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Episodic swimming behavior in the nematode *C. elegans*

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SUMMARY

Controlling the choice of behavioral output is a central function of the nervous system. Here we document a novel spontaneous behavioral transition in *C. elegans* locomotion. Upon transfer of the nematode from a solid surface into a liquid environment, swimming occurs in two phases: an initial, 1–2 h phase of continuous swimming, followed by a second phase during which swimming is episodic. During the second, episodic phase, periods of active swimming alternate in a highly regular fashion with a quiescent state lasting for several minutes. We analyzed the nature of the quiescent state and the basis for spontaneous switching between swimming and quiescence. The transition from swimming to quiescence is promoted by acetylcholine signaling and initially during quiescence body wall muscles are in a state of contraction. After the first minute, quiescent worms respond to prodding and resume swimming normally. The major command interneurons that control the locomotory circuits are not necessary for quiescence since swimming–quiescence cycling occurs after ablation of command interneurons. However, when subsets of neurons including the command interneurons are killed, the switching pattern becomes less regular, suggesting that a timer governing switching may lie within circuitry controlling motor neurons. The results show that the motor circuits have a tendency to switch spontaneously between active and inactive behavioral states. This property might be important to the animal in a uniform environment where sensory input is invariant.

Supplementary material available online at http://jeb.biologists.org/cgi/content/full/211/23/3703/DC1

Key words: locomotory behavior, episodic behavior, acetylcholine, command interneuron, behavioral quiescence.

INTRODUCTION

Animal nervous systems can generate a great variety of behavioral outputs and are presented at each moment with the problem of deciding which to express. With more complex animals this process encompasses choices and decisions governed by motivational states and drives (Kupferman et al., 2004; Schall, 2001). With less complex invertebrates, selection among stereotyped behavioral patterns may be regarded as switches between well-defined behavioral states (Calabrese, 2003; Garcia et al., 2001). Rhythm-generating circuits are examples of neural networks with simple behavioral output that undergoes well-defined state transitions, for example, between episodes of activity and inactivity. These behavioral transitions may be governed by sensory input, internal conditions *via* neuromodulators, or interactions with other oscillating circuits or with command interneurons (Briggman et al., 2005; Calabrese, 1998; Marder et al., 2005; Staras et al., 2003).

Study of the regulation of behavior in the nematode *C. elegans* takes advantage of the fact that its behaviors are relatively simple and its nervous system contains a constant number of neurons whose pattern of synaptic connectivity is known (Chen et al., 2006; White et al., 1986). During navigation on a solid surface, switches between forward and backward locomotion are controlled by command interneurons that synapse onto excitatory motor neurons, but can also occur within the motor neuron circuitry itself (Brockie et al., 2001a; Chalfie et al., 1985; Chalfie and White, 1988; Von Stetina et al., 2006; Zheng et al., 1999b). In a non-uniform environment, switching between forward and backward locomotion and a sharp turn is influenced by sensory input acting through interneuron circuitry situated upstream of the command interneurons (Gray et al., 2005a; Tsalik and Hobert, 2003; Wakabayashi et al., 2004).

Here we document a novel behavioral transition in *C. elegans* locomotion. We show that when *C. elegans* and several additional nematode species swim in liquid, their locomotion is episodic: periods of active swimming and extended periods of inactivity spontaneously alternate with great regularity. We show that quiescence appears to be induced by a mechanism acting at the level or downstream of the motor neurons, and is induced by high levels of acetylcholine (ACh). The timing of swimming–quiescence cycling may be controlled in part by the command interneurons.

MATERIALS AND METHODS

Behavioral assays

Nematodes were reared at 20°C on *E. coli* 0P50 on NGM agar plates (Brenner, 1974). For the swimming assay, adults were transferred to a fresh unseeded plate at 25°C and allowed to forage for 2 min, after which they were picked singly into wells of a Costar microtiter plate containing 200 µl M9 buffer at 25°C (Brenner, 1974). The assays were carried out in a room where the temperature was maintained between 24°C and 25°C.

To score swimming *versus* quiescent behavior, worms were observed visually for 5 s every minute with a dissecting microscope (Wild Heerbrugg, Gais, Switzerland). The assay duration was at least 3 h except where noted otherwise. The cutoff for the transition from swimming to quiescence was less than or equal to 2 body bends per 5 s. Transitions in both directions were distinct. During the quiescent interval, worms remained motionless except for a slow relaxation towards a straight posture. More than 2 but less than 5 body bends per 5 s was scored as slow swimming. Greater than 5 body bends per 5 s was considered as swimming. Muscle contraction resulting in a bend on one side (dorsal or ventral) was

considered as 1 body bend. During swimming, the wild-type worm generally made 3.5–4 body bends s^{-1}, hence swimming was clearly distinguishable from quiescence. In certain mutants the swimming was poor, for example worms made fewer body bends per second or they swam with a different posture. In scoring and analyzing the data for such worms, a swimming worm was one that was moving but did not satisfy the criteria of quiescence as defined above. In prodding assays, quiescent worms were prodded 2 times at either the posterior or the anterior end with a platinum wire.

Data analysis

Histograms of swimming and quiescent bout durations include all swimming bouts excluding the first swimming bout and all quiescent bouts for at least 3 h of assay (unless mentioned otherwise) plotted with 2 min bins. In the text, average and standard error of the mean are given unless noted otherwise. The D'Agostino and Pearson omnibus normality test with α=0.05 was used to decide the subsequent statistical analysis to be performed on log-transformed data sets. In cases where the data sets did not pass the normality test, the Kruskal–Wallis test followed by Dunn's multiple comparison *post-hoc* test was performed to determine the two-tailed *P*-value. In cases where two groups were compared, a Mann–Whitney *U*-test was performed. These analyses were done using the Graphpad Prism version 4.00 software for Macintosh. If a data set passed the normality test, ANOVA with Bonferroni–Dunn *post-hoc* test was used to determine significance. These analyses were performed with Statview 5.0 software.

Measurement of body length

Worm images on a dissecting microscope were recorded with a Videolabs analogue camera attached to a Toshiba VHS recorder. Recorded movies were converted into ImageJ-compatible format using iMovie (5.0.2, Apple Computers, www.apple.com/ilife/imovie/). Animals were measured while swimming and after entering quiescence and the two measured lengths compared.

Laser ablations

Laser ablations followed published procedures (Bargmann and Avery, 1995).

Pharmacological analysis

The GABA agonist muscimol (Sigma) and the Ach agonist levamisole (Sigma) in M9 buffer were added directly to the liquid in which the worm was swimming to achieve a final concentration or included in the assay buffer initially. To determine the effect of the drug on the length of quiescence, worms were allowed to go into quiescence in 100 µl M9 buffer for 1 min and drugs were added in a volume of 100 µl. To control for the possible effect of disturbance of the liquid, experiments are compared with control data in which 100 µl of buffer without drug was added.

