Behavioral/Systems/Cognitive

Stimulation of Movement in a Quiescent, Hibernation-Like Form of *Caenorhabditis elegans* by Dopamine Signaling

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One of the characteristics of animals in hibernation is reduced behavioral activity. The *Caenorhabditis elegans* dauer state is a hibernation-like state of diapause that displays a dramatic reduction in spontaneous locomotion. A similar dauer-like quiescent state is produced in adults by relatively strong mutations in the insulin/IGF-1 receptor homolog *daf-2*. In this study, we show that mutations affecting the neurotransmitter dopamine, which regulates voluntary movement in many organisms, can stimulate movement in dauers and dauer-like quiescent adults. Surprisingly, the movement of quiescent animals is stimulated by conditions that reduce dopamine signaling and also by conditions predicted to increase dopamine signaling. Reducing dopamine signaling is likely to stimulate movement by activating a foraging response also seen in nondauers after withdrawal of food. In contrast, the stimulation of movement by increased dopamine is much more pronounced in quiescent *daf-2(−)* dauer and dauer-like adult animals than in nondauaer animals. This altered response to dopamine is primarily attributable to activity of the FOXO (forkhead box O) transcription factor DAF-16 in neurons. We suggest that dauers and dauer-like quiescent adults may have underlying changes in the dopamine system that enable them to respond differently to environmental stimulation.

Introduction

Adapting one's physiology to changing environmental conditions is essential for all living organisms. The *Caenorhabditis elegans* juvenile responds to conditions unfavorable for reproductive growth by altering its developmental trajectory and entering an alternative, hibernation-like larval stage called dauer (Cassada and Russell, 1975). Dauer larvae have unique morphological and behavioral features, including a marked reduction in movement, which are thought to allow them to survive harsh conditions. Because dauers can move when stimulated mechanically (Cassada and Russell, 1975), their quiescence appears to be a behavioral rather than a morphological adaptation. However, little is known about how their quiescence is influenced by neurotransmitter signaling.

We asked whether neurotransmitter systems that influence locomotion in *C. elegans* might also modulate dauer quiescence. We focused on the biogenic amine dopamine because dopamine regulates voluntary movement in many organisms. In *C. elegans* hermaphrodites, dopamine is produced by eight neurons (Sulston et al., 1975) and signals via at least four dopamine receptors, DOP-1 through DOP-4 (Suo et al., 2002, 2003; Chase et al., 2004; Sugiura et al., 2005). In addition, a presynaptic reuptake transporter, DAT-1, removes dopamine from the extracellular space to terminate signaling (Jayanthi et al., 1998; Nass et al., 2002).

In *C. elegans*, dopamine is best known for mediating the "basal slowing response" in which the animal's movement slows when it encounters food (Sawin et al., 2000). Dopamine is thought to be released in response to food and to act on motor neurons to modulate the animal's movement (Chase et al., 2004). Animals that cannot produce dopamine fail to slow in response to food (Sawin et al., 2000), and exogenous dopamine slows the movement of animals cultured without food (Schafer and Kenyon, 1995). The response to dopamine in this situation is complex, as the absence of specific dopamine receptors can have either positive or negative effects on movement (Chase et al., 2004).

In this study, we asked how conditions predicted to change the level of dopamine signaling influence the movement of dauers. Unexpectedly, we find that dauer movement can be stimulated either by increasing or decreasing the predicted level of dopamine signaling.

Dauers are developmentally arrested, prepubescent juveniles, but a dauer-like quiescent state can also be produced in the adult. Dauer formation is regulated by several signaling pathways, including the insulin/IGF-1 pathway (Riddle et al., 1981; Gottlieb and Ruvkun, 1994; Kimura et al., 1997; Riddle and Albert, 1997). Mutations that completely eliminate the function of DAF-2, the *C. elegans* insulin/IGF-1 receptor (InsR) (Kimura et al., 1997),

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cause constitutive dauer formation because of increased activity of the forkhead box O (FOXO) transcription factor DAF-16 (Riddle et al., 1981; Vowels and Thomas, 1992; Gottlieb and Ruvkun, 1994; Lin et al., 1997; Ogg et al., 1997; Riddle and Albert, 1997; Hu, 2007). Some slightly weaker *daf-2*/InsR mutations allow the animals to reach adulthood but produce a dauer-like quiescent behavior (Gems et al., 1998). We found that the movement of these quiescent adults was also stimulated by conditions predicted to either increase or decrease dopamine signaling.

Our findings suggest three conclusions. First, because neither reducing nor increasing the level of dopamine signaling produces dauer-like quiescence in normal animals, it is unlikely that dauer quiescence is caused simply by a change in dopamine-signaling strength. Second, because reducing food levels, like reducing dopamine levels, stimulates the movement of both dauer-like quiescent animals and nondauers, we infer that these quiescent animals respond to food withdrawal in a manner similar to nondauer animals. Third, because increasing dopamine signaling stimulates movement in *daf-2(−)* dauers and dauer-like adults, but not in nondauer animals, we infer that reductions in insulin/IGF-1 signaling change the animals' response to dopamine. This altered response to dopamine is mediated in part by increased DAF-16/FOXO transcriptional activity in the nervous system.

Materials and Methods

Strains

Nematodes were raised under standard laboratory conditions on agar plates containing a lawn of *Escherichia coli* strain OP50, as described previously (Brenner, 1974). "Wild type" was the *C. elegans* strain N2. The mutant and transgenic strains used were as follows: CB1372 *daf-7(e1372)*, CF1041 *daf-2(e1370)*, CF1085 *daf-16(mu86); daf-2(e1370)*, CF1362 *daf-16(mu86); daf-2(e1370); muEx142[rol-6(su1006)]*, CF1592 *daf-2(e1370); muEx226[rol-6(su1006)]*, CF1794 *daf-16(mu86); daf-2(e1370); muIs109[Pdaf-16::gfp::daf-16; rol-6(su1006)]*, CF2005 *daf-16(mu86); daf-2(e1370); muIs120[Pges-1::gfp::daf-16; rol-6(su1006)]*, CF2093 *daf-16(mu86); daf-2(e1370); muIs131[Punc-119::gfp::daf-16; rol-6(su1006)]*, CF2102 *daf-16(mu86); daf-2(e1370); muIs126[Pmyo-3::gfp::daf-16; rol-6(su1006)]*, CF2470 *dat-1(ok157)*, CF2532 *daf-2(e1370) dat-1(ok157)*, CF2593 *cat-2(e1112)*, CF2625 *daf-2(e1370); lin-15(n765ts); akEx248[Pdat-1::gfp; lin-15(+)]*, CF2626 *daf-2(e1370); lin-15(n765ts); akEx387[Pdat-1::gfp; Pdat-1::hICE; lin-15(+)]*, CF2646 *daf-2(e1370); dop-3(vs106)*, CF2665 *daf-2(e1370); dop-1(vs100) dop-3(vs106)*, CF2666 *daf-2(e1370); dop-1(vs100)*, CF2685 *dat-1(ok157) daf-2(e1370); dop-1(vs100)*, CF2801 *dat-1(ok157) daf-2(e1370); dop-2(vs105)*, CF2802 *dat-1(ok157) daf-2(e1370); dop-3(vs106)*, CF2803 *dat-1(ok157) daf-2(e1370); dop-4(ok1321)*, CF2805 *dop-2(vs105); dop-4(ok1321) dop-1(vs100) dop-3(vs106)*, CF2831 *daf-2(e1370); dop-2(vs105); dop-4(ok1321) dop-1(vs100) dop-3(vs106)*, CF3148 *daf-16(mu86); daf-2(e1370) dat-1(ok157)*, CF3248 *daf-2(e1370); muIs176[Pdat-1::gfp; Pdat-1::hICE; Podr-1::rfp]*, CF3376 *dat-1(ok157) daf-2(e1370); dop-2(vs105); dop-4(ok1321) dop-1(vs100) dop-3(vs106)*, CF3387 *daf-2(e1370); dop-1(vs100); ljEx131[Pmec-7::dop-1; Punc-122::gfp]*, CF3396 *daf-16(mu86); daf-2(e1370); muEx536[Pacr-2::gfp::daf-16; rol-6(su1006)]*, CF3397 *daf-16(mu86); daf-2(e1370); muEx537[Pdat-1::gfp::daf-16; rol-6(su1006)]*, CF3399 *daf-16(mu86); daf-2(e1370); muEx539[Posm-5::gfp::daf-16; rol-6(su1006)]*, CF3401 *daf-16(mu86); daf-2(e1370); muEx541[Pnmr-1::gfp::daf-16; rol-6(su1006)]*, CF3415 *daf-16(mu86); daf-2(e1370); muEx545[Pglr-5::gfp::daf-16; rol-6(su1006)]*, CF3417 *daf-16(mu86); daf-2(e1370); muEx547[Punc-47::gfp::daf-16; rol-6(su1006)]*, CF3420 *daf-2(e1370); dop-1(vs100); muEx532[Pacr-2::dop-1; Podr-1::cfp]*, CF3421 *daf-2(e1370); dop-3(vs106); muEx534[Pacr-2::dop-3; Podr-1::cfp]*, JT195 *daf-11(sa195)*. All mutations were backcrossed at least three times to our laboratory N2 strain, except the mutations in CB1372 and JT195.

Transgenic strains and constructs

Transgenic strains were created using standard methods (Mello and Fire, 1995).

pTH5 (*Pdat-1::hICE*) and pTH6 (*Pdat-1::gfp*) were kind gifts from A. Villu Maricq (University of Utah, Salt Lake City, UT) (Hills et al., 2004). They were injected into wild-type (N2) adults at a concentration of 10 ng/μl each, together with a *Podr-1::rfp* marker (40 ng/μl). The transgenes were integrated using UV exposure to create *muIs176*, and the integrated lines were backcrossed six times to wild type (N2).

Pacr-2::dop-1 and *Pacr-2::dop-3* were kind gifts from Daniel Chase (University of Massachusetts Amherst, Amherst, MA) (Chase et al., 2004). They were injected at 25 ng/μl into *dop-1(vs100)* and *dop-3(vs106)* animals, respectively, together with *Podr-1::cfp* (50 ng/μl) as a coinjection marker, to create *muEx532* and *muEx534*.

Neuron-specific GFP::DAF-16 rescue constructs were generated from the same original construct used by Libina et al. (2003) to generate tissue-specific rescue constructs. A promoterless GFP::DAF16cDNA fusion construct (pCF105) was altered to insert a multiple cloning site (creating pCF231). Promoters were amplified with specific primers (sequence available on request) flanked with restriction sites, using Pfu Ultra High Fidelity (Stratagene). The lengths of the promoters were as follows: *acr-2* (acetylcholinergic motor neurons): 3198 bp [based on Chase et al. (2004)]; *dat-1* (dopamine-producing neurons): 1400 bp [based on Hills et al. (2004)]; *glr-5* (interneurons): 1611 bp [based on Lee and Ashrafi (2008)]; *nmr-1* (command interneurons): 4471 bp [based on Brockie et al. (2001)]; *osm-5* (sensory neurons): 300 bp [based on Haycraft et al. (2001)]; *unc-47* (GABAergic motor neurons): 262 bp [based on Chase et al. (2004)]. All promoters included sequences up to (but not including) the ATG start site. All constructs were injected into *daf-16(mu86); daf-2(e1370)* double-mutant animals at 50 ng/μl, and pRF4 [*rol-6(su1009)*] (Mello and Fire, 1995) was used as a coinjection marker (100 ng/μl). The expression pattern of each strain tested was verified. In all cases, it matched reported expression patterns. Green fluorescent protein (GFP) expression driven by *Pacr-2* and *Punc-47* was only clearly detected at larval stages.

