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A tripartite flip-flop sleep circuit switches sleep states

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Abstract

Inhibitory sleep-active neurons depolarize at sleep onset to shut down the activity of wakefulness circuits. Wake-active arousal neurons in turn suppress inhibitory sleep-active neurons, thus forming a bipartite flip-flop switch. However, how sleep states are switched is unclear because neural circuits that directly depolarize inhibitory sleep-active neurons are not understood. Using optogenetics, we solved the presynaptic circuit for depolarization of the sleep-active RIS neuron in *C. elegans*. Surprisingly, we found that the PVC forward command interneuron, which is known to control wake behavior, is a major activator of RIS. The PVCs are inhibited by reverse command interneurons, which are stimulated by arousing cues. This suggests a model for sleep switch operation in which declining arousal increases activation of PVC, thus triggering activation of RIS. Depolarization of RIS in turn promotes the activation of PVC, thus forming a positive feedback loop for all-or-none sleep induction. The flip-flop sleep switch in *C. elegans* thus is tripartite and requires excitatory sleep-promoting neurons activated by wakefulness that act as an amplifier that translates reduced arousal into the depolarization of an inhibitory sleep-active neuron. A tripartite flip-flop switch likely also underlies sleep state switching in other animals including in mammals.
Introduction

Sleep is a behavior that affects many, if not all, physiological processes. Disorders and curtailment of sleep affect the lives of 10-30% of the adult population of modern societies. Sleep deprivation is detrimental to human health and is associated with an increased risk of infection, cardiovascular disease, depression, obesity, type 2 diabetes, and cancer. The high prevalence of insomnia and insufficient sleep quality thus presents a massive unmet health and economic problem[1-4]. To understand how behavior is generated, it is crucial to solve the underlying neural circuits. For example, the circuit of the crustacean stomatogastric nervous system showed that neuromodulation alters behavior without changing the connectome[5], the learning circuits in *Aplysia* revealed synaptic mechanisms of memory formation[6], and the grid cell circuit in the mammalian entorhinal cortex demonstrated how a map of the spatial environment can be encoded[7].

Sleep circuits require inhibitory sleep-active sleep-promoting neurons, which depolarize specifically at sleep onset and actively induce sleep by releasing inhibitory neurotransmitters, GABA and neuropeptides, to dampen arousal and the activity of wake circuits[8]. The depolarization of inhibitory sleep-active neurons defines the key properties of sleep behavior, which are the suppression of voluntary movements and sensory perception, reversibility, and homeostasis[9]. Inhibitory sleep-active neurons suppress wake circuits leading to a reduction of voluntary movement, sensory processing, and reduced responsiveness to stimulation. Inhibitory sleep-active neurons can be rapidly suppressed by arousing stimulation to allow for quick awakening. Forced wakefulness is followed by an increase of inhibitory sleep-active neuron depolarization, which leads to homeostatic sleep corrections. Thus, understanding sleep control requires comprehension of the circuit mechanisms that determine when and how much inhibitory sleep-active neurons depolarize[8, 10].

Circuits control the depolarization of inhibitory sleep-active neurons. For example, wake-active wake-promoting neurons promote arousal and suppress inhibitory sleep-active neurons, whereas sleep need causes tiredness and inhibitory sleep-active neuron
depolarization. Thus, inhibitory sleep-active sleep-promoting and wake-active wake-promoting neurons form a flip-flop switch, which ensures that sleep and wake exist as discrete states. This sleep switch is under the control of arousal that favors wake and inhibits sleep through the suppression of sleep-active neurons by inhibitory wake-active neurons[8, 11]. It has been proposed that sleep induction is favored by disinhibition of inhibitory sleep-active neurons [12-14], and also excitatory sleep-active neurons exist that might perhaps present activators of inhibitory sleep-active neurons [15]. However, the forces and mechanisms that flip the sleep switch from wake to sleep when an organism gets tired cannot be satisfactorily explained by the present circuit models as it is unclear how inhibitory sleep-active neurons are turned on when the system is set to sleep.

Sleep is under circadian and homeostatic controls that determine the timing of sleep and ensure that enough of this essential physiological state takes place[16]. Sleep homeostasis comprises multiple mechanisms that act on different time scales. On long time scales sleep is a function of prior wakefulness, i.e. prolonged wakefulness leads to increased sleep propensity and sleep loss triggers compensatory increases in the intensity or duration of sleep. This chronic sleep homeostasis likely is mediated by several parallel mechanisms. For example, in mammals, somnogens such as adenosine accumulate during wakefulness leading to the inhibition of wake-promoting neurons[17, 18]. In Drosophila, activity-dependent plasticity of sleep-promoting neurons increases during wakefulness to increase subsequent sleep[19, 20]. On short time scales, acute homeostasis determines whether the system’s actual state matches the system’s set point and carries out corrective action if those values do not match. For example, to homeostatically maintain sleep despite disturbance, micro arousals need to be compensated for. In humans, homeostatic sleep maintenance can be seen in EEG recordings in the form of k-complexes, where a spontaneous or evoked short cortical up state is followed by a down state[21-23]. Homeostatic sleep maintenance is also found during sleep in C. elegans, where sleep bouts are interrupted by short motion bouts, with the length of a motion bout correlating with the length of the subsequent sleep bout[24, 25]. Thus, across systems, homeostatic sleep maintenance requires constant surveillance of sleep and corrective action.
Sleep-active sleep-promoting neurons are conserved regulators of sleep and have been found both in vertebrates as well as in invertebrates[10, 26]. Mammals possess several populations of sleep-active neurons across the brain, most of which are inhibitory, including neurons in the anterior hypothalamus, brain stem, and cortex [8, 14]. Excitatory sleep-active neurons were found in the periocular midbrain that project to inhibitory sleep-active neurons in the anterior hypothalamus, whose role could be to activate inhibitory sleep-active neurons, but this hypothesis has yet to be tested experimentally[15]. Studying sleep in less complex brains facilitates sleep circuit analysis. In Drosophila, sleep-promoting neurons are found at several locations in the brain. A well-characterized population of sleep-promoting neurons is formed by ExFl1 neurons residing in the dorsal fan-shaped body. R2 neurons accumulate homeostatic sleep pressure over time to promote activation of ExFl1 neurons, probably by an indirect mechanism [19, 20]. C. elegans possesses a single inhibitory sleep-active neuron called RIS. Like its mammalian counterparts, RIS depolarizes at sleep onset. RIS is crucial for sleep induction as its ablation leads to a virtually complete loss of detectable sleep bouts[27-29]. The small, invariant nervous system, its mapped connectome, and the transparency of C. elegans facilitates neural circuit analysis[30]. However, the specific neural circuits that control RIS activity are not yet understood.

C. elegans shows sleep behavior during many stages and conditions. Here we analyzed sleep behavior during lethargus, the stage prior to each of the four molts during larval development [10, 29, 31-33]. We used optogenetics to dissect the neural circuit that controls the activation of the inhibitory sleep-active RIS neuron in C. elegans. We found that the PVC command interneurons, which are activated by wakefulness, are sleep-promoting by turning on the RIS sleep-active neuron. PVC activity is inhibited by reverse arousal command interneurons that thus inhibit sleep. This tripartite flip-flop circuit can explain how arousing stimulation inhibits RIS depolarization, how RIS depolarization is homeostatically controlled, and how reduced arousal can induce RIS depolarization.

Results
A command interneuron circuit controls RIS activity

RIS is crucially required for sleep and activates during each sleep bout (Figure 1A) [27]. However, the presynaptic driver neurons that activate and control this neuron are not known. To identify the circuits controlling RIS activation transients, we optogenetically tested the role of neurons that are presynaptic to RIS according to the C. elegans connectome [30]. The neurons AVJL, CEPDL, URYVL, RIMR, PVCL, and SDQL have been shown to be presynaptic to RIS[30]. To find out how these presynaptic neurons control RIS, we activated them with ReaChR and green light, and followed RIS calcium activity during and outside of lethargus. Because there were no specific promoters available for the expression in SDQL and PVC, we expressed ReaChR using semi-specific promoters and selectively illuminated only the presynaptic neuron class. We used L1 larvae for most of the optogenetic experiments to dissect the circuit. As SDQL is born postembryonically and likely is not yet functional during the L1 stage, we used L4 larvae to assay its function[34]. We compared the effects of optogenetic stimulation outside and during lethargus, defined as the period prior to the molt during which the animals do not feed[35]. Before lethargus, we measured an activation of RIS upon depolarization of AVJ, CEP and SDQL, whereas activation of the other neurons tested caused no significant change in RIS activity. During lethargus, the activation of CEP, PVC and SDQL caused RIS activation (Figure 1B, Supplementary Figure 1A). All neurons showed consistent effects on RIS depolarization except RIM. While RIM caused only a minor and non-significant net activation of RIS in the averaged measurements, RIM activation either activated or inhibited RIS depending on the trial (Figure S1B-C).

PVC appeared to be a strong activator of RIS only during lethargus. This suggested that either the PVC to RIS connection might be specific for lethargus or that it has not yet matured during the mid L1 stage. We hence repeated the experiment and activated PVC in L2 larvae. PVC activated RIS both during and outside of lethargus, but the activation during lethargus was much stronger, suggesting that the activation of RIS by PVC is strongly enhanced during lethargus (Figure S1C). To find out which presynaptic neurons are required for inhibition or activation of RIS during lethargus, we tested the effect of...
optogenetic inhibition of the presynaptic neurons on RIS activation. We used ArchT, which
hyperpolarizes neurons by pumping protons out of the cell [36, 37]. As before, we
specifically illuminated each presynaptic neuron class and quantified RIS activation using
calcium imaging. Before lethargus, inhibition of AVJ and PVC led to an inhibition of RIS,
whereas inhibition of the other neurons tested had no acute significant effect on RIS.
During lethargus, only optogenetic inhibition of PVC led to significant RIS inhibition,
whereas there was no effect seen for the other neurons (Figure 1C, Figure S2A). While
inhibition of RIM had no significant net effect on RIS, in roughly one quarter of the
measured trials, the inhibition of RIM led to RIS inhibition before and during lethargus
(Figure S2B).

