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Report

DAF-16/FOXO Regulates Homeostasis of Essential Sleep-like Behavior during Larval Transitions in *C. elegans*

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Summary

Sleep homeostasis, which refers to the maintenance of sleep amount or depth following sleep deprivation, indicates that sleep and sleep-like states serve fundamental functions that cannot be bypassed [1]. Homeostasis of sleep-like behavior is observed during *C. elegans* lethargus, a 2–3 hr behavioral quiescent period that occurs during larval state transitions [2]. Here, we report a role for DAF-16/FOXO, a transcription factor that is active under conditions of stress [3], in the response to deprivation of lethargus quiescence. Forced locomotion during lethargus results in nuclear translocation of DAF-16. The formation of dauer larvae, a developmental state promoted by *daf-16*, is increased in response to quiescence deprivation. *daf-16* mutants show an impaired homeostatic response to deprivation of lethargus quiescence and are hypersensitive to the lethal effects of forced locomotion during lethargus. DAF-16 expression in muscle cells, but not in neurons, is sufficient to restore a homeostatic response to deprivation of quiescence, pointing to a role for muscle in sleep homeostasis. These findings are relevant to clinical observations of altered metabolic signaling in response to sleep deprivation and suggest that these signaling pathways may act in non-neuronal tissue to regulate sleep behaviors.

Results and Discussion

Elevated *daf-16* Signaling in Response to Deprivation of Lethargus Quiescence

We hypothesized that, given the adverse consequences of sleep deprivation in other species [4, 5], deprivation of lethargus quiescence would be a stressor in *C. elegans*. DAF-16, a FOXO transcription factor involved in multiple stress responses [3, 6, 7], is partially activated by nuclear translocation [3, 8, 9]. The absence of food for more than 1 hr results in nuclear translocation of DAF-16 [3]. Although the animal is surrounded by food during lethargus, it cannot eat, because there is a plug of extracellular material that occludes the buccal opening to the pharynx [10, 11] and because pharyngeal pumping, which is essential for ingestion [12], ceases [10, 11]. Because worms do not feed during lethargus, which can last up to 3 hr, we were surprised to observe DAF-16 distributed in the cytoplasm during the fourth larval (L4) lethargus period (Figures 1A and 1B). This observation extends to the first larval (L1) lethargus period. DAF-16 nuclear localization was low at hatching but increased to a maximum during the mid-L1 stage. During the L1 lethargus period, DAF-16 showed less nuclear localization than during the surrounding larval stages (Figure 1C). These observations indicate that there is reduced nuclear DAF-16 during lethargus despite the absence of feeding.

Does depriving worms of lethargus quiescence affect the subcellular distribution of DAF-16? We stimulated L4 lethargus animals to move in a liquid bacterial suspension for 30 min. L4 lethargus worms that had been stimulated for 30 min showed greater nuclear-localized DAF-16 than age-matched control animals (Figures 1A and 1B). Adult worms stimulated at the same frequency did not show an increase in nuclear DAF-16 in comparison to age-matched controls (Figures 1A and 1B). Stimulation of animals without liquid immersion, by touching them with a wire every 20 s for 30 min, also led to DAF-16 nuclear translocation during lethargus (see Figure S1B available online). We observed nuclear translocation of DAF-16 in both body muscle and intestinal cells (Figures 1A, 1B, and S1B). In the nervous system, we did not see a difference in DAF-16 subcellular distribution between deprived and control animals (data not shown), although due to the high nucleus-to-cytoplasm ratio of neuron cell bodies, an effect would be difficult to detect.

DAF-16 nuclear translocation is positively regulated by the stress-activated c-Jun N-terminal kinase JNK-1 [13] and by the dafachronic acid nuclear receptor DAF-12 [14]. Although the overall ratio of nuclear to cytoplasmic DAF-16 was reduced in *jnk-1(gk7)* mutants, as reported [13], DAF-16 was more nuclear in worms deprived of lethargus quiescence than in nondeprived animals (Figure S1C). By contrast, in *daf-12(rh61rh411)* mutants, the nuclear-to-cytoplasmic ratio of DAF-16 was not different between deprived and control animals (Figure S1C), implicating DAF-12 in this response.