Movement assays

Wild-type animals versus daf-2 *mutant dauers.* Wild-type (N2) and *daf-2(e1370)* mutant animals were grown from eggs at 25°C. Wild-type L1/L2 larvae were scored the day after moving the eggs to 25°C and wild-type L3/L4 the second day after transferring the eggs. Wild-type adults and *daf-2(e1370)* dauers were scored 3 d after transferring the eggs. All populations were scored at least three times.

Visual assay: mutant dauers. Movement of dauers was scored visually using a dissecting microscope. Dauers of strains carrying *daf-2(e1370)*, *daf-7(e1372)*, and *daf-11(sa195)* mutations were obtained by growing animals from eggs at 25°C for 3 d on plates containing food (*E. coli* OP50). Dauers were scored as moving if they moved at least one body bend forward or backward. A minimum of 30 animals per strain was scored in each repeat of the experiment. Experiments represented in Figures 1*B*, 2*B*, 6*D*, and 7*C* were part of a set of experiments done in parallel, in which each strain was scored at least 11 times. Other dauer motility assays were repeated at least five times. The experiment in Figure 2*A* includes both transgenic and nontransgenic animals, as it is not possible to distinguish *lin-15(−)* and *lin-15(+)* animals at the dauer stage. This is the case for both the test and the control strain.

For *daf-2(−) dat-1(−)* mutant dauers, we checked that the morphology of the pharynx, cuticle, and gonad were those of a fully formed dauer larva, and found that they were. Also, *dat-1* mutations did not impair the ability of worms to respond to dauer pheromone, and *dat-1(−)* dauers formed after exposure to dauer pheromone were also more motile than wild-type dauers. These observations indicate that there is no reason to think *dat-1* mutations impair dauer formation, or that their effect is very specific to *daf-2*/InsR mutant dauers.

Visual assay: adults. Animals were raised until the L4 stage at 15°C, and then moved to 25°C on plates with *E. coli*, and scored 3 d later. CF2625 *daf-2(e1370); lin-15(n765ts); akEx248[Pdat-1::gfp; lin-15(+)]* and CF2626 *daf-2(e1370); lin-15(n765ts); akEx387[Pdat-1::gfp; Pdat-1::hICE; lin-15(+)]* were grown at 20°C, instead of 15°C, to pick out transgenic animals. Animals were scored as moving if they moved at least one body bend forward or backward. If animals were rollers (see Fig. 5*C*; supplemental Fig. S3, available at www.jneurosci.org as supplemental material),

they were scored as moving if their head moved forward or backward in a circle. A minimum of 30 animals per strain was scored in each repeat of the experiment. In all experiments, each strain was tested a minimum of six times. Experiments in Figures 1*C*, 5*A*, 6*E*, and 7*D* were part of a set of experiments done in parallel, in which each strain was scored at least 12 times.

For the longitudinal studies (supplemental Fig. S1*A*,*B*, available at www.jneurosci.org as supplemental material), animals were grown at 15°C, shifted to 25°C as L4, and scored every day. On the third day, they were shifted back to 15°C and scored every day for 2 more days. Each strain was tested four times.

Adult assays using the worm tracker ("Tracker assay"). The parallel worm tracker platform developed by the laboratory of Miriam Goodman (Stanford University, Stanford, CA) was used to characterize adult movement as described previously (Ramot et al., 2008). Animals were grown in the same conditions used for the visual assays, and tested 3 d after the L4 stage. At the beginning of the experiment, animals were transferred on NGM plates seeded with *E. coli* OP50 as described previously (Ramot et al., 2008). The only exceptions were the "off food" assays, in which animals were transferred to unseeded plates instead. One minute videos were taken every 10 min for 90 min and analyzed using the parallel worm tracker tools (Ramot et al., 2008). The "identify paralyzed" function was used to determine the average speed of the population and the fraction of animals that were moving. The average speed was defined as the average of the instantaneous speed measurement for all tracks in a given video (Ramot et al., 2008). To determine the fraction of animals moving, animals were deemed "paralyzed" if 80% of the instantaneous speed measurements collected during the track were <15 μm/s. Each strain was tested four to six times. For the tracking assay (see Fig. 6*A*; supplemental Fig. S4, available at www.jneurosci.org as supplemental material), CF3248 *daf-2(e1370); muIs176[Pdat-1::gfp; Pdat-1::hICE; Podr-1::rfp]* adults were used as *daf-2(−)* without dopamine neurons. These animals also moved more in a visually scored adult assay (data not shown).

We noticed that, right after the transfer ($t = 0$ min), more animals moved, and at a faster speed. Over time, both the speed and the percentage of animals moving decreased, and reached a stable value. We considered the initial movement response to be movement in response to a stimulus, whereas we reasoned that the stable level of locomotion represented baseline (unstimulated) movement.

Long-term dopamine addition

Dopamine plates were prepared as previously described (Schafer and Kenyon, 1995; Chase et al., 2004). A final concentration of 30 mM dopamine hydrochloride (Sigma-Aldrich) was used. Both 30 mM and control ("0 mM") dopamine plates contained a final concentration of 2 mM glacial acetic acid. Animals were grown at 15°C and shifted to 25°C as L4 larvae. Three days later, animals were moved to dopamine or control plates seeded with *E. coli* OP50 for 2.5 h. They were then shifted to fresh plates also seeded with *E. coli* containing the same dopamine concentrations and tested using the parallel worm tracker. One minute videos were taken every 10 min for 60 min. Each strain–concentration combination was tested at least three times.

Dopamine-induced paralysis

Acute dopamine treatment was performed as described previously (Schafer and Kenyon, 1995), with minor modifications. Animals were grown at 15°C, shifted to 25°C as L4 larvae, and tested the following day. They were transferred to unseeded 1.5% Difco agar (BD Biosciences) plates containing 2 mM glacial acetic acid and the indicated concentration of dopamine, and kept at 25°C for 1 h. The fraction of animals moving was then determined visually using a dissecting microscope.

Pumping

Animals were grown at 15°C and transferred to 25°C at L4 stage. Pumping rates were scored 3 d later. The contractions of the pharynx in a 30 s interval were counted for five animals per strain on 3 separate days. Animals were counted whether or not they pumped, as 20% of the *daf-2(e1370)* mutant animals were observed not to pump at all.

HPLC analysis

Animals were grown at 15°C until the L4 stage and shifted to 25°C for 3 d. Samples were collected in M9 buffer and frozen immediately. HPLC analysis was done by the Neurochemistry Core facility at the Center for Molecular Neuroscience of Vanderbilt University (Nashville, TN). Each strain was tested at least three times.

Statistics

Statistical analysis was performed using XLSTAT 8.0 add-in software for Excel (Addinsoftware). Appropriate tests were chosen as follows: (1) Student's *t* test was used when two strains were compared with each other in visual assays; (2) one-way ANOVA followed by *post hoc* Dunn–Sidak's corrected *t* test or Dunnet's corrected *t* test (for comparison with a single control) was used when multiple strains were compared with each other in visual assays (the correction for multiple comparisons was applied for each figure panel separately); (3) two-way ANOVA was used to analyze the tracker data, setting as variables time and strain, time and condition for the on–off food comparisons or time and concentration for the experiments with dopamine addition; *p* values reported are for the strain/condition/dopamine concentration component of the analysis (the time component had, as expected, a significant effect in all comparisons); when single data points are reported (e.g., for $t = 0$ min), Student's *t* test or Dunn–Sidak's corrected *t* test was used; (4) Kruskal–Wallis test with Dunn's test was used for the pumping data, as these data were not normally distributed.

Results

Dauer larvae move less than nondauer animals

C. elegans dauer larvae share certain features with animals in hibernation, as both are states of dormancy characterized by suppressed metabolism and increased resistance to stress (Carey et al., 2003). Consistent with this, Cassada and Russell (1975) reported that dauer larvae have reduced spontaneous locomotion but respond to stimuli. We tested whether we could quantify the quiescence of dauer larvae by observing nondauers and dauers visually and comparing the fractions of animals that moved. We found that this was the case: whereas 56–60% of nondauer, wild-type animals moved freely, only 14% of the dauer larvae did (Fig. 1*A*). (Henceforth, we refer to this method of scoring as the "visual assay.")

Several signaling pathways interact to regulate dauer formation. In addition to the insulin/IGF-1 signaling pathway, a TGF-β pathway and a cGMP-dependent pathway also influence dauer formation, and mutations in either of these pathways can cause constitutive dauer formation (for review, see Riddle and Albert, 1997; Hu, 2007). To test whether the dauer quiescence phenotype was specific to *daf-2*/InsR mutant dauers, we assayed the movement of dauer larvae formed in response to mutations in *daf-7*, a gene encoding a TGF-β ligand (Ren et al., 1996), and in *daf-11*, a gene encoding a guanylate cyclase (GC) that acts in the cGMP pathway (Birnby et al., 2000). Dauers caused by all three mutations were quiescent (Fig. 1*B*). We also examined wild-type dauers induced by exposure to dauer pheromone, which is a natural cue that induces dauer formation, and confirmed that these animals were quiescent as well (data not shown). We conclude that locomotory quiescence is a general feature of dauer larvae.

daf-2(e1370) adults display a quiescent behavior reminiscent of dauer larvae

To better characterize the quiescence phenotype, we sought to examine quiescence independently of dauer formation. To this end, we took advantage of the dauer-like locomotory phenotype produced by class II mutations in *daf-2*/InsR, which are thought to cause a strong reduction in insulin/IGF-1-like signaling (Gems