Our optogenetic analysis revealed a complex set of presynaptic inputs for regulation of RIS
activity. The optogenetic gain-of-function experiments suggest that CEP, PVC, and SDQL
present the most potent presynaptic activators of RIS. The capacity of PVC to activate RIS
is strongly increased during lethargus, indicating that this neuron is involved in the
lethargus-specific activation of RIS. The optogenetic loss-of-function experiments suggest
that PVC is an essential presynaptic activator of RIS during lethargus, but additional
neurons might contribute to RIS activation during lethargus or other conditions. RIM has
the potential to both inhibit and activate RIS. While the activation could be direct, RIM is
known to be an inhibitor of PVC through activation of the reverse command interneurons
AVA/AVD/AVE and could thus perhaps inhibit RIS indirectly [38-40]. Consistent with
the lack of net effects of RIM on RIS, RIM ablation does not change spontaneous sleep
amounts (Figure S2C). Therefore, the majority of synaptic inputs into RIS is activating and
inhibition appears to be predominantly indirect. The CEP, URY, and SDQL neurons
present sensory receptors and might play a role in activating RIS in response to stimulation
[41, 42]. Because of the strong and lethargus-specific effects of the PVC neurons on RIS
activation we focused our analysis on these key neurons.

As the PVC neurons are crucial activators of RIS we tested their role in inducing sleep
behavior. We used a strain that ablated PVC and other command interneurons by
expressing the pro-apoptosis regulator ICE from the nmr-1 promotor and measured sleep
and RIS activation[43]. Command interneuron ablation reduced sleep bouts during lethargus by about 76% (Figure S3A). The command interneuron-ablated worms also generally moved much slower (Figure S3B) and RIS activation was reduced by 63% (Figure S3C). Quiescence bouts did not occur at the beginning of the lethargus phase as defined by cessation of feeding, and were only observed around the middle of the lethargus phase (Figure S3D). An independently generated strain that ablates command interneurons using egl-1 expression caused a reduction of sleep by 81% (Figure S2A). Because the command interneurons are controlled by glutamatergic signaling we also tested mutants in which this type of signaling is impaired and also observed impaired sleep consistent with ablating command interneurons (Figure S4).

To be able to more specifically manipulate PVC and to test the effects of PVC inhibition on behavior without affecting the other command interneurons, we used a more specific promoter for expression in PVC, which had been identified from single-cell RNA sequencing data [44, 45](Jonathan Packer, personal communication). There was no gene in the available datasets that was expressed only in the cluster of cells containing PVC, but the previously uncharacterized gene zk673.11 was expressed strongly in PVC and in only a few other neurons excluding other command interneurons and therefore we used the promoter of this gene for PVC expression (Figure S5). Hyperpolarization of PVC through activation of ArchT led to an acute inhibition of RIS, an increase in locomotion, and a reduction of sleep (Figure 1D). These experiments show that RIS is controlled by a command interneuron circuit. The major command interneuron activator of RIS during L1 lethargus are the PVC forward command interneurons (Figure 1E). While PVC has previously been shown to promote forward locomotion[46], its inhibition leads to increased mobility implying that the reduction of locomotion after simultaneous ablation of most command interneurons stems from the reverse command interneurons that promote mobility, whereas PVC appears to play a predominant role in dampening motion behaviors through activation of RIS.

**RIS and PVC activate each other forming a positive feedback loop**
PVC presents a major activator of RIS, but how a forward command interneuron can cause massive activation of the RIS neuron during sleep bouts is not clear. We hence tested how optogenetic RIS activation affects PVC activity. We selectively activated RIS using ReaChR and measured calcium activity in PVC (Figure 2A). Because the calcium transients observable in PVC are typically small we used immobilized worms to reduce measurement noise[47]. Upon RIS stimulation, PVC immediately displayed unexpectedly strong calcium transients, which were slightly stronger during lethargus (Figure 2B, Figure S6A). These results show that PVC and RIS activate each other, thus forming a positive feedback loop. The strong calcium activation of PVC by RIS depolarization is striking because activation transients in PVC have hitherto only been observed after mechanical stimulation, whereas spontaneous PVC activity transients have not yet been reported[47]. The sleep-inducing RIS neuron has so far only been shown to inhibit other neurons making PVC the first neuron which is not inhibited but activated by RIS. For example, command interneurons such as AVE/AVA and other neurons are not activated by RIS but inhibited[27]. While PVC is presynaptic to RIS, RIS is not presynaptic to PVC. The activation of PVC by RIS could involve diffusional mechanisms or could be indirect through other neurons, perhaps mediated by the inhibition of a PVC inhibitor such as AVA/AVD/AVE. It is currently not possible to measure spontaneous calcium transients in PVC, but our results would predict that PVC should spontaneously activate during sleep bouts, which would characterize PVC not only as a neuron active during wakefulness as suggested by previous studies[46, 47] but also as a sleep-active neuron. It would thus differ from previously identified sleep-active neurons in that it does not induce sleep directly by inhibiting wake circuits but indirectly through activation of an inhibitory sleep-active sleep-inducing neuron. As PVC is activated by wakefulness and also promotes sleep, it would thus be ideally suited to mediate the transition from active to quiescent states.

Together, these data suggest that PVC and RIS rely on positive feedback to achieve strong transients during a sleep bout.

If depolarization of RIS activates PVC, what consequences does hyperpolarization of RIS have on PVC activity? To answer this question, we measured the response of PVC to RIS inhibition. We hyperpolarized RIS optogenetically for one minute using ArchT and...
measured the activity of PVC. Interestingly, PVC showed a small but significant activity increase during RIS inhibition, an effect which was increased during lethargus (Figure 2C). The disinhibition of PVC by RIS inactivation is likely not direct and may reflect a general increase in neuronal and behavioral activity that is caused by RIS inhibition and that extends to the PVC neurons. Since PVC is a major activator of RIS, its disinhibition could be part of a homeostatic feedback regulation.

Our results suggest that there is a positive feedback from sleep induction onto RIS activation and that full RIS activation is only possible when sleep is successfully induced, explaining the strong correlation of RIS depolarization and sleep bout induction[29]. This model would predict that RIS transients are dampened if RIS is not able to induce sleep bouts. To test this idea we analyzed RIS calcium transients in aptf-1(-) mutant worms in which RIS still shows depolarization transients during lethargus, but cannot efficiently induce quiescence behavior because its inhibitory neuropeptides are not expressed[27, 48]. In aptf-1(-) mutant animals, calcium transients’ maxima were reduced by about 35% (Figure 2 D-E). These results are consistent with the idea that sleep induction is a self-enforcing process in which RIS-mediated inhibition of brain activity promotes further RIS activation (Figure 2F).

**RIS depolarization is under homeostatic control**

The design of the sleep circuit suggests an intimate mutual control mechanism of RIS and command interneurons that could allow homeostatic control of sleep. Arousing stimulation is known to inhibit sleep-active neurons and to increase subsequent sleep[24, 25, 27, 29]. We thus hypothesized that inhibition of RIS leads to its subsequent depolarization, forming a homeostat that allows maintaining or reinstating sleep bouts. We tested this hypothesis by optogenetically hyperpolarizing RIS and following its activity using calcium imaging. We inhibited RIS directly for 60 seconds by expressing the light-driven proton pump ArchT specifically in this neuron and used green light illumination to activate ArchT. We followed RIS calcium activity using GCaMP during the experiment and quantified behavior. Optogenetic hyperpolarization of RIS led to a decrease in intracellular calcium
and increased behavioral activity. Approximately one minute after the end of the inhibition, RIS showed a rebound activation transient during which calcium activity levels increased strongly and rose well above baseline levels, concomitant with a decrease in behavioral activity. Overall brain activity measurements showed that behavioral activity and brain activity correlated throughout the experiment (Figure 3A). Strikingly, while the rebound transient was also measurable outside of lethargus, the strength of the RIS rebound depolarization was three-fold stronger during lethargus than before lethargus, indicating that the propensity for RIS rebound activation is strongly increased during lethargus.

To test whether rebound activation of RIS mediates acute or chronic homeostasis, we tested whether the strength of the rebound activation is a function of length of prior inhibition. For this experiment we increased the length of the RIS inhibition and quantified the time it took after the end of the stimulation until the rebound transient started as well as the peak maximum of the rebound. After inhibiting RIS for five minutes, the rebound initiated immediately after the end of the stimulation and the maximum that was reached exceeded that observed after about one minute of RIS stimulation. Inhibiting RIS for ten minutes did not further increase the occurrence or strength of the rebound transient. These results show that RIS activation rebound transients rapidly saturate with increasing length of inhibition (Figure 3B, Figure S6B-D) Thus, RIS shows a rebound activation upon inhibition that leads to its own subsequent activation. A rebound activation was not only seen when RIS was inhibited directly, but we also saw a reactivation of RIS after it was inhibited indirectly using a blue light stimulus when the worms returned to sleep (Figure S7). The rebound activation presents the translation of RIS inhibition into subsequently increased RIS activity and thus sleep induction. Rebound activation of RIS does not seem to constitute a chronic integrator of wake time but presents an acute homeostatic regulatory phenomenon to induce, maintain, or reinstate sleep bouts.

If RIS is part of a homeostatic system and RIS inhibition triggers its subsequent activation, does activation of RIS also trigger its own inhibition? To test this idea, we optogenetically activated RIS and measured its activity before, during, and after the manipulation. We optogenetically depolarized RIS for one minute using green light and the light-activated
cation channel ReaChR expressed specifically in RIS. Activity of RIS was followed using calcium imaging and increased strongly during optogenetic depolarization but was also reduced significantly after stimulation compared with baseline activity, indicating that RIS activity also shows a negative rebound. Again, this effect was highly increased, about five-fold, in worms that were in lethargus compared with worms outside of lethargus (Figure 3C). Thus, polarization of RIS during lethargus is under homeostatic control in both directions. Hyperpolarization leads to subsequent depolarization, perhaps through a reactivation of wake-active circuits that ultimately lead to the reactivation of PVC. Depolarization leads to subsequent hyperpolarization, perhaps through inhibition of wake-active circuits that cause PVC activation. This homeostatic regulation is highly enhanced during lethargus and can thus contribute to the repetitive pattern of RIS activation and thus the oscillating induction of consecutive sleep bouts[24].

