LETTERS

Lethargus is a *Caenorhabditis elegans* sleep-like state

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There are fundamental similarities between sleep in mammals and quiescence in the arthropod *Drosophila melanogaster*, suggesting that sleep-like states are evolutionarily ancient[1–3]. The nematode *Caenorhabditis elegans* also has a quiescent behavioural state during a period called lethargus, which occurs before each of the four moults[4]. Like sleep, lethargus maintains a constant temporal relationship with the expression of the *C. elegans* Period homologue LIN-42 (ref. 5). Here we show that quiescence associated with lethargus has the additional sleep-like properties of reversibility, reduced responsiveness and homeostasis. We identify the cGMP-dependent protein kinase (PKG) gene *egl-4* as a regulator of sleep-like behaviour, and show that *egl-4* functions in sensory neurons to promote the *C. elegans* sleep-like state. Conserved effects on sleep-like behaviour of homologous genes in *C. elegans* and *Drosophila* suggest a common genetic regulation of sleep-like states in arthropods and nematodes. Our results indicate that *C. elegans* is a suitable model system for the study of sleep regulation. The association of this *C. elegans* sleep-like state with developmental changes that occur with larval moults suggests that sleep may have evolved to allow for developmental changes.

Behavioural quiescence is concentrated during lethargus—a period at larval-stage transitions (Fig. 1 and Supplementary Table 1). Each lethargus can be characterized by total quiescence, by the peak frequency of quiescent epochs in a 10-min period, and by the mean quiescence bout duration (Supplementary Fig. 2 and Supplementary Table 1). There is a rhythm to this process, with distinct lethargus periods that are consistent across animals (Fig. 1b and Supplementary Table 1).

A key feature of sleep is reduced sensory responsiveness. To determine if arousal threshold is increased during *C. elegans* lethargus, we tested responses to mechanical and olfactory stimuli, which are sensed by distinct neurons[6–8] (Fig. 2a).

The predominant response to dish-tap—a mechanical stimulus[9]—was a brief backward movement, both during and outside lethargus (Fig. 2b and Supplementary Video 3). Outside lethargus, the worm also frequently responded with complex behaviours (Fig. 2b). Therefore, lethargus represents a period of reduced responsiveness to mechanical stimulation. The fact that the worm always showed a response to this stimulation indicates that the mechanosensory circuit can function during lethargus.

We subjected the animal's nose to the chemical 1-octanol, which produces a withdrawal response. The response latency to diluted 1-octanol was increased during lethargus (Fig. 2c), yet animals remained responsive (Fig. 2d). After strong mechanical stimulation of the worms during lethargus, the response latency to 1-octanol was as short as during the fourth larval stage before lethargus (Fig. 3f). Therefore, the ASH sensory neurons can function normally during lethargus, and the reduced responsiveness is probably due to altered processing of sensory information.

Behavioural quiescence observed during lethargus is a reversible behavioural state. During lethargus, quiescent periods are interrupted by brief movements in which the animal assumes a sinusoidal posture—a posture assumed during normal locomotion (Supplementary Videos 1 and 2). Furthermore, response latency to 1-octanol is reduced to levels seen outside lethargus after strong mechanical stimulation of the animals (Fig. 3f). Finally, in response to strong mechanical stimulation, forward movement is as fast during lethargus as outside lethargus (Fig. 2e).

The homeostatic property of sleep[10] is manifested when, after a period of enforced wakefulness, subsequent sleep occurs with a reduced latency and is deeper, where depth of sleep is reflected by increased consolidation and reduced responsiveness[3,11]. To test for homeostasis, we stimulated worms mechanically beginning at nine hours after the end of the L3 lethargus—a time when most animals would display quiescent behaviour (Supplementary Table 1). On the basis of the behaviour of the unperturbed control group, our stimulation protocol is predicted to deprive the animals of 31% of the total quiescence in L4 lethargus. After the one-hour stimulation, the peak quiescence and the mean quiescence bout duration—two measures of consolidation—are increased (Fig. 3b, c). The timing of the end of the quiescent period is unaltered by the stimulation (Fig. 3d), indicating a probable temporal constraint on the timing of lethargus.