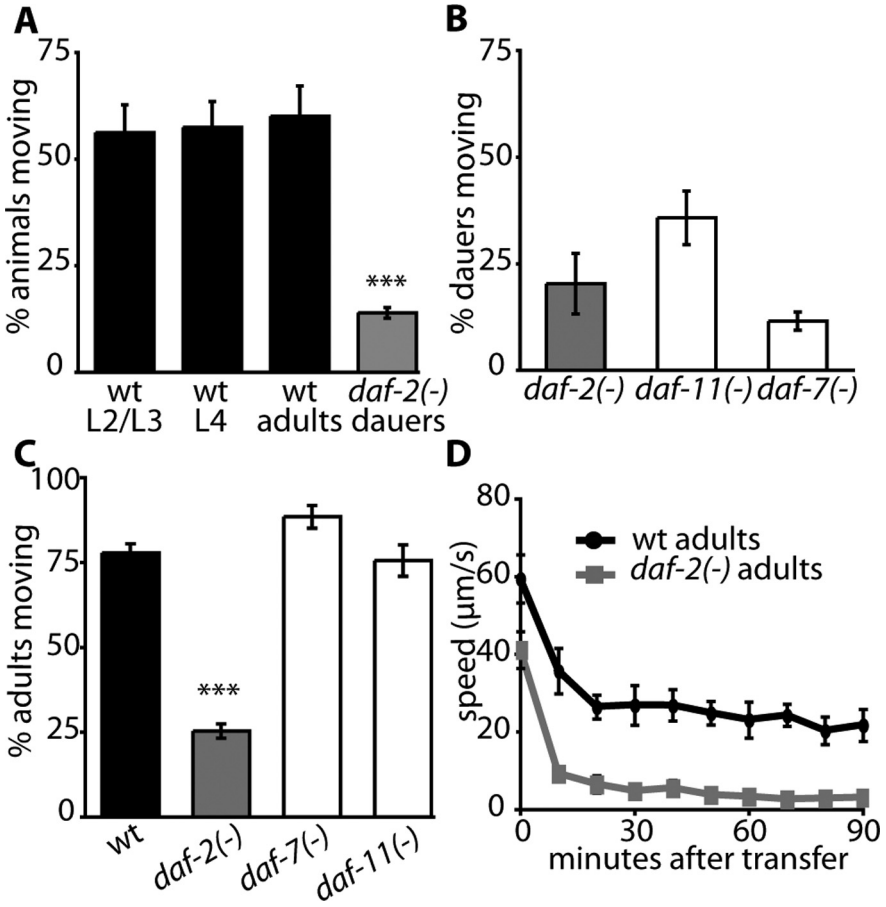

Figure 1. Dauer larvae have reduced spontaneous movement and mutations in the *daf-2*/InsR pathway can produce dauer-like quiescence in adult animals. ***A***, The percentage of *daf-2(e1370)* dauer larvae that moved on visual observation was compared with that of wild-type (wt) larvae and adults also grown at 25°C. Percentage moving ± SEM: wild-type L2/L3 larvae, 56 ± 6%; wild-type L4 larvae, 57 ± 6%; wild-type adults, 60 ± 7%; *daf-2(−)* dauer larvae, 14 ± 1%. ***$p < 0.0001$ for *daf-2(−)* versus wt (any stage), Dunn–Sidak's corrected *t* test. ***B***, The movement of dauer larvae formed at 25°C because of mutations in genes representative of the three major dauer formation pathways was assayed: the insulin/IGF-1 receptor homolog (InsR) *daf-2* for the insulin/IGF-1-like pathway, the TGF-β homolog *daf-7* for the TGF-β pathway, and the GC *daf-11* for the cGMP pathway. Dauers formed by all three mutations were quiescent. Percentage moving ± SEM: *daf-2(e1370)*, 20 ± 6%; *daf-11(sa195)*, 36 ± 6%; *daf-7(e1372)*, 12 ± 2%. ***C***, Mutations in *daf-2*/InsR, but not mutations in *daf-7*/TGF-β or in *daf-11*/GC, decreased the percentage of adult animals that moved. Percentage moving ± SEM: wt, 78 ± 3%; *daf-2(e1370)*, 25 ± 2%; *daf-7(e1372)*, 88 ± 2%; *daf-11(sa195)*, 76 ± 5%. ***$p < 0.0001$ versus wt, Dunn–Sidak's corrected *t* test. ***D***, *daf-2(e1370)* adults were compared with wild-type animals using the tracker. Both animals responded to stimulation, but the average baseline speed of the population was lower in *daf-2(e1370)* adults. $p < 0.0001$ (two-way ANOVA) for *daf-2(−)* versus wt. Error bars represent SEM. Animals in Figure 1, *C* and *D*, were grown at 15°C, shifted to the quiescence-inducing temperature, 25°C, at the L4 stage (after the dauer-decision point), and tested at day 3 of adulthood on food.

et al., 1998). Because the process of dauer formation is facilitated by high temperature, these mutations cause constitutive dauer formation at high temperature (25°C). However, when raised at low temperature (15°C) until the last stage of larval development and then shifted to 25°C, these animals develop into adults but have several phenotypes that are reminiscent of dauers, including reduced locomotion. We confirmed that *daf-2(e1370)* mutant adults progressively became quiescent when shifted to 25°C at the last stage of larval development, the L4 stage (Gems et al., 1998) (supplemental Fig. S1*A*, available at www.jneurosci.org as supplemental material). (All subsequent adult assays were performed 3 d after the shift to 25°C.) The defect could be readily reversed by shifting the animals back to 15°C (Gems et al., 1998) (supplemental Fig. S1*B*, available at www.jneurosci.org as supplemental material). We also found that *daf-2(e1370)* animals had dramatically reduced rates of pharyngeal pumping 3 d after the shift to the restrictive temperature (supplemental Fig. S1*C*, available at www.jneurosci.org as supplemental material), which is reminiscent of the lack of pumping in dauer larvae. These dauer-like phenotypes can be uncoupled from the increased longevity of *daf-2(−)* animals, as weaker (class I) mutations in *daf-2*/InsR do not affect movement but still increase the life span of the worms (Gems et al., 1998).

Like class I *daf-2*/InsR mutations, mutations in genes from other dauer-formation pathways did not cause dauer-like phenotypes as adults. For example, both *daf-7*/TGF-β and *daf-11*/GC mutant adults moved as much as wild-type animals (Fig. 1*C*) and had pumping rates similar to wild-type animals (supplemental Fig. S1*C*, available at www.jneurosci.org as supplemental material). Although it is possible that changing both the cGMP and the TGF-β pathways in the same animal would trigger adult quiescence [these double mutants have an enhanced dauer-constitutive phenotype (Thomas et al., 1993)], the adult data suggest that the *daf-2*/InsR pathway may have a direct effect on the expression of dauer-like quiescence.

Because of their small size, dauer larvae are difficult to track with automated systems. However, we were able to use an automated tracker (Ramot et al., 2008) to analyze the quiescence of *daf-2(e1370)* adults. (We will refer to assays done using the automated tracker as "tracker assays.") Tracker assays allowed us to observe both the average speed and the percentage of animals moving over an extended period of time.

We noticed that, regardless of the strain, we could now clearly distinguish two stages of movement (Fig. 1*D*; supplemental Fig. S1*D*, available at www.jneurosci.org as supplemental material). When first transferred to the assay plate, the animals moved faster, but later the average speed of the animals and the percentage that were moving decreased and subsequently remained more or less constant. We reasoned that the first stage represented movement in response to stimulation (transferring to new plates) (see Materials and Methods), whereas the second stage represented baseline, unstimulated movement. In principle, mutations that alter movement rates could affect the stimulated movement, the baseline movement, or both. We found that *daf-2*/InsR mutations affected baseline rates of movement, because 60 min after the beginning of the assay the average speed of the *daf-2(−)* population had dropped to 3.3 ± 0.5 μm/s, whereas the speed of wild-type animals was 23.1 ± 4.6 μm/s (Fig. 1*D*) ($t = 60$ min; $p < 0.05$, Dunn–Sidak's corrected *t* test). In contrast, wild-type and *daf-2(−)* animals both moved much faster immediately after stimulation (Fig. 1*D*) [average population speed ± SEM at $t = 0$ min: wt, 59.4 ± 6.2 μm/s, vs *daf-2(−)*, 41.1 ± 4.7 μm/s; $p < 0.05$, Dunn–Sidak's

corrected *t* test], although wild-type animals still moved faster than did *daf-2*/InsR mutant animals. In addition, in both populations almost all the animals were classified as moving (supplemental Fig. S1*D*, available at www.jneurosci.org as supplemental material) [percentage moving ± SEM at $t = 0$ min: wt, 88 ± 6%, vs *daf-2(−)*, 90 ± 6%]. These data indicate that *daf-2*/InsR mutant adults, like dauers, are capable of moving after stimulation, consistent with the interpretation that their quiescence is likely attributable to an active modulation of the locomotory circuit, rather than to permanent defects in muscles or neurons or to a debilitating lack of energy.

Mutations that affect dopamine can reverse the quiescence of dauer larvae

Previous studies have shown that dopamine modulates the locomotion of *C. elegans* (Schafer and Kenyon, 1995; Sawin et al., 2000; Chase et al., 2004; Hills et al., 2004). Therefore, we asked whether changes in dopamine signaling could affect dauer movement. To test the effect of decreased dopamine signaling, we examined a strain in which dopamine-producing neurons were genetically ablated by cell-specific expression of a cell death caspase (Hills et al., 2004). To test the effects of increased dopamine signaling, we examined animals with a loss-of-function mutation in the dopamine-reuptake transporter *dat-1* (Nass et al., 2002), which is needed for termination of the dopamine signal (Gainetdinov and Caron, 2003). In mammals, dopamine transporter (DAT) mutations increase extracellular dopamine levels (Giros et al., 1996), and previous behavioral analysis in *C. elegans* suggests this is likely to be the case in the worm too (Kindt et al., 2007; McDonald et al., 2007). Neither treatment appeared to affect the ability of the animals to form dauers (data not shown) (see Materials and Methods). Surprisingly, we found that both conditions stimulated dauer movement: *daf-2(−)* dauers with no dopamine-producing neurons moved more than control *daf-2(−)* dauers (Fig. 2*A*) and *daf-2(−) dat-1(−)* double-mutant dauers moved more than *daf-2(−)* dauers (Fig. 2*B*). We also found that *dat-1* mutations could increase movement in dauers obtained by exposure to dauer pheromone (data not shown). Together, these data suggest that dopamine may influence two pathways that stimulate dauer movement, one activated by reduced dopamine signaling and the other activated by increased dopamine signaling.

Decreases in dopamine levels in *C. elegans* have previously been correlated with increased locomotion in the absence of food (Sawin et al., 2000), which could potentially explain why loss of dopamine-producing neurons can increase movement in dauer larvae. In contrast, increases in dopamine signaling have not been reported to stimulate locomotion in *C. elegans* before. Therefore, we will discuss the effects of mutations predicted to increase or decrease dopamine-signaling levels separately.

dat-1 mutations stimulate the movement of *daf-2(e1370)* adults

To study the effects of increasing dopamine signaling, we first asked whether *dat-1* mutations could also affect the quiescence of *daf-2*/InsR mutant adults. We found that *dat-1* mutations dramatically increased the percentage of *daf-2(−)* animals moving in the visual assay [percentage moving ± SEM: *daf-2(e1370)*, 25 ± 2%; *daf-2(e1370) dat-1(ok157)*, 67 ± 3%; $p < 0.0001$, Student's *t* test].