RESULTS

In a liquid environment, worms alternate between swimming and quiescence

When suspended in liquid, a worm swims with an undulatory motion that resembles its crawling motion on a solid substrate (Fig. 1A) (Burr and Forest Robinson, 2004; Gray and Lissmann, 1964; Korta et al., 2007; Tsechpenakis et al., 2008). By observing worms in liquid over longer periods, we found that after transfer from a solid surface into liquid, a worm initially swam continuously under our assay conditions for a period of 92.6±2.5 min (s.e.m., *N*=109), after which it stopped and lay quiescent (Fig. 1B). The transition from swimming to quiescence was distinct and abrupt, lasting an average of 6.9±1.3 s (*N*=7). During quiescence, a worm maintained a straight rather than sinusoidal posture, often with a slight bend that gradually straightened (Fig. 1B). The quiescent interval lasted for several minutes, after which the worm spontaneously and equally abruptly started swimming again (supplementary material Movie 1). After the initial longer swimming bout and first quiescent bout, worms entered a period of alternating swimming and quiescence, a pattern that has been observed to go on for over 7 h. Over longer durations (>4 h), periods of swimming gradually declined and periods of quiescence became longer (Fig. 1C). We focused our study on the initial 4 h interval after transfer into liquid, during which time, after the first swimming bout, there was little change. During this interval, the periods of swimming and quiescence were highly regular (Fig. 2A). Excluding the first swimming bout, intervals of swimming were on average 14.0±0.5 min and quiescent periods were 4.8±0.1 min (Table 1). Three additional *C. elegans* strains and five nematode species tested all entered and exited a quiescent state in liquid, although none of the other species had the regularity of *C. elegans* (Fig. 1D). We conclude that, in a liquid environment, swimming and quiescence are alternative behavioral states of the nematode motor system. Similar extended periods of quiescence were not seen for adult worms crawling on an agar surface (data not shown).

Quiescence is a regulated, neural state

Touching worms on the tail with a wire 1 min after entering quiescence, but not on the head, caused them to commence swimming (supplementary material Movie 2). Thus worms in quiescence are capable of swimming. Moreover, this observation implicates specific neuronal circuitry in the induction of swimming from the quiescent state. After being 'awakened' by prodding following 1 min of the first quiescent bout, worms swam for the same amount of time as if they had resumed swimming spontaneously after 4.8 min (prodded, 16.1±1.5 min, *N*=64; spontaneous, 17.1±1.4 min, *N*=42; *P*=0.3). There was no correlation between the length of the quiescent period and the length of the preceding swimming period in *C. elegans* or the other species (data not shown). The timing of the transition between swimming and quiescence was strongly influenced by environmental variables and sensory input (data not shown). Notably, inclusion of food in the swimming buffer (*E. coli* OD=0.6) decreased the initial swimming period (69±5 min, 32 bouts) and increased the average length of quiescent periods (9.3±1.1 min, 167 bouts). All these observations argue against simple fatigue and recovery as the reason for quiescence.

During quiescence muscles are initially in a state of equal tension requiring ACh

As an approach to understand the nature of the quiescent state, we investigated the status of the motor system during quiescence in order to gain insight into why periodic muscle contractions stopped. We examined three of the principal elements of the motor system: the excitatory neurotransmitter ACh, the inhibitory neurotransmitter GABA and the muscles (Chalfie and White, 1988; Von Stetina et al., 2006).

To determine whether muscle contractions stopped because motor neurons ceased to secrete ACh, we compared animals lacking this neurotransmitter with animals in quiescence. In liquid, animals lacking ACh due to mutations in the ACh transporter *unc-17* or the biosynthetic enzyme choline acetyltransferase *cha-1* (Alfonso et al., 1993; Rand, 1989; Rand and Russell, 1984; Rand and Russell, 1985)