Modest dampening of brain arousal occurs upstream of RIS

Our results demonstrate that PVC plays a major role in activating RIS involving self-enforcing positive feedback, resulting in strong RIS activation and thus sleep induction. RIS calcium transients during development outside of lethargus are small, whereas transients are high during lethargus. What determines that RIS calcium transients are limited outside of lethargus but promoted during lethargus? With other words, what tips the balance that allows the self-enforcing feedback loop of PVC and RIS to set off? As an important principle of command interneuron control, forward and reverse command interneurons inhibit each other to allow discrete forward and reverse locomotion states. The AVA/AVD/AVE/RIM interneurons initiate reverse locomotion by activating premotor interneurons while inhibiting the forward command circuit including AVB/PVC. By contrast, during forward movement reverse command interneurons are inhibited [46, 49].

Small changes in arousal and activity of the command interneurons can change the equilibrium of forward and reverse command interneurons [43]. Hyperactive mutants suppress sleep across species including C. elegans [50-52]. Many arousal cues trigger backwards escape movements and inhibit RIS [27, 29, 53]. Thus, previous studies on the
command interneuron circuit together with our results suggest that arousal inhibits RIS through inhibiting PVC. This model of RIS activation would predict that there are changes during lethargus that are upstream of RIS activity that change the properties of the command circuit leading to increased PVC and thus RIS activation.

We reasoned that it should be possible to measure these changes that occur in command interneuron activity upstream of RIS by characterizing neural activity and behavior in aptf-1(-) mutant worms. We quantified behavior and command interneuron calcium levels across lethargus in aptf-1(-) mutant worms. Wild type animals showed successive sleep bouts and a 70% reduction in locomotion speed by during lethargus. By contrast, aptf-1(-) mutant animals almost never showed quiescence bouts (Figure 2E), but nevertheless locomotion speed was decreased by 25% during the lethargus phase (Figure 4). Consistent with the behavioral activity reduction, there was a significant reduction of command interneuron activity during lethargus also in aptf-1(-) mutant animals (Figure 4, Figure S8). To further characterize the neuronal changes upstream of RIS-mediated sleep induction, we imaged the activity of RIM as exemplary neurons during lethargus in aptf-1(-) mutants. In wild type animals, RIM regularly showed activation transients before lethargus but did not show many transients during lethargus. RIM showed not only a change in transient frequency across the lethargus cycle but also a reduction in baseline calcium activity. In aptf-1(-) mutant worms, RIM continued showing RIS transients during lethargus, indicating that RIS inhibits RIM transients during sleep bouts. However, reduction of baseline calcium activity was preserved in aptf-1(-), indicating that RIM activity is dampened during lethargus independently of RIS at the level of baseline calcium activity. Together these experiments indicate that a dampening of behavioral and neural baseline activity occurs during lethargus that is independent of RIS. This neuronal baseline and behavioral dampening itself appears not to be sufficient to constitute normal sleep bouts, but could hypothetically lead to a weaker activity of reverse arousal neurons thus allowing stronger or longer activation of forward command interneurons including PVC and thus RIS activation[43, 54].

**An arousing stimulus inhibits RIS through RIM**
Arousal plays a major role in inhibiting sleep but the circuits that mediate the effect of arousing stimuli on RIS inhibition are not well understood. We hence studied the circuit by which stimulation of a nociceptor, the ASH neurons, leads to a reverse escape response and inhibition of RIS[55]. We optogenetically stimulated ASH using ReaChR and green light and followed RIS and RIM activities. ASH activation led to the activation of the RIM neuron and triggered a backwards response as previously described [55, 56]. Simultaneously, RIS was inhibited (Figure 5A). RIM can inhibit PVC through reverse interneurons which it synchronizes, making it ideally suited to mediate the effects of ASH stimulation[39, 46]. To test whether RIM is required for RIS inhibition, we ablated RIM genetically by expression of egl-1 from the tdc-1 promoter, which is specific to RIM and RIC, and repeated the optogenetic stimulation of ASH. We used L4 stage animals as the genetic ablation was not yet effective in L1 animals. In RIM-ablated animals, activation of ASH caused the opposite effect on RIS activity. Instead of inhibiting RIS, ASH activated RIS, while still increasing behavioral activity (Figure 5B). Consistent with our calcium imaging data, ASH stimulation after RIM ablation predominantly caused a forward locomotion response (Figure 5C). Further supporting the idea that reverse interneurons inhibit and forward PVC neurons activate RIS during stimulation, gentle tail touch increased RIS activity more strongly when RIM was ablated (Figure S9). These results delineate a circuit model for how sensory stimulation can control RIS activation. By activating the reverse response that is mediated by RIM, PVC is suppressed leading to RIS inhibition. In addition, sensory stimulation can also promote RIS activation if the forward command circuit is activated.

Discussion

A tripartite flip-flop circuit controls sleep

Optogenetic activation and inhibition showed how the activity of presynaptic neurons affects RIS depolarization during developmental sleep. Several presynaptic neurons can activate RIS, but potent direct inhibitors were not identified. Loss-of-function experiments
showed that the command circuit controls activation of RIS, with PVC presenting the key activator of RIS. PVC has long been known to mediate the forward escape response by transmitting information from posterior sensory neurons to activate AVB pre-motor neurons to trigger forward locomotion[46, 47, 57]. Consistent with promoting the forward escape response, optogenetic activation of PVC leads to an, albeit modest, increase in forward movement[57, 58]. Reverse movement, in turn, is mediated by AVA, AVE, and AVD command premotor interneurons, which activate reverse motor neurons. Forward PVC and reverse AVA/AVE/AVD command interneurons are presynaptic to and mutually inhibit each other, which ensures discrete forward and reverse locomotion states analogous to a flip-flop switch [40, 43, 46].

Our finding that PVC presents a key activator of RIS immediately suggests a model for how RIS is controlled and provides a potential mechanism for linking sleep induction to decreasing arousal and for homeostatically maintaining a series of sleep bouts. According to this model, during conditions of high arousal, such as during development outside of lethargus, larvae are constantly awake. The command interneuron circuit cycles between forward and reverse states, leading to the activation of forward or reverse motor programs, respectively[40, 46, 59]. Arousal promotes reverse command interneurons activity and thus limits PVC and RIS activation. Independently of RIS, lethargus induces a modest neural dampening that includes command interneuron activity that is evident as a decrease in baseline calcium activity and reduction of locomotion speed. Consistent with the view that reduction of neuronal activity can occur partially independently of RIS, the dampening of sensory neurons during lethargus is not fully reversible upon stimulation[60, 61]. This modest but broad activity dampening could cause a modest shift of command interneuron activity that should lead to a modest favoring of PVC activity, perhaps through disinhibition. Consistent with this model, sleep bouts are typically induced from long forward movement bouts or periods of reduced locomotion activity and not from reverse movements [54, 59, 62]. Mutations that increase arousal and suppress sleep increase the activity of reversal neurons, whereas conditions that decrease arousal decrease the activity of the reversal neurons and increase sleep[40, 51, 52]. Also, the ablation of reverse command interneurons such as AVE reduces reversals and leads to ectopic quiescence[47,
The increased activation of PVC in turn depolarizes RIS. Because PVC and RIS form
a self-reinforcing positive feedback loop, a modest increase of PVC activity could translate
non-linearly to a strong activation of RIS, which in turn induces the shutdown of behavior
that is required for the sleep state [24, 31, 54, 60, 61]. The activation of RIS leads to the
secretion of inhibitory neuropeptides that inhibit key arousal neurons [27, 48]. While PVC
is presynaptic to RIS and likely activates it directly, RIS is not presynaptic to PVC,
suggesting an indirect mechanism that perhaps could involve inhibition of reverse
command interneurons including AVA/AVE, which are inhibited by RIS [27] and could
thus lead to further activation of PVC through disinhibition. Thus, the process of sleep
induction, once it reaches a critical threshold, could become self-enforcing. In summary,
our model allows a circuit understanding of the mutual control of the wake-active central
pattern generator formed by the command interneurons and the sleep-active RIS neuron
and provides the mechanistic basis for understanding the correlation of sleep and
locomotion patterns observed in pan-neuronal imaging studies [54, 62]. Our model suggests
that the sleep switch is tripartite and includes self-enforcing inhibitory sleep-active sleep-
promoting neurons as well as excitatory wake-activated sleep-promoting neurons as
mediators of switch flipping. This sleep switch acts as an amplifier that can translate a
modest reduction of arousal into a massive shutdown of behavioral activity during sleep.
Dampening of neural activity independently of sleep-active neurons could be interpreted
as a neural equivalent of tiredness that leads to an increased propensity to activate sleep-
active neurons and to induce sleep bouts. Thus, the sleep switch could present an amplifier
that translates reduced brain activity into sleep bouts (Figure 6). Further experiments will
be necessary to find out how PVC activity is controlled by sleep-promoting signals such
as reduced arousal.

What causes the termination of sleep bouts? The PVC and RIS neurons might not be able
to sustain prolonged activity leading to the cessation of a sleep bout. The RIS activation
transient and thus sleep bout can be blunted by a sensory or optogenetic arousing stimulus
[27, 29, 54, 61]. Arousing stimulation, for instance by activating the nociceptive sensory
neurons, triggers a reverse escape response through backwards command and RIM
interneurons [39, 56, 59, 64]. Optogenetic RIM depolarization variably causes activation
or inhibition of RIS but the cause of this probabilistic outcome is unclear. Potentially, the activating effect of RIM on RIS could be mediated by direct synaptic innervation and might serve a buffering or probabilistic function [39], consistent with the almost zero net effects of RIM depolarization on RIS activity. However, stimulation of the nociceptive ASH neurons causes consistent inhibition of RIS that depends on RIM, suggesting that a main physiological role of RIM is to inhibit sleep upon arousing stimulation, perhaps by synchronizing the reverse interneurons[39]. RIM activation can inhibit sleep also in response to acute food deprivation [65, 66]. Thus, RIM might present an arousal module that can be activated upon sensing various external conditions that signal the need to suppress sleep (Figure 6).