When we characterized these mutants further using the tracker assay, we found that *dat-1* mutations caused an increase in movement after the animals were transferred to the assay plates, suggesting that increasing dopamine signaling affected movement in response to stimulation. However, by 40 min, most of the animals had stopped moving, suggesting that *dat-1* mutations did not affect baseline movement of *daf-2(−)* animals (Fig. 3). Specifically, at time 0, *dat-1* mutations dramatically increased the average speed of the *daf-2(−)* population [average speed ± SEM at $t = 0$ min: *daf-2(−)*, 39.5 ± 3.8 μm/s vs *daf-2(−) dat-1(−)*, 81.3 ± 8.6 μm/s; $p < 0.01$, Dunn–Sidak's corrected *t* test],

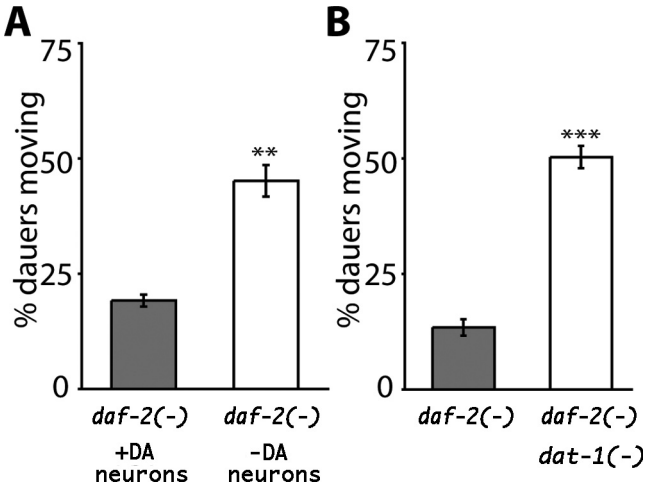

Figure 2. Ablation of the dopamine-producing neurons and mutations in the dopamine transporter *dat-1* increase the percentage of moving dauers. ***A***, *daf-2(e1370)* strains expressing GFP (+DA neurons) or a human homolog of *C. elegans* caspase (interleukin-converting enzyme) (−DA neurons) in the dopamine-producing neurons (Hills et al., 2004) were tested to examine the effect of genetically ablating dopamine-producing neurons. Loss of the dopamine-producing neurons increased the number of dauers that moved. Percentage moving ± SEM: *daf-2(−); lin-15(−); akEx248[Pdat-1::gfp; lin-15(+)]* [*daf-2(−)* +DA neurons], 19 ± 3%; *daf-2(−); lin-15(−); akEx387[Pdat-1::gfp; Pdat-1::hICE; lin-15(+)][daf-2(−)* −DA neurons], 45 ± 5%. $^{**}p < 0.01$, Student's *t* test. ***B***, The effect of loss of the dopamine transporter was tested by comparing spontaneous movement in *daf-2(e1370)* and *daf-2(e1370) dat-1(ok157)* dauer larvae. *dat-1* mutations increased the percentage of dauers that were moving. Percentage moving ± SEM: *daf-2(−)*, 14 ± 2%; *daf-2(−) dat-1(−)*, 50 ± 3%. $^{***}p <$ 0.0001, Student's *t* test. Error bars represent SEM.

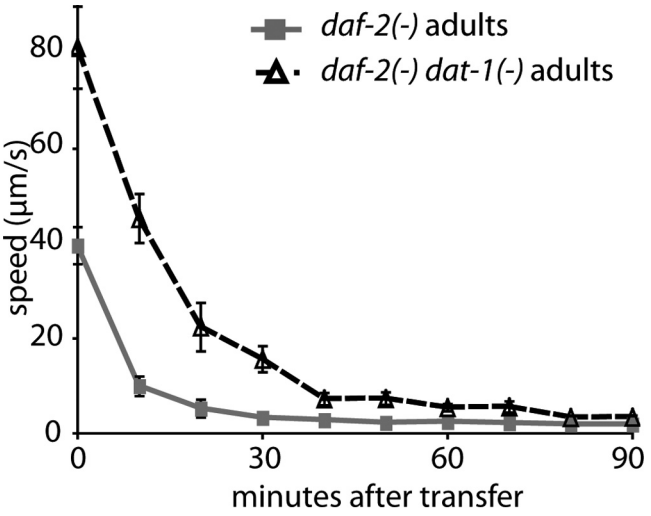

Figure 3. Mutations in *dat-1* increase movement in *daf-2*/InsR mutant adults by affecting their locomotory response to stimulation. When stimulated, *daf-2(e1370) dat-1(ok157)* animals initially moved faster than *daf-2(e1370)* mutants alone. $p < 0.0001$, two-way ANOVA. Error bars represent SEM. All animals were grown at 15°C, shifted to 25°C at the L4 stage, and tested at day 3 of adulthood on food.

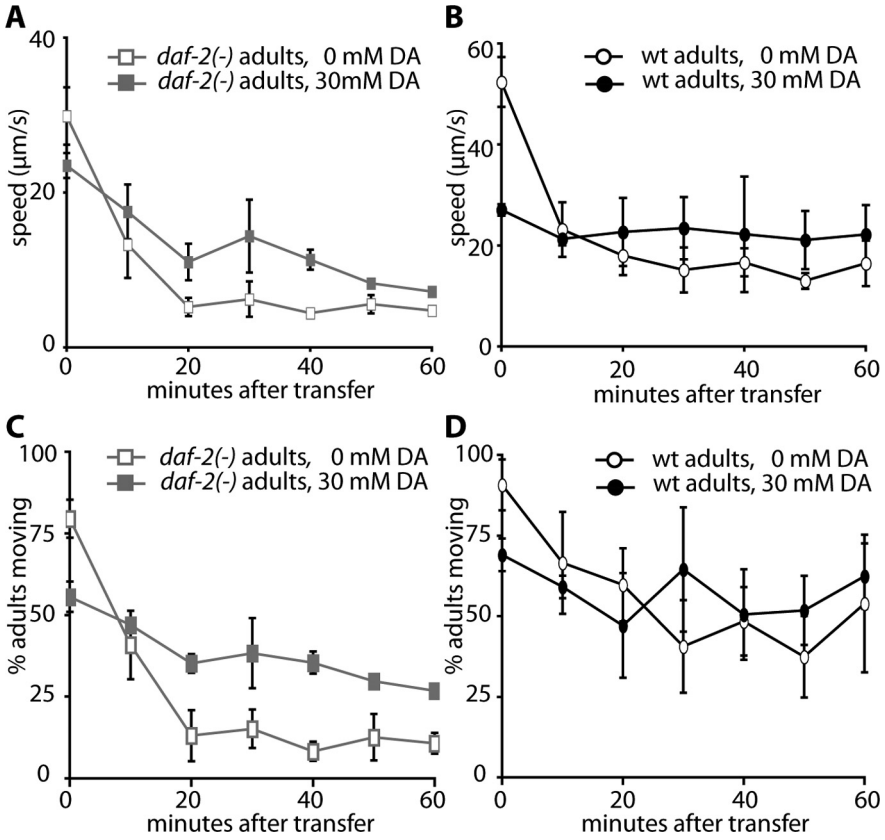

Figure 4. Long-term exogenous addition of dopamine suppresses quiescence in *daf-2*/InsR mutant animals. *daf-2(e1370)* (***A***, ***C***) and wild-type (wt) (***B***, ***D***) animals were incubated on plates containing 0 or 30 mM dopamine for 3.5 h (2.5 h of preincubation and 1 h of recording). Exogenous dopamine increased the average speed (***A***) ($p < 0.05$, two-way ANOVA) and the percentage of animals moving (***C***) ($p < 0.001$, two-way ANOVA) in *daf-2*/InsR mutant adults but had no effect on the speed (***B***) ($p = 0.8$, two-way ANOVA) and percentage of animals moving (***D***) ($p = 0.9$, two-way ANOVA) in wild-type adults. All animals were grown at 15°C, shifted to 25°C at the L4 stage, and tested at day 3 of adulthood on food. Error bars represent SEM.

but the difference decreased over time [average speed ± SEM at $t = 90$ min: *daf-2(−)*, 2.8 ± 0.1 μm/s, vs *daf-2(−) dat-1(−)*, 3.4 ± 0.3 μm/s; $p < 0.01$, Dunn–Sidak's corrected t test]. We conclude that *dat-1* mutations, presumably by increasing dopamine signaling, allow *daf-2*/InsR mutant adults to respond more vigorously to external stimuli.

dat-1 mutations have little effect on wild-type movement

Finding that mutations predicted to increase dopamine signaling increased movement in response to stimulation was surprising, because in *C. elegans* loss of dopamine is known to increase movement in the presence of food (Sawin et al., 2000). However in other organisms, mutations in genes encoding DAT, which prevent reuptake of the neurotransmitter and thus prolong dopamine signaling, cause hyperactivity (Giros et al., 1996; Kume et al., 2005). *dat-1* mutations in *C. elegans* have been reported not to have an effect on wild-type locomotion under standard culture conditions (McDonald et al., 2007). To investigate this further, we tested what effect *dat-1* mutations had on wild-type movement rates in the tracker assay. We found that *dat-1* mutations did not change the average population speed of *daf-2(+)* worms (supplemental Fig. S2*A*, available at www.jneurosci.org as supplemental material), despite their dramatic effect on the speed of *daf-2(−)* animals (Fig. 3). *dat-1* mutations did increase the percentage of *daf-2(+)* animals moving after stimulation (supplemental Fig. S2*C*, available at www.jneurosci.org as supplemental material) (a measure that is clearly visible only with the tracker); however, the effect was relatively modest [supplemental Fig. S2*C* (available at www.jneurosci.org as supplemental material), percentage moving ± SEM at time $t = 20$ min: wt, 62 ± 4%, vs *dat-1(−)*, 77 ± 5%; compare with supplemental Fig. S5*A* (available at www.jneurosci.org as supplemental material), percentage moving ± SEM at time $t = 20$ min: *daf-2(−)*, 10 ± 6%, vs *daf-2(−) dat-1(−)*, 46 ± 10%].

Exogenous dopamine stimulates the movement of *daf-2*/InsR mutant adults

These data suggest that a prolonged increase in dopamine signaling can stimulate the movement of *C. elegans*, particularly that of quiescent insulin/IGF-1-receptor mutants. We decided to test this more directly by asking whether we could mimic the effect of the *dat-1* mutation by prolonged exposure to exogenous dopamine. We found that exposing the worms to 30 mM dopamine (DA) for 3.5 h (which included a 2.5 h preexposure and 1 h of recording on the tracker) increased the movement of quiescent *daf-2(−)* adults, both in terms of the average speed of the population (Fig. 4*A*) and the percentage of worms moving (Fig. 4*C*). No significant effect was detected in wild-type animals (Fig. 4*B*,*D*). We conclude that prolonged exposure to dopamine increases the movement of quiescent *daf-2*/InsR mutant adults.

Notably, right after stimulation, at time 0, wild-type animals treated with dopamine actually moved less than control-treated animals (Fig. 4*D*) (percentage moving ± SEM at $t = 0$ min: 0 mM DA, 91 ± 11%, vs 30 mM DA, 69 ± 7%; $p < 0.05$, Student's t test), a decrease that may reflect the paralysis reported previously in acute assays (Schafer and Kenyon, 1995; Chase et al., 2004).