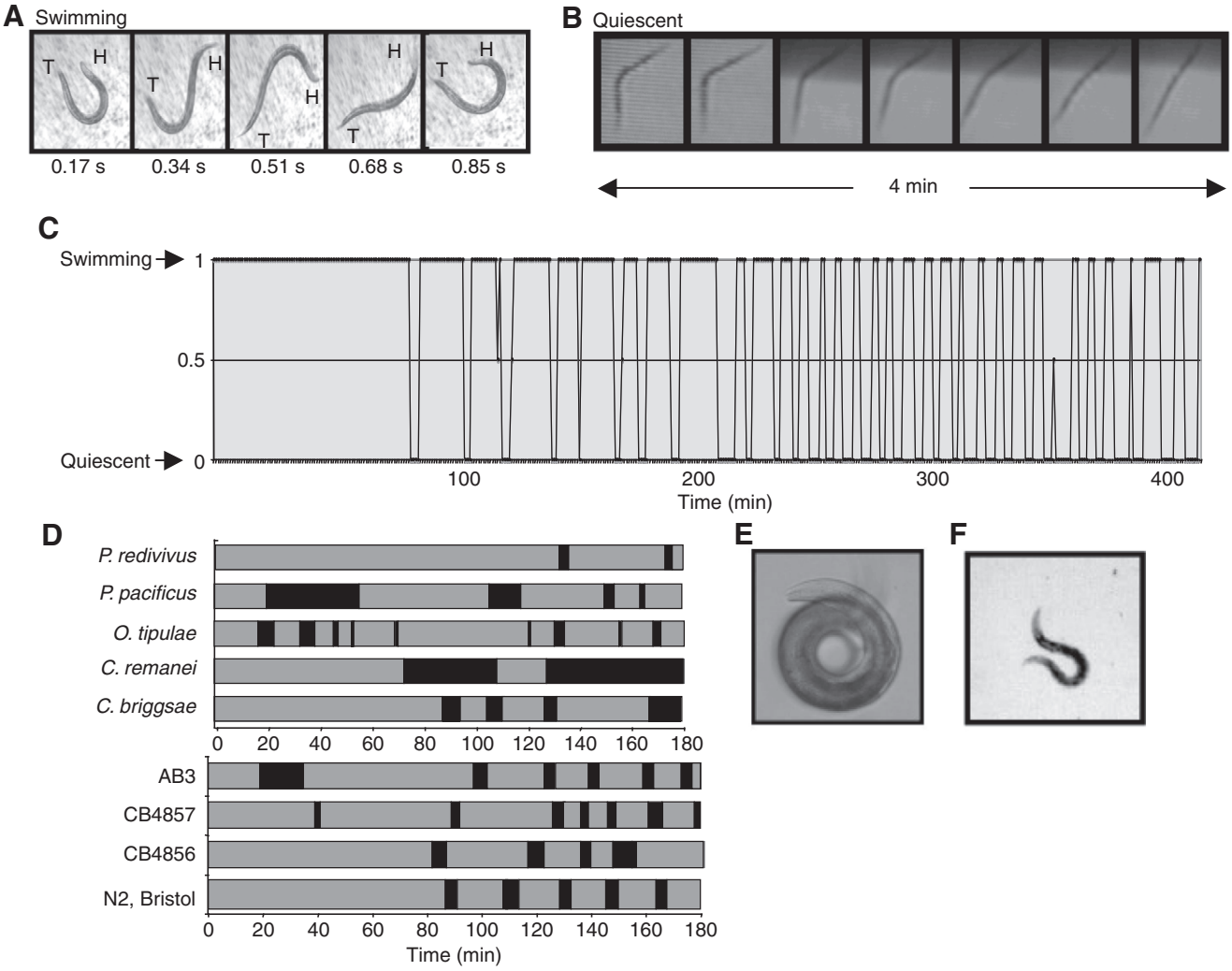

Fig. 1. In a liquid environment, after a long initial swim, worms alternate between swimming (A) and quiescence (B). (C) Typical swimming–quiescent pattern for an individual worm. 1, swimming; 0.5, slow swimming; and 0, quiescence (see Materials and methods). At time 0 the worm was transferred from the surface of solid media into liquid. (D) Swimming (gray) and quiescent (black) behavior for typical worms of five species and four *C. elegans* strains. N2 (Bristol) is the wild-type strain used in this study; the N2 data are from C. (E) Coiled posture in liquid (25°C) of a worm lacking ACh [*cha-1(y226ts)*]. (F) Sinusoidal quiescent posture of *egl-4(ks60)* mutant worms.

assumed and maintained a ventrally coiled posture (Fig. 1E). This posture was different from the straight, slightly kinked posture of wild-type worms in quiescence (Fig. 1B). To eliminate the possibility that coiling was due to a developmental defect, we examined a temperature-sensitive allele of *cha-1*. *cha-1(y226ts)* worms move apparently normally at permissive temperature on plates, but when transferred to liquid at 25°C they curl up and cease movement (98% after 10 min, 30 worms). Because of internal hydrostatic pressure, in the absence of muscle contraction worms assume a straight posture. A coiled posture indicates that, in the absence of ACh, there is some degree of spontaneous muscle contraction with differential tension between the ventral and dorsal sides of the animal. From these observations we conclude that, while ACh is necessary for swimming as expected, it is also necessary for the straightened posture maintained by wild-type worms in quiescence.

Since ACh released at the neuromuscular junctions between the excitatory motor neurons and the body wall muscles stimulates the inhibitory GABAergic motor neurons (Chalfie and White, 1988; Von Stetina et al., 2006), it was possible that the maintenance by ACh of a straight posture during quiescence occurred through the stimulation of GABA release and relaxation of muscle. To assess this possibility, we examined the behavior of three GABA-deficient mutants: *unc-25(sa94ts)*, *unc-25(e156)* and *unc-30(e318)*. *unc-25* encodes the GABA biosynthetic enzyme glutamate decarboxylase and *unc-30* encodes a homeodomain transcription factor necessary for expression of *unc-25* as well as a GABA vesicular transporter (McIntire et al., 1993). In liquid, animals homozygous for GABA mutations underwent swimming–quiescence cycling. In quiescence, they maintained a posture similar to that of wild-type animals. Thus GABA is not necessary for quiescence or for the maintenance of straight posture during quiescence.

However, absence of GABA did have an effect on the timing of transitions between swimming and quiescence. For *unc-25(sa94ts)* and *unc-30(e318)* worms, the average length of swimming bouts was shorter than that of wild-type worms, while for *unc-25(e156)* there was no significant difference in the length of swimming bouts (Fig. 2A; Table 1). For all three mutations the average length of quiescent bouts was increased (Fig. 2A; Table 1). Thus GABA

Table 1. Swimming and quiescent bouts for nematode worms

	Mean (min)	s.d.	s.e.m.	No. of bouts/worms	*P*-values
Swimming bout					
Wild-type	14.1	10.29	0.5	419/74	n.a.
unc-25(sa94ts)	4.1	4.62	0.3	308/24	<0.001
unc-25(e156)	13.0	13.44	1.1	152/20	>0.05 n.s.
unc-30(e318)	8.8	14.13	0.8	279/24	<0.001
0.01 mmol l^{-1} aldicarb	3.9	3.89	0.2	246/23	<0.001
0.1 mmol l^{-1} aldicarb	4.2	3.98	0.6	52/20	<0.001
ace-1(g72);ace-2(p1000)	8.1	6.79	0.4	278/25	<0.001
dgk-1(nu62)	4.1	3.44	0.1	433/23	<0.001
glr-1::ICE(kyIs36)	5.2	9.38	0.4	508/29	<0.0001
nmr-1::ICE(akIs11)	16.4	17.00	0.9	343/65	=0.99 n.s.
Mock ablation *(glr-1::GFP)*	13.1	9.59	1.1	77/12	n.a.
AVB ablated	5.8	4.17	0.3	206/16	<0.0001*
glr-1::GLR-1(A/T)	10.1	7.93	0.6	152/30	<0.0001
nmr-1::GLR-1(A/T)	12.7	11.89	0.5	470/67	<0.0001
egl-4(ks60)	9.0	11.37	0.9	141/24	<0.001
egl-4(ks61)	4.9	3.34	0.2	196/24	<0.001
egl-4(ks62)	9.9	11.98	0.7	292/46	<0.001
egl-4(n477)	8.6	9.19	0.8	144/22	<0.001
Quiescent bout					
Wild-type	4.8	2.17	0.1	489/74	n.a.
unc-25(sa94ts)	9.6	11.71	0.7	309/24	<0.001
unc-25(e156)	8.5	7.58	0.6	159/20	<0.001
unc-30(e318)	8.6	9.09	0.5	292/24	<0.001
0.01 mmol l^{-1} aldicarb	10.4	12.59	0.8	250/23	<0.001
0.1 mmol l^{-1} aldicarb	29.3	42.18	4.9	74/20	<0.001
ace-1(g72);ace2(–1000)	10.2	13.07	0.8	287/25	<0.001
dgk-1(nu62)	7.2	6.71	0.3	436/23	<0.001
glr-1::ICE(kyIs36)	7.3	14.64	0.7	491/29	<0.0001
nmr-1::ICE(akIs11)	5.6	6.89	0.4	349/65	=0.1 n.s.
Mock ablation *(glr-1::GFP)*	7.3	3.02	0.3	86/12	n.a.
AVB ablated	9.2	10.29	0.7	213/16	>0.05 n.s.*
glr-1::GLR-1(A/T)	25.9	29.84	2.4	158/30	<0.0001
nmr-1::GLR-1(A/T)	7.4	7.92	0.3	520/67	=0.0006
egl-4(ks60)	2.7	2.49	0.2	173/24	<0.001
egl-4(ks61)	2.2	0.96	0.1	216/24	<0.001
egl-4(ks62)	2.9	3.28	0.2	330/46	<0.001
egl-4(n477)	2.8	2.01	0.2	162/22	<0.001

P-values are for comparison with wild-type or mock ablation (*).
n.s., non-significant difference; n.a., not applicable.

promoted swimming – it maintained worms in a swimming state for a longer time and caused worms in quiescence to begin swimming again sooner. The reason for the difference between *unc-25(e156)* and the other GABA-deficient mutants is unknown but might be because of developmental compensation that does not occur for the temperature-sensitive allele raised at 20°C or for the transcription factor mutation.