Does the molecular response to sleep deprivation dissipate as the animals are allowed to sleep? Over a 20 min recovery period following the 30 min swimming deprivation of quiescence, we observed a redistribution of DAF-16 from the nucleus to the cytoplasm (Figure 1D). Therefore, the *C. elegans* molecular response to deprivation of sleep-like behavior is transient, as has been observed in mammals and *Drosophila* [15–17].

To test whether DAF-16 signaling is increased by deprivation of lethargus quiescence, we used a dauer formation assay as a readout of DAF-16 signaling. The dauer is a third larval stage that forms under unfavorable conditions [18]. The decision to enter the dauer stage is made partially during L1 lethargus [19]. Because DAF-16 promotes dauer formation [20], we asked whether deprivation of L1 lethargus quiescence increases dauer formation. To increase the propensity to form dauers, we used animals mutant for *daf-8* [21]. In *daf-8* mutants, increased DAF-16 signaling further increases the

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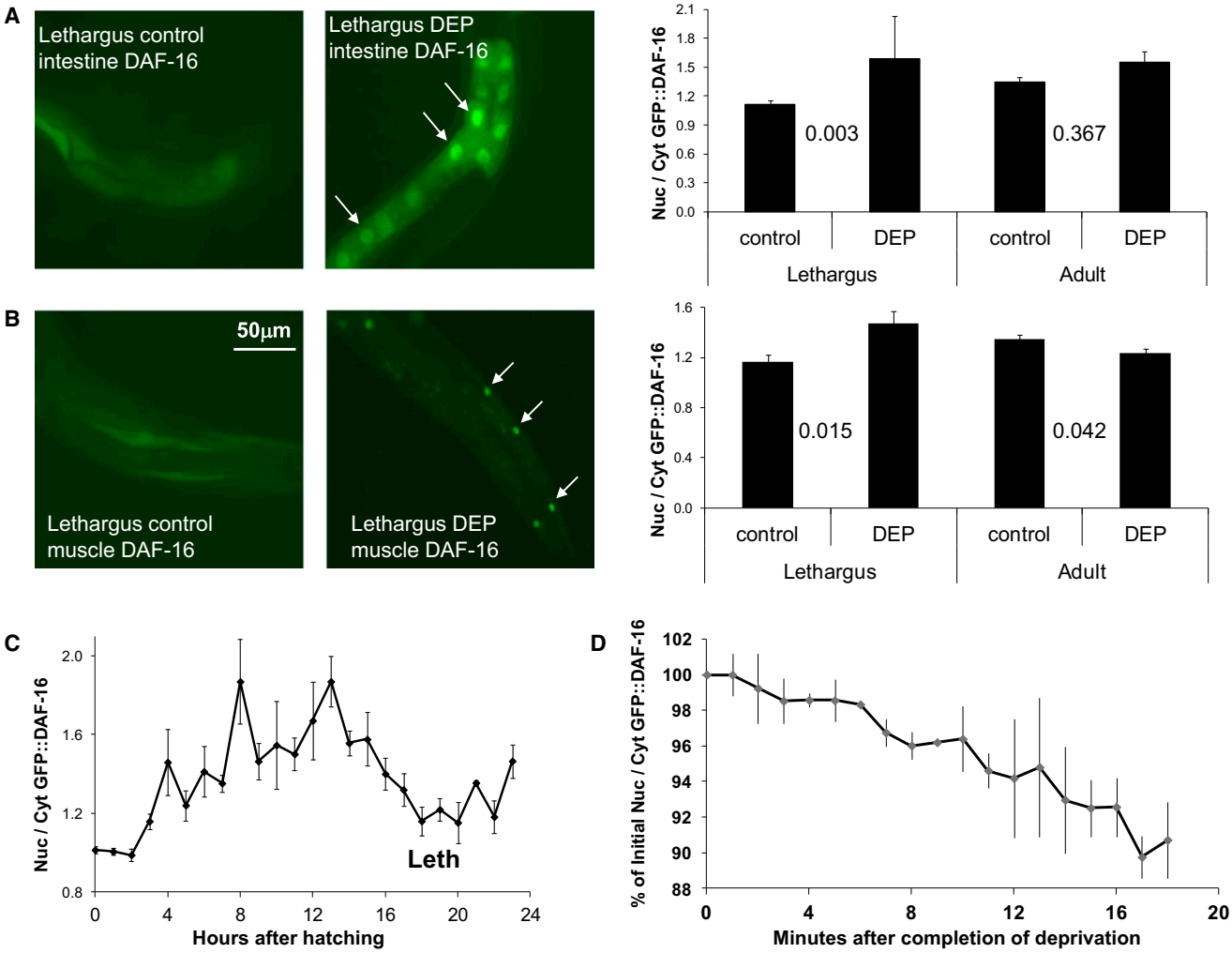