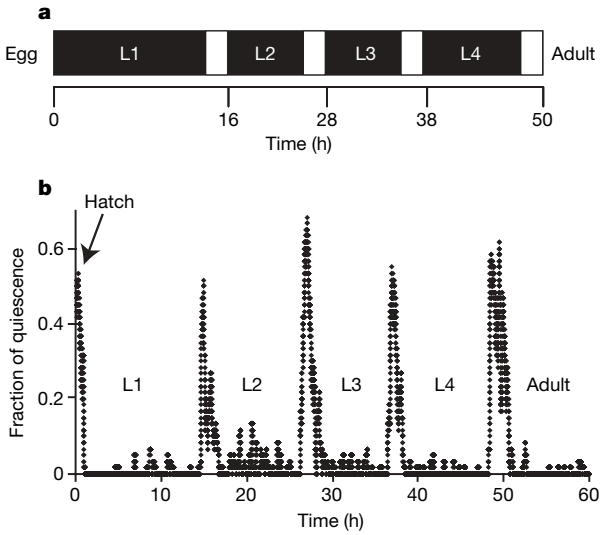

Figure 1 | Behavioural quiescence is concentrated during the lethargus periods. L1–L4 corresponds to larval stage 1 to larval stage 4. **a**, Postembryonic development of *C. elegans* at 20 °C. Lethargus is designated by the white rectangles. **b**, Shown is the fraction of quiescence of a single wild-type worm in a 10-min time window that is moved 10 s for each data point.

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This constraint would explain the overall reduction in total quiescence during L4 lethargus in deprived animals (Fig. 3a).

To assess for homeostasis further, we used the 1-octanol response latency to measure the time course of recovery to a sleep-like state. Animals that were deprived of quiescence and were kept continuously moving for 30 min during lethargus had, subsequently, an earlier occurrence of the long 1-octanol response latencies typical of lethargus in comparison to animals allowed to go through lethargus unperturbed (Fig. 3f). In addition, these animals showed an earlier cessation of locomotion in comparison to non-deprived animals (Fig. 3e). Finally, these deprived animals showed 1-octanol response latencies that were further increased compared to the latencies observed during lethargus (Fig. 3f), demonstrating a deeper sleep-like behaviour. Animals that were deprived of quiescence for 20 min during lethargus showed a latency to sleep-like behaviour that was intermediate to that seen after 30 min deprivation (Fig. 3e, f), indicating that this latency depends on the duration of previous deprivation as predicted for a sleep homeostatic process. Thirty minutes of activity during the early adult stage had no effect on subsequent 1-octanol response latencies and had a minimal effect on locomotion (Fig. 3e, f). We conclude that the sleep-like behaviour during lethargus is under homeostatic regulation.