In wild-type animals treated with 30 mM dopamine, we observed a trend toward increased speed, but it was not statistically significant. We considered the possibility that we failed to see an effect on wild-type animals because their average speed was already high. However, food removal could double the speed of wild-type animals (supplemental Fig. S2*A*,*B*, available at www.jneurosci.org as supplemental material). Therefore, these data indicate that increases in dopamine specifically suppress the quiescence of *daf-2*/InsR mutant animals after stimulation.

daf-2/InsR mutations require *daf-16*/FOXO activity in neurons to affect movement

daf-2/InsR mutations lead to increased nuclear localization and activation of the FOXO transcription factor DAF-16 (Henderson and Johnson, 2001; Lee et al., 2001; Lin et al., 2001). *daf-16*/FOXO is required for *daf-2*/InsR mutant animals to become dauers (Riddle et al., 1981; Vowels and Thomas, 1992; Gottlieb and Ruvkun, 1994), and Gems et al. (1998) showed that *daf-16*/FOXO is also required for the quiescence phenotype of *daf-2(e1370)* adults. Therefore, we wanted to test whether *daf-16*/FOXO was required for the enhanced response of *daf-2*/InsR mutants to *dat-1* mutations.

First, we confirmed that *daf-16*/FOXO mutations increased movement in *daf-2(e1370)* adults (Fig. 5*A*). When we analyzed *daf-16(−); daf-2(−)* double mutants with the tracker, we found that *daf-16*/FOXO mutations partially rescued the decreased baseline movement of *daf-2*/InsR mutant adults (Fig. 5*B*). At $t = 60$ min, when the worms have reached a stable speed, the average speed of wild-type animals was 23.1 ± 4.6 μm/s, whereas that of *daf-2(−)* animals was 3.3 ± 0.5 μm/s. In contrast, *daf-16(−); daf-2(−)* mutants moved at 12.5 ± 2.2 μm/s. We conclude that, although there may be additional pathways involved, DAF-16/FOXO has a role in controlling quiescence in response to changes in insulin/IGF-1 signaling.

We then asked in which tissue DAF-16/FOXO acts to prevent animals from moving. To this end, we used transgenic lines expressing a *daf-16* cDNA under the control of tissue-specific promoters in a *daf-16(−); daf-2(−)* mutant background. Libina et al. (2003) previously showed that neuronal *daf-16*/FOXO expression is sufficient to restore a constitutive dauer-formation phenotype to *daf-16(−); daf-2(−)* double mutants. As expected, expression of *daf-16*/FOXO under its own promoter restored the quiescence phenotype of the *daf-2*/InsR mutant adults. Expression from the neuronal promoter *unc-119* also restored the quiescence, whereas expression from intestinal (*Pges-1*) and muscle (*Pmyo-3*) promoters did not (Fig. 5*C*). These data suggest that DAF-16/FOXO acts in neurons to slow the movement of *daf-2*/InsR mutants. We noticed that expression from the pan-neuronal promoter did not restore quiescence as well as expression from the *daf-16* promoter did. It is possible that DAF-16/FOXO activity in other tissues contributes to the regulation of quiescence or that the *unc-119* promoter we used does not drive sufficient levels of expression to give a complete rescue.

We attempted to further define the neuronal population in which DAF-16/FOXO acts to regulate dauer-like quiescence by expressing *daf-16*/FOXO under promoters specific for neuronal subpopulations in *daf-16(−); daf-2(−)* animals. We created transgenic lines in which *daf-16*/FOXO was expressed from promoters specific for acetylcholinergic (*Pacr-2*) and GABAergic (*Punc-47*) motor neurons, a wide range of interneurons (*Pglr-5*), command interneurons (*Pnmr-1*), a range of sensory neurons (*Posm-5*) and dopamine-producing neurons (*Pdat-1*). We confirmed that GFP::DAF-16 protein was de-

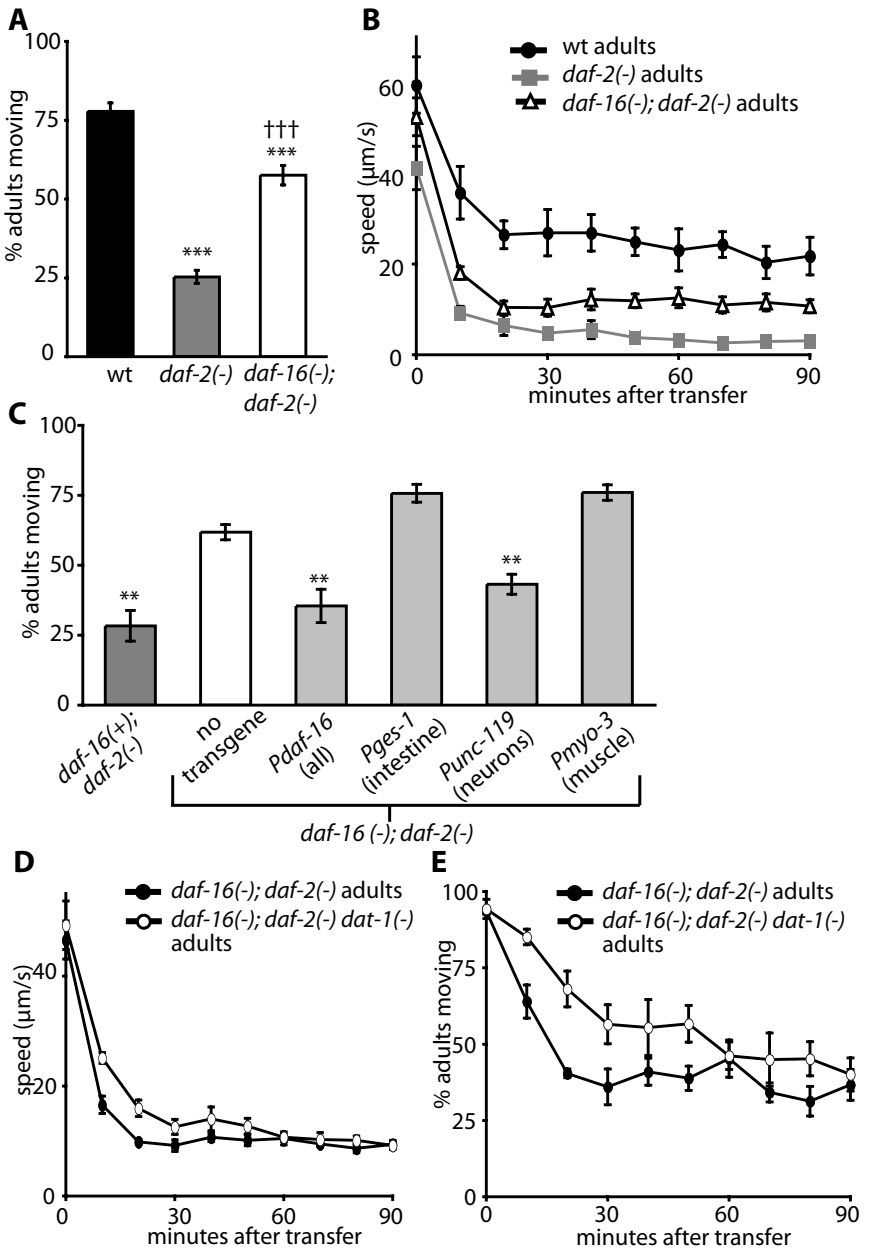

Figure 5. The role of *daf-16*/FOXO in the regulation of *daf-2*/InsR mutant dauer-like quiescence. ***A***, ***B***, *daf-16(mu86); daf-2(e1370)* double-mutant animals were more active than *daf-2(e1370)* mutants, both in the visual (***A***) and the tracker (***B***) assays, but were still less active than wt animals. ***A***, Percentage moving ± SEM: wild-type, 77 ± 3%; *daf-2(−)*, 25 ± 2%; *daf-16(−); daf-2(−)*, 57 ± 3%. ***$p < 0.0001$ versus wt control, $^{\dagger\dagger\dagger}p < 0.0001$ versus *daf-2(−)*, Dunn–Sidak's corrected *t* test. ***B***, The average speed of *daf-16(mu86); daf-2(e1370)* animals was higher than that of *daf-2(e1370)* animals but lower than that of wild-type animals, suggesting that *daf-16*/FOXO mutations can only partially suppress *daf-2(e1370)* quiescence. $p < 0.0001$ (two-way ANOVA) for *daf-16(−); daf-2(−)* versus *daf-2(−)* and *daf-16(−); daf-2(−)* versus wt. Error bars represent SEM. ***C***, Neuronal expression of *daf-16*/FOXO was sufficient for *daf-2*/InsR mutant worms to become inactive. *daf-16(−); daf-2(−)* animals carrying transgenes expressing *daf-16* cDNA under different promoters were tested against *daf-2(−)* and *daf-16(−); daf-2(−)* strains expressing only the coinjection marker *rol-6*. Expression of *daf-16*/FOXO under the control of its own promoter or the neuronal *unc-119* promoter led to quiescence, whereas intestinal (*Pges-1*) or muscle (*Pmyo-3*) expression had no effect. Percentage moving ± SEM: *daf-16(+); daf-2(−)*, 28 ± 5%; *daf-16(−); daf-2(−)*, 62 ± 3%; *daf-16(−); daf-2(−); Pdaf-16::gfp::daf-16*, 35 ± 6%; *daf-16(−); daf-2(−); Pges-1::gfp::daf-16*, 76 ± 3%; *daf-16(−); daf-2(−); Punc-119::gfp::daf-16*, 43 ± 4%; *daf-16(−); daf-2(−); Pmyo-3::gfp::daf-16*, 76 ± 3%. **$p < 0.01$ (Dunnet's corrected *t* test) versus *daf-16(−); daf-2(−)*. ***D***, ***E***, *dat-1(ok157)* mutations had a small effect on the speed (***D***) of *daf-16(mu86); daf-2(e1370)* animals but increased the percentage of animals moving (***E***). This is similar to the effect that *dat-1(ok157)* mutations had on wild-type animals. Compare Figure 5*D* with Figure 3 and supplemental Figure S2*A* (available at www.jneurosci.org as supplemental material), and Figure 5*E* with supplemental Figures S5*A* and S2*C* (available at www.jneurosci.org as supplemental material). ***D***, $p < 0.001$, two-way ANOVA. ***E***, $p < 0.0001$, two-way ANOVA. All animals were grown at 15°C, shifted to 25°C at the L4 stage, and tested at day 3 of adulthood on food. Error bars represent SEM.