We further tested the effect of GABAergic signaling on the reinitiation of swimming by adding the GABA agonist muscimol to the liquid surrounding a worm that had entered quiescence. Addition of muscimol (2 mmol l^{-1}) 1 min after a worm entered quiescence caused reinitiation of swimming significantly sooner than if buffer alone was added (after addition of drug, 1.1±0.2 min, N=30; after addition of buffer, 2.4±0.3 min, N=12; P<0.001). Thus pharmacological treatment also suggested that GABAergic signaling promoted the termination of quiescence and the reinitiation of swimming.

We next examined the status of the muscles during quiescence. Although the straight posture of worms in quiescence could not be due to relaxation of the muscles by GABA, it could be caused by another muscle relaxant. Alternatively, the cessation of movement and straight posture might come about because all the body wall muscles were exerting equal tension. To discriminate between these two possibilities, we measured the lengths of worms during swimming and quiescence. We found that quiescent worms were initially contracted to 95±1% of their length while swimming before entering quiescence. This initial shortening attenuated during the quiescent period. To gain an indication of the change in body length that would occur if there were different degrees of muscle relaxation or contraction, we measured worms treated with the GABA agonist muscimol or the ACh agonist levamisole. Muscimol (20 mmol l^{-1}) caused worms to relax but produced no measurable change in length, while levamisole (2 mmol l^{-1}) caused a contraction to 77±1% of untreated length. We interpret these results to indicate that at the start of quiescence the body wall muscles are under some degree of contraction. Hence, the straight posture of worms is not caused by a complete relaxation of the body wall muscles but apparently by a uniform degree of tension all along the body wall.

Increased cholinergic activity promotes quiescence

To test further the role of ACh in swimming–quiescence cycling, we examined the swimming behavior of animals with increased ACh signaling. The inhibitor aldicarb increases the level of ACh at the neuromuscular junction by blocking the activity of the degradative

