CONSERVATION OF GENES REQUIRED FOR *D. MELANOGASTER* AND *C. ELEGANS* SLEEP

Deep Conservation of Genes Required for Both *Drosophila melanogaster* and *Caenorhabditis elegans* Sleep Includes a Role for Dopaminergic Signaling

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Objectives: Cross-species conservation of sleep-like behaviors predicts the presence of conserved molecular mechanisms underlying sleep. However, limited experimental evidence of conservation exists. Here, this prediction is tested directly.

Measurements and Results: During lethargus, *Caenorhabditis elegans* spontaneously sleep in short bouts that are interspersed with bouts of spontaneous locomotion. We identified 26 genes required for *Drosophila melanogaster* sleep. Twenty orthologous *C. elegans* genes were selected based on similarity. Their effect on *C. elegans* sleep and arousal during the last larval lethargus was assessed. The 20 most similar genes altered both the quantity of sleep and arousal thresholds. In 18 cases, the direction of change was concordant with *Drosophila* studies published previously. Additionally, we delineated a conserved genetic pathway by which dopamine regulates sleep and arousal. In *C. elegans* neurons, G-alpha S, adenylyl cyclase, and protein kinase A act downstream of D1 dopamine receptors to regulate these behaviors. Finally, a quantitative analysis of genes examined herein revealed that *C. elegans* arousal thresholds were directly correlated with amount of sleep during lethargus. However, bout duration varies little and was not correlated with arousal thresholds.

Conclusions: The comprehensive analysis presented here suggests that conserved genes and pathways are required for sleep in invertebrates and, likely, across the entire animal kingdom. The genetic pathway delineated in this study implicates G-alpha S and previously known genes downstream of dopamine signaling in sleep. Quantitative analysis of various components of quiescence suggests that interdependent or identical cellular and molecular mechanisms are likely to regulate both arousal and sleep entry.

Keywords: sleep, *C. elegans*, *Drosophila*, dopamine

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INTRODUCTION

Sleep is observed in all animal species, but the genetic underpinnings of mammalian sleep remain largely unknown.[1] However, the recent identification of several genes and DNA polymorphisms affecting human sleep strongly suggests that this behavior is genetically regulated.[2-4] Sleep in model organisms, including zebrafish, fruit flies, and nematodes, has been defined based on behavioral changes shared with human sleep. These changes include sleep-specific posture, spontaneous cessation of movement/feeding, increased arousal thresholds, rapid reversibility, and homeostatic response to sleep deprivation.[4-6] Although genetic studies have identified many genes required for sleep in these species,[5,7-11] generally the cross-species relevance of these genes has not been examined. Given the shared characteristics of sleep and the conservation of this behavior across the animal kingdom, it seemed likely that the genes and pathways required for sleep are conserved. Studies in mice, fruit flies, and nematodes have established a role for the epidermal growth factor (EGF) signaling pathway, protein kinase G (PKG), cyclic adenosine monophosphate (cAMP)/protein kinase A (PKA)/cAMP response element binding (CREB), dopamine, neuropeptides, and the Notch signaling pathway in sleep in two or more of these model systems.[6,12-26] Here, we more comprehensively address the conservation of genes required for sleep using two invertebrate model organisms.

Caenorhabditis elegans sleep is called quiescence,[6] whereas *Drosophila melanogaster* sleep is called rest.[27] In *Drosophila*, rest is temporally coordinated with the solar light-dark cycle via circadian proteins such as Period, whose abundance oscillates rhythmically on a 24-h cycle.[28] Under standard culture conditions, *C. elegans* quiescence occurs during a period called lethargus and is temporally coordinated with molting of the larval cuticle, which occurs at the end of each larval stage.[6] Interestingly, the *C. elegans* Period ortholog, *lin-42*, regulates both cuticle molting and quiescence.[29] Although the roles of circadian genes in sleep regulation do vary between these invertebrate species, the role of other genes required for entry, maintenance, and exit from sleep may be more directly conserved. However, little experimental evidence exists to support this hypothesis.

To test this putative conservation, we focused on two well-known invertebrate model systems, *Drosophila* and *C. elegans*. Published *Drosophila* literature was reviewed to shortlist genes required for rest. The effect of orthologous *C. elegans* genes on quiescence and arousal was assessed. We found that *C. elegans* orthologs of *Drosophila* genes required for rest also altered both the amount of *C. elegans* quiescence and arousal during quiescence. Correlation analysis for the *C. elegans* genes tested herein revealed a simple and direct correlation between arousal thresholds and quiescence bout entry. Finally, the comprehensive analysis presented here allowed us to delineate a conserved genetic pathway acting downstream of the DOP-1 D1 receptors that regulates *C. elegans* quiescence.

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MATERIALS AND METHODS

C. elegans strains

Strains and alleles examined are listed in Table S1 (supplemental material). Animals were reared on standard nematode growth media (NGM) media seeded with OP50 *Escherichia coli* at 25°C and assayed at 24°C. Animals from the RNA interference (RNAi) sensitized strain, HA2518, were transferred to RNAi feeding plates as L4 larvae and their progeny were assayed for quiescence and arousal defects. In some cases, the effect of RNAi was not seen until the F2 generation. To control for potential off-target effect of the RNAi knockdown, RNAi clones for nonoverlapping regions of the gene of interest were tested. The only potential *inso-1(RNAi)* off-target gene is *csn-1*, encoding a component of a COP9 signalosome complex. RNAi studies used NGM plates with 1 mM ampicillin and 1mM isopropyl β-D-1-thiogalactopyranoside (IPTG).[30]

Plasmids and transgenic strains

Transgenic animals were generated by microinjection of plasmids using standard methods.[31] Co-injection markers were transcriptional fusions pGH#8[32] *rab-3p::mCherry* (25ng/µl) or pPD48.33[33] *myo-2::gfp* (5 ng/µL) (gfp = green fluorescent protein). Primers are listed in Table S2 (supplemental material). Results for at least two independent transgenic lines were determined and combined for each transgenic genotype reported.

dop-1p::gfp pHA#651, 4.3 kb of sequence upstream the *dop-1* start codon was polymerase chain reaction (PCR) amplified and cloned into the pPD49.26[34] *Xma*I site. GFP was cloned behind the *dop-1* promoter using KpnI and EcoRI sites.

dop-1p::kin-2(RNAi) pHA#652, 2.5 kb of *kin-2* genomic sequence containing exons was PCR amplified and cloned in reverse orientation behind the *dop-1* promoter of pHA#581, using KpnI and SacI sites.

dop-1p::crh-1 pHA#653, the *crh-1* complementary DNA (cDNA) sequence of 945 bp was PCR amplified and cloned behind the *dop-1* promoter of pHA#581, using NheI and KpnI sites.

Total quiescence: RNAi feeding clones described below:

crh-1(RNAi) feeding clone pHA#665 469 bp of the *crh-1* cDNA sequence was cloned into pL4440[30] vector using NheI and NcoI sites.

egl-4(RNAi) feeding clone pHA#666 2.1 kbp of the *egl-4* genomic sequence containing exons was PCR-amplified and cloned into pL4440 vector using the SphI site.

cul-3(RNAi) feeding clones pHA#657 and pHA#661 each contain a nonoverlapping, PCR-amplified *cul-3* cDNA sequence cloned into XmaI site of pL4440.

lgc-38(RNAi) feeding clones pHA#654 and pHA#664 contain a nonoverlapping PCR-amplified *lgc-38* genomic and cDNA sequence, respectively cloned into NheI and SacI sites of pL4440.

cya-1(RNAi) feeding clones pHA#662 and pHA#663 contain a nonoverlapping, PCR-amplified *cya-1* cDNA sequence cloned into NheI and XmaI sites of pL4440 vector.

inso-1(RNAi) feeding clones pHA#659 and pHA#660 each contain a nonoverlapping, PCR-amplified *inso-1* cDNA sequence cloned into the SpeI site of pL4440 vector.

jnk-1(RNAi) feeding clone pHA#667 1.8 kbp of the *jnk-1* genomic sequence containing exons was PCR-amplified and cloned into pL4440 vector using NheI and SacI sites.

Arousal threshold: RNAi feeding clones described below. The effect on quiescence of these clones was virtually identical to the effect of clones above that altered quiescence (Figure 2A).

inso-1(RNAi) feeding clone pHA#656, 624 bp of *inso-1* genomic sequence containing exons was PCR-amplified and cloned into pL4440 vector using the SpeI site.

cul-3(RNAi) feeding clone pHA#657 (described above).

cya-1(RNAi) feeding clone pHA#655, 1.47 kbp of *cya-1* genomic sequence containing exons was PCR-amplified and cloned into pL4440 vector using NheI and XmaI sites.

lgc-38(RNAi) feeding clone pHA#654 (described above).

crh-1(RNAi) feeding clone pHA#665 (described above).

jnk-1(RNAi) feeding clone pHA#667 (described above).

Quiescence and arousal threshold assays

Quiescence during the L4-to-adult molting lethargus was determined using the previously published microfluidic chamber-based assays.[19] Image subtraction was performed using the previously published Matlab analysis program.[19] A single pixel change above camera noise was the metric for movement detection (lack of quiescence). Cameras used for image acquisition were AxioCam ICc1 (Zeiss, Oberkochen, Germany) (pixel size: 4.65 µm × 4.65 µm), AxioCam MRc Rev3 (Zeiss) (pixel size: 6.45 µm × 6.45 µm) and Stingray F201c (Allied Vision Technologies, Stadtroda, Germany) (pixel size: 4.4 µm × 4.4 µm). Unless indicated otherwise, early-L4 stage larvae were loaded into 1 × 4 mm microfluidic chambers 4-6 h prior to lethargus and the activity was monitored for 12 h. HA2518 animals were loaded in the microfluidic chambers as black-dot/late-L4 stage larvae, just as lethargus began and activity was recorded for 8 h. Images were acquired at 10-sec interval. Unlike the previous analysis presented in Singh et al.,[19] in the current study quiescence onset time was defined as a time point after which the fractional quiescence remains > 0.1 for at least 20 minutes. The quiescence exit time was defined as the time point after which the fractional quiescence had remained < 0.1 for at least 20 minutes. The total quiescence was calculated by counting the number of images where no pixel change was detected (compared to the previous image, above camera noise) between quiescence onset and exit time points. Each image subtraction that did not detect motion represents 10 sec of quiescence. Quiescence onset time was subtracted from quiescence exit time to determine the duration of lethargus quiescence and was reported in hours. The number of quiescent bouts that were 10 sec or longer was determined and total number of bouts occurring between the quiescence onset and exit time was reported. Bout frequency was calculated by dividing total number of bouts by lethargus duration for each animal. The duration of all the quiescent bouts occurring between the quiescence onset and exit time was measured and the average bout duration was calculated and reported in sec. We calculated bout durations for each animal and then averaged the results of all animals in a genotype/treatment. Other bout metrics were calculated similarly. *goa-1(lf)* and *gsa-1(gf)* animals had profoundly decreased total quiescence. As a result, the fractional quiescence cutoff of > 0.1 for 20 min was never exceeded. Therefore, these

animals have total quiescence = 0 min, bout number = 0, and lethargus duration = 0 h (see Table S3, supplemental material). Because lethargus duration for *goa-1(lf)* and *gsa-1(gf)* animals was equal to zero, bout number and bout duration for these genotypes could not be determined and were excluded from the correlation analysis presented in Figure 4. Quiescence and arousal thresholds of *pde-4(ok1439)* animals were presented as *pde-4(ce268)* animals exhibited normal quiescence (total quiescence of *pde-4(ce268)* = 59 ± 6 min, P = 0.27). *pde-4(ce268)* changes a single amino acid, whereas *pde-4(ok1439)* deletes 1100 bp and likely results in a complete loss of function. For genotypes with most and least total quiescence, bout duration was reexamined at one frame per sec interval. In this reanalysis bout duration > 1 sec was measured and total quiescence determined. This analysis is described in Table S4 (supplemental material).