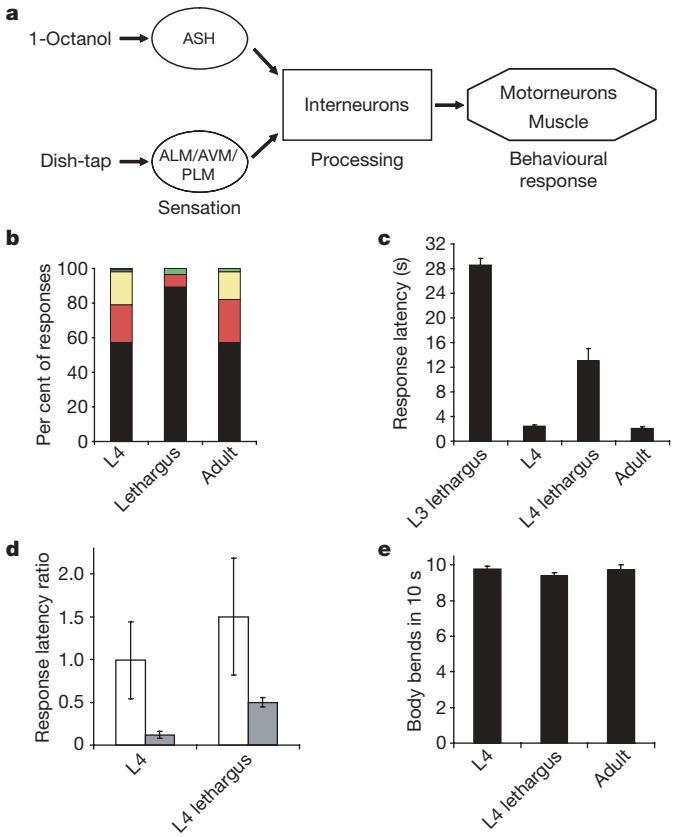

Figure 2 | Responsiveness is reduced during lethargus. **a**, Dish-tap is sensed by the mechanosensory neurons ALM, PLM and AVM[6], and dilute 1-octanol is sensed by the polymodal sensory neuron ASH[7]. **b**, In response to dish-tap, five behavioural responses were observed: brief backing (black); sustained backing (red); complex reorienting response (yellow); acceleration (green); and shrinking (blue). Shrinking was observed only once. The difference in the frequency distribution in the five categories between lethargus and the other two stages was significant at $P < 0.0001$ (chi-squared test). See Supplementary Information for additional details. **c**, Response latency to 30% 1-octanol is increased during lethargus. The mean ± s.e.m. response latency is shown for the L3 lethargus ($n = 20$), the L4 stage before lethargus ($n = 109$), the L4 lethargus ($n = 34$) and the adult stage ($n = 48$). Differences in response latency between the L3 lethargus and L4 stages, the L4 lethargus and L4 stages, and the L4 lethargus and adult stages were all significant at $P < 0.0001$, two-tailed Student's *t*-test with unequal variance. **d**, Worms respond to 30% 1-octanol during lethargus. Shown is the mean ± s.e.m. ratio of response latencies to two stimulations. The first stimulation consisted of 100% ethanol, and the second stimulation consisted either of 30% 1-octanol (grey) or of 100% ethanol (white). 'L4' and 'L4 lethargus' denote the fourth larval stage before and during lethargus, respectively. The effect of 1-octanol in comparison to that of ethanol was significant during both the L4 and the L4 lethargus stages at $P < 0.00001$ and $P = 0.01$, respectively (two-tailed Student's *t*-test, unequal variances). **e**, Continuous 10-s stimulation of the worms' tail during lethargus results in normal movement, as assessed by the number of anterior body bends. $n = 45$, 45 and 16 for the L4, L4 lethargus and adult stages, respectively. There was no difference between the speed during the L4 stage before lethargus and L4 lethargus ($P = 0.10$) or between adults and L4 lethargus ($P = 0.28$), two-tailed Student's *t*-tests. Shown is the mean ± s.e.m.