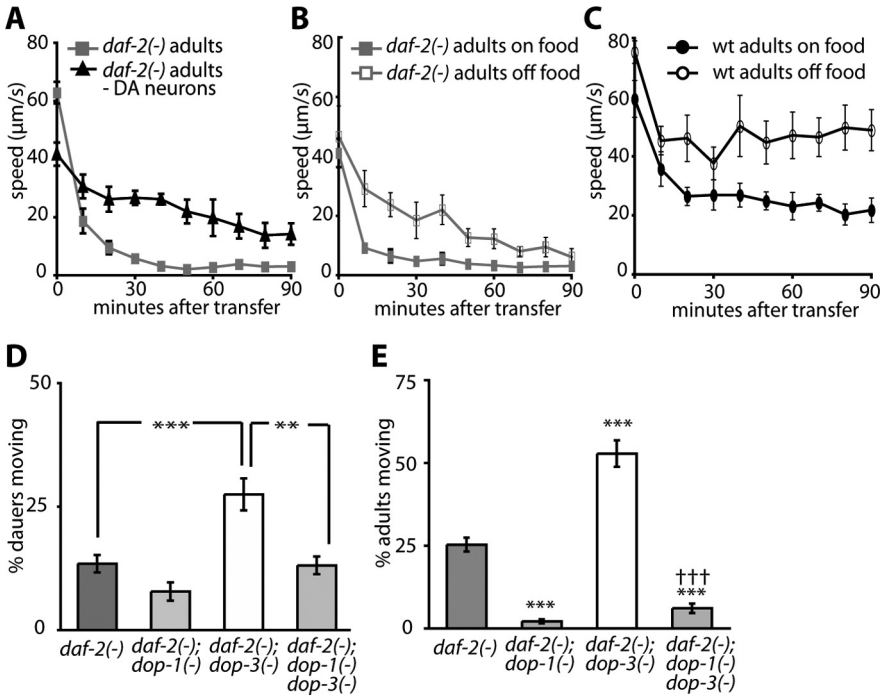

Figure 6. *daf-2(e1370)* animals display a basal slowing response and are stimulated by mutations that mimic lack of food. ***A***, Ablation of the dopamine-producing neurons by expression of a human interleukin-converting enzyme (Hills et al., 2004) in *daf-2(e1370)* animals [*daf-2(−)* −DA neurons] increased their movement as adults. $p < 0.0001$, two-way ANOVA. ***B***, ***C***, *daf-2(e1370)* (***B***) and wt (***C***) animals were transferred to plates with food ("on food") or without food ("off food") and observed for 90 min. The average speed of the worms is plotted as a function of time after transfer. As previously reported for younger wild-type animals (Sawin et al., 2000), the speed of wild-type animals was higher in the absence of food. In *daf-2(−)* animals, the average speed off food was also higher right after stimulation, but decreased over time. $p < 0.0001$ for wild type on food versus off food and *daf-2(−)* on food versus off food (two-way ANOVA). ***D***, *daf-2(e1370); dop-3(vs106)* dauers moved more than *daf-2(e1370)* dauers, but their increased movement was suppressed by *dop-1(vs100)* mutations. Percentage moving ± SEM: *daf-2(−)*, 14 ± 2%; *daf-2(−); dop-1(−)*, 8 ± 2%; *daf-2(−); dop-3(−)*, 27 ± 3%; *daf-2(−); dop-1(−) dop-3(−)*, 13 ± 2%. ***$p < 0.0001$ and **$p < 0.01$, Dunn–Sidak's corrected *t* test. ***E***, Mutations in the dopamine receptor *dop-3* caused *daf-2(e1370)* adults to move more. Conversely, mutations in the dopamine receptor *dop-1* caused them to move even less. *daf-2(e1370); dop-1(vs100) dop-3(vs106)* animals moved similarly to *daf-2(e1370); dop-1(vs100)* animals, suggesting that *dop-1* acts downstream of *dop-3* in the regulation of *daf-2(e1370)* adult quiescence. Percentage moving ± SEM: *daf-2(−)*, 25 ± 2%; *daf-2(−); dop-1(−)*, 2 ± 1%; *daf-2(−); dop-3(−)*, 53 ± 4%; *daf-2(−); dop-1(−) dop-3(−)*, 6 ± 1%. ***$p < 0.0001$ versus *daf-2(−)*, $^{\dagger\dagger\dagger}p < 0.0001$ for *daf-2(−); dop-3(−)* versus *daf-2(−); dop1(−) dop-3(−)*, Dunn–Sidak's corrected *t* test. All adult animals were grown at 15°C, shifted to 25°C at the L4 stage, and tested at day 3 of adulthood. Error bars represent SEM.

tectable in the expected cells in all the lines we tested. Unlike the pan-neuronal *daf-16*/FOXO-expressing line, none of these neuron-specific lines showed a significant reduction of movement compared with *daf-16(−); daf-2(−)* animals (supplemental Fig. S3, available at www.jneurosci.org as supplemental material), although several of them showed a small decrease in the percentage of worms moving. (For more details, see the legends of the supplemental figures, available at www.jneurosci.org as supplemental material.) These data suggest that DAF-16/FOXO may act in more than one type of neuron to regulate dauer-like quiescence; alternatively, it is possible that we have not tested the right neuronal population.

Loss of *daf-16*/FOXO causes *daf-2*/InsR mutants to respond more like wild type to *dat-1* mutations

Because *daf-16*/FOXO and *dat-1* mutations both partially suppress the quiescence phenotype of *daf-2*/InsR mutant adults, and both of them act in neurons, we tested how *daf-16(−); daf-2(−)* animals behaved when the *dat-1* dopamine transporter was removed. We found that the average speed of *daf-16(−); daf-2(−) dat-1(−)* mutants was only slightly higher than that of *daf-16(−); daf-2(−)* mutants (Fig. 5*D*), which is similar to the situation in wild-type animals (supplemental Fig. S2*A*, available at www.jneurosci.org as supplemental material). In addition, as in wild type (supplemental Fig. 2*C*, available at www.jneurosci.org as supplemental material), a greater percentage of *daf-16(−); daf-2(−) dat-1(−)* triple mutants moved at early time points compared with *daf-16(−); daf-2(−)* double mutants (Fig. 5*E*). These data indicate that *dat-1* mutations affect *daf-16(−); daf-2(−)* mutants similarly to the way they affect wild type. These data suggest that *daf-16*/FOXO is necessary for *daf-2*/InsR mutant animals to respond to increased dopamine signaling with increased movement.

daf-2/InsR mutants have a normal basal slowing response

Our data indicate that *dat-1* mutations and exogenous dopamine addition can stimulate movement in *daf-2*/InsR mutant dauers and adults. However, as mentioned previously, previous studies of the dopamine system in *C. elegans* have focused on the animals' requirement for dopamine to slow their movement in response to food, an effect that has been called the "basal slowing response" (Sawin et al., 2000). In this context, reduction of dopamine increases the speed of the animals. We wondered what role this response played in dauer and dauer-like animals. Indeed, we found that reduction in dopamine signaling because of ablation of dopamine-producing neurons also increased the movement of *daf-2*/InsR mutant dauers (Fig. 2*A*) and adults [Fig. 6*A*, as well as by visual scoring: percentage moving ± SEM: *daf-2(−)* +DA neurons, 39 ± 10%; *daf-2(−)* −DA neurons, 80 ± 3%; $p < 0.05$, Student's *t* test].

We confirmed that the behavioral change seen in *daf-2(−)* animals with no dopamine neurons is attributable to the loss of dopamine, because when we compared the movement of *daf-2(−)* animals to *daf-2(−)* animals with no dopamine neurons in the presence of exogenous dopamine (30 mM), there was no longer any difference between the two strains (supplemental Fig. S4, available at www.jneurosci.org as supplemental material).

To explain the apparent paradox that both increasing and decreasing dopamine signaling leads to increased movement in dauers and dauer-like adults, we hypothesized that there are two different mechanisms at play. On the one hand, for reasons that are not understood, increases in dopamine preferentially stimulate the movement of *daf-2*/InsR mutant dauers and adults. However, loss of dopamine-producing neurons speeds up *daf-2*/InsR mutant adults, just as it does wild-type animals, because loss of dopamine mimics the absence of food, which in turn stimulates movement.

To test this hypothesis, we asked whether *daf-2*/InsR mutant animals have a basal slowing response, that is, whether they move more in the absence of food. We found that this was the case, as

the average speed of *daf-2(−)* adults was higher when the animals were off food than when they were on food (Fig. 6*B*). However, *daf-2(−)* animals still eventually stopped moving in the absence of food (Fig. 6*B*) (average population speed ± SEM at $t = 90$ min: on food, 3.1 ± 0.3 μm/s, vs off food, 6.1 ± 2.7 μm/s; $p = 0.35$, Student's *t* test), which indicated that absence of food did not alter baseline movement but rather the response of animals to stimuli. Interestingly, loss of the dopamine neurons seemed to have some effect on both the stimulation-induced movement and the baseline movement of the animals (Fig. 6*A*) [average population speed ± SEM at $t = 90$ min: *daf-2(−)*, 3.0 ± 0.1 μm/s, vs *daf-2(−)* −DA neurons, 14.2 ± 3.7 μm/s; $p = 0.05$, Student's *t* test]. It is thought that food removal reduces dopamine secretion in the animals and that in turn this causes the increase in movement (Sawin et al., 2000). Perhaps baseline movement is not affected in *daf-2(−)* animals in the absence of food because there is residual dopamine secretion or because dopamine secretion can resume at a later time. In contrast, no dopamine secretion is ever present in animals without dopamine neurons.

Next, we tested whether other mutations in dopamine-signaling genes known to affect the basal slowing response in wild-type animals also affected the locomotion of *daf-2*/InsR mutant animals. We found that mutations in the dopamine receptor *dop-3* (Chase et al., 2004), which prevent slowing in response to food, increased the percentage of *daf-2(e1370)* dauers and dauer-like adults that were moving (Fig. 6*D*,*E*). Furthermore, we found that in both types of animals the increased motility produced by *dop-3* mutations was dependent on another dopamine receptor, *dop-1* (Fig. 6*D*,*E*). In the basal slowing response of nondauer animals, *dop-3* mutations have a similar epistatic relationship with mutations in *dop-1* (Chase et al., 2004). In addition, we tested whether DOP-1 and DOP-3 acted in acetylcholine-producing motor neurons to modulate *daf-2*/InsR mutant quiescence and found that they did: *daf-2(−); dop-1(−)* adults expressing *dop-1* in the motor neurons and *daf-2(−); dop-3(−)* adults expressing *dop-3* in the motor neurons moved as much as *daf-2(−)* mutants alone (supplemental Fig. S5*D*, available at www.jneurosci.org as supplemental material). DOP-1 and DOP-3 also act in the motor neurons in the context of the basal slowing response (Chase et al., 2004). Together, these data suggest that dopamine-pathway mutations that prevent the slowing response to food may affect the quiescence of *daf-2*/InsR mutant animals because they mimic food withdrawal. They also suggest that *daf-2*/InsR-mutant quiescence is not simply an exaggerated response to food, as quiescence is displayed in the absence of food as well. Instead, it appears that a reduction in dopamine signaling can stimulate movement equally in *daf-2*/InsR mutant animals and in wild-type animals. In contrast, *daf-2*/InsR mutant animals may respond in a different manner from wild type to increases in dopamine signaling.