For arousal threshold determination, response to body touch[35] was used; exceptions noted later in this paper. A hair was placed on the agar plate evenly seeded with OP50 bacteria and flexed to contact the animal behind the pharynx; locomotion within 1 sec was scored as a response and percentage of animals failing to respond is reported. Arousal thresholds of HA2518 RNAi sensitized animals and *cnb-1(lf)* animals were performed using 1-octanol diluted to 60% concentration with ethanol. Average response time to 60% octanol of quiescent animals reared on experimental and control RNAi strains is reported in sec. During quiescence, animals responded to touch or dilute 1-octanol by initiating backward locomotion. For touch, any immediate locomotion was scored as response. For dilute octanol, time to initiate locomotion was recorded. Octanol assays stopped at 20 sec. For all genotypes, at least half of the trials were done by an observer blinded as to the genotypes/treatment. Control genotypes were always run in parallel (also blinded). Nonquiescent animals in lethargus were used as controls to determine the basal response to mechanical or chemosensory stimuli. Any immobile, nonfeeding animal was considered quiescent for arousal threshold determination. For *gsa-1* and *goa-1*, rare quiescent animals identified during the appropriate developmental stage were scored for arousal. Arousal threshold results are presented for at least three independent trials with N ≥ 6 per trial for each genotype tested. The following exceptions should be noted: *goa-1*: N = 1 and *kin-2*: N = 5 animals (because of scarcity of quiescent animals).

Statistics

Genes examined here are *C. elegans* orthologs of *Drosophila* genes that alter the quantity of *Drosophila* rest. Genes that affect circadian behavior or genes that only alter homeostasis were not included in the analysis. Orthologs were identified and similarity was assessed based on E values from BLAST at the National Center for Biotechnology Information.[36] The Expect (E) values for all the *C. elegans* genes compared to *Drosophila* genes are listed in Table S5 (supplemental material). The least similar was RCN-1 (E = 3e-27). At least 8 or 20 animals were tested for each quiescence or arousal threshold determination, respectively. For each of the 20 genes examined, independent sets of wild type or control animals were tested in parallel and these were used to determine significance by the Student *t* test (Table S3 and figure legends). Because multiple comparisons were made, we calculated the false discovery rate (FDR).[37,38] The FDR q-value was set at the standard 0.05 to ascribe significance (Table S5). A similar statistical analysis was performed for arousal thresholds (Table S5).

RESULTS

Lethargus quiescence requires the function of conserved genes

The *Drosophila* rest and *C. elegans* quiescence share behavioral characteristics of sleep including cessation of movement, altered arousal, and homeostatic regulation.[4-6,39] The presence of these common hallmarks suggests that conserved processes regulate sleep in these invertebrate species. For clarity herein, we will refer to sleep in *Drosophila* as rest and sleep in *C. elegans* as quiescence.

To determine if orthologs of *Drosophila* genes required for rest also play a conserved role in *C. elegans* quiescence, we first identified genes that altered the quantity of *Drosophila* rest. Genes involved in circadian regulation or solely in homeostasis were excluded from this analysis. *C. elegans* orthologs of *Drosophila* genes were identified based on protein sequence similarity using BLAST (Table S5), and the closest *C. elegans* ortholog was examined in this study. No unambiguous orthologs could be found for six *Drosophila* genes: *Hyperkinetic*,[40] *Fragile X mental retardation*,[41] *homer*,[42] *Relish*,[43] *sleepless*,[44] and *Regulator of cyclin A*.[45] Publicly available deletion, missense, or nonsense alleles were obtained for the remaining *C. elegans* genes, whenever possible (Figure 1, Table S1). If no alleles were available, then RNAi by feeding was used to test the effect of genes on *C. elegans* quiescence (see Figure 2). *C. elegans* L4-to-adult lethargus quiescence was assessed using a microfluidic chamber-based assay. Total time spent in quiescence in minutes, which will be referred to as total quiescence, was reported[19] (Figure 1, see Methods). All genes tested altered *C. elegans* quiescence. For 18 genes, the change in *C. elegans* total quiescence was as would be predicted from *D. melanogaster* literature. When disruption of *Drosophila* gene function increased rest, disruption of the *C. elegans* ortholog increased total quiescence, and *vice versa*. Exceptions were the *C. elegans* ortholog of *Drosophila basket* and *cyclin A* (described in the following paragraphs). Results are summarized in Tables S3 and S6 (supplemental material), presented graphically in Figures 1 and 2, and detailed in the following paragraphs.

Shaker and the calcineurin pathway (*shk-1*, *tax-6*, *cnb-1*, *rcn-1*)

Drosophila Shaker encodes a voltage-gated potassium channel, which regulates membrane repolarization and synaptic signaling.[46] Disruption of *Drosophila Shaker* function reduces rest.[47] Consistent with this, decreased total quiescence was observed in animals carrying a deletion allele of the *C. elegans Shaker* ortholog, *shk-1* (Figure 1A). The calcineurin signaling pathway has also been implicated in *Drosophila* rest.[48,49] Calcineurin is a Ca^{2+}/calmodulin dependent protein phosphatase complex containing both a catalytic subunit and a regulatory subunit. Disruption of either catalytic subunit, *Pp2B-14D* or *CanA-14F*, or either regulatory subunit, *CanB* or *CanB2*, results in reduced *Drosophila* rest. Additionally, loss of the calcineurin regulator *sarah* reduces *Drosophila* rest.[48] In *C. elegans*, the catalytic and regulatory subunits of calcineurin are encoded

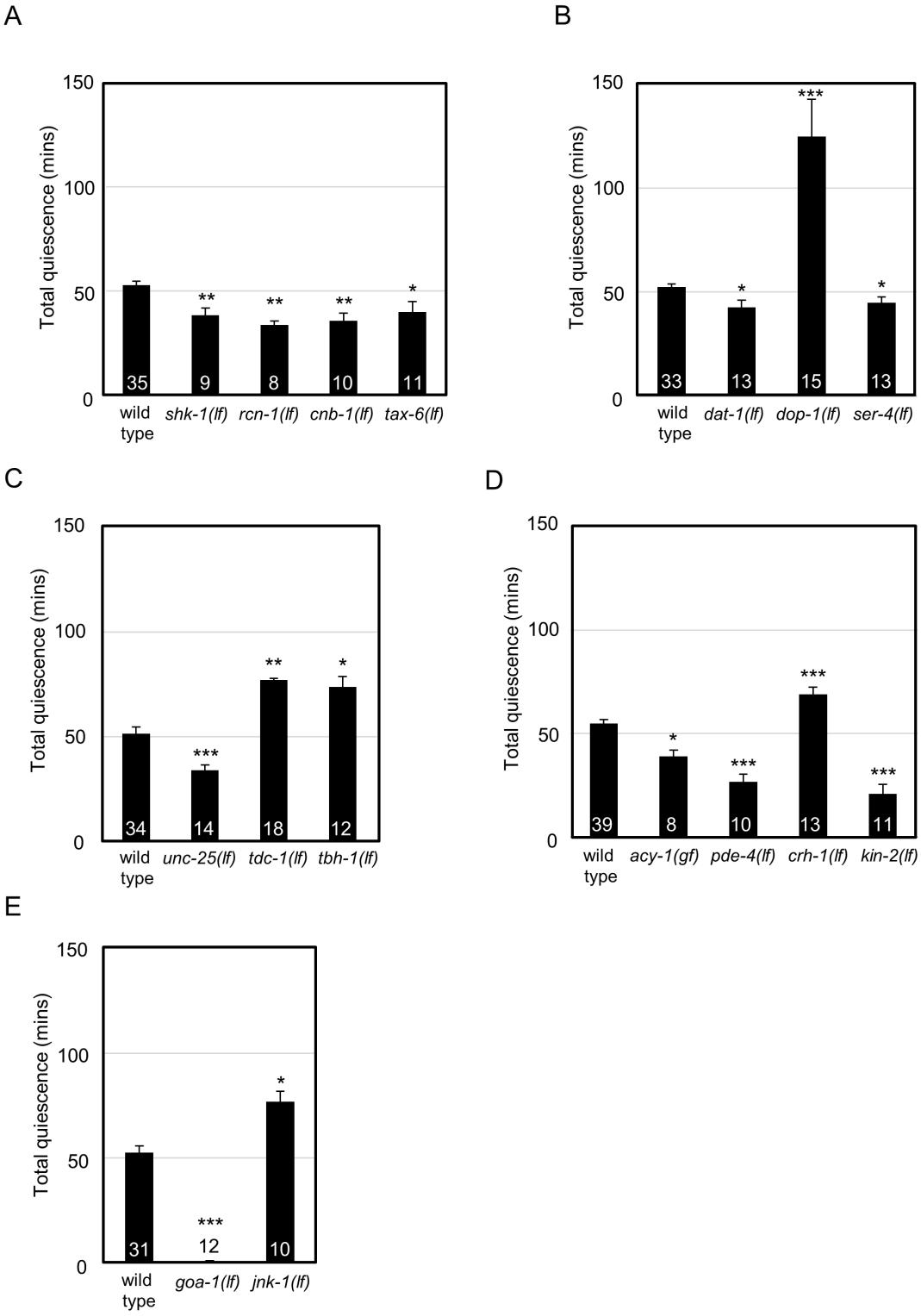

Figure 1—Lethargus quiescence requires the function of conserved genes. L4-to-adult lethargus quiescence was determined. Total quiescence was reported in min. Error bars represent standard error of the mean (s.e.m.). Numbers inside or above the bar indicate sample size. For each gene examined, independent sets of wild-type control animals were tested in parallel and these were used to determine significance by Student *t* test. P < 0.05* < 0.01** and < 0.001*** versus wild-type. Results were grouped by pathway/function and controls were pooled for concise presentation. Control is *dop-1* promoter driving gfp (*dop-1p::gfp*). **(A)** Loss of calcineurin signaling components (*rcn-1, cnb-1, tax-6*) or Shaker potassium channel (*shk-1*) function decreased total quiescence. **(B)** Loss of dopamine transporter (*dat-1*) or serotonin receptor (*ser-4*) function decreased total quiescence. Loss of D1 dopamine receptor (*dop-1*) function increased total quiescence. **(C)** Loss of γ-aminobutyric acid synthesis (*unc-25*) decreased total quiescence. Loss of genes required for octopamine biosynthesis increased total quiescence: tyrosine decarboxylase (*tdc-1*) and dopamine beta hydroxylase (*tbh-1*). **(D)** Adenylyl cyclase (*acy-1*) gain of function or loss of phosphodiesterase (*pde-4*) decreased total quiescence. Loss of CREB (*crh-1*) function increased total quiescence. Loss of protein kinase A regulatory subunit (*kin-2*) function decreased total quiescence. **(E)** Loss of G_o (*goa-1*) decreased total quiescence. Loss of C-Jun N-terminal kinase (*jnk-1*) function increased total quiescence.