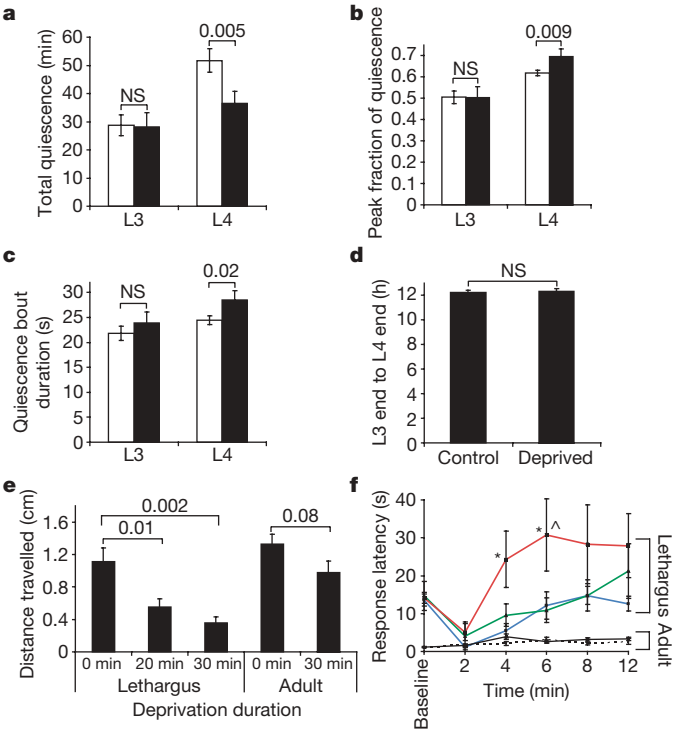

Figure 3 | Homeostatic regulation of lethargus. **a**–**d**, Mechanical stimulation for one hour beginning nine hours after the end of the L3 lethargus period results in reduced total quiescence (**a**) and increased quiescence consolidation (**b**, **c**) in the L4 lethargus period, but no change in the timing of the end of L4 lethargus (**d**). Shown are the mean ± s.e.m values from analysis of 18 unperturbed animals that began L4 lethargus at least nine hours after the end of L3 lethargus (white), and of 13 animals that were deprived of quiescence for one hour beginning nine hours after the end of the L3 lethargus period (black). NS denotes $P > 0.1$. Statistical significance was assessed with ANCOVA in **a**–**c** and with Student's *t*-test in **d**. **e**, The distance travelled by the worm in two minutes is reduced after deprivation of quiescence during L4 lethargus. *P* values are based on two-tailed Student's *t*-tests. $n = 10$ for each group. **f**, Prolonged 1-octanol response latencies observed during lethargus are reversible by strong stimulation, and recur with a faster time course and with further prolongation of the response latency after previous deprivation of quiescence. The *x* and *y* axes denote the time after strong stimulation of the worm, and the mean ± s.e.m. 1-octanol response latency, respectively. Values of deprived worms that were different ($P < 0.05$, two-tailed Student's *t*-test) from stage-matched controls at the same time point are designated with an asterisk. Values that were greater than the baseline 1-octanol response latencies were tested with a one-tailed Student's *t*-test with unequal variance, and the value that is significantly different from the baseline response at $P < 0.05$ is designated with an arrowhead. Blue line, worms in lethargus that were not deprived of quiescence; green line, worms in lethargus that were deprived for 20 min; red line, worms in lethargus that were deprived for 30 min; black dashed line, adult worms that were not deprived; black solid line, adult worms that were deprived for 30 min. $n = 10$ for each group.

To identify genetic regulators of lethargus, we initially focused on *egl-4*. The mutant *egl-4*(*ad450*sd), which contains a gain-of-function (gf) mutation in a cGMP-dependent protein kinase (PKG)[12], has been noted during its adult stage to stop moving and feeding[12,13]—behaviours normally observed to stop during lethargus. We measured the quiescence associated with lethargus in the *egl-4*(*gf*) mutant as well as in the *egl-4* loss-of-function (lf) null mutant *egl-4*(*n479*) (ref. 14). The *egl-4*(*gf*) mutants showed a time-dependent increase in behavioural quiescence, whereas the *egl-4*(*lf*) mutants showed reduced behavioural quiescence (Fig. 4a, b). *egl-4*(*gf*) mutants have quiescence outside of lethargus, during the normally active periods (Fig. 4a, b).