Figure 1. DAF-16 Moves to the Nucleus in Response to Deprivation of Lethargus Quiescence

(A–C) Nuclear translocation of DAF-16 as a function of developmental time (C) and in response to deprivation of lethargus quiescence in intestine (A) and body muscle (B). DEP denotes worms that had been stimulated during lethargus (protocol 2) for 30 min. White arrows in the fluorescence images point to nuclei showing DAF-16::GFP localization. The average pixel fluorescence intensity ratio between the nucleus and the cytoplasm is shown. p values (two-tailed t test) are shown in (A) and (B) between conditions compared. n = 15 for each condition.

(D) The nuclear/cytoplasmic ratio decreases with time after completion of deprivation. Shown is the average of three worms, each mounted for imaging within 5 min of being deprived of L4 lethargus quiescence for 30 min and then imaged for 20 min.

Error bars denote SD. See also Figure S1.

formation of dauers [22]. We therefore used a change in dauer formation propensity of *daf-8* mutants to infer effects of DAF-16 signaling.

We deprived *daf-8* mutants of quiescence by forcing them to swim in a bacterial suspension for 1 hr beginning at the start of L1 lethargus. Three control groups of animals were treated identically, except that they (1) were not forced to swim, (2) were forced to swim before L1 lethargus, or (3) were forced to swim after L1 lethargus. We observed a higher percentage of dauers among animals that had been forced to swim during L1 lethargus than among control animals (Figure 2). A similar result was observed with deprivation of mutants for *daf-7*, which encodes a transforming growth factor β (TGF-β) acting upstream of *daf-8* [23] (Figure 2). These results were not due to the buffer in which the worms swam, because we also observed an enrichment in dauer formation when *daf-8* mutants were kept moving in a lawn of bacteria by touching them with a wire every 20 s for 1 hr (23 of 63 deprived animals formed dauers; 6 of 100 control animals formed dauers, $p < 0.001$, Fisher's two-tailed test). Consistent with the notion that increased dauer formation is partially explained by increased DAF-16 activity in response to deprivation of L1 lethargus quiescence, the effect of deprivation on *daf-7* dauer formation was attenuated by introducing the *daf-16* loss-of-function mutation *mu86* into the strain (Figure 2).

