. A., M. J. Jutras, B. W. Connors, and R. D. Burwell, 2005 Electrical synapses coordinate activity in the suprachiasmatic nucleus. Nat. Neurosci. 8: 61–66. https://doi.org/10.1038/nn1361
Meelkop, E., L. Temmerman, T. Janssen, N. Suetens, I. Beets *et al.*, 2012 PDF receptor signaling in *Caenorhabditis elegans* modulates locomotion and egg-laying. Mol. Cell. Endocrinol. 361: 232–240. https://doi.org/10.1016/j.mce.2012.05.001
Meng, L., C. H. Chen, and D. Yan, 2016 Regulation of gap junction dynamics by UNC-44/ankyrin and UNC-33/CRMP through VAB-8 in *C. elegans* neurons. PLoS Genet. 12: e1005948. https://doi.org/10.1371/journal.pgen.1005948
Mok, D. Z., P. W. Sternberg, and T. Inoue, 2015 Morphologically defined sub-stages of *C. elegans* vulval development in the fourth larval stage. BMC Dev. Biol. 15: 26. https://doi.org/10.1186/s12861-015-0076-7
Monsalve, G. C., C. Van Buskirk, and A. R. Frand, 2011 LIN-42/PERIOD controls cyclical and developmental progression of *C. elegans* molts. Curr. Biol. 21: 2033–2045. https://doi.org/10.1016/j.cub.2011.10.054
Nichols, A. L. A., T. Eichler, R. Latham, and M. Zimmer, 2017 A global brain state underlies *C. elegans* sleep behavior. Science 356: eaam6851. https://doi.org/10.1126/science.aam6851
Park, E. C., and H. R. Horvitz, 1986 Mutations with dominant effects on the behavior and morphology of the nematode *Caenorhabditis elegans*. Genetics 113: 821–852.
Patel, L. S., C. K. Mitchell, W. P. Dubinsky, and J. O'Brien, 2006 Regulation of gap junction coupling through the neuronal connexin Cx35 by nitric oxide and cGMP. Cell Commun. Adhes. 13: 41–54. https://doi.org/10.1080/15419060600631474
Pogoda, K., P. Kameritsch, M. A. Retamal, and J. L. Vega, 2016 Regulation of gap junction channels and hemichannels by phosphorylation and redox changes: a revision. BMC Cell Biol. 17: 11. https://doi.org/10.1186/s12860-016-0099-3
Raizen, D. M., J. E. Zimmerman, M. H. Maycock, U. D. Ta, Y. J. You *et al.*, 2008 Lethargus is a *Caenorhabditis elegans* sleep-like state. Nature 451: 569–572 (erratum: Nature 453: 952). https://doi.org/10.1038/nature06535
Ren, D., 2011 Sodium leak channels in neuronal excitability and rhythmic behaviors. Neuron 72: 899–911. https://doi.org/10.1016/j.neuron.2011.12.007
Schwarz, J., and H. Bringmann, 2013 Reduced sleep-like quiescence in both hyperactive and hypoactive mutants of the Galphaq gene *egl-30* during lethargus in *Caenorhabditis elegans*. PLoS One 8: e75853. https://doi.org/10.1371/journal.pone.0075853

Schwarz, J., I. Lewandrowski, and H. Bringmann, 2011 Reduced activity of a sensory neuron during a sleep-like state in *Caenorhabditis elegans*. Curr. Biol. 21: R983–R984. https://doi.org/10.1016/j.cub.2011.10.046

Sedensky, M. M., and P. M. Meneely, 1987 Genetic analysis of halothane sensitivity in Caenorhabditis elegans. Science 236: 952–954. https://doi.org/10.1126/science.3576211

Senatore, A., and J. D. Spafford, 2013 A uniquely adaptable pore is consistent with NALCN being an ion sensor. Channels (Austin) 7: 60–68. https://doi.org/10.4161/chan.23981

Senatore, A., A. Monteil, J. van Minnen, A. B. Smit, and J. D. Spafford, 2013 NALCN ion channels have alternative selectivity filters resembling calcium channels or sodium channels. PLoS One 8: e55088. https://doi.org/10.1371/journal.pone.0055088

Singh, K., M. Y. Chao, G. A. Somers, H. Komatsu, M. E. Corkins *et al.*, 2011 *C. elegans* Notch signaling regulates adult chemosensory response and larval molting quiescence. Curr. Biol. 21: 825–834. https://doi.org/10.1016/j.cub.2011.04.010

Singh, K., J. Y. Ju, M. B. Walsh, M. A. DiIorio, and A. C. Hart, 2014 Deep conservation of genes required for both *Drosophila melanogaster* and *Caenorhabditis elegans* sleep includes a role for dopaminergic signaling. Sleep (Basel) 37: 1439–1451. https://doi.org/10.5665/sleep.3990

Starich, T. A., R. K. Herman, and J. E. Shaw, 1993 Molecular and genetic analysis of unc-7, a *Caenorhabditis elegans* gene required for coordinated locomotion. Genetics 133: 527–541.