The increased behavioural quiescence of *egl-4*(*gf*) adults is associated with a longer latency of response to 1-octanol (Fig. 4c), indicating that the behavioural state in adults also has sleep-like properties. After strong mechanical stimulation of *egl-4*(*gf*) adults, the mutants resume normal adult locomotion[12,13] and respond normally to 1-octanol (Fig. 4c), indicating that they are capable of a normal sensory response. Thus, the increased 1-octanol response latency is a result of sleep-like behaviour of this mutant during the adult stage. The 1-octanol response latency of third-day adult *egl-4*(*gf*) worms that had been treated with *egl-4*(*RNAi*) for two days was shorter (7 ± 2 s) than that of control *egl-4*(*gf*) worms treated with control RNA interference (RNAi) (19 ± 5 s, mean $\pm$ s.e.m.; $n = 15$ for each group, $P = 0.02$, two-tailed Student's *t*-test), indicating that the sleep-like properties of *egl-4*(*gf*) are not the result of altered development.

In addition to implicating *egl-4* in the control of the sleep-like behaviour in *C. elegans*, the finding that sleep-like behaviours in the *egl-4*(*gf*) mutants can occur during the adult stage, after completion of all moults, indicates that sleep-like behaviour can be uncoupled from the moulting cycle.

In contrast to the behaviour of *egl-4*(*gf*), response latency to 1-octanol during lethargus is reduced in the *egl-4*(*lf*) mutant (Fig. 4d), indicating a reduction in sleep-like behaviour in this mutant. Rescue of the short 1-octanol response latency of *egl-4*(*lf*) during lethargus was achieved by expression of *egl-4* in a subset of sensory neurons under the control of either the *odr-3* or the *tax-4* promoter but not under the control of the *odr-1* promoter (Supplementary Fig. 3). These transgenic experiments indicate that sensory neurons have a role in the regulation of lethargus. In addition, because the *odr-1* promoter used in this experiment is known to promote expression in the only two neurons that share *tax-4* and *odr-3* expression (Supplementary Table 2), these results indicate that *egl-4* can function in multiple sensory neurons to promote sleep-like behaviour. Given the demonstrated role for *egl-4* in sensory adaptation[15], one interpretation of this function in sensory neurons in regulating lethargus is that *egl-4* serves to reduce the arousal state of the animal by dampening sensory input.

The effects of *egl-4* mutations on state-dependent 1-octanol responses cannot be explained as a non-specific effect of overall activity of these mutants. This is because *egl-8* mutant adults, which like *egl-4*(*gf*) show decreased movement when unperturbed[16], have normal 1-octanol response latencies (Fig. 4c), and *goa-1* mutants, which