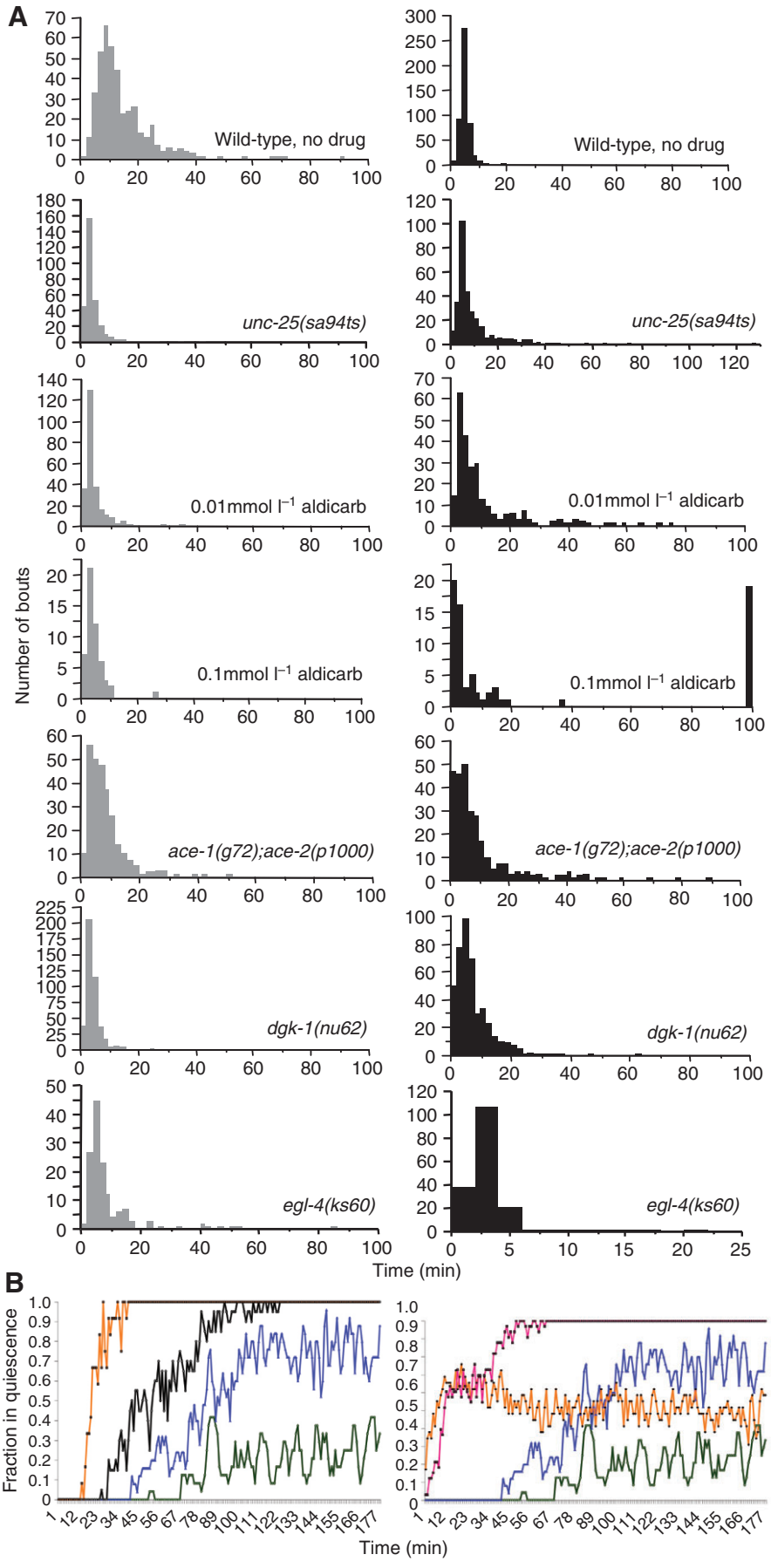

Fig. 2. (A) Effects of mutants and drug treatment on the duration of swimming (left) and quiescent (right) bouts. Data were collected starting with the first quiescent bout after the long initial swim, when the worm has entered the episodic phase of swimming. Panels 2–6: decreased GABA [*unc-25(sa94ts)*] and increased ACh [aldicarb, *ace-1(g72);ace-2(p1000)*, *dgk-1(nu62)*] decrease swimming and increase quiescence. Panel 7: Mutation in *egl-4* decreases the duration of both swimming and quiescent bouts. (B) Fraction of worms in quiescence. Left panel: in the presence of the acetylcholine esterase inhibitor aldicarb, quiescent bouts become progressively longer with time, resulting in an increasing fraction of worms in quiescence. Green, no drug; blue, 0.001 mol l^{-1} aldicarb; black, 0.01 mol l^{-1} aldicarb; yellow, 0.1 mol l^{-1} aldicarb. Right panel: worms lacking command interneurons cycle between swimming and quiescence immediately upon transfer into liquid, have increased quiescence, and remain sensitive to aldicarb. Yellow, *glr-1::ICE*, buffer; red, *glr-1::ICE*, 0.001 mol l^{-1} aldicarb; wild-type controls (green, blue) same as left panel.

enzyme acetylcholine esterase (Nguyen et al., 1995). We found that, by expression of a cell-death caspase, aldicarb decreased swimming and increased quiescence (Fig. 2A,B; Table 1). The posture of quiescent worms in aldicarb was similar to that of worms in the absence of the drug.

We also assayed several mutants with increased ACh signaling. Three acetylcholine esterase-coding genes have been identified in *C. elegans*: *ace-1*, *ace-2* and *ace-3*. A mutant of all three is lethal (Johnson et al., 1988). Mutation of two of the three in the *ace-1(g72);ace-2(p1000)* double mutant reduced the average duration of swimming bouts and increased the average duration of quiescent bouts (Fig. 2A; Table 1). In a second test, we examined a mutation in a diacylglycerol kinase, DGK-1. The mutation *dgk-1(nu62)* enhances the release of ACh from motor neurons in the ventral cord by elevation of DAG levels (Nurrish et al., 1999). Like *ace* mutants, *dgk-1(nu62)* mutants also exhibited shortened swimming bouts and longer quiescent bouts (Fig. 2A; Table 1).

Thus genetic and pharmacological experiments gave similar results and suggested that elevated levels of ACh signaling both promoted the transition to quiescence, shortening the average length of swimming bouts, and prolonged the average duration of quiescence.

An initial prodding-insensitive phase of quiescence is prolonged by ACh

The above observations are consistent with the hypothesis that high ACh signaling inhibits swimming and causes the worms to go into quiescence. The origin of this ACh is not known but a likely source is the excitatory motor neurons of the ventral cord. Once a worm enters quiescence, release of ACh by the excitatory motor neurons might stop or decrease. ACh concentration at neuromuscular junctions should then fall due to the action of acetylcholine esterase. The level of ACh must fall to a level permissible for swimming within 1 min of entering quiescence, because worms are capable of swimming at this time if prodded (100%, N=42).

To test for an initial refractory period during which worms could not swim if prodded, we prodded worms 10 s after they entered quiescence. Only 40% of the worms resumed swimming (N=38), demonstrating an initial refractory period as predicted. If this refractory period was due to the continuing presence of ACh, it should be extended by addition of aldicarb. Indeed we found that when worms in 0.01 mmol l^{-1} aldicarb were prodded after 1 min of quiescence, only 70% (N=27) and 58% (N=26) resumed swimming at the first and second quiescent bouts, respectively. When they were prodded similarly after 5 min in quiescence, 100% (N=18) and 90% (N=28) resumed swimming. Therefore, blocking acetylcholine esterase activity prolonged a refractory period during which the worms were not responsive to prodding. ACh signaling appeared to prevent quiescent worms from undergoing the transition from a prodding-insensitive to a prodding-sensitive state.

Command interneurons are dispensable for cycling

Initially after entering quiescence a worm is in a state insensitive to prodding that appears to be induced by ACh signaling. However, the state of the worm soon changes and after 1 min it can swim if prodded, yet it remains in quiescence for several minutes longer. Thus a secondary mechanism, possibly independent of ACh signaling, extends the quiescent period. Command interneurons are logical candidates for providing this secondary mechanism. To assess the role of the command interneurons in both swimming and quiescent intervals, we examined the behavior of animals (*glr-1::ICE*) in which all five pairs of command interneurons are killed along with 12 other classes of neurons (Zheng et al., 1999a). In spite of this drastic loss of upstream circuitry, *glr-1::ICE* worms continued to undergo swimming–quiescence cycling. Their posture during quiescence resembled that of non-transgenic wild-type worms rather than the coiled posture of ACh-deficient mutants. Thus command interneurons are not necessary for swimming–quiescence cycling.

However, the timing of swimming and quiescent intervals was affected in this strain. *glr-1::ICE* worms began cycling immediately upon transfer to liquid (Fig. 2B). Thus one or more of the neurons killed in this strain were necessary to maintain swimming during the first swimming bout. During cycling, the average length of swimming bouts was decreased while the average length of quiescent bouts was increased (Fig. 3A; Table 1). However, the distribution of both swimming and quiescent intervals was more highly skewed due to a relative increase in the frequency of short bouts. For quiescent intervals, although the average length was increased, the shortest bouts were the most frequent class. Hence one or more *glr-1*-expressing neurons inhibit the reinitiation of swimming during the first few minutes of quiescence and evidence for a timing mechanism is lost. *glr-1::ICE* worms were sensitive to aldicarb, similar to non-transgenic animals (Fig. 2B). Along with the straight posture of the *glr-1::ICE* worms in quiescence, this observation is consistent with the hypothesis that induction of quiescence in worms lacking command interneurons occurs by the same ACh-mediated mechanism as in non-transgenic worms.

To help identify which of these neurons was responsible for inhibiting short quiescent bouts, we examined a strain in which a smaller subset of neurons is killed. A strain carrying the *nmr-1::ICE* transgene lacks all of the command interneurons, with the exception of AVBL and AVBR, plus three additional neurons of two types, RIML, RIMR and AVG (Zheng et al., 1999a). In this strain there was no significant difference from non-transgenic worms in the average duration of swimming or quiescent periods (Fig. 3A; Table 1). However, the distributions of both swimming and quiescent intervals were broadened compared with wild-type, indicating that the neurons killed in the *nmr-1::ICE* strain contributed to the accuracy of the timing mechanism.