RIS inactivation leads to disinhibition of arousal and brain activity starting anew the cycle of command interneuron activity and locomotion behavior. Depending on the arousal levels, PVC activity should increase either immediately or after a delay until it reaches a critical level sufficient to cause substantial RIS reactivation and thus a return to sleep. The timing at which the rebound activation occurs can be controlled by the level of arousal, with strong arousal leading to longer wake periods before the return to sleep, whereas milder stimulations cause the immediate return to sleep[25]. Consistent with this circuit model of recurrent RIS activation, RIS activity oscillates, resulting in the typical pattern of sleep bouts that are interrupted by activity bouts[24]. This circuit design allows homeostatic sleep maintenance of a series of consecutive sleep bouts with sensory stimulation restarting the cycle of RIS activation thus prompting an acutely increased RIS activation causing the return to sleep (Figure 6A)[25, 54].

How can PVC mediate seemingly contrary behaviors, the forward escape response and sleep bout induction? Sensory mechanical stimulation causes general arousal. For example, tail touch not only activates the forward PVC neurons to trigger a forward locomotion response, but also activates the reverse AVA neurons, with a net dominance of the PVC activation [46, 57]. Because the reverse command interneurons inhibit the forward interneurons, the sensory stimulation should lead to only a limited activation of PVC that is sufficient to trigger a forward movement, but that is insufficient to cause strong RIS
activation and thus a sleep bout. Consistent with this idea, weakening of the reverse circuit by RIM ablation changes the behavioral response of tail touch during lethargus, and PVC activation outside of lethargus only causes a modest increase in forward movement [57, 58]. During normal waking behavior, modest RIS activation may thus function to dampen the behavioral response to sensory stimulation and to regulate the speed of locomotion [67, 68].

Here we have identified a circuit controlling sleep-active neuron depolarization in *C. elegans*. This work built on the neural connectome and was facilitated by the small size and invariance of the nervous system as well as the transparency of the organism. While the *C. elegans* sleep circuit clearly is built from less cells than the human sleep circuit, there are many conceptual similarities. For instance, in both *C. elegans* and humans, sleep is controlled by inhibitory sleep-active sleep-promoting neurons that depolarize at sleep onset to actively induce sleep by inhibiting wake circuits. A main difference is that humans have many brain centers each consisting of thousands of sleep-active neurons [14]. The single RIS neuron is the major inhibitory sleep-active neuron required for sleep induction in *C. elegans* [27]. Work in mammals revealed the general principles of wake-active wake-promoting neurons and sleep-active sleep-promoting neurons as well as their mutual inhibition. While this information explains the flip-flop nature of sleep and wake states, there is no satisfactory understanding of what flips the sleep switch, i.e. how wakefulness is detected when the system is set to sleep, prompting the activation of inhibitory sleep-active neurons [8]. Our model for the operation of the *C. elegans* sleep circuit indicates that flipping of the sleep switch can be understood if excitatory wakefulness-activated sleep-promoting neurons and a positive feedback of sleep-active neuron depolarization are added to the switch model. In this tripartite flip-flop sleep switch model, the sleep-active sleep-promoting center is activated by an excitatory sleep-promoting center that is activated by wakefulness. Wake-activated sleep-promoting neurons and wake-active wake-promoting neurons inhibit each other thus forming the actual flip-flop switch. Arousing stimulation increases the activity of the wake-active wake-promoting neurons and thus suppresses excitatory wake-activated sleep-promoting neurons and thus sleep. According to this model, reducing arousal increases the activity of excitatory wake-
activated, sleep-promoting neurons and thus favors inhibitory sleep-active sleep-promoting neuron depolarization, causing sleep behavior (Figure 6). Therefore, reduced arousal and activity of wake-active wake-promoting neurons could present a neural correlate of tiredness. Excitatory neurons in the periocular midbrain of mammals are similar to PVC in that they may be activators of inhibitory sleep-active neurons an could perhaps be part of circuits that operate by mechanisms analogous to the tripartite sleep switch of *C. elegans* [15].

Sleep is reversible by stimulation and hyperarousal is the major cause for insomnia in humans[2, 69, 70]. Homeostatic sleep maintenance is an essential feature of sleep and is found from worms to humans[21-23, 25]. R2 ring neurons in *Drosophila* present an integrator of wake time causing subsequently increased depolarization of ExFl1 sleep-inducing neurons, thus forming a chronic sleep homeostat [71, 72]. Our model of a tripartite flip-flop circuit suggests that excitatory wake-activated sleep-promoting neurons are part of an acute sleep homeostat that translates acute brain activity into increased sleep neuron activity when the system is set to sleep. Excitatory wake-activated sleep-promoting neurons measure systemic activity, i.e. they become active as a consequence of a global brain activity increase, and directly activate the inhibitory sleep-active neuron transiently. Thus, the interplay of inhibitory sleep-active sleep-promoting and excitatory wake-active sleep-promoting neurons form an oscillator that periodically sends out sleep-inducing pulses. Macroscopically, sleep in mammals exists as cortical oscillations of global down states, known as slow waves[73]. Micro arousals trigger cortical upstates that are followed by cortical down states, known as k-complexes[21-23]. Both slow wave activity as well as k-complexes could be hypothetically explained by the concept of excitatory wake-activated sleep-promoting circuits.

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Author Contributions

E.M, I.B, F.M., J.B., and M.T. designed, performed, and analyzed experiments. E.M and I.B edited the manuscript. H.B. acquired funding, conceived the project, designed experiments, supervised the work, and wrote the manuscript.

Declarations of Interest

The authors declare that they have no competing interests.

Supplemental Information

Supplemental Information includes 9 figures and 1 table and can be found with this article online.

Materials and Methods

Worm maintenance and strains

*C. elegans* worms were grown on Nematode Growth Medium (NGM) plates seeded with *E. coli* OP50 and were kept at 15-25°C [74]. Crossed strains were genotyped through Duplex PCR genotyping of single worms [75]. The primer sequences that were used for Duplex PCR can be found below. To confirm the presence of transgenes after crossings, fluorescent markers were used. The following *C. elegans* strains were used:

N2 Wild type (Bristol)
HBR430  goeIs64[aptf-1p::SL1-GCaMP3.35-SL2::mKate2-unc-54-3'utr, unc-119(+)].

HBR448  aptf-1(gk794) II; goeIs64[aptf-1p::SL1-GCaMP3.35-SL2::mKate2-unc-54-3'utr, unc-119(+)].

HBR531  yIs1[glt-1p::GCaMP3, unc-122p::gfp]; aptf-1(gk794) II.

HBR560  goeIs120[tdc-1p::SL1-GCaMP3.35-SL2::mKate2-unc-54-3'utr, unc119(+)].

HBR1118 aptf-1(gk794) II; goeIs120[tdc-1p::SL1-GCaMP3.35-SL2::mKate2-unc-54-3'utr, unc-119(+)].

HBR1361 goeIs304[flp-11p::SL1-GCaMP3.35-SL2::mKate2-unc-54-3'UTR, unc-119(+)].

HBR1374 goeIs307[flp-11p::ArchT::SL2mKate2-unc-54-3'utr, unc-119(+)];
goIs304[flp-11p::SL1-GCaMP3.35-SL2::mKate2-unc-54-3'UTR, unc-119(+)].

HBR1466 goeIs268[aptf-1p::SL1-GCaMP3.35-SL2::aptf-1-3'utr, unc-119(+)];
goIs315[flp-11p::ReaChR::mKate2-unc-54-3'UTR, unc-119(+)].

HBR1472 goeIs268[aptf-1p::SL1-GCaMP3.35-SL2::aptf-1-3'utr unc-119(+)];
goIs307[flp-11p::ArchT::SL2mKate2-unc-54-3'utr, unc-119(+)].

HBR1478 goeIs268[aptf-1p::SL1-GCaMP3.35-SL2::aptf-1-3'utr unc-119(+)];
  goeEx557[gcy-13p::ArchT::mKate2-unc-54-3'-utr, unc-119(+)].

HBR1482 goeIs268[aptf-1p::SL1-GCaMP3.35-SL2::aptf-1-3'utr unc-119(+)];
HBR1537  goeIs268[aptf-1p::SL1-GCaMP3.35-SL2::aptf-1-3'utr, unc-119(+)];
goIs308[dat-1p::ReaChR::mKate2-unc-54-3'UTR, unc-119(+)].

HBR1589  goeIs268[aptf-1p::SL1-GCaMP3.35-SL2::aptf-1-3'utr, unc-119(+)];
goIs330[nmr-1p::ArchT::mKate2-unc-54-3'utr, unc-119(+)].

HBR1597  goeIs268[aptf-1p::SL1-GCaMP3.35-SL2::aptf-1-3'utr, unc-119(+)],
goIs332[nmr-1p::ReaChR::mKate2-unc-54-3'utr, unc-119(+)].

HBR1659  unc-119(ed3) III; goeIs364[tdc-1p::egl-1::SL2-mKate2-unc-54-3utr, unc-119(+)].

HBR1753  wtfIs5[rab-3p::NLS::GCaMP6s; rab-3p::NLS::tagRFP]

HBR1776  wtfIs5[rab-3p::NLS::GCaMP6s; rab-3p::NLS::tagRFP]; goeIs307[flp-11p::ArchT::SL2mKate2-unc-54-3'utr, unc-119(+)]

HBR1793  goeIs268[aptf-1p::SL1-GCaMP3.35-SL2::aptf-1-3'utr, unc-119(+)];
goIs293[tol-1p::ReaChR::mKate2-unc-54-3'utr, unc-119(+)].