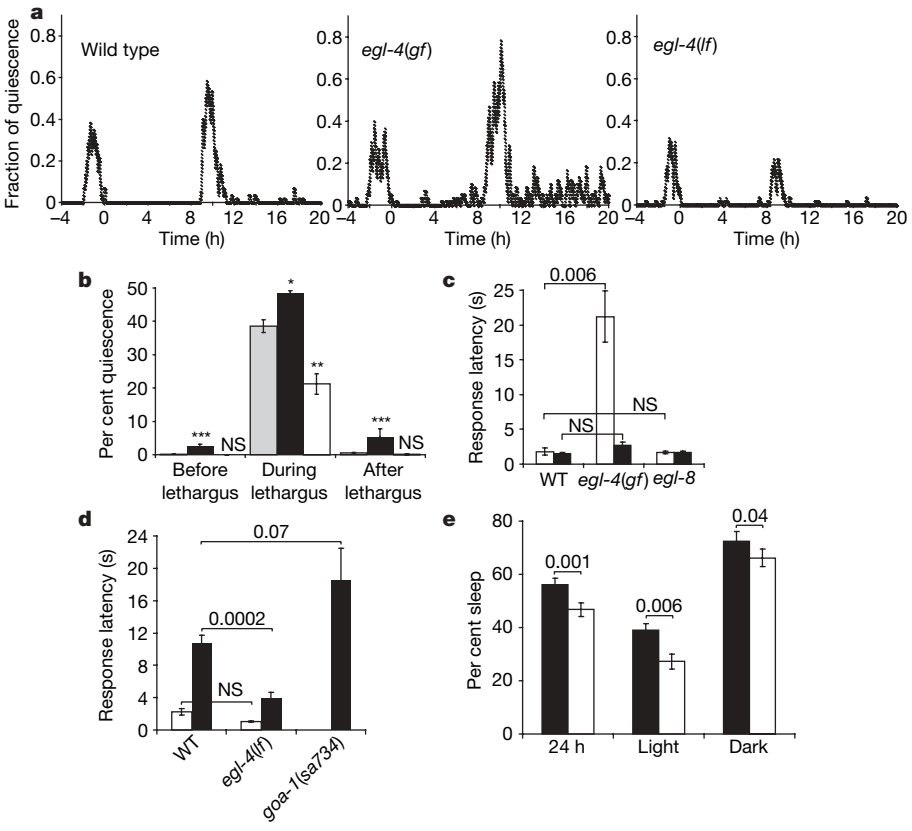

Figure 4 | The *egl-4* cGMP-dependent protein kinase promotes sleep-like behaviour. a, Quiescence measurement of a wild-type worm, the *egl-4*(*gf*) mutant *ad450* and the *egl-4*(*lf*) mutant *n479*. The zero time point represents the end of the L3 lethargus period. The *egl-4*(*gf*) mutant shows increased L4 quiescence as well as quiescence during the adult stage, whereas the *egl-4*(*lf*) mutant shows a reduction of behavioural quiescence associated with the L3 and L4 lethargus stages. **b**, Comparison of mean $\pm$ s.e.m. percentage quiescence in one hour in wild type (grey), *egl-4*(*gf*) (black) and *egl-4*(*lf*) (white) worms. 'Before lethargus', 'During lethargus' and 'After lethargus' are defined in Supplementary Table 1. Differences between mutant and wild type are designated $*P < 0.05$, $**P < 0.005$, $***P < 0.0005$ (Student's *t*-test). 'NS' denotes $P > 0.1$. $n = 7$ for each mutant and $n = 21$ for wild type. **c**, Adult *egl-4*(*gf*) mutants, when unperturbed, show sleep-like behaviour in their response to 30% 1-octanol (white), whereas *egl-8* mutants do not. Ten minutes after strong stimulation of the animal (black), the latency is not different from that of wild-type adult worms. Error bars represent s.e.m. Comparisons were made between genotypes using a two-tailed Student's *t*-test with unequal variance. 'NS' denotes $P > 0.1$. $n = 15$ worms for each condition. **d**, During lethargus (black), *egl-4*(*lf*) mutants show a reduction in 1-octanol response latencies, whereas *goa-1*(*sa734*) mutants do not. During the L4 stage before lethargus (white), the response latencies are not different between the genotypes. Values are mean $\pm$ s.e.m. Comparisons were made using a two-tailed Student's *t*-test with unequal variance. $n = 15$ worms for each condition. **e**, Increased activity of the cGMP-dependent protein kinase gene *foraging* is associated with increased sleep in *Drosophila*. Shown is the mean $\pm$ s.e.m. percentage time spent asleep during a two-day video recording of 24–26 for^{s2} (white) and for^{R} (black) flies in the 24-h period, in the 12-h light period (Light) and in the 12-h dark period (Dark). Comparisons were made using a two-tailed Student's *t*-test with unequal variance.

like *egl-4*(*lf*) show increased movement outside of lethargus[17], have normal response latencies during lethargus (Fig. 4d). The timing of lethargus, as reflected by the duration between the quiescence peaks of the L3 and L4 lethargus, was not different in wild-type (11.3 ± 0.2 h, $n = 30$), *egl-4*(*gf*) (11.1 ± 0.6 h, $n = 6$) and *egl-4*(*lf*) (11.4 ± 0.5 h, $n = 5$) worms, indicating that *egl-4* affects the expression and not the timing of sleep-like behaviour.