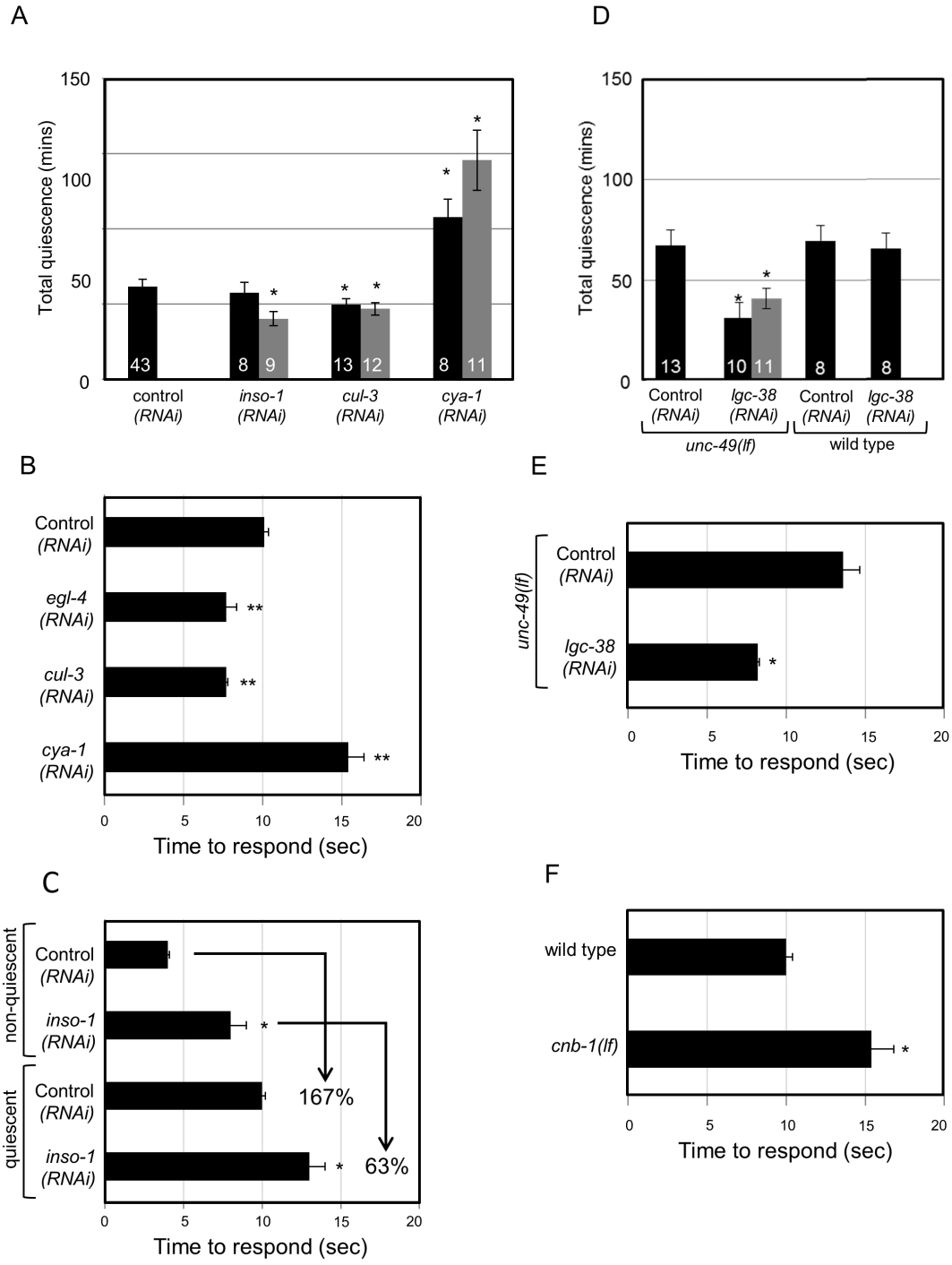

Figure 2—Lethargus quiescence and altered arousal during sleep requires the function of conserved genes. For RNA interference (RNAi) feeding experiments, HA2158 animals expressing the SID-1 double- stranded RNA channel in neurons were used.[77] Total quiescence during L4-to-adult lethargus reported in min. Arousal thresholds were determined by measuring the time to respond to 60% dilute 1-octanol. Response time is reported in sec. Error bars represent standard error of the mean (s.e.m.). Gray and black bars represent independent clones in A and D. Numbers inside the bar indicate sample size. For each gene examined, independent sets of control animals were tested in parallel and these were used to determine significance by Student *t* test. P < 0.05* < 0.01** and < 0.001*** versus control (RNAi), *unc-49(lf)*, or wild-type. Results were grouped and controls were pooled for concise presentation. **(A)** RNAi knockdown using two independent clones of insomniac (*inso-1*) and cullin-3 (*cul-3*) decreased total quiescence with one exception. See Methods for a possible off-target gene for *inso-1(RNAi)*. RNAi knockdown of cyclin A (*cya-1*) increased total quiescence. **(B)** RNAi knockdown of *egl-4* and *cul-3* in sensitized HA2158 animals lowered arousal thresholds during quiescence in comparison with their respective controls. RNAi knockdown of *cya-1* increased arousal thresholds. **(C)** Nonquiescent *inso-1(RNAi)* animals were mildly 1-octanol sensing defective. Quiescent wild-type animals' response time to dilute 1-octanol increased by 167 ± 11%. However, *inso-1(RNAi)* animals increased their response time by 63 ± 13%, suggesting that these animals maintain inappropriately low arousal thresholds during quiescence. **(D)** RNAi knockdown of the *lgc-38* γ-aminobutyric acid (GABA) A receptor decreased quiescence in animals lacking the *unc-49* GABA A receptor. **(E)** RNAi knockdown of the *lgc-38* in *unc-49(lf)* animals also decreased arousal thresholds. **(F)** Nonquiescent *cnb-1(lf)* animals were defective in their response to mechanosensory stimuli, but responded normally to dilute 1-octanol. Therefore, dilute 1-octanol response was used to demonstrate heightened arousal thresholds during quiescence.

by *tax-6* and *cnb-1*, respectively[50,51]; the calcineurin regulator *sarah* is encoded by *rcn-1*.[52] We found that loss of *rcn-1*, *cnb-1*, or *tax-6* function decreased *C. elegans* quiescence. Combined, these results suggest that *Shaker* potassium channels and calcineurin play conserved roles in sleep across species (Figure 1A).

Neurotransmitter systems (*dop-1*, *dat-1*, *ser-4*, *unc-25, unc-49, lgc-38, tbh-1, tdc-1*)

In *Drosophila*, loss of dopamine signaling increases rest; therefore dopamine is said to have a wake-promoting effect.[53,54] For example, increasing synaptic dopamine by disrupting the dopamine transporter *fumin* significantly decreases *Drosophila* rest.[12] Conversely, loss of the D1 dopamine receptor *DopR* increases *Drosophila* rest.[54] The *C. elegans* orthologs of *fumin* and *DopR* genes are *dat-1* and *dop-1*, respectively.[55,56] Consistent with the *Drosophila* results, *dat-1* loss of function decreased total quiescence, and as expected, the *dop-1* loss of function increased total quiescence (Figure 1B).

In *Drosophila*, loss of serotonin signaling decreases rest; serotonin is said to have a sleep-promoting effect.[57] Loss of the *Drosophila* serotonin receptor 5-HT1A decreases rest.[57] In *C. elegans*, *ser-4* [58] encodes the protein most similar to *Drosophila 5-HT1A*. Consistent with *Drosophila* results, *ser-4* loss of function decreased total quiescence in *C. elegans* (Figure 1B).

γ-Aminobutyric acid (GABA) signaling also affects *Drosophila* rest. Overexpression of the only *Drosophila* $GABA_A$ receptor, *Resistant to dieldrin* (*Rdl*), in LNvs (large ventral lateral neurons) results in increased rest.[24] In addition, inhibiting GABAergic neurons via ectopic *Shaw* channel expression decreases *Drosophila* rest.[59] To test the effect of GABA signaling on *C. elegans* quiescence, the role of GABA receptor function in quiescence was determined. Similarity searching suggested that in *C. elegans* both *unc-49* and *lgc-38* are orthologs of *Drosophila Rdl*.[60] Neither *unc-49(lf)* nor *lgc-38(RNAi)* alone affected quiescence (total quiescence = 60 ± 3 min, P = 0.66 or total quiescence = 66 ± 5 min, P = 0.26, respectively and Figure 2D), possibly because of redundant function of the *unc-49* and *lgc-38*. Because no alleles were available for *lgc-38*, the gene was knocked down using two nonoverlapping RNAi clones in *unc-49(lf)* animals and quiescence determined. *unc-49(lf)* animals reared on *lgc-38(RNAi)* feeding clones had significantly decreased total quiescence compared to those reared on empty vector control (Figure 2C). Complete loss of GABA biosynthesis does not cause lethality in *C. elegans*. Therefore, we tested animals completely lacking the function of *unc-25,* which encodes glutamic acid decarboxylase.[61] These animals have decreased total quiescence, which is consistent with the effect of GABA loss on *Drosophila* rest (Figure 1C).

Octopamine, a biogenic amine, also regulates *Drosophila* sleep.[62] Loss of octopamine biosynthesis pathway genes, tyrosine decarboxylase (*Tdc*) and tyramine beta hydroxylase (*TβH*), increases rest.[62] *tdc-1* and *tbh-1* are the *C. elegans* orthologs of *Drosophila Tdc* and *TβH* genes.[63] Consistent with the *Drosophila* sleep defects observed in *Tdc* and *TβH* flies, *tdc-1(lf)* and *tbh-1(lf) C. elegans* had increased total quiescence (Figure 1C).

cAMP signaling (*acy-1*, *pde-4*, *kin-2*, *crh-1*)

The duration of *Drosophila* sleep is inversely proportional to cAMP signaling, PKA activity, and CREB activity.[64] The *Drosophila rutabaga* and *dunce* genes encode adenylyl cyclase and phosphodiesterase, respectively, and they antagonistically regulate cAMP levels. Loss of *rutabaga* increases rest, whereas loss of *dunce* decreases rest.[64] *C. elegans* cAMP levels are regulated by two orthologous genes: *acy-1* and *pde-4*.[65] Complete loss of *acy-1* causes lethality. Therefore, a previously described *acy-1* gain of function allele was tested.[66] Either *acy-1* gain of function or *pde-4* loss of function likely increases cAMP levels.[67] We found that *acy-1(gf)* or *pde-4(lf)* animals exhibited decreased total quiescence, consistent with results in *Drosophila* (Figure 1D).

Two conserved downstream components of cAMP signaling pathway are PKA and cAMP response element binding protein (CREB).[68] In *Drosophila*, overexpression of PKA panneuronally or with a heat shock promoter decreases rest.[64,69] Conversely, blocking *Drosophila* CREB activity increases rest.[64] *C. elegans kin-2* encodes the regulatory subunit of PKA and *kin-2* loss of function increases PKA catalytic activity.[66] As predicted from the *Drosophila* study,[64] *kin-2* loss of function decreased *C. elegans* total quiescence (Figure 1D). *crh-1* encodes the *C. elegans* CREB ortholog.[70] *crh-1(lf)* animals had increased total quiescence, which is consistent with CREB function in *Drosophila* rest (Figure 1D). Combined, these results suggest that the cAMP signaling cascade plays a conserved role in *Drosophila* and *C. elegans* sleep.