HBR1807  goeIs323[sra-6p::ReaChR::mKate2-unc-54-3'utr, unc-119(+)];
goIs304[flp-11p::SL1-GCaMP3.35-SL2::mKate2-unc-54-3'UTR, unc-119(+)].

HBR1844  goeIs268[paptf-1p::SL1-GCaMP3.35-SL2::aptf-1-3'utr, unc-119(+)];
goIs340[dat-1p::ArchT::SL2mKate2-unc-54-3'UTR].

HBR1845  goeIs268[paptf-1p::SL1-GCaMP3.35-SL2::aptf-1-3'utr, unc-119(+)];
goeIs370[lad-2p::ReaChR::mKate2-unc-54-3'UTR, unc-119(+)].

HBR1849 goeIs304[flp-11p::SL1-GCaMP3.35-SL2::mKate2-unc-54-3'UTR, unc-119(+)]; goeIs364 [tdc-1p::egl-1::SL2-mKate2-unc-54-3'utr, unc-119(+)]; goeIs232[sra-6p::ReaChR::mKate2-unc-54-3'utr, unc-119(+)].

HBR1873 goeIs268[paptf-1p::SL1-GCaMP3.35-SL2::aptf-1-3'utr, unc-119(+)];
goIs373[lad-2p::ArchT::SL2-mKate2-unc-54-3'UTR, unc-119(+)].

HBR1889 goeIs120[tdc-1p::SL1-GCaMP3.35-SL2::mKate2-unc-54-3'utr, unc119(+)];
goIs32[tdc-1p::SL1-GCaMP3.35-SL2::mKate2-unc-54-3'utr, unc-119(+)].

HBR1951 ynIs40[flp-11p::GFP] V; goeIs359 [nmr-1p::egl-1::SL2-mKate2-unc-54-3'utr, unc-119(+)].

HBR1982 goeIs402[tol-1p::ArchT::SL2-mKate2-unc-54-3'UTR,unc-119(+)];
goIs304[flp-11p::SL1-GCaMP3.35-SL2::mKate2-unc-54-3'UTR, unc-119(+)].

HBR2019 akIs11[nmr-1p::ICE];
goIs307[flp-11p::ArchT::SL2mKate2-unc-54-3'utr, unc-119(+)];
goIs304[flp-11p::SL1-GCaMP3.35-SL2::mKate2-unc-54-3'UTR, unc-119(+)].

HBR2021 goeIs307[flp-11p::ArchT::SL2mKate2-unc-54-3'utr,unc-119(+)];
goIs304[flp-11p::SL1-GCaMP3.35-SL2::mKate2-unc-54-3'UTR, unc-119(+)];
nmr-1(ak4) II.

HBR2033 goeIs195[nmr-1p::SL1-GCaMP6s::mKate2-unc-54-3'utr, unc-119(+)];
goIs403[flp-11p::ArchT::mKate2-flp-11-3'utr, unc-119(+)].
HBR2109  goels195[nmr-1p::SL1-GCaMP6s::mKate2-unc-54-3'utr, unc-119(+)];
goels315[flp-11p::ReaChR::mKate2-unc-54-3'UTR, unc-119(+)].

HBR2128  eat-4(ky5) III; goels304[flp-11p::SL1-GCaMP3.35-SL2::mKate2-unc-54-3'UTR, unc-119(+)].

HBR2169  goeEx718[hlh-34p::ReaChR::mKate2-unc-54-3'UTR, unc-119(+); myo-2p::mCherry]; goels304[flp-11p::SL1-GCaMP3.35-SL2::mKate2-unc-54-3'UTR, unc-119(+)].

HBR2180  goeEx725[hlh-34p::ArchT::SL2mKate2-unc-54-3'UTR, unc-119(+); myo-3p::mCherry]; goels304[flp-11p::SL1-GCaMP3.35-SL2::mKate2-unc-54-3'UTR, unc-119(+)].

HBR2231  goels445[ZK673.11p::ArchT::SL2mKate2-unc-54 3' UTR, unc-119(+)].

HBR2243  goels445[ZK673.11p::ArchT::SL2mKate2-unc-54 3' UTR, unc-119(+)];
goel5[nmr-1p::SL1-GCaMP3.35-SL2::unc-54-3'utr, unc-119(+)].

ZC1148   yxIs1[glr-1p::GCaMP3.35, unc-122p::gfp].

Primer sequence 5’-3’

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**aptf-1(gk794)**

| Primer sequence 5’-3’ |
|-----------------------|
| CGACAATCTTCCAAAGACC  |
| CGGATCGATTGCTAGAGGG  |
| GCTTGGACGCTTTAGTTGA  |

ArchT

| Primer sequence 5’-3’ |
|-----------------------|
| ACTTCATCGTCAAGGGATGG |
| CATGCGATGCTGGGAGAAGA |
Strain generation

DNA constructs were cloned with the three fragments Gateway System (Invitrogen, Carlsbad, CA) into pCG150 to generate new strains [76]. The ArchT, the ReaChR and the egl-1 genes were expression-optimized for C. elegans [77]. The tdc-1::egl-1 transgene specifically expresses the apoptosis inducing protein EGL-1 in RIM and RIC. Hence, RIM and RIC are genetically ablated in worms carrying this transgene. The ablation is probably incomplete in L1 worms. The nmr-1::egl-1 transgene leads to the expression of egl-1 in all command interneurons causing their genetic ablation. Similar to the tdc-1::egl-1 transgene, ablation might be incomplete in L1 worms. Transgenic strains were generated by microparticle bombardment or by microinjection. For microparticle bombardment unc-119(ed3) was used. The rescue of the unc phenotype was hence used as a selection marker [78, 79]. The transgenes were backcrossed twice against N2 wild-type worms to remove the unc-119(ed3) background. Extrachromosomal arrays were generated by DNA microinjection. DNA was injected in wild-type, mutant or transgenic worms. For injection, DNA was prepared as follows: construct 30-100 ng/µl, co-injection marker 5-50 ng/µl, pCG150 up to a concentration of 100 ng/µl if required. Positive transformants were
selected according to the presence of coinjection markers. The following plasmids were generated for this study:

K31  nmr-1p::SL1-GCaMP3.35-SL2::mKate2-unc-54-3UTR, unc-119(+)
K78  tdc-1p::SL1-GCaMP3.35-SL2::mKate2-unc-54-3UTR, unc-119(+)
K183  nmr-1p::ReaChR::mKate2-unc-54-3UTR, unc-119(+)
K196  gcy-13p::ArchT::SL2 mKate2 unc-54 3'UTR, unc-119(+)
K197  gcy-13p::ReaChR::mKate2 unc-54 3'UTR, unc-119(+)
K200  nmr-1p::ArchT::SL2 mKate2 unc-54 3'UTR, unc-119(+)
K204  tol-1p::ReaChR::mKate2-unc-54-3UTR, unc-119(+)
K215  flp11p::ReaChR::SL2mKate2-unc-54-3UTR, unc-119(+)
K249  dat-1p::ReaChR::mKate2-unc-54-3UTR, unc-119(+)
K257  lad-2p::ReaChR::mKate2-unc-54-3UTR, unc-119(+)
K259  dat-1p::ArchT::SL2 mKate2 unc-54 3'UTR, unc-119(+)
K260  tol-1p::ArchT::SL2 mKate2 unc-54 3'UTR, unc-119(+)
K300  lad-2p::ArchT::SL2 mKate2 unc-54 3'UTR, unc-119(+)
K308  tdc-1p::egl-1::SL2mKate2-unc-54-3UTR, unc-119(+)
K309  nmr-1p::egl-1::SL2mKate2-unc-54-3UTR, unc-119(+)
K355  hlh-34p::ReaChR::mKate2-unc-54-3UTR, unc-119(+)
K356  hlh-34p::ArchT::SL2 mKate2 unc-54 3'UTR, unc-119(+)
K364  ZK673.11p::ArchT::SL2 mKate2 unc-54 3'UTR, unc-119(+)

Imaging

Cameras and software

All imaging experiments were conducted using either an iXon EMCCD (512x512 pixels), an iXon Ultra EMCCD (1024x1024 pixels), a Photometrics Prime 95B back-illuminated sCMOS camera (1200x1200 pixels) or a Nikon DS Qi2 (4908x3264 pixels). For the iXon cameras the EM Gain was set between 100-200. The exposure times used were between 5-30ms. Andor IQ 2 and 3 and NIS Elements 5 were used for image acquisition.
Illumination systems

A standard 100W halogen lamp together with an infrared filter (Semrock Brightline HC 785/62) were used for Differential Interference Contrast (DIC) microscopy. For calcium imaging and blue light stimulation an LED illumination (CoolLED) with a 490nm LED and standard GFP filter set (EGFP, Chroma) were used. Optogenetic stimulations and RFP imaging were performed with an LED illumination (CoolLED) with a 585nm LED and standard TexasRed filter set (Chroma).

Agarose Microchamber Imaging

Long-term imaging experiments were conducted in agarose microchambers as previously described [80, 81]. To summarize, a PDMS mold was used to cast box-shaped indentations in a hydrogel, which consisted of 3% agarose dissolved in S-Basal [82]. Two different sizes were used. We imaged L1 larvae in 190 x 190 x 15 µm microchambers and L4 larvae were imaged in 370 x 370 x 25 µm microchambers. Depending on the developmental state of the worm that was imaged, either pretzel stage eggs or L3 larvae were picked into the chambers with OP50 bacteria. For optogenetic experiments, worms were kept at 25°C before filming. For other experiments, worms were kept at either 20°C or 25°C.

For time lapse calcium imaging experiments, L1 worms were filmed every 5s (Figure 1A, 2D-E, S1E, S2A, S7C), every 8s (Figure S2B-D, S3, S8) or every 10 s (Figure 4) with differential interference contrast (DIC) and widefield fluorescence. The DIC light source was left on continuously and was blocked by a shutter during fluorescence image acquisition. LED illumination was triggered by the EMCCD camera using the TTL exposure output of the camera. An objective with 20x magnification, an LED with 480 nm (light intensity was between 0.15-2 mW/mm²), and EM gain of 100-200 was used.