To test for the possibility that the quiescence-promoting effects of PKG are phylogenetically conserved, we compared sleep in *D. melanogaster* strains that differed in the activity of the *foraging* (*for*) gene, which encodes a *Drosophila* PKG[18] similar in sequence and function to *egl-4* (ref. 19; Supplementary Information). Cyclic GMP has previously been implicated in the signalling events that control insect pre-ecdysis behaviour[20]. We found that the *Drosophila* strain for^{s2}, which has low PKG levels, slept less than the for^{R} strain from which it was derived[21] (Fig. 4e). Therefore, as in *C. elegans*, greater PKG activity is associated with more sleep in *Drosophila*.

To explore further the idea that there is some conservation of genetic regulation of sleep-like behaviour, we studied worms that carry reduction-of-function mutations in *pde-4* and worms that carry a gain-of-function mutation in *acy-1*—genes that encode *C. elegans* homologues of *Drosophila dunce*[22] and *rutabaga*[23], respectively. Studies of *dunce* and *rutabaga* have led to the conclusion that cAMP signalling promotes *Drosophila* wakefulness[24]. The 1-octanol response latency during lethargus of *pde-4* and *acy-1* mutants was reduced (Supplementary Fig. 4). This increased sensory responsiveness during a normally sleep-like period suggests that cAMP signalling antagonizes worm sleep-like behaviour. These effects of *pde-4*, *acy-1* and *egl-4* mutations in *C. elegans* indicate that there is some conservation in the genetic control of sleep. Independent evidence of such conservation was reported recently[25].

The reason for the evolution of sleep is unknown. The temporal relationship between *C. elegans* lethargus and the moult, which is required for animal growth and development and is a time of biosynthetic activity[26,27], suggests that this sleep-like state has a role in growth and development. Synaptic changes occur during a lethargus period[28,29], suggesting that lethargus promotes nervous system change. A role in nervous system development is interesting in light of data suggesting that sleep is necessary for changes in the nervous system[30].

METHODS SUMMARY

A digital video analysis method based on a frame subtraction principle was used to identify 10-s epochs of behavioural quiescence. This method has a spatial resolution for movement detection that approaches 5 μm. Additional details of this method as well as methods for deprivation of quiescence are described in the Methods. *C. elegans* strains used as well as methods for sensory stimulation, for statistical analysis, for *Drosophila* sleep measurements, for transgenesis and for RNAi are detailed in the Supplementary Information.

Full Methods and any associated references are available in the online version of the paper at www.nature.com/nature.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

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Author Contributions D.M.R. designed and performed research, J.E.Z. performed *Drosophila* experiments, M.H.M. wrote computer programs, U.D.T. performed behavioural experiments involving 1-octanol response measurements, Y.Y. showed that *tax-4p::egl-4* can rescue sleep-like behaviours of *egl-4*(*lf*), and M.V.S. and A.I.P. provided input into research design and drafted this manuscript along with D.M.R.

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METHODS

Quiescence measurements. To measure worm quiescence, either a single egg or a single third-larval-stage hermaphrodite was transferred without bacteria and placed onto an NGM agar surface next to a circle of OP50 bacteria (~0.5 cm in diameter). After 5–30 min of exploration, a larva would typically find the bacterial lawn and then stay within the confines of this lawn until it reached the adult stage. Direct observations of worms revealed that when they occasionally departed the bacterial lawn, they remained moving continuously if the departure was less than 20 min. Therefore, during periods of less than 20 min in which the worm was not seen within the image of the camera, it was considered to have moved during these periods. Experiments in which the worm left for more than 20 min were discontinued and the data discarded. This limitation precluded quiescence analysis of mutants that left the field of view frequently such as *pde-4* and *goa-1* mutants. The worm was placed on a Diagnostics Instruments microscope base, illuminated with continuous white light, and visualized using a Zeiss Stemi 2000 microscope at objective magnifications ranging from ×2.0 to ×3.2. Video frames were captured every 10 s for 24–96 h. A Spot Insight B/W digital camera (Diagnostic Instruments) at 533 × 400 pixel resolution and a Hitachi B/W analogue camera at 640 × 480 pixel resolution were used to capture the images and stored with 8-bit greyscale resolution. At the magnifications used, a single pixel corresponds to 7 μm^2 when using the Spot camera and 5 μm^2 when using the Hitachi camera. There was no significant difference in the analysis results obtained using the two cameras. During the fourth larval stage the worm grows in length from 600 μm to 800 μm, and therefore the resolution of movement is approximately 1% of the length of the worm.