Taken together, these experiments provide evidence that increased DAF-16/FOXO signaling is a physiological consequence of the deprivation of lethargus quiescence.

daf-16 Is Required for the Normal Behavioral Response to Deprivation of Lethargus Quiescence

Previous analysis indicated that the time at which quiescence of locomotion ends is not affected by deprivation of the early part of lethargus [2]. Consistent with this observation, we detected no difference in the timing of pharyngeal pumping resumption, defecation resumption, or ecdysis between

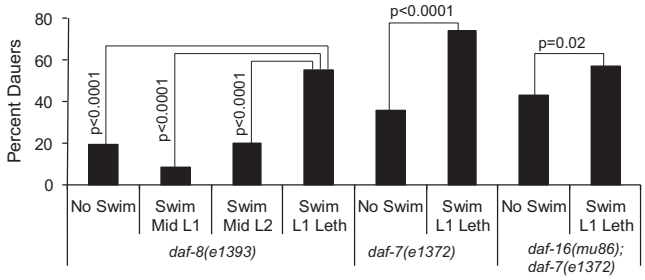


Figure 2. Dauer Formation Is Increased following Deprivation of Lethargus Quiescence

Forced swimming of *daf-8(e1393)* and *daf-7(e1372)* mutants during L1 lethargus leads to a greater percentage of dauers. Introducing *daf-16(mu86)* into the *daf-7* mutant attenuates the dauer-inducing effects of lethargus quiescence deprivation. n = 86–181 in each condition. p values were calculated with a two-tailed Fisher's exact test.

unperturbed L4 lethargus worms and L4 lethargus worms that were forced to swim for 30 min starting in the first 5 min of L4 lethargus (Figure 3B).

In contrast to the timing of the events marking the completion of lethargus, which were unaffected by deprivation of quiescence, the arousal threshold of animals was strongly affected by deprivation (Figure 3C). Wild-type animals forced to swim for 30 min during lethargus showed an accelerated return to sleep-like behavior, as demonstrated by an increased response latency to the noxious chemical 1-octanol [2]. The increased response latency after deprivation is not explained by irreversible damage to the animal, because the 1-octanol response latency of ten animals 4 hr after the completion of deprivation during L4 lethargus, during their early adult stage (3.3 ± 3.0 s, mean ± SD), was not different from that of ten age-matched control animals (2.3 ± 1.1 s, p = 0.7, Mann-Whitney U test). Therefore, the quality, but not the duration, of quiescent behavior is affected by deprivation of quiescence.

In contrast to wild-type worms, *daf-16(mu86)* and *daf-16(mgDf50)* mutants [22, 24] did not demonstrate elevated 1-octanol response latencies following deprivation of lethargus quiescence (Figure 4). This defect in the homeostatic response is not explained by reduced baseline quiescence, because total quiescence during L4 lethargus (107.1 ± 17.9 min) and L4 lethargus duration (3.08 ± 0.24 hr) of 16 *daf-16(mu86)* mutant animals were not different from these measurements in 104 wild-type animals (97.9 ± 15.3 min and 3.04 ± 0.22 hr, respectively; mean ± SD). Arousal threshold was also not defective in these mutants: baseline 1-octanol response latencies of unperturbed worms in L4 lethargus were not reduced in *daf-16(mgDf50)* (11.5 ± 4.7 s, n = 80, p = 0.19) and were slightly increased in *daf-16(mu86)* mutants (15.8 ± 4.4 s, n = 60, p = 0.001) in comparison to wild-type worms (12.8 ± 5.6 s, n = 70). Therefore, *daf-16* is required for the normal homeostatic response to deprivation of lethargus quiescence without impairing the baseline sleep-like behavior. This suggests that baseline sleep behavior and the behavioral response to sleep deprivation can be genetically separated, as has been shown for sleep in other animals [25, 26].

To ask whether constitutively nuclear DAF-16 affects the homeostatic response to deprivation of lethargus quiescence, we tested animals with a reduction-of-function mutation in *daf-2*. The *daf-2* mutant *e1370* had an elevated baseline 1-octanol response latency both during L4 lethargus (Figure 4B) as well as during the adult stage [5.9 ± 2.4 s in *daf-2(e1370)* versus 3.9 ± 2.0 s in wild-type adults, p = 0.01, Mann-Whitney U test]. The response latency was further elevated after deprivation of lethargus quiescence (Figure 4B), indicating that nuclear DAF-16 does not occlude a homeostatic response.