During the continuous experiments in Figure S1E and S2A only DIC images were taken. With the 20x objective and a 0.7 lens 4 worms could be imaged simultaneously in one
field. 1-4 fields could be filmed in parallel in one experiment. These image sequences gave measurable neuronal calcium transients and clear DIC images to identify pumping or non-pumping phases.

**Optogenetic experiments**

Optogenetic experiments were either conducted in agarose-microchambers as described above or the worms were immobilized. For immobilization experiments the agarose was solved in S-Basal. We used three methods of immobilization for optogenetic experiments.

1) Immobilization on a 3% agarose pad with 25mM Levamisole (Sigma Aldrich) (Figure S5).

2) Immobilization on a 10% agarose pad with 0.1µm Polybead microspheres (Polyscience) [83] (Figure 1B-C/SDQL, S1A/SDQL, S1D, S2A/SDQL).

3) Immobilization on a 10% agarose pad with 0.1µm Polybead microspheres (Polyscience) [83] and 25mM Levamisole (Figure 1B-C/PVC, 2B-C, S1A/PVC, S2A/PVC, S6A).

Worms were imaged within 30 min of immobilization. A 100x oil objective was used for illumination and imaging. For images in Figure S5A-B a 1.5 lens was added.

ReaChR for neuronal depolarization or ArchT for hyperpolarization was utilized. For optogenetic stimulation, a 585nm LED and a standard TexasRed (Chroma) filter set were used.

For optogenetic experiments with L1 larvae, either L4 stage worms or young adult worms were pre-picked onto NGM plates with all-trans-retinal (ATR, Sigma Aldrich) and grown at 25°C. During the two days after exposure to ATR, pretzel stage eggs or L1 worms were taken from this plate for optogenetic experiments. For optogenetic experiments with L4 larvae an agar chunk containing a mixed population of growing worms was added to NGM plates containing ATR. Worms for optogenetic experiments were taken from this plate within the next two days.
Calcium imaging was conducted with an interval of 3s and with an exposure time of 5-30ms. A standard optogenetic protocol included calcium imaging during a baseline. This was followed by a stimulation time, in which the worms were optogenetically stimulated. The 585nm light exposure was continuous except for brief interruptions during the time calcium imaging was conducted. After the optogenetic stimulation, calcium images were acquired during a recovery period.

In mobile worms this standard protocol was preceded by 20 DIC frames that were taken every 500ms to determine if the worm was pumping. The overall protocol was repeated every 15-30min. L1 mobile worms were imaged with a 20x objective and a 0.7 lens. Mobile L4 worms were imaged with either a 10x objective (Figure 5A-C) or a 20x objective (Figure1B/CEP, 1C/URY, S1A/CEP, S2A/URY). Fixed worms were usually imaged between 1-4 trials. A delay preceded the standard protocol to allow the worm to recover between trials. A 100x oil objective was used for the experiments. To specifically manipulate PVC and SDQL in Figure 1B-C, S1A, S2A, the stimulating illumination was restricted to the neuronal areas. The details for optogenetic experiments can be found in Supplementary Table S1.

**Blue light stimulation experiments**

L1 worms were placed in microfluidic-chambers for blue light stimulation experiments. The protocol was repeated every 15min. First, 20 DIC frames were taken every 500ms to determine whether the worm was pumping or not. Next, baseline GCaMP was imaged for 3 min, the stimulation phase then lasted 18s and a recovery phase was imaged for 3 min. The 490nm intensity for calcium imaging was 0.07mW/mm². The 490nm intensity for stimulation was set to 1.01 mW/mm² with a 20x objective. The same LED was used for calcium imaging and stimulation. The intensity levels were controlled with Andor IQ2 software.
The RFP signal of the pan-neuronal strain was imaged in addition to the GCaMP signal during the protocol every 3s with 585nm LED illumination, which was set to 0.17mW/mm².

**Spinning Disc Confocal Microscopy**

L4 worms were fixed with Levamisole. Spinning disc imaging was done with an Andor Revolution disc system using a 488nm (0.34 mW/mm²) and a 565nm (0.34 mW/mm²) laser and a Yokogawa (Japan) CSU-X1 spinning disc head. Worms were imaged through a 100x oil objective. In Figure S5A-B an additional 1.5 lens was used. Z-stacks with z-planes 0.5µm apart spanning a total distance of 10µm were taken and a maximum intensity projection calculated in ImageJ.

**Tapping experiment**

L4 worms were grown and filmed on NGM plates with OP50 bacteria at 20°C. An eyelash was used to gently touch the tail of the worms during L4 lethargus. The time from tail touch until the worms were immobile again was measured with a timer. If worms did not mobilize upon tail touching the time was counted as zero. For GCaMP intensities worms were imaged before and after tail touch each second for a total of 30s. They were illuminated with a Leica EL6000 LED.

**Image Analysis**

Image sequences for analysis were selected either based on lethargus or molting timepoints. Lethargus was determined through the DIC images as the non-pumping phase before molting. Time points were classified to be in or outside of lethargus. Typically, the entire lethargus time and two hours before lethargus were analyzed. For some experiments, a certain timespan before molting was analyzed. This timespan was 3h (Figure 2C-D) or 4h (Figure S3D-E). Immobilized worms were classified according to their pumping behavior.
on NGM plates directly before imaging. Two parameters were extracted from the image sequences.

1) Calcium transients were extracted in two different ways.

A) If only a specific neuron expressed GCaMP, a custom-written MATLAB code automatically detected the position and intensities of the neuron pixels and subtracted the background intensities.

B) If more than one neuron expressed GCaMP, a custom-written MATLAB code was used to manually track the intensities and the position of the desired neuron or neuron groups (Figure S8). Background intensities were additionally subtracted from the signal.

For all stimulation experiments, optogenetic and blue light stimulation experiments, the baseline measurement of each time point was utilized for signal normalization and $\Delta F/F$ generation, except for Figure 3A. In Figure 3A, a mean of all baseline intensities for wake time points for each worm was calculated. The mean was then utilized for normalization for all time points for each worm to better show the different RIS activities during wake and sleep. The pan-neuronal signal in Figure S5 was normalized over the measured RFP signal to retrieve $\Delta R/R$. For the transient alignments in Figure 2D, peaks and corresponding speeds were extracted through a custom-written MATLAB script and aligned as time point zero.

2) The speeds of the worms were calculated from the positions of the tracked neuron, except for the experiments in Figures S2C, S3A. Here, no GCaMP intensity was measured. Frame subtraction of the DIC images was done with a custom-written MATLAB routine instead.

Baseline extraction

In Figure 4A, C baseline of RIM GCaMP data was extracted by excluding the 95-100 percentile range for wild type and by excluding the 75-100 percentile range for aptf-1(gk794) mutants. The baseline was smoothed through a second-degree polynomial local
regression model and with weighted linear least squares. Zero weight was assigned to data points six means outside the absolute deviation. The amount of data points used for smoothing was three percent.

**Sleep bout analysis**

In experiments in Figure 4A, C, S2A, C-D, S8 only the lethargus time was analyzed.

Sleep bouts were extracted from selected parts of the time-lapse movies. Dependent on the experiment, a specific period of the movie sequence was selected and processed:

1) the lethargus period (Figure 4, S2A, C-D, S8)

2) the period from two hours before lethargus up to the end of lethargus (Figure 1A, D, S1E, S3A-C, S7)

3) either three (Figure 2D-E) or four hours (Figure S3D-E) before molting until molting

To extract sleep bouts, speeds and subtraction values were first smoothed. In Figure 1A, D, 2D-E, 5, and S5 speeds were smoothed through a 1st degree polynomial local regression model over 20 time points. Other experiments were smoothed through a second-degree polynomial local regression model and with weighted linear least squares. Zero weight was assigned to data points six means outside the absolute deviation. Data was smoothed either over three percent of all data (Figure S2C-D, S8) or over 40 data points (Figure 4A, C, S3). This was achieved with the *smooth* function in MATLAB. Smoothed speeds were normalized between 0 and 1, with 0 representing the lowest and 1 the highest smoothed speed value of each worm. In order to be scored as a sleep bout, the normalized speed had to be under a defined percentage threshold of the normalized speed for a minimum time. The exact speed and time thresholds were adjusted empirically to represent the worms’ behavior [84]. In Figure 2D-E worms had to have a speed below 5% of their maximum smoothed speeds for at least 2min in order to be counted as sleeping. For all other experiments the speed threshold was 10% and the time threshold was 2min. The sleep bout analysis was carried out with a custom-written MATLAB script.
For stimulation experiments, the baseline and recovery time measurements were too short to include a minimum time threshold in the sleep bout analysis. Hence, immobility was used as a proxy for sleep. A mean of the wake speeds was calculated for each worm. In most experiments a worm was counted as sleeping when its speed was below 10% of the calculated mean of the wake speeds. In Figure 3A, C the threshold was adjusted to 30% and in Figure S6 to 50% to account for a different locomotor behavior of the worms. RIS signals and speeds of wild type and mutants were aligned to sleep bout onset for comparison in Figure S2C, S3C, D. For GCaMP normalization ten data points before sleep bout onset were taken as baseline in order to calculate ΔF/F. In Figure S7C motion bouts were assigned whenever there was no detected sleep bout.

Fitting

The data in Figure 3B was fitted to an asymptote with Origin software. The data in Figure S5 was fitted to a logistic regression using Origin software. Exact functions and R² values can be found in the respective figures.

Statistics

Sample sizes were determined empirically based on previous studies. If possible, experiments were carried out with internal controls. If this was not possible, control and experimental condition were alternated. Researchers were not blinded to the genotype for data analysis, as data analysis was performed by automated routines. Sample exclusion is described in the respective methods section. To compare GCaMP intensities and speeds of one sample group at different time points the Wilcoxon signed-rank test was utilized. The Fisher’s Exact Test was used to compare the sleep fractions of one sample group at different time points. The entirety of the baseline was compared to the entirety of the stimulation period unless otherwise stated through significance bars. Data from different strains was either compared with the Kolmogorov-Smirnov-Test or the Welch test. The p-values can be taken from the respective Figure descriptions. In the graph depicted is the mean +/- SEM unless otherwise stated. The box in the box plots represents the inter quartile
range with the median. The whiskers show the 10-90-percentile range. The individual data points are furthermore plotted on top of the box.
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Presynaptic neurons control the activity of the sleep-active RIS neuron

A) Sample trace of RIS activity and worm locomotion behavior outside of and in lethargus. RIS is relatively inactive outside of lethargus and shows strong activity transients in lethargus. Upon RIS activation, worms enter sleep bouts.