Analysis of quiescence was performed using a combination of Image Pro Plus 5.0 (Media Cybernetics) and custom programs developed in C++. After normalization of image illumination intensity, the difference in greyscale values between each pair of successive frames for individual pixels was calculated and added to a greyscale value of 128. If the worm did not move between the two frames, all pixels had a greyscale value close to 128, and the image therefore appeared grey (Supplementary Fig. 1). If the worm moved, a greyscale value darker than 128 (typically less than 115) was identified for one or more pixel. Two-hundred and fifty images containing a dead worm were analysed by this method to determine the minimum greyscale value that can occur in the absence of any movement; thus, when analysing a live worm, a greyscale value less than this minimum was considered to reflect worm movement between the two frames.

Visual inspection of the images showed that rarely (< 0.1% of the time) a tiny movement of the nose is perceptible to the human eye yet is not detected as movement using our computer algorithm. Because the nose is less dark than the rest of the body, these small movements do not result in any pixels with sufficiently low greyscale values to consider the worm to have moved.

Deprivation of quiescence. In studies of the effect of deprivation of quiescence on quiescence consolidation, the worms were deprived by stimulating them mechanically on an agar surface. In studies of the effect of deprivation of the latency to resumption of quiescence and sleep-like 1-octanol responses, the worms were stimulated in solution as described below.

For the quiescence consolidation experiment, 9 h after the end of the L3 lethargus the worm was transferred to a fresh NGM agar surface covered completely with a lawn of OP50 bacteria. Every 40–60 s, the worm was mechanically stimulated by touching it on the posterior end of its body with an eyelash tethered to the end of a Pasteur pipette—a standard method for mechanically stimulating worms (http://www.wormbook.org/chapters/www_behavior/behavior.html). If the touch did not induce worm movement, the worm was prodded again more strongly until movement was apparent. Approximately 5% of stimulations were applied to the anterior end of the worm to induce locomotion reversal if the worm approached the edge of the agar plate. This procedure was continued for 1 h, after which the worm was transferred back to the agar surface plated with a small bacterial lawn in the centre of the plate. Initial attempts to deprive worms for two hours in an effort to eliminate most to all of the quiescence associated with L4 lethargus were unsuccessful because, after an hour of stimulation, the animals typically would become virtually unresponsive to continued stimulation and would be injured by the harsh stimulations needed to induce movement. We therefore restricted the stimulation to 1 h from 9–10 h after the end of the L3 lethargus period.

To measure the rate of return to sleep-like behaviour, wild-type worms in lethargus were identified within 20 min of the start of their fourth-larval-stage lethargus period. Worms in lethargus were identified as those with reduced movement, cessation of feeding behaviour and elevated response latencies to 30% 1-octanol. Worms were then deprived of quiescence for durations ranging from 0 min to 30 min by placing 10 μl of M9 buffer on the worm to induce thrashing behaviour. The worms were monitored continuously to ensure that they moved continuously during the complete deprivation period. If they stopped moving or if the buffer was absorbed into the agar, an additional 10 μl was added to agitate the buffer and to induce resumption of thrashing. After this period of deprivation, the worms were dragged out of the buffer using an eye lash, and were strongly stimulated by briefly lifting them from the agar surface with a worm pick. The response latency to 1-octanol was then measured every 2 min for 10 min for all the worms.

To quantify the duration of locomotion before resumption of behavioural quiescence, we measured the distance the worm moved within 2 min after placement on the centre of the agar surface.