DOP-3 receptors may also mediate the effect of increased dopamine on *daf-2*/InsR mutant quiescence

Because we showed that loss of some dopamine receptors affects the motility of *daf-2*/InsR mutant dauer and dauer-like adults, we wanted to examine the relative contributions of the receptors to the reduced locomotion of *daf-2(−)* animals and to the stimulated locomotion of *daf-2(−) dat-1(−)* animals. First, we tested animals carrying mutations in all four known *C. elegans* dopamine receptors [henceforth we will refer to all the *dop-2(−); dop-4(−) dop-1(−) dop-3(−)* animals as "*dop-(1–4)(−)*" for simplicity] (Suo et al., 2002, 2003; Chase et al., 2004; Sugiura et al., 2005). Loss of the four receptors, like loss of dopamine neurons, stimulated movement in *daf-2(−)* animals [compare *daf-2(−); dop-(1–4)(−)* animals with *daf-2(−)* single mutants (Fig. 7*A*)]. These animals could not be further stimulated to move by increases in dopamine [compare *daf-2(−) dat-1(−); dop-(1–4)(−)* animals with *daf-2(−); dop-(1–4)* animals (Fig. 7*A*)]. This suggests that one or more of the dopamine receptors identified so far mediates the effect of *dat-1* mutation on *daf-2*/InsR mutant adult quiescence.

In *daf-2(−)* dauers, loss of the dopamine receptors also decreased the effect that the *dat-1* mutation had on movement (Fig. 7*B*), although the effect was less dramatic than in dauer-like adults (Fig. 7*A*). Another important difference between dauers and dauer-like adults was that *daf-2(−); dop-(1–4)(−)* dauers did not move significantly more than did *daf-2(−)* single mutant dauers (Fig. 7*B*). Because the *dop-(1–4)(−)* genotype did not produce the same effect as loss of dopamine neurons (compare, for example, Figs. 2*A*, 7*B*), there may be additional, yet unidentified, dopamine receptors. This interpretation is consistent with another observation we made. Namely, we found that *dop-(1–4)(−)* animals still became paralyzed in a dopamine-induced paralysis assay (supplemental Fig. S6, available at www.jneurosci.org as supplemental material). Alternative explanations for these data are that some of these mutations may not be null or that one of the receptors has ligand-independent activity.

We then compared *daf-2(−)* and *daf-2(−) dat-1(−)* mutant animals that lacked single receptors. Our finding differed slightly between dauer larvae and dauer-like adults (Fig. 7*C*,*D*). *dop-3* receptor mutations had dramatic effects on both dauers and adult animals, as *daf-2(−) dat-1(−); dop-3(−)* dauers and adults did not move any more than did *daf-2(−); dop-3(−)* dauers and adults (Fig. 7*C*,*D*). This suggests that DOP-3 has an important role not only in *daf-2*/InsR mutant quiescence, as mentioned in the previous section, but also in the stimulation of locomotion caused by increase in dopamine. Conversely, we found that loss of the DOP-2 receptor appeared to no effect on dauer quiescence and a minor effect on adult quiescence; also, it did not prevent the stimulation of movement caused by dopamine (Fig. 7*C*,*D*).

The two other receptors, DOP-1 and DOP-4, had striking effects only in the adult animals. *dop-4* mutations completely blocked the increased movement of *daf-2(−) dat-1(−)* adults (Fig. 7*D*), suggesting that DOP-4 plays an important role. *dop-1* receptors further decreased the motility of *daf-2(−)* adults and *daf-2(−) dat-1(−)* adults (Fig. 7*D*). Because the effect of *dop-1* mutations on both *daf-2(−)* animals and *daf-2(−) dat-1(−)* animals appeared similar in magnitude, we suggest that this effect is independent of any increase in dopamine levels. We do not know the reason for the discrepancy between the effects of dopamine receptor mutations on dauer and adult animals, but these data suggest that there are some significant differences between dauers and dauer-like adults.

Discussion

In this study, we investigated the effects of dopamine signaling on a behavioral condition reminiscent of hibernation in *C. elegans*; namely, the quiescence of *daf-2*/InsR mutant dauer larvae and dauer-like adults. Both types of animals display a remarkable and mostly uncharacterized decrease in spontaneous movement. We found that perturbations in dopamine signaling have dramatic effects on *daf-2*/InsR mutant dauer and adult movement. Paradoxically, both lack of dopamine-producing neurons, which should prevent dopamine production, and reduced clearance of dopamine from the extracellular medium, which should increase

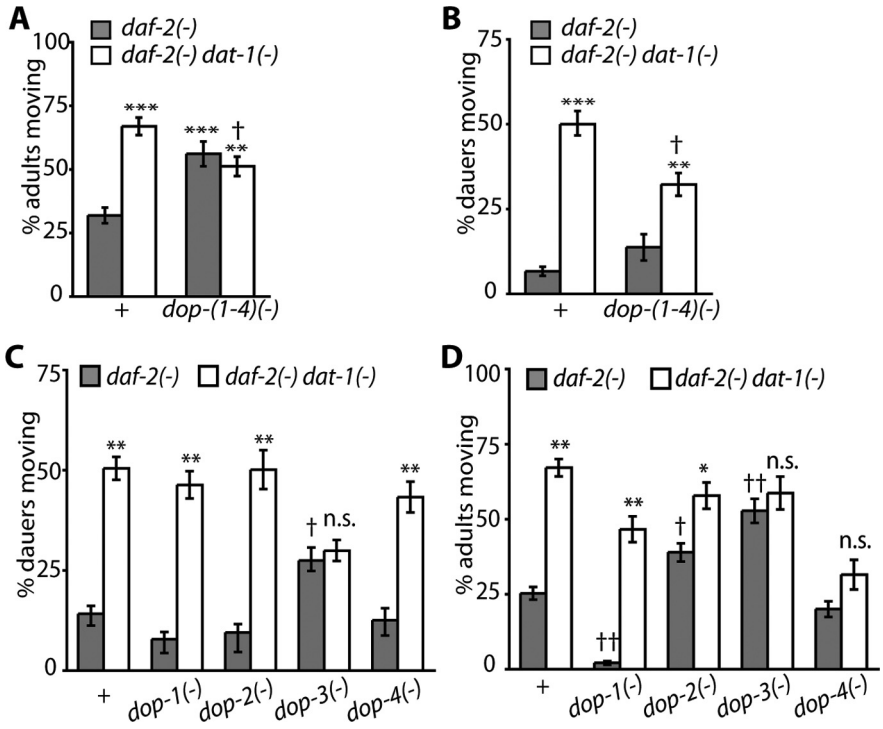

Figure 7. The DOP-3 receptor may be required for *dat-1* mutations to increase *daf-2*/InsR mutant dauer and dauer-like adult motility. ***A***, Mutating all four known dopamine receptors [*dop-(1–4)(−)*] increased the movement of *daf-2(−)* adults. The effects of *dat-1(−)* mutation and of mutation of the four dopamine receptors on *daf-2(−)* adult quiescence were not additive. Percentage moving ± SEM: *daf-2(e1370)*, 32 ± 3%; *daf-2(e1370); dop-2(vs105); dop-4(ok1321) dop-1(vs100) dop-3(vs106)*, 56 ± 3%; *daf-2(e1370) dat-1(ok157)*, 67 ± 5%; *daf-2(e1370) dat-1(ok157); dop-2(vs105); dop-4(ok1321) dop-1(vs100) dop-3(vs106)*, 51 ± 4%. $^{***}p < 0.0001$ and $^{**}p < 0.01$ versus *daf-2(−)* strain; $^{\dagger}p < 0.05$ versus *daf-2(−) dat-1(−)*, Dunn–Sidak's corrected *t* test. *daf-2(−); dop-(1–4)(−)* and *daf-2(−) dat-1(−); dop-(1–4)(−)* were not significantly different from each other ($p = 0.376$). ***B***, Mutating all the four known *C. elegans* dopamine receptors [*dop-(1–4)(−)*] partially prevented the increase in motility of *daf-2(−) dat-1(−)* dauers. Percentage moving ± SEM: *daf-2(−)*, 7 ± 1%; *daf-2(e1370); dop-2(vs105); dop-4(ok1321) dop-1(vs100) dop-3(vs106)*, 14 ± 4%; *daf-2(e1370) dat-1(ok157)*, 50 ± 4%; *daf-2(e1370) dat-1(ok157); dop-2(vs105); dop-4(ok1321) dop-1(vs100) dop-3(vs106)*, 32 ± 3%. $^{***}p < 0.0001$ and $^{**}p < 0.01$ for each *daf-2(−) dat-1(−)* strain versus the corresponding *daf-2(−)* strain. $^{\dagger}p < 0.05$ versus *daf-2(−) dat-1(−)*, Dunn–Sidak's corrected *t* test. ***C***, Loss of the DOP-3 receptor prevented *dat-1* mutations from increasing the motility of *daf-2*/InsR mutant dauers. Percentage moving ± SEM: *daf-2(e1370)*, 14 ± 2%; *daf-2(e1370) dat-1(ok157)*, 50 ± 3%; *daf-2(e1370); dop-1(vs100)*, 8 ± 2%; *daf-2(e1370) dat-1(ok157); dop-1(vs100)*, 46 ± 3%; *daf-2(e1370); dop-2(vs105)*, 10 ± 2%; *daf-2(e1370) dat-1(ok157); dop-2(vs105)*, 50 ± 5%; *daf-2(e1370); dop-3(vs106)*, 27 ± 3%; *daf-2(e1370) dat-1(ok157); dop-3(vs106)*, 30 ± 3%; *daf-2(e1370); dop-4(ok1321)*, 13 ± 3%; *daf-2(e1370) dat-1(ok157); dop-4(ok1321)*, 43 ± 4%. $^{**}p < 0.01$ and $^{n.s.}p > 0.05$ for each *daf-2(−) dat-1(−)* strain versus the corresponding *daf-2(−)* strain; $^{\dagger}p < 0.05$ versus *daf-2(−)*, Dunn–Sidak's corrected *t* test. ***D***, Loss of the receptors DOP-3 and DOP-4 prevented *dat-1* mutations from increasing the motility of *daf-2*/InsR mutant adults, whereas *dop-1* mutations decreased the motility of both *daf-2(−)* and *daf-2(−) dat-1(−)* adults. Percentage moving ± SEM: *daf-2(e1370)*, 25 ± 2%; *daf-2(e1370) dat-1(ok157)*, 67 ± 3%; *daf-2(e1370); dop-1(vs100)*, 2 ± 1%; *daf-2(e1370) dat-1(ok157); dop-1(vs100)*, 47 ± 4%; *daf-2(e1370); dop-2(vs105)*, 39 ± 3%; *daf-2(e1370) dat-1(ok157); dop-2(vs105)*, 58 ± 4%; *daf-2(e1370); dop-3(vs106)*, 53 ± 4%; *daf-2(e1370) dat-1(ok157); dop-3(vs106)*, 59 ± 5%; *daf-2(e1370); dop-4(ok1321)*, 20 ± 3%; *daf-2(e1370) dat-1(ok157); dop-4(ok1321)*, 31 ± 5%. $^{**}p < 0.01$, $^{*}p < 0.05$, and $^{n.s.}p > 0.05$ for each *daf-2(−) dat-1(−)* strain versus the corresponding *daf-2(−)* strain. $^{\dagger\dagger}p < 0.01$ and $^{\dagger}p < 0.05$ versus *daf-2(−)*, Dunn–Sidak's corrected *t* test. All adult animals were grown at 15°C, shifted to 25°C at the L4 stage, and tested at day 3 of adulthood. Error bars represent SEM.

dopamine signaling, increase the percentage of *daf-2*/InsR mutant dauers and adults that move. Changes in dopamine signaling affect the animals' ability to sustain an increase in motion after stimulation, rather than their unstimulated, baseline quiescence. This finding is significant because it suggests that dopamine specifically affects the response of these animals to environmental cues. In addition, it indicates that changes in dopamine do not affect the movement of *daf-2(−)* animals by somehow suppressing the primary *daf-2-(−)* defect through upregulation of insulin/IGF-1 signaling.