B) Presynaptic neurons activate RIS outside of and in lethargus. For statistical calculations neural activities before the stimulation period (0 - 0.95 min) were compared to activity levels during the stimulation period (1 - 1.95 min); *p < 0.05, **p < 0.01, ***p < 0.001, Wilcoxon Signed Rank Test.

C) PVC is essential for lethargus-specific RIS activation; Statistical calculations were performed as described in B, but in experiments, in which SDQL was stimulated baseline activity levels were calculated over the time interval from 0.6 - 0.95 min; *p < 0.05, Wilcoxon Signed Rank Test.

D) PVC hyperpolarization inactivates RIS and induces behavioral activity; **p < 0.01, ***p < 0.001, Wilcoxon Signed Rank Test for GCaMP and speed, Fisher’s Exact Test for sleep fraction.

E) Circuit model of the RIS presynaptic regulatory network. Presynaptic neurons present mostly activators of RIS. PVC is essential for lethargus-specific RIS activation. Effects of presynaptic neurons might be mostly direct, except for RIM.
Figure 2

A. 1. baseline PVC GCaMP
2. RIS depolarization/hyperpolarization simultaneously imaging PVC GCaMP
3. recovery PVC GCaMP

B. RIS depolarization

C. RIS hyperpolarization

D. Wild Type n=34, apaf-1(ok974) n=30

E. Sleep fraction (%)
Figure 2

**RIS and PVC activate each other forming a positive feedback loop**

A) The experimental set up. First, a baseline of PVC was measured through calcium imaging. Then, RIS was optogenetically manipulated while PVC was simultaneously calcium imaged. Finally, a recovery period was imaged of PVC.

B) RIS depolarization leads to a strong PVC depolarization outside and during lethargus; *p < 0.05, **p < 0.01, Wilcoxon Signed Rank Test.

C) RIS hyperpolarization leads to a weak PVC depolarization outside and in lethargus. For statistical calculations neural activities before the stimulation period (0 - 1 min) were compared to activity levels during the stimulation period (1 - 2 min); *p < 0.05, **p < 0.01, compared before and during stimulation, Wilcoxon Signed Rank Test.

D) RIS does not reach the same activation levels in *aptf-1*(gk794) mutants compared to wild-type worms. *aptf-1*(gk794) mutants do neither immobilize nor sleep during RIS activation; ***p < 0.001, Welch Test.

E) *aptf-1*(gk794) mutants do not show sleep during lethargus; **p < 0.01, Kolmogorov-Smirnov-Test.

F) A circuit model for the positive feedback loop between RIS and PVC. During wakefulness, reverse command interneurons inhibit PVC so that PVC does not activate RIS. In lethargus, PVC is directly activating RIS, which then inhibits reverse command interneurons. This may speculatively disinhibit PVC, leading to a positive feedback.
Figure 3

A

outside lethargus lethargus, n=10

RIS hyper-polarization

RIS GcAMP (ΔFF)

pan-neuronal GcAMP (ΔFF)

speed (μm/s)

sleep fraction

time (min)

B

RIS signal
asymptotic fit, \( R^2 = 0.55628 \)

max. ΔFF change after stimulation

stimulus length (min)

time until maximum ΔFF change (min)

C

outside lethargus lethargus, n=16

RIS depolarization

RIS GcAMP (ΔFF)

speed (μm/s)

sleep fraction

time (min)
**Figure 3**

**RIS depolarization is homeostatically controlled**

A) RIS shows rebound activation following hyperpolarization. Behavioral and brain activity measurements correlate throughout the whole experiment; *p < 0.05, **p < 0.01, ***p < 0.001, Wilcoxon Signed Rank Test for GCaMP and speed, Fisher’s Exact Test for sleep fraction.

B) Dose-response curve of optogenetic RIS hyperpolarization with different stimulus lengths. RIS activation rebound transients saturate with increasing length of inhibition. Worms not showing a rebound activation transient after RIS optogenetic hyperpolarization were excluded from the analysis.

C) RIS shows a negative rebound following its optogenetic depolarization; *p < 0.05, **p < 0.01, ***p < 0.001, Wilcoxon Signed Rank Test for GCaMP and speed, Fisher’s Exact Test for sleep fraction.
Figure 4

A

B

C
Figure 4

**RIM activity levels are dampened in lethargus independently of RIS**

A) Sample traces of RIM transient frequencies, RIM baseline activities and worm locomotion behaviors outside of and in lethargus in wild-type worms and *aptf-1*(gk794) mutants.

B) Wild type worms, but not *aptf-1*(gk974) mutants display changes in RIM transient frequencies across lethargus. Transient frequencies were assessed manually. To be counted as a transient, RIM activity levels had to be at least twice as high as baseline activity levels. ***p < 0.001 Kolmogorov-Smirnov-Test for wild-type condition, Welch Test for mutant condition.

C) The reduction of RIM baseline activity levels in lethargus is preserved in *aptf-1*(gk974) mutants; **p < 0.01, Wilcoxon Signed Rank Test.
Figure 5

A

Wild type n=21  ASH depolarization

B

RIM(-) n=20  ASH depolarization

C

response direction (%)

Wild type, n = 21  RIM(-), n = 20

D

stimulus

AVF  AVE  PVC  RS

AVF

RIM

AVA

RS
**Figure 5**

**Arousing stimulation inhibits RIS and sleep through RIM**

A) ASH depolarization in wild-type *C. elegans* leads to mobilization, awakening and RIS inhibition; *p < 0.05, **p < 0.01, ***p < 0.001, Wilcoxon Signed Rank Test for GCaMP and speed, Fisher’s Exact Test for sleep fraction.

B) ASH depolarization in RIM ablated *C. elegans* leads to weaker mobilization, awakening and RIS activation; *p < 0.05, **p < 0.01, ***p < 0.001, Wilcoxon Signed Rank Test for GCaMP and speed, Fisher’s Exact Test for sleep fraction.

C) The response direction following ASH activation in wild-type worms is predominantly backward, while in RIM ablated worms it is predominantly forward. This suggests that the forward direction is connected to RIS activation; ***p < 0.001, Fisher’s Exact Test.

D) A speculative circuit model for RIS regulation through arousal by ASH. RIM serves as synchronizer of AVE and AVA to regulate PVC and therefore RIS inhibition.
Figure 6
A) the circuit for sleep control in *C. elegans*

Model of the tripartite flip-flop sleep switch
A) Hypothetical model of the *C. elegans* sleep switch that is consistent with our data explaining the switch states.
Supplementary Information

Supplementary Figures
Figure S1

A

B

C

D

AVJ,
n=10

CEP,
n=8

PVC,
n=8

RIM,
n=8

SDQL,
n=11

URY,
n=8

lethargus

n=13, n=12/67

lethargus

n=13, n=8/57

lethargus,
n=3

lethargus,
n=3

minus ATR

depolarization

RIS GC-AMP (ΔF/F)

RIS GC-AMP (ΔF/F)

speed (μm/s)

sleep fraction

RIS GC-AMP (μF/F)

RIS GC-AMP (μF/F)

speed (μm/s)

sleep fraction

RIS GC-AMP (μF/F)

RIS GC-AMP (μF/F)

speed (μm/s)

sleep fraction

n.s.

n.s.

n.s.

n.s.

n.s.

n.s.

n.s.

n.s.
Figure S1

**RIM and PVC control RIS activity levels in lethargus**

A) Control experiments. Optogenetic depolarization of RIS presynaptic neurons without the addition of ATR. For statistical calculations baseline neural activities (0 - 0.95 min) were compared to neural activity levels during the stimulation period (1 - 1.95 min); *p < 0.05, compared before and during stimulation, Wilcoxon Signed Rank Test.

B-C) RIM optogenetic depolarization can induce both RIS activation and inhibition. Single trials were classified as activating, if an activity increase in RIS correlated with onsets of optogenetic stimulation periods. Trials were classified as inhibitory, if an activity decrease in RIS correlated with onsets of optogenetic stimulation periods. n represents the number of animals tested, r represents the number of trials. For statistical testing baseline neural activities (0 - 0.95 min) were compared to neural activity levels during the stimulation period (1 - 1.55 min); *p < 0.05, **p < 0.01, ***p < 0.001, Wilcoxon Signed Rank Test for GCaMP and speed, Fisher’s Exact Test for sleep fraction.

D) Optogenetic PVC depolarization in L2 larvae leads to RIS activation outside of and in lethargus. RIS activation during lethargus is strongly enhanced. Plotted data represent the average over all experimental trials. Neural activity levels before the stimulation (0 - 0.95 min) were compared to activity levels during the stimulation (1 - 1.95 min); *p < 0.05, **p < 0.01, Wilcoxon Signed Rank Test.
Figure S2

A

B

C

outside lethargus, lethargus, n=14, n=18/50
outside lethargus, lethargus, n=14, n=15/59

RIM hyperpolarization

RIS GcAMP (ΔFF)

speed (mm/s)

sleep fraction

Wild type, n=11
RIM(-), n=15
Figure S2

Hyperpolarization of RIS presynaptic neurons

A) Control experiments. Optogenetic hyperpolarization of RIS presynaptic neurons without the addition of ATR. Neural baseline activity levels (0 - 0.95 min) were compared to neuronal levels during the stimulation (1 - 1.95 min), except for experiments in which SDQL was stimulated. In these experiments the baseline was taken over the time interval of 0.6-0.95min; **p<0.01, Wilcoxon Signed Rank Test.