Finally, we tested the homeostatic response to deprivation of lethargus quiescence in *daf-12(rh61rh411)* mutants. Like *daf-16* mutants, *daf-12* mutants had a defective homeostatic response to deprivation of lethargus quiescence (Figure 4B). A recent report showed that *daf-12* positively regulates DAF-16 via its negative regulation of two DAF-16 negative regulators, AKT-1 and LIN-14 [27]. AKT-1 is a particularly attractive candidate to mediate a sleep homeostatic response because it is a target of the metabolically sensitive TOR kinase [28], an enzyme that is negatively regulated by sleep deprivation in mice [29].

Role for Muscle Cells in the Response to Deprivation of Lethargus Quiescence

Mechanical stimulation during lethargus has two effects: the animal is awake at a time when it normally sleeps, and muscle contractions occur at a time when the animal is normally still. These effects occur in every experiment where a laboratory animal is forced to move during its quiescent sleep time.

To test the role of muscle in the homeostatic response to deprivation of lethargus quiescence, we restored DAF-16 function in muscle and other tissues. As expected, expression of *daf-16* under the control of its own promoter rescued the defective homeostatic response to deprivation of lethargus quiescence (Figure 4B). In addition, *daf-16* expression in body muscle cells partially restored the homeostatic response of *daf-16* mutants to deprivation of lethargus quiescence (Figure 4B). This rescue was abrogated when these transgenic animals were treated with *daf-16* (RNAi), indicating that it was the muscle expression of *daf-16* that was restoring the homeostatic response (Figure 4B). In contrast to its effect in muscle, *daf-16* expression in neurons did not restore the homeostatic response of *daf-16* mutants (Figure 4B).

The homeostatic behavioral response may be explained by a physiological change in sensory neurons, interneurons, motor neurons, or muscle, or by a change in a combination of these sites. To study the integrity of neuromuscular function in response to deprivation of lethargus quiescence, we stimulated cholinergic motor neurons optogenetically. This stimulation, which causes a muscle contraction and therefore shortening of the worm's body [30], caused the same magnitude of muscle contraction in lethargus worms that had not been perturbed (4.6% ± 1.8% of initial body length, n = 8) as in lethargus worms that had been forced to move for 30 min (5.0% ± 1.8%, n = 8, p = 0.60, Mann-Whitney U test). Therefore, although body muscle cells are involved in the homeostatic response to deprivation of lethargus quiescence, the reduced responsiveness to 1-octanol is likely to be explained by effects outside the muscle or the neuromuscular junction.

Deprivation of Lethargus Quiescence Is Lethal

We noted that 11% (n = 265) of wild-type worms did not recover after forced movement for 30 min during L4 lethargus. Microscopic inspection of these arrested animals showed that, although they had secreted and assembled an adult cuticle as evidenced by the presence of a new cuticle lining the buccal cavity (Figure S3A) and adult-specific alae (Figure S3E), they were unsuccessful in escaping from the L4 cuticle (Figures S3A–S3D). That is, these worms had a molting-defective (Mlt) phenotype [31].