We also found that *daf-16*/FOXO, a transcription factor that mediates most of the effects of *daf-2*/InsR mutations, controls the unstimulated baseline movement in *daf-2*/InsR mutant animals. Therefore, mutations in *daf-16*/FOXO and dopamine signaling affect *daf-2*/InsR mutant quiescence in different ways.

Dauer-like quiescence is probably not caused by changes in dopamine signaling

If the quiescence of dauers and that of dauer-like adults were directly caused by a change in dopamine signaling within the animal, it should be possible to produce dauer-like quiescence simply by altering dopaminergic pathways. However, neither the addition of exogenous dopamine, the *dat-1* mutation, loss of dopamine-producing neurons, nor mutation of any dopamine receptors produced a quiescent dauer-like phenotype. Although quiescence could conceivably be caused by a more subtle change in dopamine signaling, it seems most likely that dauer-like quiescence is caused by changes in nondopaminergic pathways. Also, our results suggest that perhaps different neuronal populations are required for the distinct regulation of baseline and stimulated movement in quiescent *daf-2*/InsR mutant dauers and adults, because the dopaminergic system appears to have preferential effects on movement in response to stimulation.

The basal slowing response is intact in *daf-2*/InsR mutants

The loss of dopamine-producing neurons increases the movement of dauer-like animals, and we attribute this effect to mimicry of a food-deprived state. Dopamine is required for worms to slow down when they encounter food, and as a consequence, the increased locomotion of animals that lack dopamine consistently resembles that of animals deprived of food (Sawin et al., 2000; Chase et al., 2004; Li et al., 2006). The slowing response to food, called the basal slowing response, is thought to ensure that animals stay on food when they encounter it. We found that *daf-2*/InsR mutant animals display a basal slowing response, as their average speed is higher off food than on food. (However, food withdrawal is not sufficient to prevent quiescence, as *daf-2*/InsR mutant animals eventually become quiescent whether they are on food or not.) In addition, we found that mutations in the *dop-3*-receptor and *dop-1*-receptor genes affect *daf-2*/InsR mutant quiescence in a similar manner to the way they affect the basal slowing response in wild-type nondauer animals (Chase et al., 2004). We conclude that, in *daf-2*/InsR mutant animals, the dopamine circuit that controls the basal slowing response is mostly intact.

dat-1 mutations affect the quiescence of *daf-2*/InsR mutants through a novel mechanism

Our results suggest that *dat-1* mutations increase the locomotion of dauer-like *daf-2*/InsR mutant animals by a different mechanism, one that involves higher extracellular dopamine levels. Because *dat-1* mutant animals can modulate their locomotion in response to food similarly to wild-type animals (supplemental Fig. S2*A*,*B*, available at www.jneurosci.org as supplemental material), it is unlikely that the increase in extracellular dopamine simply leads to a compensatory decrease in dopamine signaling that mimics the basal slowing response (Jones et al., 1998). In addition, other phenotypes displayed by *dat-1* mutants are consistent with their having elevated levels of dopamine signaling, such as slowed habituation on food (Kindt et al., 2007) and swimming-induced paralysis (McDonald et al., 2007). Therefore, we conclude that *dat-1* mutations increase movement in *daf-2*/InsR mutants by a mechanism that is novel and unrelated to the basal slowing response.

Because wild-type worms move more frequently and faster than *daf-2*/InsR animals, one possibility is that an increase in movement may be easier to detect in the quiescent *daf-2(−)* animals. However, it is possible to increase the speed of locomotion in wild type: for example, by food withdrawal, ablation of dopamine-producing neurons, and mutations in *dop-3* (Chase et al., 2004). Therefore, we reasoned that a stimulation of movement by dopamine should be detectable. We hypothesize that *daf-2*/InsR mutant dauers and adults are programmed to respond differently from nondauers to increases in dopamine signaling. The dopamine receptor DOP-3 appears to mediate the stimulating effect of increased dopamine signaling in *daf-2*/InsR mutant animals, and another receptor, DOP-4, may also be important, although only in adult animals.

In mammals, dopamine has been studied intensively both for its control of voluntary movement and for its role in reward pathways that underlie, among other things, drug-seeking behavior. It is possible that considering dopamine to be a reward signal in the context of dauer movement might explain how opposite interventions, decreasing and increasing dopamine, both result in similar behavioral outcomes. Also, it may explain why, whereas in *C. elegans* dopamine is generally thought to decrease movement (Schafer and Kenyon, 1995), in most other organisms dopamine is known to increase movement (Giros et al., 1996; Kume et al., 2005). Increases in dopamine when the worm encounters food (i.e., during the basal slowing response) may serve as a "reward" stimulus, encouraging the worm to consume the food rather than to keep moving. In dauers, however, where energy conservation and quiescence are the basal state, an additional system of rewards may be necessary to stimulate the worms to move in the first place, and thus dopamine release may be activated by particularly strong stimuli. For these two systems to coexist, the dopamine signaling system would need to be altered in dauer and dauer-like animals so as to make the animals more sensitive to the stimulatory effects of dopamine. Our data suggest that this alteration may be attributable to changes in the activity of the *C. elegans daf-2*/InsR pathway.

Our findings indicate that the DAF-16/FOXO transcription factor, which is known to be activated by reduction of insulin/IGF-1 signaling, is required for *daf-2* mutants to move faster in response to increased dopamine signaling (i.e., *dat-1* mutations). We observed that, in *daf-16(−); daf-2(−)* double mutants, which move more like wild type, *dat-1* mutations increase the percentage of animals moving, but have only a small effect on their speed. This resembles the situation in wild-type animals. In addition, restoring *daf-16*/FOXO in the neurons is sufficient for *daf-16(−); daf-2(−)* double mutants to become quiescent. Thus, transcriptional changes controlled by DAF-16/FOXO in neurons may underlie the different responses to dopamine displayed by *daf-2*/InsR mutants. DAF-16/FOXO and DAF-2/InsR are both expressed widely in the nervous system and thus could affect dopamine signaling by changing gene expression in cells that release or respond to dopamine. However, the insulin/IGF-1 signaling pathway could also regulate the dopamine pathway in a cell-nonautonomous manner, as it does in its regulation of life span and dauer formation (Apfeld and Kenyon, 1998; Wolkow et al., 2000; Libina et al., 2003).

We tested whether any of the known components of the dopamine pathway are targets of DAF-16/FOXO. Two genes were downregulated in *daf-2*/InsR mutant animals compared with wild-type animals: *bas-1*, an aromatic amino acid decarboxylase required for synthesis of dopamine and serotonin, and *dop-2*, one of the dopamine receptors (data not shown) [also reported in McElwee et al. (2004)]. However, these changes do not explain our findings, as there was no detectable difference in the overall levels of dopamine in wild-type versus *daf-2(e1370)* animals (supplemental Fig. S7, available at www.jneurosci.org as supplemental material) (tested as day 3 adults by HPLC analysis), although the data do not rule out more subtle changes in distribution of dopamine in the animal. In addition, *daf-2(−) dat-1(−); dop-2(−)* animals still moved faster than *daf-2(−); dop-2(−)* animals (Fig. 7*C*,*D*). It would be interesting to test all candidate DAF-16-regulated genes for their effects on quiescence, but because RNA interference does not work well in neurons (Timmons et al., 2001), this is not a simple task. Perhaps critical genes could be recovered in a screen for mutations that stimulate the movement of quiescent *daf-2(−)* animals.

Insulin regulation of dopamine signaling in other organisms

The idea that the insulin/IGF-1 signaling pathway can alter the activity of the dopamine pathway in *C. elegans* is compatible with the emerging idea that, in the mammalian brain, hormonal signals involved in energy balance, including insulin, can affect the dopamine reward pathway to modulate feeding behavior (Figlewicz et al., 2007; Palmiter, 2007). Food-restricted animals, which have lower circulating insulin levels, behave as though they have elevated dopamine signaling. However, the mechanism by which insulin signaling acts at the cellular level to influence dopamine signaling is still unclear.

In the mammalian brain, some of the effects of dopamine are not attributable to simple increase or decrease of dopamine, but rather to changes in the kinetics of dopamine release (Goto et al., 2007). In *C. elegans*, many of the subtleties of dopamine signaling kinetics that are important for dopamine signaling in other organisms (Goto et al., 2007) are still unexplored, because all studies, including this one, have relied on generalized loss of dopamine production or unspecific increases in dopamine levels. Therefore, the study of *daf-2*/InsR mutant movement in response to stimulation may be an interesting paradigm in which to explore the worm's dopamine system in more detail.

Implications for hibernation

Quiescent *C. elegans* dauers resemble animals in hibernation in many ways (Carey et al., 2003), although, unlike in *C. elegans*, hibernation in higher animals does not occur only at a specific prepubescent developmental stage. Interestingly, neurons of hibernating mammals have decreased activity of Akt (Lee et al., 2002; Eddy and Storey, 2003; Cai et al., 2004; Abnous et al., 2008),

which is one of the kinases that inhibits DAF-16/FOXO under replete conditions. Thus, hibernation and the dauer diapause may be similar at a molecular level. It is even possible that hibernation is potentiated, in part, by a reduction in insulin/IGF-1 signaling caused by nutrient deprivation or other stressful conditions. During hibernation, suppression of movement is important to prevent energy expenditure, but little is known about how suppression of movement is regulated in this context. It would be interesting to know whether hibernating animals are particularly susceptible to the stimulatory effects of dopamine. Likewise, in *C. elegans*, it would be interesting to know whether there are environmental stimulants that activate quiescent dauers by elevating dopamine signaling.

It seems possible that the ability to suppress movement is an ancient response to prolonged environmental stress that is expressed in the context of the dauer in *C. elegans* and in the context of hibernation (and possibly sleep) in other animals. If so, then understanding the neural pathways that regulate dauer behavior, which are genetically accessible, may have more general implications for the behavior of higher organisms.

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