Other signaling (*goa-1*, *jnk-1*)

The *Drosophila* G_o protein is encoded by the *G_oα47a* gene and has been implicated in rest. Pan-neuronal expression of G_o increases rest, whereas RNAi knockdown of G_o decreases rest.[71] In *C. elegans*, G_o is encoded by *goa-1*.[72] Complete loss of *goa-1* function decreased *C. elegans* quiescence supporting a conserved role for G_o protein in invertebrate sleep (Figure 1E).

The *Drosophila* gene *basket* encodes a C-Jun N-terminal kinase that is required for rest.[73] RNAi knockdown of *basket* decreases rest.[73] In *C. elegans*, the closest ortholog of *Drosophila basket* is encoded by *jnk-1*.[74] *C. elegans jnk-1* loss of function caused increased total quiescence (Figure 1E). This gene is one of only two *C. elegans* orthologs that had discordant results between species, which is discussed in the next paragraphs.

BTB, cullin, and other signaling (*inso-1, cul-3, cya-1*)

RNAi knockdown of either *Drosophila insomniac* or *Cullin-3* decreases rest. Consistent with this result, *insomniac* loss of function alleles also decreases rest.[75] In *C. elegans*, *cul-3* is the predicted ortholog of *Cullin-3*.[76] The *C. elegans* gene most similar to *insomniac* is C52B11.2, which is named *inso-1* here. These *C. elegans* genes have not been previously characterized. A *cul-3* mutant allele is not available. A previously uncharacterized *inso-1(gk344)* deletion allele exists, which removes the predicted first exon of *inso-1,* but this mutation did not alter quiescence (total quiescence = 50 ± 3 min, P = 0.09). However, examination of *inso-1* cDNA clones suggested that translation in *inso-1(gk344)* animals might initiate at methionine (22) in exon 2 and might not dramatically alter gene function. Therefore, we used RNAi knockdown to test the effect of these two genes on *C. elegans* quiescence. Expression of double- stranded RNA channels in neurons increases *C. elegans* sensitivity to RNAi by feeding.[77] We confirmed that *egl-4(RNAi)* in this

sensitized background resulted in decreased total quiescence (total quiescence = 42 ± 6 min, P = 0.047), which is consistent with previous work using *egl-4(lf)* mutant animals.[6] The impact of *cul-3*, the *C. elegans* ortholog of *Cullin-3*, on quiescence was tested using two independent RNAi clones. Knockdown of *cul-3* by RNAi decreased total quiescence (Figure 2A). Two independent *inso-1* RNAi clones were tested. One decreased quiescence compared with that of the control RNAi animals (Figure 2A).[75] Although decreased quiescence is consistent with the effect of the Drosophila *insomniac* on rest, potential off-target effects of *inso-1* RNAi cannot be ruled out. See Methods.

Neuronal depletion of *Drosophila CycA*, which encodes cyclin A, decreases rest.[45] The *C. elegans* ortholog of cyclin A is *cya-1*.[78] Because no deletion alleles were available for *cya-1*, RNAi knockdown in the sensitized background was used to test the effect on quiescence. RNAi knockdown of *cya-1* using two independent clones increased *C. elegans* total quiescence compared with that in animals raised on control bacteria containing the empty vector (Figure 2A). C-Jun N-terminal kinase and cyclin A are the only two genes for which the change in sleep was inconsistent between the two species.

In summary, all 20 *C. elegans* genes tested here affected quiescence. For 18 genes, the effect on quiescence was consistent with predictions based on the *Drosophila* literature. Taken together, these results suggest deep conservation of genes required for invertebrate sleep.

Conserved genes also regulate arousal during *C. elegans* quiescence

Altered arousal is an essential component of sleep. *C. elegans* and *Drosophila* also exhibit increased arousal thresholds during sleep as their responsiveness to sensory stimuli is reduced.[4,5,77] In *Drosophila*, disruption of *fumin*, $G_o\alpha47A$, or *basket* gene function perturbs arousal thresholds during rest.[12,71] To independently assess the effect of these genes on arousal during *C. elegans* quiescence, we examined response using chemosensory and/or mechanosensory stimuli.[19,35] Failure to respond to touch with a hair during quiescence is presented for all *C. elegans* alleles and is reported as percent failed to respond to touch (Figure 3). Response to 1-octanol during quiescence is reported for RNAi studies (Figures 2B, 2C, and 2E) as the requisite genetic background and/or bacterial strain causes hypersensitivity to mechanosensory stimuli. Response to 1-octanol during quiescence is reported for *cnb-1(lf)* animals (Figure 2F) because these animals exhibited defective response to mechanosensory stimuli. In RNAi studies, nonquiescent animals responded to dilute 1-octanol as quickly as control animals with one exception. Nonquiescent *inso-1(RNAi)* animals were defective in their response to dilute 1-octanol (8 ± 1 sec) compared with nonquiescent wild type animals (4 ± 0.1 sec), complicating analysis. Quiescent wild type animals increased their response time by 167 ± 11% but *inso-1(RNAi)* animals only increased their response time by 63 ± 13%, suggesting that *inso-1* loss decreases arousal thresholds during quiescence (Figure 2C).

All 20 genes that affected *C. elegans* quiescence also altered arousal thresholds (Figures 2B, 2C, 2E, 2F, 3, and Tables S3 and S6). We found decreased arousal thresholds in *dat-1* and *goa-1* loss of function animals, which is consistent with the decreased arousal thresholds of *Drosophila fumin* and $G_o\alpha47A$ animals.[12,71] The only other comparison possible across species was for C-Jun N-terminal kinase orthologs. *C. elegans jnk-1* loss of function decreased arousal thresholds, whereas *Drosophila basket* RNAi is reported to have no effect on arousal during rest.[73] For the 17 other orthologous genes, additional studies in *Drosophila* will be required to test if their function in arousal across species is conserved.

Assessing correlations between arousal and quiescence

Arousal thresholds likely reflect depth and/or quality of sleep. We considered the possibility that arousal might correlate with other metrics of *C. elegans* sleep, which include total time spent in quiescence, lethargus duration, and/or quiescence bout characteristics (number and duration). Total quiescence, lethargus duration, and bout number are intrinsically correlated based on the methods used to detect and calculate *C. elegans* quiescence. Therefore, we only considered possible correlations of arousal with bout duration and bout number. The effect of *C. elegans* genes previously tested on these parameters is reported in Table S3. For *lgc-38(RNAi)*, *cul-3(RNAi)*, *inso-1(RNAi)*, *cya-1(RNAi)*, and *cnb-1(lf)* animals, arousal thresholds were determined by chemosensory response (see Figures 2B, 2C, 2E, and 2F), which complicated comparison to the other 15 genes, whose arousal thresholds were determined by mechanosensory response. Therefore, we excluded these five genes from the correlation analysis described in the next paragraphs.

We noted that genotypes with increased arousal thresholds usually had both increased total quiescence, and *vice versa* (Table S3). Total quiescence is a function of quiescent bout duration and bout number. One possibility is that arousal threshold changes might affect bout duration. We tested this by looking for correlation between these two parameters and found that bout duration did not directly correlate with arousal thresholds (Figure 4A). This is not surprising because there is remarkably little variation in bout duration across genotype. Another possibility was that arousal threshold changes might affect bout number, as altered arousal might influence entry into quiescence. We found that bout number directly correlated with arousal thresholds (Figure 4B). In summary, we conclude that mechanisms governing bout duration are likely independent from the mechanisms regulating arousal thresholds. Increased bout number reflects increased propensity to enter a quiescent state. Together, these results make it likely that common genetic pathways likely regulate arousal thresholds and quiescence bout entry.

Genetic pathway for dopamine regulation of *C. elegans* lethargus quiescence

Combined with the previously published *Drosophila* and murine studies, our cross-species comparison of genes required for sleep (Figure 1B) confirms that decreased dopamine signaling increases sleep[12,13,79] (Figure 1B). Past studies have determined the site of action of D1 receptor as well as the neuronal circuitry of dopamine signaling in *Drosophila* sleep.[80,81] However, little is known about the signaling cascade acting downstream of the D1 receptor in sleep. We undertook genetic and phenotypic rescue experiments to assemble a signaling pathway downstream of dopamine regulating quiescence in *C. elegans*.