Comparison of quiescent bouts in *glr-1::ICE* and *nmr-1::ICE* worms suggested that AVB might be important in suppressing short quiescent bouts. To test the function of the AVB neurons, we killed the AVB neurons with a laser microbeam. Elimination of AVBL and AVBR decreased the average length of swimming bouts, while there was no effect on quiescent bouts (Table 1). Therefore, while AVB may be important in sustaining swimming, any role that it plays in promoting quiescence overlaps that of other neurons. We searched for other neurons that might be responsible for timing by ablating them individually. The neurons we tested included AVA, AVE, PVC, AVD and RIM. Ablation of AVA, AVD and RIM resulted in broadened distributions of quiescent bouts, but loss of any single neuron did not affect timing drastically (supplementary material Fig. S1 and Table S1).

Constitutive depolarization of upstream interneurons prevents worms from recovering from quiescence

Since ACh signaling appeared to promote quiescence over swimming, depolarization of the command interneurons, which is expected to depolarize the excitatory motor neurons and hence increase their release of ACh, might also promote quiescence over swimming. To test this prediction, we examined a series of strains in which the command interneurons along with additional neurons are constitutively depolarized by expression of a leaky form of the

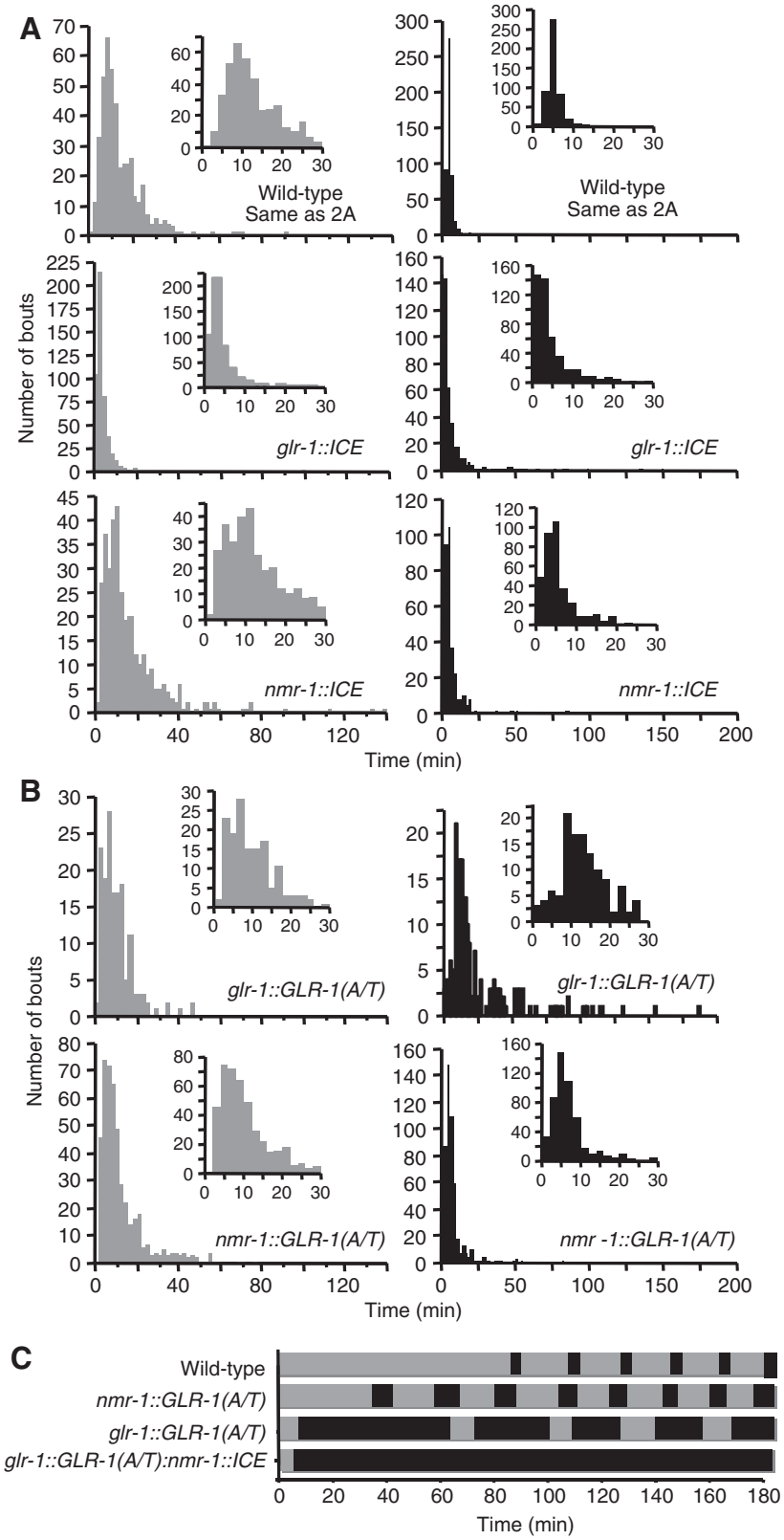

Fig. 3. Effects of killing or depolarizing the command interneurons. (A) Command interneurons sustain both swimming (left panels) and quiescence (right panels). (B) Depolarization of command interneurons increases quiescence. Left and right panels as in A. (C) Progressive increase in quiescence with all command interneurons except AVB depolarized [*nmr-1::GLR-1(A/T)*], all command interneurons including AVB depolarized, [*glr-1::GLR-1(A/T)*] and AVB depolarized and the remaining command interneurons killed [*glr-1::GLR-1(A/T):nmr-1::ICE*]. Typical single worm swimming (gray)–quiescence (black) behavior. Wild-type data are the same as in Fig. 1D (N2, Bristol).

glutamate channel GLR-1, denoted GLR-1(A/T) (Zheng et al., 1999b). As predicted, in strains where the command interneurons are depolarized [(*glr-1::GLR-1(A/T), nmr-1::GLR-1(A/T)*], the length of the swimming bouts decreased and the length of the quiescent bouts increased (Fig. 3B; Table 1). These effects were progressively more severe through a series of three strains – all command interneurons but AVB plus RIML, RIMR and AVG depolarized [*nmr-1::GLR-1(A/T)*], all command interneurons plus 12 classes of additional neurons depolarized [*glr-1::GLR-1(A/T)*], AVB depolarized and the remaining command interneurons plus 12 classes

of additional neurons killed [*glr-1::GLR-1(A/T);nmr-1(ICE)*] – such that in the last of these strains, worms went into a quiescent state from which they rarely or never emerged (Fig. 3C). Although they can crawl on plates (Zheng et al., 1999b), these worms cannot swim.