Starich, T. A., R. Y. Lee, C. Panzarella, L. Avery, and J. E. Shaw, 1996 eat-5 and unc-7 represent a multigene family in *Caenorhabditis elegans* involved in cell-cell coupling. J. Cell Biol. 134: 537–548. https://doi.org/10.1083/jcb.134.2.537

Starich, T. A., J. Xu, I. M. Skerrett, B. J. Nicholson, and J. E. Shaw, 2009 Interactions between innexins UNC-7 and UNC-9 mediate electrical synapse specificity in the *Caenorhabditis elegans* locomotory nervous system. Neural Dev. 4: 16. https://doi.org/10.1186/1749-8104-4-16

Takahashi, M., and S. Takagi, 2017 Optical silencing of body wall muscles induces pumping inhibition in *Caenorhabditis elegans*. PLoS Genet. 13: e1007134. https://doi.org/10.1371/journal.pgen.1007134

Trojanowski, N. F., and D. M. Raizen, 2016 Call it worm sleep. Trends Neurosci. 39: 54–62. https://doi.org/10.1016/j.tins.2015.12.005

Trojanowski, N. F., M. D. Nelson, S. W. Flavell, C. Fang-Yen, and D. M. Raizen, 2015 Distinct mechanisms underlie quiescence during two *Caenorhabditis elegans* sleep-like states. J. Neurosci. 35: 14571–14584. https://doi.org/10.1523/JNEUROSCI.1369-15.2015

Trojanowski, N. F., D. M. Raizen, and C. Fang-Yen, 2016 Pharyngeal pumping in *Caenorhabditis elegans* depends on tonic and phasic signaling from the nervous system. Sci. Rep. 6: 22940. https://doi.org/10.1038/srep22940

Troup, M., M. H. Yap, C. Rohrscheib, M. J. Grabowska, D. Ertekin *et al.*, 2018 Acute control of the sleep switch in Drosophila reveals a role for gap junctions in regulating behavioral responsiveness. eLife 7: e37105. https://doi.org/10.7554/eLife.37105

Van Buskirk, C., and P. W. Sternberg, 2007 Epidermal growth factor signaling induces behavioral quiescence in *Caenorhabditis elegans*. Nat. Neurosci. 10: 1300–1307. https://doi.org/10.1038/nn1981

van der Linden, A. M., M. Beverly, S. Kadener, J. Rodriguez, S. Wasserman *et al.*, 2010 Genome-wide analysis of light- and temperature-entrained circadian transcripts in *Caenorhabditis elegans*. PLoS Biol. 8: e1000503. https://doi.org/10.1371/journal.pbio.1000503

Venkatachalam, V., N. Ji, X. Wang, C. Clark, J. K. Mitchell *et al.*, 2016 Pan-neuronal imaging in roaming *Caenorhabditis elegans*. Proc. Natl. Acad. Sci. USA 113: E1082–E1088. https://doi.org/10.1073/pnas.1507109113

Xie, L., S. Gao, S. M. Alcaire, K. Aoyagi, Y. Wang *et al.*, 2013 NLF-1 delivers a sodium leak channel to regulate neuronal excitability and modulate rhythmic locomotion. Neuron 77: 1069–1082. https://doi.org/10.1016/j.neuron.2013.01.018

Yeh, E., S. Ng, M. Zhang, M. Bouhours, Y. Wang *et al.*, 2008 A putative cation channel, NCA-1, and a novel protein, UNC-80, transmit neuronal activity in *C. elegans*. PLoS Biol. 6: e55. https://doi.org/10.1371/journal.pbio.0060055

Zhang, L., B. Y. Chung, B. C. Lear, V. L. Kilman, Y. Liu *et al.*, 2010 DN1(p) circadian neurons coordinate acute light and PDF inputs to produce robust daily behavior in *Drosophila*. Curr. Biol. 20: 591–599. https://doi.org/10.1016/j.cub.2010.02.056

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