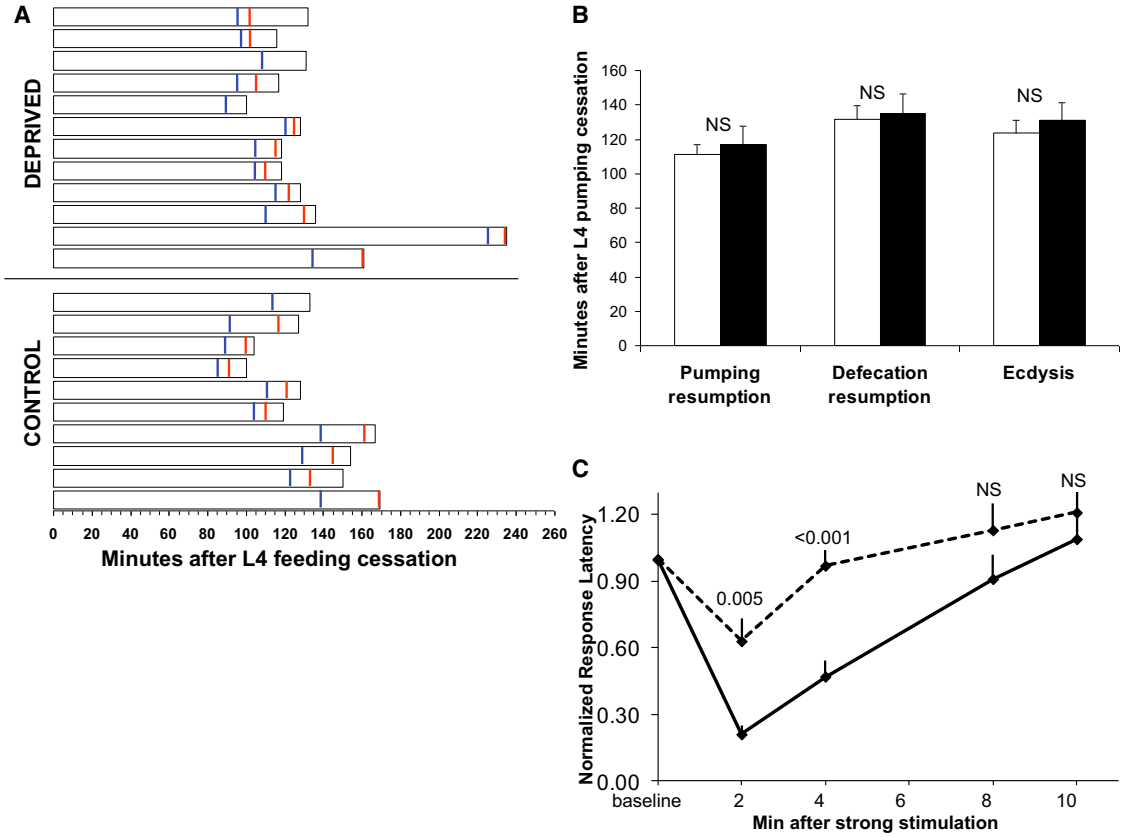


Figure 3. Deprivation of Early L4 Lethargus Quiescence Does Not Delay the Timing of Lethargus End Points but Does Affect Subsequent Sleep Quality

(A) Duration from onset of pumping cessation to ecdysis of individual worms. Blue vertical lines denote resumption of pharyngeal pumping; red vertical lines denote resumption of defecation movements.

(B) Mean durations of the data presented in (A). Black columns denote deprived animals. Two worms, which did not recover from the deprivation and were therefore censored (Table S2), were observed to resume body movements but not pharyngeal pumping or defecation movements and remained trapped in their prior stage cuticle. Error bars denote SEM. NS denotes not significant, $p > 0.1$ (two-tailed Student's t test).

(C) Wild-type worms have shortened 1-octanol response latencies following strong stimulation during lethargus but then return to baseline elevated response latencies over 10 min. In contrast, worms that had been stimulated for 30 min during lethargus (dashed line, protocol 2) show an accelerated return to baseline response latencies after a strong stimulus. p values shown at each time point were calculated with a two-tailed Student's t test. n = 10 worms. Error bars denote SEM. NS denotes $p > 0.05$.

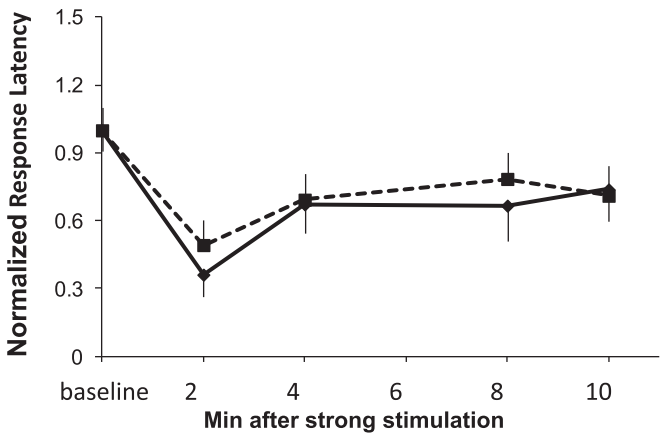
See also Figures S2 and S3.