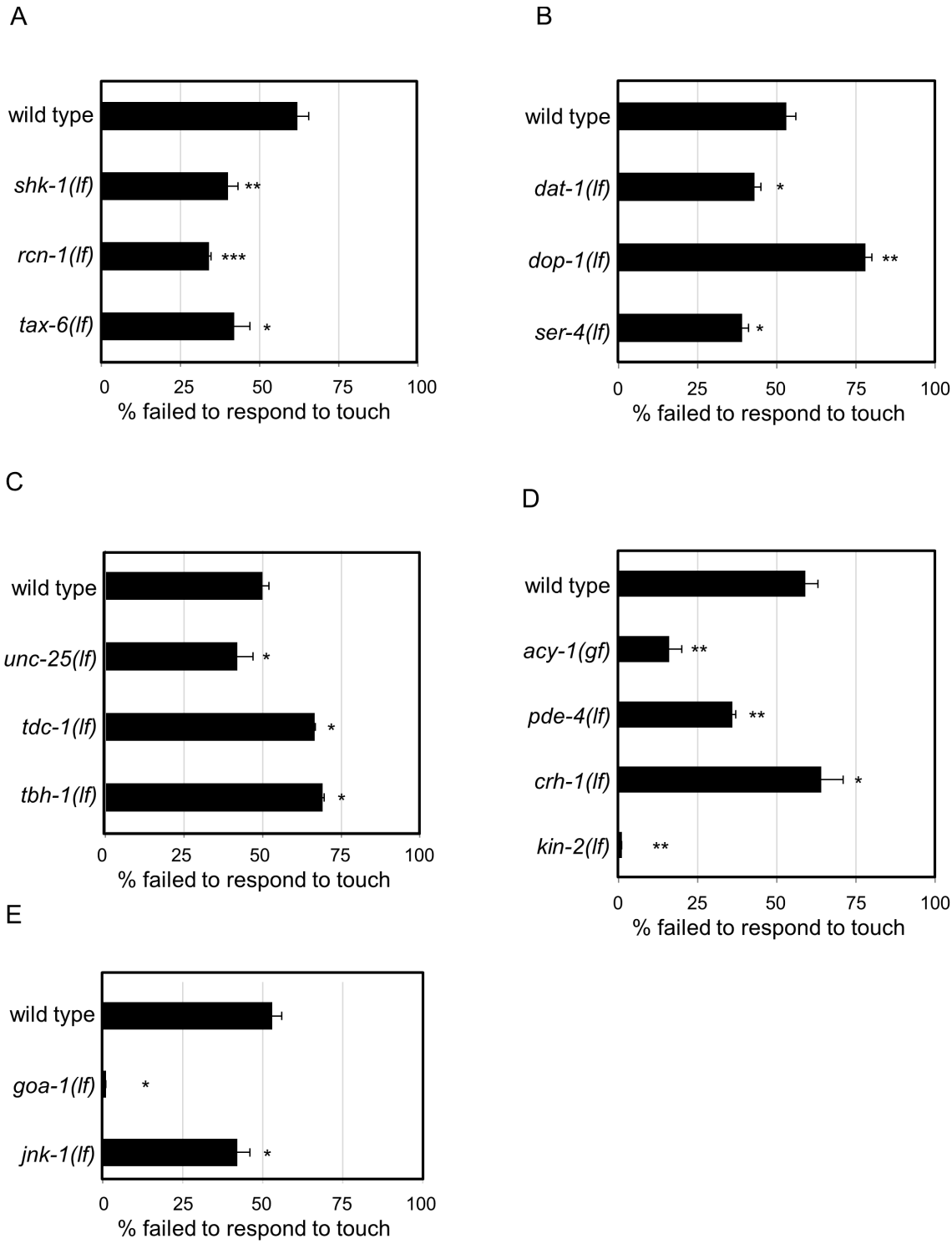

Figure 3—Conserved genes regulate arousal. Arousal thresholds were determined by counting the number of quiescent animals responding to body touch. Percent of animals failing to respond to body touch is reported in all the figure panels. Error bars represent standard error of the mean (s.e.m.). Non-quiescent animals rarely failed to respond to body touch with a failure rate of 0-10% for all genotypes. Because of their profound quiescence defects, very few quiescent *goa-1(lf)* (n = 2) and *kin-2(lf)* (n = 5) animals were found, even when more than 100 animals were sampled. For each gene examined, independent sets of wild-type control animals were tested in parallel and these were used to determine significance by Student *t* test. P < 0.05* < 0.01** and < 0.001*** versus wild-type. Results were grouped by pathway/function and controls were pooled for concise presentation. **(A)** Loss of either *C. elegans* calcineurin signaling genes (*rcn-1* and *tax-6*) or loss of *C. elegans* Shaker (*shk-1*) decreased arousal thresholds. **(B)** Loss of dopamine transporter (*dat-1*) or serotonin receptor (*ser-4*) function decreased arousal thresholds. Loss of D1 dopamine receptor (*dop-1*) function increased arousal thresholds. **(C)** Loss of γ-aminobutyric acid (GABA) synthesis gene (*unc-25*) decreased arousal thresholds. Loss of octopamine biosynthesis genes tyrosine decarboxylase (*tdc-1*) and dopamine beta hydroxylase (*tbh-1*) increased arousal thresholds. **(D)** Loss of phosphodiesterase (*pde-4*) or protein kinase A regulatory subunit (*kin-2*) function decreased arousal thresholds. Gain of adenylyl cyclase (*acy-1*) function decreased arousal thresholds. Loss of CREB increased arousal thresholds (*crh-1(lf)* = 63 ± 0.6 versus parallel wild type control = 51 ± 0.4, P = 0.005). **(E)** Loss of G_o (*goa-1*) or C-Jun N-terminal kinase (*jnk-1*) function decreased arousal thresholds.

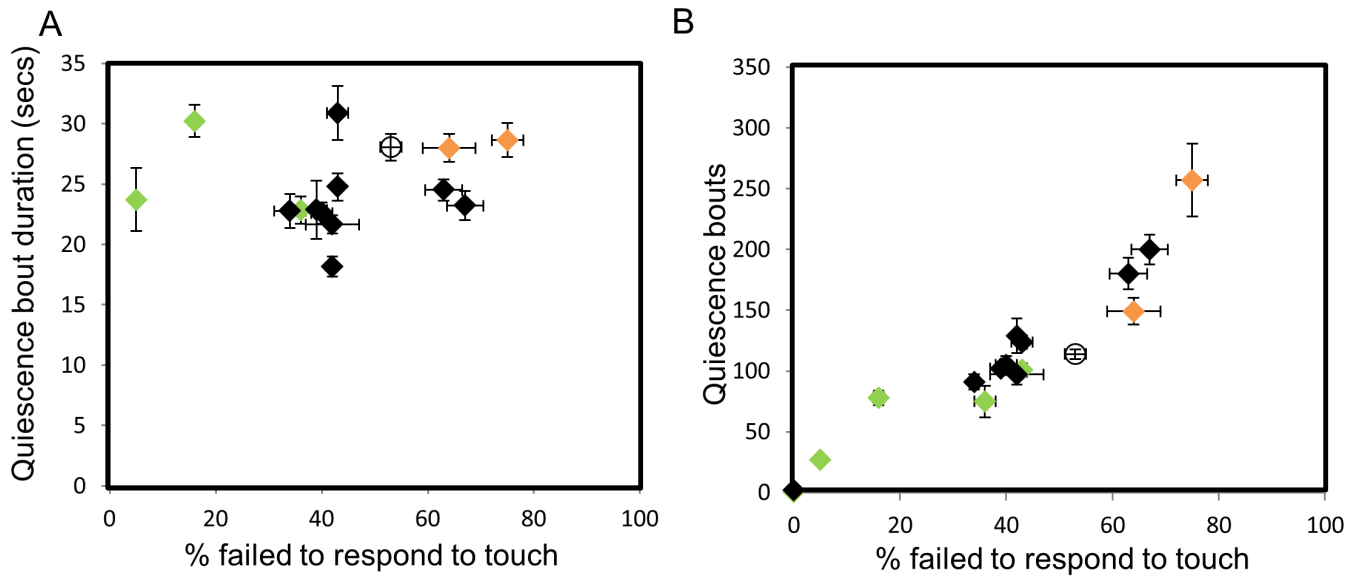

Figure 4—Assessing correlations between arousal and quiescence. Quiescence bout number and bout duration during lethargus were examined versus arousal thresholds for genotypes tested in Figures 1 and 3. Wild-type is represented as an empty circle. Genes implicated in the dopamine signaling pathway are represented as green and orange rhombi, which represent decreased and increased quiescence, respectively. **(A)** No correlation was observed between quiescent bout duration and arousal thresholds (r = -0.051, P = 0.580). **(B)** A direct correlation was detected between quiescent bout numbers and arousal thresholds. Correlation coefficient r = 0.887, P = 1e-08. See Methods for statistical details.

In humans, D1 dopamine receptors couple with various Gα proteins, including G_s and G_o.[82] Loss of the DOP-1 D1 receptor increased quiescence (Figure 1B, *dop-1(lf)*). The *C. elegans* G_o ortholog *goa-1* is unlikely to act downstream of the DOP-1 D1 receptor as loss of *goa-1* decreased total quiescence (Figure 1E, *goa-1(lf)*). Therefore, the role of *C. elegans* G_s ortholog GSA-1 was assessed. Loss of *gsa-1* function causes lethality; therefore, a previously characterized *gsa-1(gf)* gain of function allele was tested.[65] *gsa-1* gain of function decreased total quiescence (Figure 5A, *gsa-1(gf)*), which was consistent with increased signaling downstream of the DOP-1 D1 receptor. If *gsa-1* functions downstream of *dop-1*, then *gsa-1(gf)* should suppress the increased quiescence of *dop-1(lf)* animals. We found that *gsa-1(gf);dop-1(lf)* double mutant animals had decreased total quiescence compared to *dop-1(lf)* animals (Figure 5A). Combined, these results suggest that *gsa-1* functions downstream of dopamine and D1 dopamine receptors in *C. elegans* quiescence.

The *C. elegans* adenylyl cyclase *acy-1* is known to act downstream of *gsa-1* in synaptic signaling and locomotion.[66] Similar to the *gsa-1(gf)* effect on quiescence, *acy-1(gf)* decreased total quiescence (Figure 1D, *acy-1(gf)*). If *acy-1* functions downstream of *dop-1* and *gsa-1*, then *acy-1* gain of function should suppress the increased quiescence of *dop-1(lf)* animals. Indeed, *acy-1(gf); dop-1(lf)* double mutant animals have decreased quiescence compared to the *dop-1(lf)* animals (Figure 5A). Together, these results suggest that both *gsa-1* and *acy-1* function downstream of or in parallel to *dop-1* D1 receptor in quiescence.

Next, we determined if the components of the cAMP signaling pathway, adenylyl cyclase, PKA, and CREB, function in the DOP-1 D1 receptor expressing neurons. The effect of adenylyl cyclase activity in DOP-1 D1 receptor expressing neurons was assessed first. Overexpression of adenylyl cyclase in DOP-1 D1 receptor expressing neurons of wild-type animals was sufficient to decrease quiescence (Figure 5B). Additionally, overexpressing adenylyl cyclase in the D1 dopamine receptor expressing neurons of *dop-1(lf)* animals suppressed their quiescence defects (Figure 5B). Increased PKA activity in *kin-2(lf)* animals resulted in decreased total quiescence (Figure 1D, *kin-2(lf)*), which was concordant with the effect of *acy-1(gf)* on quiescence (Figure 1D, *acy-1(gf)*). To determine if increased PKA activity in DOP-1 D1 receptor expressing neurons was sufficient to regulate quiescence, we knocked down *kin-2* in these neurons. As predicted, RNAi knockdown of *kin-2* in DOP-1 D1 receptor expressing neurons partially recapitulated the quiescence defects of *kin-2(lf)* animals (Figure 5C). Additionally, RNAi knockdown of *kin-2* in DOP-1 D1 receptor expressing neurons of *dop-1(lf)* animals suppressed their quiescence defects (Figure 5C). These results suggest that manipulating adenylyl cyclase and PKA activity in these neurons was sufficient to regulate quiescence.

We found that global loss of CREB function in *crh-1(lf)* animals increased quiescence (Figure 1D, *crh-1(lf)*). We confirmed this by RNAi knockdown of *crh-1* (Table S3). However, it is not clear if CRH-1 CREB functions in *C. elegans* DOP-1 D1 neurons. Simultaneous overexpression of two *crh-1* splice isoforms in DOP-1 D1 expressing neurons did not rescue the quiescence defects of *dop-1(lf)* animals (total quiescence of *dop-1(lf);dop-1p::crh-1(cDNA)* = 82 ± 7 min, P = 0.88). This might be because of insufficient phosphorylation of CRH-1 in *dop-1(lf)* animals.[68] Expression of these two *crh-1* splice isoforms did not ameliorate the total quiescence defects of *crh-1(lf)* animals (total quiescence of *crh-1(lf);dop-1p::crh-1(cDNA)* = 85 ± 7 min, P = 0.79). As described in other systems, CRH-1 may function downstream of other receptors or