The cGMP-dependent protein kinase EGL-4 prolongs both swimming and quiescent states

Two previously studied quiescent states of *C. elegans* are promoted by activity of the cGMP-dependent protein kinase EGL-4 (Avery, 1993; Raizen et al., 2006; Raizen et al., 2008; Van Buskirk and Sternberg, 2007). We examined the effect of mutation in *egl-4* on swimming–quiescence cycling by scoring worms carrying four different loss-of-function or null alleles of *egl-4*, with similar results. All four mutants underwent swimming–quiescence cycling, indicating that *egl-4* function is not necessary for spontaneous quiescence during swimming. However, the average length of both swimming and quiescent bouts was shorter than in wild-type (Fig. 2A; Table 1). In quiescence, *egl-4* worms had a different posture from wild-type worms; rather than a straightened posture, *egl-4* worms stopped moving while maintaining a deeply bent posture (Fig. 1F; supplementary material Movie 3). Further, while the body was immobilized in this bent posture, the nose of the animal continued to move rapidly back and forth. Thus while *egl-4* function was not necessary for cycling between swimming and quiescent states, it was necessary for the straightened posture during quiescence and for quiescence of the head muscles.

DISCUSSION

We have characterized a previously undescribed behavioral phenomenon in *C. elegans*. We found that in a liquid environment, worms do not swim continuously; instead, after an initial phase of sustained swimming, they abruptly enter a second phase in which bouts of swimming alternate with bouts of quiescence. The regularity of the cycling between swimming and quiescence during the episodic phase is unique for a *C. elegans* behavioral transition. Previous behavioral studies focusing on transitions between forward and backward locomotion and turning have shown that these transitions appear to occur with a probabilistic distribution (Brockie et al., 2001b; Fujiwara et al., 2002; Gray et al., 2005b). Our results indicate that during swimming, transitions into and out of quiescence are governed by a deterministic timing mechanism.

The origin or target of the ACh signaling that induces quiescence is not known. Among the several cholinergic neurons in the nervous system, the excitatory motor neurons are obvious candidates for the source of ACh. Possibly quiescence is induced during swimming because of a higher rate of release of ACh by the excitatory motor neurons accompanying the higher rate of motion of the worm in liquid compared with its motion on a solid surface. Such greater release of ACh might have its effect directly on muscle, or the released ACh might act on receptors in neurons, stimulating a secondary process involved in stopping muscle contractions. A higher rate of ACh release could lead to ACh levels rising above a threshold level or to activation of remote receptors by spillover within the ventral cord or elsewhere in the nervous system. The equal state of body wall muscle tension might result from a mechanism within the muscles themselves, such as an increase in their electrical coupling. Alternatively, it could be due to a mechanism that causes equal release of ACh by all the excitatory motor neurons. One or more additional neurotransmitters, for example peptide neurotransmitters, could be triggered and act on either the motor neurons or the muscles, or on both, to bring about the state of inactivity and equal tension.

While they were not necessary for swimming–quiescence cycling, upstream neurons including the command interneurons were necessary for the regularity of cycling. We could identify no single interneuron responsible for this effect, consistent with the distributed nature of the control circuitry observed by others (Brockie et al., 2001b). The upstream neurons served to stabilize the behavioral choice. Command interneurons serve a similar stabilizing function in transitions between forward and backward locomotion during crawling (Zheng et al., 1999b).

Upstream interneurons impose regularity by prolonging quiescence beyond the initial prodding-insensitive period. Thus they provide a latch that prevents a change in behavioral state for a specified period. One possibility consistent with activation by prodding is that during the latch phase of quiescence the command interneurons are hyperpolarized and thereby inhibit the excitatory motor neurons.

The nature of the mechanism that 'times out' after about 5 min, lifting the latch and allowing spontaneous reinitiation of swimming, remains to be determined. It is also unclear how the spontaneous mechanism relates to the mechanism that operates on prodding. There is a high degree of interconnectivity among the interneurons upstream of the motor neurons which stands in the way of any obvious explanation for why posterior but not anterior prodding reawakens the system (White et al., 1986). We examined the swimming behavior of worms lacking functional touch neurons [*mec-3(e1338)* and *mec-4(e1611*) (Chalfie and Sulston, 1981)] and found they have quiescent bouts of normal length but somewhat shortened swimming bouts (data not shown). Normal quiescent bout length in these mutants suggests the spontaneous mechanism does not function through activity of the touch neurons.

The straight body posture and the ability to be awakened by prodding are similar to the characteristics of other described quiescent states of *C. elegans*, raising the possibility that spontaneous quiescence during swimming might be the same behavioral state as quiescence during lethargus or quiescence induced by EGF (Avery, 1993; Raizen et al., 2006; Van Buskirk and Sternberg, 2007). Indeed, our observation that *egl-4* activity promotes the quiescent state in liquid strengthens this conjecture. A significant difference between quiescence during swimming and that during lethargus or induced by EGF is that prodding in the last two cases induces only transient locomotion, whereas in the first it induces a normal swimming period before quiescence is resumed. It is possible that a humoral factor inducing the quiescent state in lethargus or EGF-induced quiescence, but not during swimming, persists and soon reinitiates the quiescent state. This supposition is consistent with our suggestion that ACh is the inducer of quiescence during swimming and is quickly lost once a worm enters quiescence, with quiescence being maintained thereafter by a neural latch.

We have shown that the function of the *C. elegans* motor circuit is inherently unstable when a worm swims in liquid. It spontaneously switches between two behavioral states, a swimming state and a quiescent state. While *C. elegans* is thought to normally inhabit two-dimensional liquid films on solid surfaces in soil or on an invertebrate host, it is likely that from time to time it may encounter a flooded environment in which it is obliged to swim. Hence the behavior we have observed is likely to be adaptive. Possibly the quiescent state is a reset state that allows the system to recover after running too fast. Or perhaps periods of no swimming aid a worm in escaping the liquid environment. An intrinsic ability to change behavioral state in the absence of a change in sensory input might be important to the animal in adaptively coping with an unchanging environment.

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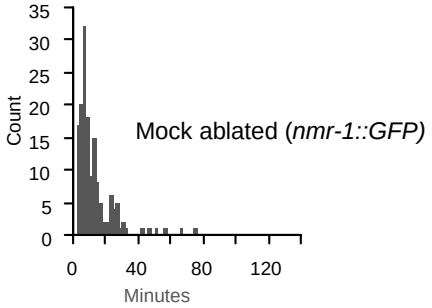