We used *daf-16* mutants to sensitize the animals to the lethal effects of deprivation of lethargus quiescence. 18% of *daf-16(mu86)* (n = 55), 57% of *daf-16(mgDf50)* (n = 44), 53% *daf-16(m26)* (n = 55), and 38% of *daf-16(m27)* (n = 63) mutants died after 30 min of continuous forced swimming movement during lethargus. To address the possibility that the lethality was caused by mechanical injury, we mechanically stimulated L1 *daf-16* mutants. Beginning 14 hr after first exposure to food (bacteria) and repeating once every hour for a different cohort of worms, *daf-16* L1 worms were transferred from an agar surface into buffer, where they were stimulated for an hour using a vortex to agitate the buffer. To ensure that all worms were receiving identical stimulation, we agitated worms of various ages simultaneously on the vortex. We observed a peak in lethality for L1 worms that were agitated at a time that corresponded to L1 lethargus in *daf-16* mutants (Figure S3F).

It is possible that worms in lethargus are more sensitive to mechanical stimulation due to fragile properties of their exoskeleton at that stage. To control for the stage of stimulation, we mechanically stimulated pairs of *daf-16* worms that were at the identical stage of L4 lethargus. We stimulated one worm, which we term "experimental," to move continuously by mechanically stimulating it each time it stopped moving. We stimulated the second animal, which we term "yoked control," each time the experimental worm was stimulated but irrespective of whether or not this yoked control animal was quiescent. The experiment was continued until the experimental animal no longer responded to stimulation. Thus, the experimental worm was totally deprived of quiescence, whereas the yoked control animal was only partially deprived of quiescence. Six of eight experimental animals died as a consequence of this stimulation, which had a duration of 59 ± 5 min (mean ± SEM), whereas zero of eight yoked control animals died ($p = 0.02$, one-tailed Fisher's exact test). These results suggest that it is the prevention of lethargus quiescence, and not solely the mechanical stimulation during lethargus, that results in the lethal phenotype.

We consider four explanations for the lethality induced by deprivation of lethargus quiescence. First, we may be injuring the worm by frequent mechanical stimulation during lethargus. However, the lethality observed in animals stimulated identically to yoked controls during lethargus suggests a more specific mechanism. It is also possible that locomotion during lethargus prevents the animal from engaging in movements

A



B

Row	Genotype	Transgene and other treatment	Octanol response latency (sec) mean ± SD		Octanol response latency (sec) mean ± SD		DEP/CONT Ratio of normalized latencies at 4 min^	p value (rows compared)
			CON Baseline	CON 4 min	DEP Baseline	DEP 4 min		
1	*wild type*	---	12.0 ± 4.5	7.2 ± 4.0	13.8 ± 6.5	16.3 ± 7.2	2.02 ± 0.10	---
2	*daf-16(mu86)*	---	15.4 ± 4.9	10.6 ± 6.0	16.3 ± 4.0	11.4 ± 5.9	1.02 ± 0.06	<0.005* (2, 1)
3	*daf-16(mgDf50)*	---	11.6 ± 4.7	7.8 ± 4.7	11.4 ± 4.8	7.9 ± 4.2	1.04 ± 0.17	<0.005* (3, 1)
4		$P_{daf\text{-}16}$:*daf-16*	14.8 ± 4.8	9.4 ± 4.3	10.0 ± 4.1	14.1 ± 7.7	2.20 ± 0.41	<0.001* (4, 3)
5		$P_{unc\text{-}119}$:*daf-16*	12.4 ± 4.6	9.0 ± 4.9	13.4 ± 5.0	9.8 ± 5.4	1.01 ± 0.08	NS (5, 3)
6		$P_{myo\text{-}3}$:*daf-16*	14.1 ± 5.4	11.5 ± 4.7	13.3 ± 4.4	16.2 ± 6.2	1.50 ± 0.27	0.009* (6, 3)
7		$P_{myo\text{-}3}$:*daf-16 on daf-16(RNAi)*	19.2 ± 4.3	11.0 ± 5.3	20.0 ± 6.9	14.6 ± 7.1	1.29 ± 0.01	NS (7, 3)
8	*daf-12(rh61rh411)*	---	18.0 ± 8.3	12.5 ± 6.4	16.4 ± 7.2	13.9 ± 8.0	1.29 ± 0.28	<0.005* (8, 1)
9	*daf-2(e1370)*	---	16.6 ± 6.2	16.3 ± 4.6	15.0 ± 5.7	23.6 ± 8.4	1.60 ± 0.27	NS (9, 1)