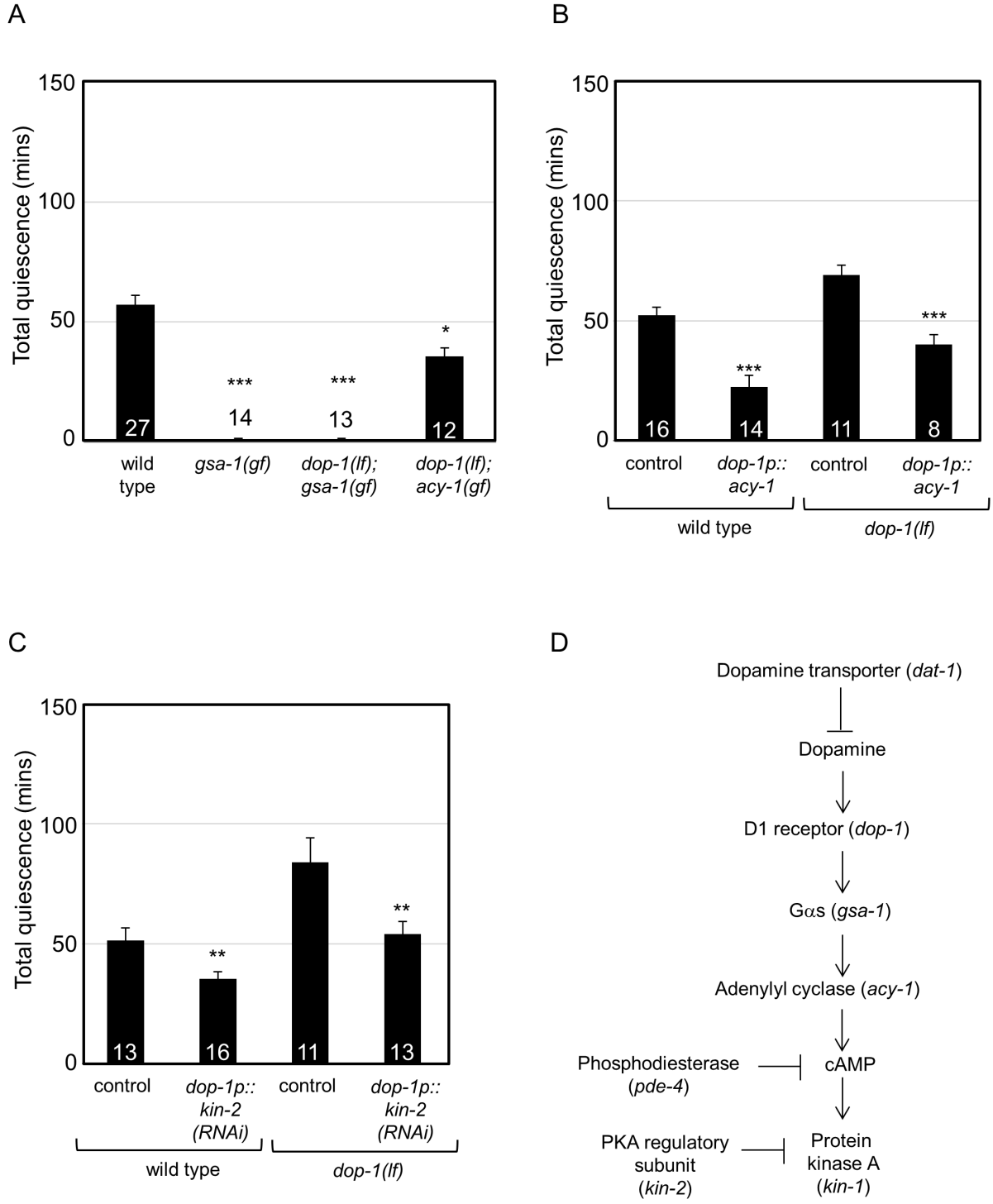

Figure 5—Genetic pathway for dopamine regulation of *C. elegans* lethargus quiescence. Genetic epistasis and rescue experiments delineate a dopamine signaling pathway functioning in the regulation of *C. elegans* quiescence. Total quiescence was reported in min. Error bars represent standard error of the mean (s.e.m.). Numbers inside or above the bar indicate sample size. Results were grouped and controls were pooled for concise presentation. Control is *dop-1* promoter driving gfp (*dop-1p::gfp*). **(A)** Gain of Gs (*gsa-1*) function or *dop-1;gsa-1* double mutant animals had decreased total quiescence. *dop-1;acy-1* double mutant animals had decreased total quiescence. **(B)** Expression of *acy-1* complementary DNA (cDNA) in DOP-1 D1 receptor expressing neurons decreased total quiescence in wild-type animals. Expression of *acy-1* cDNA in DOP-1 D1 receptor expressing neurons of *dop-1(lf)* animals suppressed the quiescence defect. **(C)** RNA interference (RNAi) knockdown of *kin-2* in DOP-1 D1 receptor expressing neurons decreased total quiescence in wild-type animals. In *dop-1(lf)* animals, RNAi knockdown of *kin-2* in DOP-1 D1 receptor expressing neurons suppressed the quiescence defect. *P < 0.05 **P < 0.01 ***P < 0.005 versus wild-type in A; versus transgenic control for each genotype in B and C. **(D)** A model for dopamine signaling mediated regulation of quiescence. Dopamine binding to D1 dopamine receptor DOP-1 activates downstream signaling *via* Gs (*gsa-1*). This results in the activation of adenylyl cyclase (*acy-1*) that produces cyclic adenosine monophosphate (cAMP). cAMP activates protein kinase A (PKA) to promote wakefulness. Consistently, knockdown of the PKA regulatory subunit (*kin-2*) in D1 receptor expressing neurons decreased quiescence. It is possible that phosphodiesterase 4 (*pde-4* in *C. elegans*) regulates cAMP levels in this pathway.

signaling cascade that may increase or decrease quiescence.[70,83] Combined, these results suggest that cAMP/adenylyl cyclase and PKA function downstream of or parallel to dopamine and the D1 dopamine receptor in *C. elegans* neurons to regulate quiescence.

DISCUSSION

Sleep has many shared characteristics and is ubiquitous across the animal kingdom, suggesting that the genes and pathways required for this behavior are conserved. However, experimental evidence to support this hypothesis is limited. Previous work has independently identified roles in sleep for PKG, neuropeptides, EGF, and Notch signaling in both flies and nematodes.[6,14,15,17-19,22,23] To test molecular conservation of sleep more broadly, we took advantage of two well-defined invertebrate model systems.[4,5] A *Drosophil*a literature survey allowed us to shortlist 26 genes required for rest. *C. elegans* orthologs of 20 of these genes were systematically tested for roles in quiescence and arousal. Disruption of nearly all of the orthologous *C. elegans* genes increased or decreased quiescence as would be predicted from *Drosophila* studies. We found few exceptions to this, which are discussed in the next paragraphs. Although only one allele was tested for most genes in the cross-species comparison, the results presented here demonstrate that conserved genes play critical roles in sleep in two well-defined invertebrate species.

Diminished response to sensory stimuli is an essential feature of sleep. Twenty genes altered *C. elegans* total quiescence, allowing a comprehensive analysis of their effect on arousal. In every genotype, arousal thresholds during quiescence were significantly different from wild-type *C. elegans*. Because few *Drosophila* studies report changes in arousal during rest,[12,47,73,84] we can only speculate regarding cross-species roles for these genes in arousal. For genes encoding the dopamine transporter or G_o, the effect on arousal thresholds was clearly conserved between these two species. It seems likely that many of the other genes described here will play a conserved role in arousal in *Drosophila* and other animals.

Only two of the genes tested here, *jnk-1* and *cya-1*, had a discordant effect on *C. elegans* and *Drosophila* sleep. RNAi knockdown of *Drosophila basket*, which encodes C-Jun N-terminal kinase, results in decreased rest with no change in arousal thresholds.[73] This is not concordant with either RNAi knockdown or loss of function of the *C. elegans basket* ortholog *jnk-1*; both manipulations increased total *C. elegans* quiescence and decreased arousal thresholds[73] (and this study). Also, RNAi knockdown of *Drosophila cycA*, which encodes cyclin A, decreases rest, but knockdown of *C. elegans cya-1* increased quiescence[45] (and this study). It is unclear why these two genes had discordant effects on sleep in flies and nematodes. Possibly, their downstream targets differ in these species or these genes regulate different aspects of sleep in *C. elegans* and *Drosophila*. Previous work established that PKG and EGF play concordant roles in invertebrate sleep,[6,17,18] but the Notch pathway affects different aspects of *C. elegans* and *Drosophila* sleep.[15,19] We conclude that most genes play conserved roles in sleep across species, but a subset of genes play species-specific roles.

Given the large number of genes characterized here, we were able to examine for the first time possible correlations among quiescence metrics, such as bout number, bout duration, and arousal thresholds. Examination of *C. elegans* quiescence suggested that genotypes with increased total quiescence also had an increased number of quiescent bouts and had an increased lethargus duration, and *vice versa* (Table S3). There was little change in bout duration or frequency despite the large number of genotypes tested. In other words, increased quiescence generally occurs when mutant animals have more quiescent bouts and spend more time in lethargus. Therefore, common genetic mechanisms likely affect arousal thresholds and bout entry. However, bout duration may be independently regulated from arousal thresholds and may require other genes that will be identified in future studies.

We did discover a simple and relatively direct correlation between arousal thresholds and total time spent in quiescence for 15 genes. In other words, most of the genetic perturbations that decreased total quiescence also resulted in lowered arousal thresholds. Also, genetic perturbations that increased quiescence were almost always associated with higher arousal thresholds. Based on these observations, we considered two possibilities. Low arousal thresholds might result in an inability either to establish or to maintain quiescence during a bout. Counterintuitively, genotypes with aberrantly high arousal thresholds did not have increased bout duration, but they did have increased number of quiescent bouts late in lethargus and had increased lethargus duration. High arousal thresholds may indicate an inappropriate propensity to establish the quiescent state, resulting in more quiescent bouts. Low arousal thresholds may reflect an inability to enter the quiescent state. Alternatively, genes altering arousal thresholds may regulate lethargus duration. However, it is unclear if quiescence changes are the cause or the result of altered arousal thresholds. Also, it is unclear if quiescence and arousal are independently regulated by these 15 genes. This may be clarified in future studies that directly examine molecular and cellular mechanisms underlying arousal and sleep.

Dopamine plays a conserved role in sleep in flies and mice.[12,13,53] Here, we extend this role to *C elegans*. However, the signaling pathways downstream of dopamine and dopamine receptors relevant to sleep were largely unknown. To establish a coherent model of dopamine signaling in sleep, we assembled a genetic pathway based on phenotype and confirmed action in a subset of *C. elegans* neurons using a combination of genetic epistasis and rescue experiments. Double mutant analysis suggested that G_s and adenylyl cyclase function in the dopamine signaling pathway, downstream of the DOP-1 D1 receptors. Additionally, activation of PKA or overexpression of adenylyl cyclase, only in neurons expressing DOP-1 D1 receptors, was sufficient to rescue the *dop-1* quiescence defects (Figure 5), suggesting that adenylyl cyclase and PKA act in parallel or downstream of D1 receptors. DOP-1 D1 receptors are widely expressed in the *C. elegans* nervous system[85]; further studies will be needed to determine precisely which *dop-1* expressing neurons are critical for sleep. It is likely that dopamine signaling *via* G_s, adenylyl cyclase, PKA, and perhaps CREB are required for sleep in all animals.

Results presented here confirm that the genetic underpinnings of sleep are broadly conserved in invertebrates. Some vertebrate orthologs of genes required for invertebrate sleep

have been implicated previously in sleep.[1,4] However, the connections between these genes and signaling pathways in vertebrate sleep remain mysterious. Invertebrate model systems can be used to rapidly identify and delineate these conserved pathways.