B) RIM optogenetic hyperpolarization causes a decrease in RIS activity levels. Single trials were classified as activating, if an activity increase in RIS occurred at the onset of the optogenetic stimulation period. Trials were classified as inhibitory, if an activity decrease in RIS occurred at the onset of the optogenetic stimulation period. n represents the number of animals tested, r represents the number of trials. For statistical calculations neural baseline activity levels (0-0.95min) were compared to levels during the stimulation period (1-1.75min); *p < 0.05, **p < 0.01, ***p < 0.001, Wilcoxon Signed Rank Test for GCaMP and speed, Fisher’s Exact Test for sleep fraction.

C) RIM genetic ablation does not change spontaneous sleep amounts. n.s. p > 0.05. Kolmogorov-Smirnov-Test.
Figure S3

**PVC and other command interneurons are required for sleep induction.**

A) Genetic ablation of PVC and other command interneurons via expression of *ICE* or *egl-1* strongly reduced the occurrence of sleep bouts in lethargus. ***p < 0.001, Welch Test.

B) PVC and other command interneuron ablated worms move much slower than wild-type worms. ***p < 0.001, Welch Test for the wake condition and Kolmogorov-Smirnov-Test for the sleep condition.

C) RIS activation in sleep bouts is strongly reduced in PVC and other command interneuron ablated worms compared to wild-type worms. *p < 0.05, Welch Test.

D) Sample traces of RIS activity levels and worm locomotion behaviors outside of and in lethargus in PVC and other command interneurons ablated worms and wild-type worms. In PVC and other command interneurons ablated worms sleep bouts occur only around the middle of the lethargus period.
Figure S4

A

B

C

D

E

1445

1446

1447
Glutamatergic signaling is required for sleep induction

A-C) Sleep bout analysis of nmr-1(ak4) mutants. RIS activity levels in sleep bouts are reduced in the mutant background. Because RIS still activates in sleep bouts, nmr-1(ak4) mutants do not show a reduced amount of quiescence in lethargus. *p < 0.05, Welch Test for comparisons of sleep bout frequencies, sleep fractions, maximum RIS activity levels in sleep bouts and RIS activity levels at the end of sleep bouts. Kolmogorov-Smirnov-Test for the comparison of sleep bout lengths.

D-E) Sleep bout analysis of eat-4(ky5) mutants. eat-4(ky5) mutants lack a significant RIS activation at sleep bout onsets. Consequently, mutants display a strong reduction in quiescence during lethargus. **p < 0.01, ***p < 0.001, Welch Test for comparisons of sleep bout lengths, sleep bout frequencies and sleep fractions. Wilcoxon Signed Rank Test for quantifications of RIS activity levels in sleep bouts.
**Figure S5**

**ZK673.11 is expressed in PVC, RID and cholinergic motor neurons**

A-B) Expression of *nmr-1* and *ZK673.11* only overlaps in PVC in the tail.

C-D) Expression of *nmr-1* and *ZK673.11* does not overlap in head neurons.
Figure S6

RIS rebound activation transients represent an acute phenomenon

A) RIS depolarizes during optogenetic activation in fixed animals. Experiments were repeated a second time without the addition of ATR. ***p < 0.001, Wilcoxon Signed Rank Test.

B-D) RIS was optogenetically hyperpolarized with stimuli lasting for 48 seconds, 5 minutes or 10 minutes. Worms not showing a rebound activation transient were excluded from the analysis. Data from these plots were used to generate a dose-response curve of optogenetic RIS hyperpolarization (Figure 3B). *p < 0.05, **p < 0.01, ***p < 0.001, Wilcoxon Signed Rank Test for GCaMP and speed, Fisher’s Exact Test for sleep fraction.
Figure S7
Figure S7

RIS is homeostatically regulated

A-B) A blue light stimulus leads to awakening and mobilization of C. elegans. Worms that go back to sleep after the stimulus show a rebound: pan-neuronal inhibition below baseline levels and RIS activation above baseline levels; *p < 0.05, **p < 0.01, ***p < 0.001, Wilcoxon Signed Rank Test for GCaMP and speed, Fisher’s Exact Test for sleep fraction.

A) The peak RIS GCaMP intensities in wild-type worms are homeostatically regulated by the preceding motion bouts. The longer the preceding motion bout, the stronger the RIS activation.
The dampening of neural and behavioral baseline activity levels during lethargus is RIS-independent. Reduction of command interneuron activity levels in lethargus occurs in wild-type worms and *aptf-1(gk974)* mutants. In the wild-type condition activity levels are reduced to -0.16 ± 0.02. In the mutant condition activity levels are reduced -0.08 ± 0.02. **p < 0.01, Wilcoxon Signed Rank Test.
Gentle tail touch reveals an inhibitory role of RIM on RIS

A) RIM ablation increases the reinstating of immobility following gentle tail touch during lethargus. *p < 0.05. Kolmogorov-Smirnov-Test.

B) RIM ablation increases RIS activation in response to gentle tail touch. **p < 0.01. Kolmogorov-Smirnov-Test.

Supplementary Table

Table S1: Description of conditions used for optogenetics experiments
| Figure | Optogenetic Manipulation | Age | Mobile (m)/Immobile (i) | AT R (mM) | Light Intensity (mW/mm²) | Protocol |
|--------|-------------------------|-----|------------------------|-----------|-----------------------|----------|
|        | Genetic Tool            | Neuron |                         | 490nm m   | 585nm m               | Cycles | Delay | baseline | stimulation | recovery |
| 1B     | tol-1:: ReaChR          | URY   | L1                      | m         | 50                    | 0.33    | 0.37  | 15min     | -           | 1min 1min 1min 1min |
|        | lad-2:: ReaChR          | SDQL  | L4                      | i         | 0.5                   | 0.05    | 0.04  | 4         | 2min        | 1min 1min 1min 1min |
|        | gcy-13:: ReaChR         | RIM   | L1                      | m         | 50                    | 0.33    | 0.37  | 30min     | -           | 1min 1min 1min 1min |
|        | nmr-1:: ReaChR          | PVC   | L1                      | i         | 50                    | 0.05    | 0.26  | 1         | 5min        | 2min 1min 1min 2min |
|        | dat-1:: ReaChR          | CEP   | L4                      | m         | 0.5                   | 0.14    | 0.09  | 30min     | -           | 1min 1min 1min 1min |
|        | hid-34:: ReaChR         | AVJ   | L1                      | m         | 50                    | 0.33    | 0.37  | 15min     | -           | 1min 1min 1min 1min |
| 1C     | tol-1:: ArchT           | URY   | L4                      | m         | 0.5                   | 0.11    | 0.39  | 30min     | -           | 3min 3min 8min  |
|        | lad-2:: ArchT           | SDQL  | L4                      | i         | 0.5                   | 0.05    | 0.09  | 4         | 5min        | 1min 1min 1min 1min |
|        | gcy-13:: ArchT          | RIM   | L1                      | m         | 50                    | 0.33    | 0.37  | 30min     | -           | 1min 1min 1min 1min |
|        | nmr-1:: ArchT           | PVC   | L1                      | i         | 50                    | 0.12    | 0.26  | 1         | 5min        | 2min 1min 2min 1min |
|        | dat-1:: ArchT           | CEP   | L1                      | m         | 50                    | 0.33    | 0.17  | 15min     | -           | 1min 1min 1min 1min |
|        | hid-34:: ArchT          | AVJ   | L1                      | m         | 50                    | 0.33    | 0.37  | 15min     | -           | 1min 1min 1min 1min |
| 1D     | ZK673.11:: ArchT         | PVC   | L1                      | m         | 10                    | 0.07    | 0.17  | 20min     | -           | 2min 1min 3min  |
| 2B     | flp-11:: ReaChR         | RIS   | L1                      | i         | 10                    | 0.28    | 3.39  | 2         | 1min        | 2min 5min 1min  |
| 2C     | flp-11:: ArchT          | RIS   | L1                      | i         | 10                    | 0.05    | 0.09  | 2         | 1min        | 1min 1min 1min  |
| 3A     | flp-11:: ArchT          | RIS   | L1                      | m         | 50                    | 0.07    | 0.17  | 30min     | -           | 3min 3min 8min  |
| 3B, 6  | flp-11:: ArchT          | RIS   | L1                      | m         | 50                    | 0.15    | 0.17  | 15min     | -           | 1min 48s 1min  |
|        | flp-11:: ArchT          | RIS   | L1                      | m         | 50                    | 0.15    | 0.17  | 15min     | -           | 3min 5min 3min  |
|        | flp-11:: ArchT          | RIS   | L1                      | m         | 50                    | 0.15    | 0.17  | 15min     | -           | 3min 10min 3min |
| 3C     | flp-11:: ReaChR         | RIS   | L1                      | m         | 50                    | 0.15    | 0.09  | 15min     | -           | 1min 1min 1min  |
| 5A     | sra-6:: ReaChR          | ASH   | L4                      | m         | 0.2                   | 0.6     | 0.14  | 15min     | -           | 1.5min 3s 87s  |
| 5B     | sra-6:: ReaChR          | ASH   | L4                      | m         | 0.2                   | 0.6     | 0.14  | 15min     | -           | 1.5min 3s 87s  |
| S1A,B  | gcy-13:: ReaChR         | RIM   | L1                      | m         | 50                    | 0.33    | 0.37  | 30min     | -           | 1min 1min 1min  |
| S1C    | gcy-13:: ArchT          | RIM   | L1                      | m         | 50                    | 0.33    | 0.37  | 30min     | -           | 1min 1min 1min  |
| S1D    | nmr-1:: ReaChR          | PVC   | L2                      | i         | 50                    | 0.05    | 0.01  | 2-3       | -           | 1min 1min 1min  |
| S5A    | tol-1:: ReaChR          | URY   | L1                      | m         | 0                    | 0.33    | 0.37  | 15min     | -           | 1min 1min 1min  |
|        | lad-2:: ReaChR          | SDQL  | L4                      | i         | 0                    | 0.05    | 0.04  | 4         | 2min        | 1min 1min 1min  |

**Table S1. Optogenetic Experimental Details.**