Figure 4. *daf-16* Mutants Are Defective in the Homeostatic Behavioral Response to Deprivation of Lethargus Quiescence

(A) *daf-16(mgDf50)* mutants have 1-octanol response latencies following 30 min of stimulation (protocol 2) during lethargus (dashed line) similar to those of nondeprived animals (solid line). At all time points, $p > 0.2$, Student's t test. n = 10 worms. Error bars denote SEM.

(B) 1-octanol response latencies at baseline and at 4 min after strong stimulation without (CON) and with (DEP) a 30 min deprivation of lethargus quiescence (protocol 2). ^Average of three to five trials, with ten worms per trial. *Significant after Bonferroni correction for multiple testing.

See also Figures S2 and S3.

required for ecdysis. Flipping movements, where the animal rotates along its longitudinal axis, have been observed during the final 10–15 min of lethargus and have been proposed to be required for ecdysis [11]. However, this explanation does not account for the increased incidence of ecdysis defects in *daf-16* mutants, which do not show enhanced flipping movements. Third, it is possible that the ecdysis defect is caused by poor maturation of the new cuticle when the animal is forced to move continuously. Maturation of the cuticle may require immobility, much like wet mortar requires immobility in order to harden appropriately to a concrete. Although adult cuticle forms in sleep-deprived molting-defective animals, we cannot be certain that this cuticle forms entirely properly. Finally, the defect in ecdysis may be caused by a defect in regulation of metabolism; that is, the sustained locomotion during the normally quiescent lethargus period consumes metabolic resources normally reserved for the molting process. This explanation is supported by our observation that mutants for *daf-16*, a key integrator of stress and metabolism [32], show enhanced sensitivity to the effects of deprivation of lethargus quiescence.

The consequences of total sleep deprivation (TSD) have been extensively examined in rats, which die when subjected to TSD. The major documented consequences of TSD are skin lesions and weight loss despite increased food intake [5]; in contrast to these systemic effects, no defects have been observed in the rat brain [33]. In *Drosophila*, a genetic perturbation outside the nervous system affects sleep [16], and the animal's fat stores affect its response to sleep deprivation [34]. Effects of exercise on sleep continuity have been documented in rats [35] as well as in humans [36]. It is therefore not surprising that in *C. elegans*, too, homeostasis of lethargus sleep-like behavior involves signaling outside the nervous system, in muscle. It will be of future interest to examine the effect of genetic perturbations in muscle on mammalian sleep homeostasis.

Total sleep deprivation promotes insulin resistance in humans [37, 38] as well as in human adipocytes [39]. Our

data extend these observations to one of the most primitive sleep-like states described and suggest that *C. elegans* lethargus can be used as model system to gain a mechanistic understanding of this clinical phenomenon of high public health importance.

Supplemental Information

Supplemental Information includes three figures, two tables, and Supplemental Experimental Procedures and can be found with this article online at http://dx.doi.org/10.1016/j.cub.2013.02.009.

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