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DISCLOSURE STATEMENT

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SUPPLEMENTAL MATERIAL

Table S1—Strains and alleles used in this study

Strain name	Genotype	Molecular lesion/backcross status
KG518	*acy-1(ce2)III*[1]	P260S
MT9974	*crh-1(n3315)III*[2]	615-base pair deletion in *crh-1* genomic sequence.
KJ300	*cnb-1(jh103)V*[3]	950-base pair deletion in *cnb-1* genomic sequence.
RM2702	*dat-1(ok157)III*[4]	Deletion size: 1836 bp. Flanking sequence: CTATTCGGATATCTTGCCAATGCTA// TAGGAATTATTTTTGCGCTCTCAGG
LX645	*dop-1(vs100)X*[5]	328 bp deletion which completely removes exons 8 and 9
MT363	*goa-1(n363)I*[6]	Deletion starting approximately 5 kb 5' of exon 1 and extending more than 5 kb 3' of the last exon of *goa-1*
KG421	*gsa-1(ce81)I*[1]	R182C
VC8	*jnk-1(gk7)IV*[a,7]	Eliminates sequences encoding amino acids 53–243 of JNK-1α and 1–182 of JNK-1β
KG532	*kin-2(ce179)X*[1]	R92C
N2	N2 (Brenner)[8]	
RB1231	*pde-4(ok1290)II*[a]	Removes 1,554 bp from the gene that includes 95 bp of coding sequence and disrupts the reading frame
AQ866	*ser-4(ok512)III*[9]	A deletion of 1,336 bp of the *ser-4* genomic sequence
RB1392	*shk-1(ok1581)II*[a]	632 bp deletion, removing exons 7, 8, and 9, and part of exon 6.
PR675	*tax-6(p675)IV*[10]	D259N
MT9455	*tbh-1(n3247)X*[11]	791 bp, deleting parts of exons 6 and 7, and causes a frameshift that leads to a premature truncation
MT10661	*tdc-1(n3420)II*[11]	803 bp deletion that removes part of exons 3 and 5 and all of exon 4
CB0156	*unc-25(e156)III*[12]	W383amber
CB0382	*unc-49(e382)III*[13]	G189E
HA2518	*uIs72[myo-2p::RFP unc-119p::sid-1 unc-119p::mec-18]; sid-1(+)* generated from TU3595 by backcross into N2 three times eliminating *lin-15(n765)*	
HA2537	*acy-1(ce2)III;dop-1(vs100)X*	
HA2538	*gsa-1(ce81)III;dop-1(vs100)X*	
HA2479	N2; *rtEx746[dop-1::gfp, rab-3p::mCherry, pBluescript KS(+)]*	
HA2480	N2; *rtEx747[dop-1::kin-2(RNAi), rab-3p::mCherry,pBluescript KS(+)]*	
HA2481	N2; *rtEx748[dop-1::kin-2(RNAi), rab-3p::mCherry, pBluescript KS(+)]*	
HA2482	*dop-1(vs100)X; rtEx749[dop-1::kin-2(RNAi), myo-2p::gfp, pBluescript KS(+)]*	
HA2483	*dop-1(vs100)X; rtEx750[dop-1::kin-2(RNAi), myo-2p::gfp, pBluescript KS(+)]*	
HA2484	*dop-1(vs100)X; rtEx751[dop-1::gfp, myo-2p::gfp, pBluescript KS(+)]*	
HA2539	N2; *rtEx752[dop-1::acy-1(cDNA), myo-2p::gfp, pBluescript KS(+)]*	
HA2540	N2; *rtEx753[dop-1::gfp, myo-2p::gfp, pBluescript KS(+)]*	
HA2541	*dop-1(vs100)X; rtEx754[dop-1::acy-1(cDNA), myo-2p::gfp, pBluescript KS(+)]*	
HA2542	*dop-1(vs100)X; rtEx755[dop-1::gfp, myo-2p::gfp, pBluescript KS(+)]*	

Previously generated strains were not backcrossed to the Hart lab N2 strain for this analysis. [a] http://www.mutantfactory.ouhsc.edu/. Superscript numbers refer to references following the tables.

Table S2—Primers used in this study

Primer name	Sequence	Purpose
Dop1F1	gaaccccgggggtacttgtgtattgtgtccc	To amplify *dop-1* promoter
Dop1R1	gaaccccgggcgaatttctggaaaaaaaacattgtgagg	To amplify *dop-1* promoter. The Dop1F1 and Dop1R1 primers correspond to the genomic region 4420396-4416071 on chromosome X.[a]
Kin2F1	gaacgctagcatggggcagcaattgagcaaccg	To amplify *kin-2* coding sequence. This primer corresponds to +1 to +23 nucleotides of *kin-2b* cDNA sequence.[b]
Kin2R1	gaacggtaccttaggtcatcagtttgacgtatgagttgtagtttg	To amplify *kin-2* coding sequence. This primer corresponds to +971 to +1005 nucleotides of *kin-2b* cDNA sequence.
Crh1F1a	gaacgctagcatgtcagcgaaaggtaacggatcagc	To amplify *crh-1* cDNAs. This primer corresponds to +1 to +26 nucleotides of *crh-1a* cDNA sequence.
Crh1F1c	gaacgctagcatggccacaatggcgagcacctc	To amplify *crh-1* cDNAs. This primer corresponds to +1 to +23 nucleotides of *crh-1c* cDNA sequence.
Crh1R1	gaacccatggtcacattccgtccttttccttttcggcag	To amplify *crh-1* cDNAs. This primer corresponds to +918 to +945 nucleotides of *crh-1a* coding sequence. This region is also the present in the *crh-1c* transcript.
Lgc38_1F1	gaacgctagcagctacggctactcaaccaagg	To amplify *lgc-38* genomic sequence. This primer corresponds to +6854 to +6875 nucleotides of *lgc-38* genomic region that is a part of the exon 7 sequence.
Lgc38_1R1	gaacgagctcgaaccggtgatgcagcaggtac	To amplify *lgc-38* genomic sequence. This primer corresponds to +7594 to +7615 nucleotides of *lgc-38* genomic region that is a part of the exon 10 sequence.
Lgc38_2F1	gaaccccgggatgacgtggctattatggtcactac	To amplify part of the *lgc-38* cDNA sequence. This primer corresponds to +1 to +25 nucleotides of *lgc-38* cDNA sequence.
Lgc38_2R1	gaaccccgggcctcaagtgcacattcttgacgatc	To amplify part of the *lgc-38* cDNA sequence. This primer corresponds to +556 to +580 nucleotides of *lgc-38* cDNA sequence.
Inso1F1	gaacactagtccaaacgacaagatcaacgctg	To amplify *inso-1* genomic sequence. This primer corresponds to +2917 to +2938 nucleotides of *inso-1* genomic region that is a part of the exon 3 sequence.
Inso1R1	gaacactagtccaacgtctagctctttgctgg	To amplify *inso-1* genomic sequence. This primer corresponds to +3524 to +3545 nucleotides of *inso-1* genomic region that is a part of the exon 5 sequence.
Inso1_1F1	gaacactagtcttcaccacgacaatcttctgacac	To amplify *inso-1* N-terminal cDNA sequence. This primer corresponds to +35 to +59 nucleotides of *inso-1* cDNA sequence.
Inso1_1R1	gaacactagtgaaagtcccgggttcatgatcag	To amplify *inso-1* N-terminal cDNA sequence. This primer corresponds to +447 to +469 nucleotides of *inso-1* cDNA sequence.
Inso1_2F1	gaacactagtggaagaaggaattcttgcgga	To amplify *inso-1* C-terminal cDNA sequence. This primer corresponds to +470 to +490 nucleotides of *inso-1* cDNA sequence.
Inso1_2R1	gaacactagtccaacgtctagctctttgctgg	To amplify *inso-1* C-terminal cDNA sequence. This primer corresponds to +794 to +815 nucleotides of *inso-1* cDNA sequence.
Cul3F1	gaactctagagccacaatagacgagcaatatg	To amplify *cul-3* genomic sequence. This primer corresponds to +827 to +848 nucleotides of *cul-3* genomic region that is a part of the exon 2 sequence.
Cul3R1	gaactctagatcgctaaccgccttaagcagcg	To amplify *cul-3* genomic sequence. This primer corresponds to +1722 to +1743 nucleotides of *cul-3* genomic region that is a part of the exon 2 sequence.
Cul3_2F1	gaaccccgggctcaacgaaaccggctcgaacattgtg	To amplify part of the *cul-3* cDNA sequence. This primer corresponds to +979 to +1005 nucleotides of *cul-3* cDNA sequence.
Cul3_2R1	gaaccccgggcacgaatctcgggctccgattccac	To amplify part of the *cul-3* cDNA sequence. This primer corresponds to +2071 to +2095 nucleotides of *cul-3* cDNA sequence.
Cya1F1	gaacgctagccatcctgtgaagatccctcagcac	To amplify *cya-1* genomic sequence. This primer corresponds to +1755 to +1778 nucleotides of *cya-1* genomic region that is a part of the exon 6 sequence.
Cya1R1	gaaccccgggcgcggcgaaaagtcaagcagtg	To amplify *cya-1* genomic sequence. This primer corresponds to +304 to +326 nucleotides of *cya-1* genomic region that is a part of the exon 2 sequence.
Cya1_1F1	gaacgctagcgtcaaagcattcgtgtgaagc	To amplify *cya-1* N-terminal cDNA sequence. This primer corresponds to +685 to +705 nucleotides of *cya-1* cDNA sequence.
Cya1_1R1	gaaccccgggcgcggcgaaaagtcaagcagtg	To amplify *cya-1* N-terminal cDNA sequence. This primer corresponds to +39 to +60 nucleotides of *cya-1* cDNA sequence.
Cya1_2F1	gaacgctagccatcctgtgaagatccctcagcac	To amplify *cya-1* C-terminal cDNA sequence. This primer corresponds to +1264 to +1287 nucleotides of *cya-1* cDNA sequence.
Cya1_2R1	gaaccccgggatacaatcacaagttaatgaagagatgcg	To amplify *cya-1* C-terminal cDNA sequence. This primer corresponds to +706 to +734 nucleotides of *cya-1* cDNA sequence.
Egl4F1	gaacgcatgcggtgacgcattcttcgtaatcaactc	To amplify *egl-4* genomic sequence. This primer corresponds to +27582 to +27607 nucleotides of *egl-4* genomic region that is a part of the exon 8 sequence.
Egl4R1	gaacgcatgccttcggcagaattggaggtttcaac	To amplify *egl-4* genomic sequence. This primer corresponds to +29727 to +29751 nucleotides of *egl-4* genomic region that is a part of the exon 9 sequence.

[a] Genomic position based on WS240. [b] For all coding and genomic sequences, +1 refers to the nucleotide A of the start codon ATG. Nucleotide positions were determined by counting up from the +1 reference point.