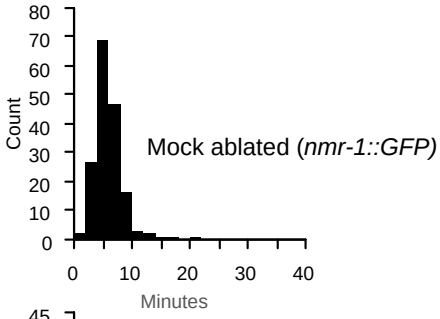

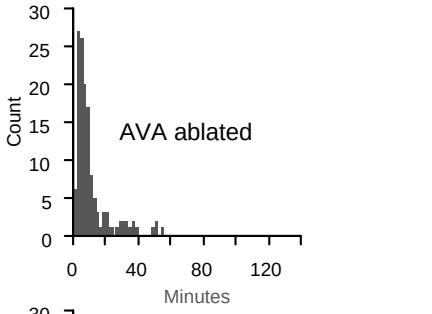

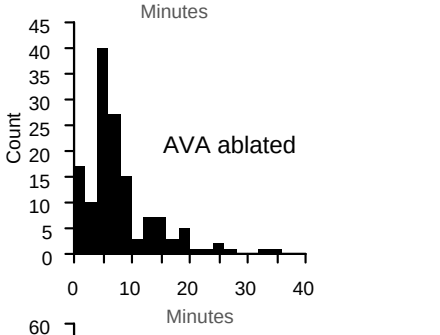

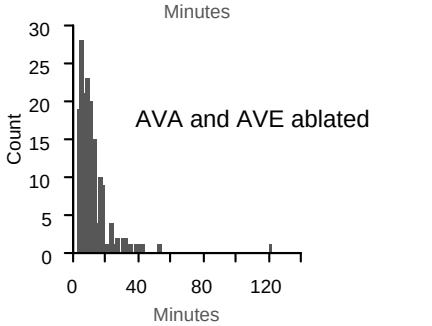

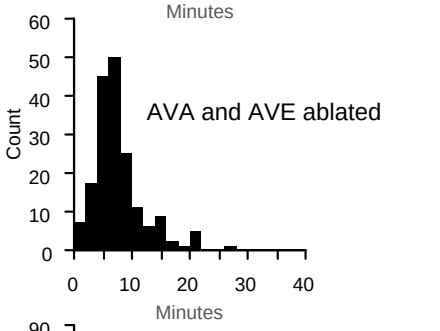

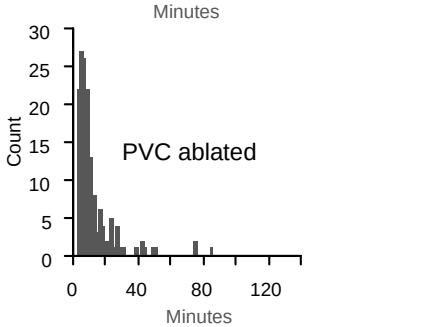

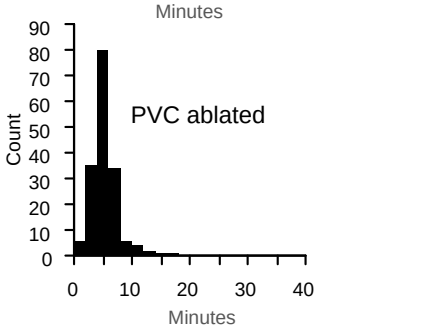

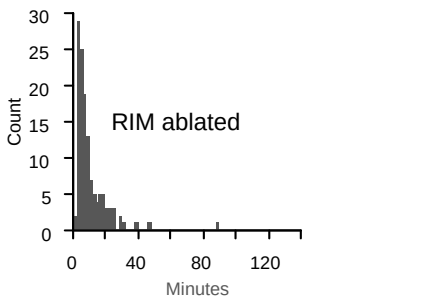

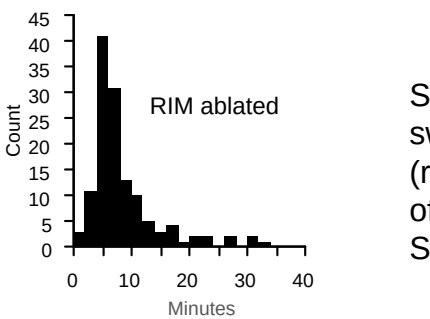

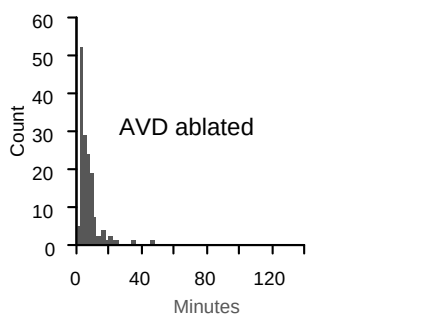

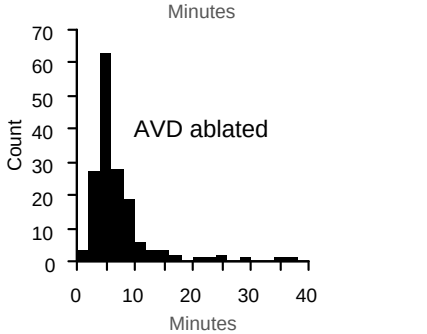

Suppl. Fig. 1. Lengths of swim (left) and quiescent (right) bouts after ablation of the neurons shown. See Suppl. Table 1 for statistics.

Table S1. Swimming and quiescent bouts for nematode worms

	Mean (min)	s.d.	s.e.m.	No. of bouts/worms	*P*-values
Swimming bout					
nmr-1::GFP mock	12.3	11.51	0.9	153/24	n.a.
AVA ablated	10.1	11.04	0.9	135/16	0.09 n.s.*
AVA and AVE ablated	11.6	11.99	0.9	167/17	0.5324 n.s.*
AVD ablated	6.4	6.04	0.5	151/17	<0.0001*
PVC ablated	11.9	13.19	1.1	154/16	0.7435 n.s.*
RIM ablated	9.6	10.66	0.9	129/16	0.04 n.s.*
Wild-type raised 20°C, assay 20°C	42.7	32.02	6.7	23/16	<0.0001†
Wild-type raised 25°C, assay 20°C	46.3	35.65	6.9	27/15	<0.0001†
Wild-type assayed in *E.coli* (Op50)	12.7	13.75	1.1	167/32	0.2860 n.s.†
Quiescent bout					
nmr-1::GFP mock	5.7	3.66	0.3	170/24	n.a.
AVA ablated	8.5	10.14	0.8	143/24	0.0003*
AVA and AVE ablated	7.2	4.35	0.3	179/17	0.0446 n.s.*
AVD ablated	7.2	7.66	0.6	163/17	0.042 n.s.*
PVC ablated	4.9	2.29	0.2	169/16	0.2639 n.s.*
RIM ablated	9.2	10.22	0.9	134/16	<0.0001*
Wild-type raised 20°C, assay 20°C	4.8	1.73	0.3	35/16	0.9965 n.s.†
Wild-type raised 25°C, assay 20°C	5.4	2.65	0.4	41/15	0.6296 n.s.†
Wild-type assayed in *E.coli* (0P50)	9.3	15.69	1.1	189/32	<0.0001†

P-values are for comparison with wild-type (†) under standard assay conditions (raised 20°C on *E. coli*, assay 25°C without *E. coli*) or mock ablation (*). n.s., non-significant difference. n.a., not applicable.