Table S3—Compilation of behavioral analysis of sleep and arousal for all genotypes

Genes	Total quiescence (min)	Lethargus duration (h)	Q. bout number	Arousal thresholds (sec or %)	Q. bout frequency (/h)	Q. bout duration (sec)
Wild-type, N2	58 ± 1	2.5 ± 0.03	114 ± 1	53 ± 2	47 ± 0.3	28 ± 0.3
acy-1(gf)	40 ± 3*	2.2 ± 1	78 ± 6*	16 ± 4*	36 ± 1*	30 ± 1
crh-1(lf)	69 ± 4*	3.2 ± 0.2*	149 ± 12*	64 ± 7*	47 ± 0.4	28 ± 1
dat-1(lf)	43 ± 4*	2.4 ± 0.1	101 ± 6	43 ± 2*	43 ± 0.3*	25 ± 1
dop-1(lf)	126 ± 16*	5.8*	257 ± 30*	78 ± 3*	44 ± 2	29 ± 1
pde-4(lf)	29 ± 4*	2.1 ± 0.1	75 ± 13*	36 ± 1*	35 ± 2	23 ± 1
kin-2(lf)	12 ± 3*	0.9 ± 0.3*	27 ± 9*	0*	28 ± 1*	21 ± 4*
gsa-1(gf)	0*	0*	0*	0*	c.b.d.	c.b.d.
rcn-1(lf)	34 ± 2*	2 ± 0.1*	91 ± 6*	34 ± 3*	46 ± 2	23 ± 1*
cnb-1(lf)	36 ± 4*	2 ± 0.2	113 ± 9	15 ± 1*sec	53 ± 2	19 ± 1*
tax-6(lf)	40 ± 5*	2.8 ± 0.3	129 ± 14	42 ± 5*	45 ± 2	18 ± 1*
ser-4(lf)	45 ± 3*	2.4 ± 0.1	102 ± 4*	39 ± 3*	43 ± 1	27 ± 2
shk-1(lf)	39 ± 3*	2.3 ± 0.1	105 ± 7	40 ± 4*	46 ± 2	23 ± 1*
unc-25(lf)	34.6 ± 3*	2.2 ± 1.1*	97 ± 9*	42 ± 5*	44 ± 1*	22 ± 0.7*
tbh-1(lf)	74 ± 6*	3.5 ± 0.3*	180 ± 13*	69 ± 1*	52 ± 1*	25 ± 1*
tdc-1(lf)	77 ± 7*	3.4 ± 0.3*	200 ± 12*	67 ± 3*	59 ± 2*	23 ± 0.4*
goa-1(lf)	0*	0*	0*	0*	c.b.d.	c.b.d.
jnk-1(lf)	63 ± 5*	2.8 ± 0.1	124 ± 5	42 ± 5	44 ± 2	31 ± 2
unc-49(lf); control*(RNAi)*	66 ± 7	3.6 ± 0.4	159 ± 15	14 ± 0.1 sec	44 ± 1	26 ± 2
unc-49(lf); lgc-38(RNAi)	42 ± 5#	2.9 ± 0.3#	116 ± 11#	8 ± 0.1# sec	39 ± 2	22 ± 1#
N2; control*(RNAi)*	69 ± 7	4 ± 0.4	180 ± 17	d.n.d.	45 ± 1	24 ± 2
N2; *lgc-38(RNAi)*	65 ± 5	3.7 ± 0.3	167 ± 12	d.n.d.	46 ± 2	24 ± 1
HA2518; control*(RNAi)*	52 ± 3	2.6 ± 0.1	120 ± 6	10 ± 0.3 sec	47 ± 1	26 ± 1
HA2518; *inso-1(RNAi)*	33 ± 3^	1.9 ± 0.1^	81 ± 6^	13 ± 1^ sec	42 ± 2^	24 ± 1^
HA2518; *cul-3(RNAi)*	49 ± 2^	2.6 ± 0.2^	124 ± 8	8 ± 0.1^	48 ± 1	26 ± 1
HA2518; *cya-1(RNAi)*	93 ± 7^	4 ± 0.3^	165 ± 11^	15 ± 1^	42 ± 1^	34 ± 6^
HA2518; *crh-1(RNAi)*	71 ± 3^	2.9 ± 0.2^	140 ± 10^	14 sec	48 ± 2	31 ± 2^
HA2518; *jnk-1(RNAi)*	68 ± 6^	2.8 ± 0.2	136 ± 10	8 sec	48 ± 1	30 ± 1

Summary of quiescence and arousal metrics is presented for all genotypes and treatments. Note that significance was determined by comparing results for each genotype to results for control animals run in parallel. Significance with $P < 0.05$ is indicated as * for strains compared to wild type, as # versus *unc-49(lf)* on control*(RNAi)* and ^ versus control*(RNAi)*. c.b.d. , cannot be determined; d.n.d., did not determine; Q. bout, quiescence bout. Arousal threshold for quiescent *inso-1(RNAi)* is underlined as nonquiescent *inso-1(RNAi)* animals were defective for response to dilute octanol; these animals should not be compared with control*(RNAi)* animals. See the main text for details.

Table S4—Analysis of correlation: arousal thresholds and bout duration (≥ 1 sec long)

Genotype	Total quiescence (min)	N	Bout duration	Arousal thresholds
Wild-type	104 ± 6	13	6.6 ± 0.4	53 ± 2
dop-1(lf)	183 ± 16***	11	8.3 ± 0.6	75 ± 3
tdc-1(lf)	140 ± 10***	8	6.3 ± 0.4	67 ± 4
acy-1(gf)	65 ± 5***	8	10.3 ± 0.9	16 ± 1
kin-2(lf)	32 ± 8***	10	5.9 ± 0.5	5 ± 0
gsa-1(gf)	11 ± 6***	8	2.0 ± 0.6***	0
goa-1(lf)	16 ± 4***	8	3.2 ± 0.4***	0

Correlation analysis presented in Figure 3A suggested that quiescence bout durations are not correlated with arousal thresholds across mutant genotypes tested. However, this analysis used data captured at a 10-sec frame rate and would have missed shorter quiescence bouts. In order to unambiguously test the absence of correlation between arousal thresholds and bout duration, genotypes with very high and very low total lethargus quiescence were reanalyzed after recording their activity at one frame per sec (fps). At this rate of image capture, quiescent bouts that are 1 sec or longer can be measured. Genotypes with increased quiescence retested at this higher frame rate were *dop-1(lf)* and *tdc-1(lf)*; decreased quiescence genotypes were *acy-1(gf)*, *kin-2(lf)*, *gsa-1(gf),* and *goa-1(lf)*. Arousal threshold results are reiterated from Figure 3. Total quiescence is increased in comparison with Figure 1 because shorter quiescence bouts are included at 1 fps. The coefficient of correlation for the genotypes in the table is r = 0.088, P = 0.264. Therefore, we conclude that there is not a direct correlation between bout duration and arousal thresholds. Student *t* test for total quiescence determination (1 fps) versus wild-type P < 0.05 < 0.01 and < 0.001 as *, **, ***, respectively.

Table S5—False discovery rate analysis and cross-species orthology

D. melanogaster	*C. elegans*	False discovery rate q value for total quiescence	False discovery rate q value for arousal threshold	Cross-species protein similarity by BLAST Expect value (E)
Shaker	*shk-1*	0.010438*	0.007610**	6e-191
sarah	*rcn-1*	0.006536**	0.000580***	3e-27
CanB2	*cnb-1*	0.011025*	0.007610**	2e-72
Pp2B-14D	*tax-6*	0.023830*	0.028936*	3e-216
fumin	*dat-1*	0.033088*	0.028339*	1e-180
DoPR	*dop-1*	0.000020***	0.005820**	5e-72
5-HT1A	*ser-4*	0.033845*	0.029122*	4e-81
rutabaga	*acy-1*	0.017052*	0.005091**	5e-147
dunce	*pde-4*	0.000008***	0.016005*	1e-158
dCREB	*crh-1*	0.000223***	0.044567*	1e-28
PKA (OE)	*kin-2*	0.000003***	0.000003***	$9e\text{-}135^{C}$; $2e\text{-}98^{R}$
Less GABA	*unc-25*	0.000759***	0.043449*	1e-153
Dieldrin resistant (OE)	*unc-49;lgc-38*	0.020669*	0.043449*	3e-93; 5e-105
$G_o\alpha47A$ (OE)	*goa-1*	0.000001***	0.000001***	4e-168
basket	*jnk-1*	0.033260*	0.049654*	1e-142
cycA	*cya-1*	0.000075***	0.016005*	1e-34
insomniac	*inso-1*	0.003515**	0.049654*	2e-49
cullin	*cul-3*	0.015139*	0.005091**	6e-191
TβH	*tbh-1*	0.016998*	0.019830*	4e-91
Tdc	*tdc-1*	0.006536**	0.007610**	1e-185

False discovery rate q values for total quiescence and for arousal thresholds of all the genotypes are presented. q value < 0.05 is significant. The Expect value for the orthologous *C. elegans* genes identified by BLAST is presented in the last column of the table. C, KIN-1 E value versus *Drosophila* Pka-C1 and R, KIN-2 E value versus *Drosophila* Pka-R1. q < 0.05, < 0.01, < 0.001 represented as *, **, ***, respectively.

Table S6—Effect of *Caenorhabditis elegans* and *Drosophila* genes on quantity of sleep and arousal.

Function	D. melanogaster	Rest	C. elegans	Quiescence	Arousal
Potassium channel	*Shaker*	↓	*shk-1*	↓	↓
Regulator of calcineurin	*sarah*	↓	*rcn-1*	↓	↓
Calcineurin subunit B	*CanB2*	↓	*cnb-1*	↓	↓
Calcineurin subunit A	*Pp2B-14D*	↓	*tax-6*	↓	↓
Dopamine transporter	*fumin*	↓	*dat-1*	↓	↓
Dopamine receptor	*DoPR*	↑	*dop-1*	↑	↑
Serotonin receptor	*5-HT1A*	↓	*ser-4*	↓	↓
Adenylyl cyclase	*rutabaga*	↑	*acy-1(gf)*	↓	↓
Phosphodiesterase	*dunce*	↓	*pde-4*	↓	↓
CREB	*dCREB*	↑	*crh-1*	↑	↑
Protein kinase A	*PKA* (OE)	↓	*kin-2*	↓	↓
GABA synthesis	less GABA	↓	*unc-25*	↓	↓
$GABA_A$ receptor	*Dieldrin resistant* (OE)	↑	*unc-49;lgc-38*	↓	↓
G_o alpha	G_o*α47A* (OE)	↑	*goa-1*	↓	↓
C-Jun N-terminal kinase	*basket*	↓	*jnk-1*	↑	↓
Cyclin A	*cycA*	↓	*cya-1*	↑	↑
Ubiquitin ligase adaptor	*insomniac*	↓	*inso-1*	↓	↓[a]
Ubiquitin ligase	*cullin*	↓	*cul-3*	↓	↓
Tyramine beta hydroxylase	*TβH*	↑	*tbh-1*	↑	↑
Tyrosine decarboxylase	*Tdc*	↑	*tdc-1*	↑	↑

The function and names of *Caenorhabditis elegans* and *Drosophila* genes examined here are listed. Drosophila genes either increase or decrease total rest (up and down arrows, respectively). The results of C. elegans studies herein are summarized as change in quiescence (Quiescence) and change in arousal threshold (Arousal) increased or decreased, indicated again by arrows. All results are based on effect of decreased gene function, unless indicated otherwise. In C. elegans, protein kinase A (PKA) activity was increased by examining animals lacking the PKA regulatory subunit, kin-2. References for Drosophila sleep genes (superscript numbers refer to references following the tables): *Shaker*,[14] *sarah*,[15] *CanB2*,[15] *Pp2B-14D*,[15] *fumin*,[16] *DopR*,[17] *5-HT1A*,[18] *rutabaga*,[19] *dunce*,[19] *dCREB*,[19] *PKA*,[19,20] less GABA,[21] *Dieldrin resistant* (OE),[22] *Goα47A*,[23] *basket*,[24] *cycA*,[25] *insomniac*,[26] *cullin*,[26] *TβH*,[27] *Tdc*.[27] [a] Nonquiescent *inso-1(RNAi)* animals are partially defective in their response to the arousal stimulus. CREB, cyclic adenosine monophosphate response element binding; GABA, γ-aminobutyric acid.

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