Calcium/Calmodulin-Dependent Protein Kinase II Regulates *Caenorhabditis elegans* Locomotion in Concert With a G_o/G_q Signaling Network

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ABSTRACT

Caenorhabditis elegans locomotion is a complex behavior generated by a defined set of motor neurons and interneurons. Genetic analysis shows that UNC-43, the *C. elegans* Ca^{2+}/calmodulin protein kinase II (CaMKII), controls locomotion rate. Elevated UNC-43 activity, from a gain-of-function mutation, causes severely lethargic locomotion, presumably by inappropriate phosphorylation of targets. In a genetic screen for suppressors of this phenotype, we identified multiple alleles of four genes in a G_o/G_q G-protein signaling network, which has been shown to regulate synaptic activity via diacylglycerol. Mutations in *goa-1*, *dgk-1*, *eat-16*, or *eat-11* strongly or completely suppressed *unc-43*(*gf*) lethargy, but affected other mutants with reduced locomotion only weakly. We conclude that CaMKII and G_o/G_q pathways act in concert to regulate synaptic activity, perhaps through a direct interaction between CaMKII and G_o.

ORGANISMS respond to the environment by modulating their behavior. To understand how behavior is modulated, the cellular and molecular components that control particular behaviors must be defined. The model organism *Caenorhabditis elegans* is particularly well suited to such analysis since it has a relatively simple nervous system and is highly amenable to both genetic manipulation and behavioral analysis. Understanding how behavior is controlled at the cellular and molecular level in a relatively simple organism can provide insight into how behavior is controlled in more complex organisms such as mammals. In *C. elegans*, environmental influences have been shown to modulate several behaviors, including locomotion, feeding, egg laying, and defecation (Avery and Thomas 1997; Bargmann and Mori 1997; Driscoll and Kaplan 1997; Jorgensen and Rankin 1997). In particular, work from several investigators has described some of the neurons and molecules that control several aspects of locomotion, including the response to food, chemical stimuli, and mechanical stimuli (Driscoll and Kaplan 1997; Jorgensen and Rankin 1997). However, despite the relative simplicity of *C. elegans*, many aspects of the neuronal and molecular control of locomotion have not yet been defined.

Neuronal connectivity maps, neuronal ablations, and the analysis of mutants that perturb locomotion have shown that at least three partially separable processes control *C. elegans* locomotion: the generation of coordinated sinusoidal body bends, selection of forward or backward movement, and determination of locomotion rate (Driscoll and Kaplan 1997; Jorgensen and Rankin 1997). *C. elegans* moves by coordinated sinusoidal body bends that propagate smoothly along the length of the animal. These bends are generated by several classes of cholinergic motor neurons and two classes of GABA-ergic motor neurons that form neuromuscular junctions with body-wall muscles. A bend is generated by the local excitation of muscles on one side of the animal (via cholinergic motor neurons) with reciprocal inhibition of corresponding muscles on the opposite side of the animal (via GABA-ergic motor neurons; White *et al.* 1986; McIntire *et al.* 1993a,b). Selection of forward or backward movement is controlled by five interconnected command interneurons, which receive input from sensory neurons and other interneurons and send output to motor neurons (Chalfie *et al.* 1985; White *et al.* 1986). These command interneurons mediate forward and backward movement in response to specific sensory cues such as food and touch, and they set the duration of forward and backward movement in the absence of specific cues (Chalfie *et al.* 1985; Zheng *et al.* 1999).

Determination of locomotion rate has also begun to be elucidated. Modulation of locomotion rate in response to several stimuli, including food, has been described (*e.g.*, Sawin 1996). Animals exhibit hyperactivity in the absence of food and reduced locomotion when returned to food, presumably to optimize foraging. These responses appear to be mediated in part by serotonergic neurons in the pharynx and dopaminergic sensory neurons (Sawin 1996). Consistent with these observations, exogenous serotonin and dopamine reduce locomotion, whereas mutants with reductions in these neurotransmitters move hyperactively (Horvitz *et al.* 1982; Schafer and Kenyon 1995; Ségalat *et al.* 1995;

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Nurrish *et al.* 1999). A G_o/G_q heterotrimeric G-protein signaling network expressed throughout the nervous system has been shown to regulate locomotion rate, partly by affecting serotonergic signaling (Mendel *et al.* 1995; Ségalat *et al.* 1995; Brundage *et al.* 1996; Hajdu-Cronin *et al.* 1999; Lackner *et al.* 1999; Miller *et al.* 1999; Nurrish *et al.* 1999). Specifically, loss-of-function (*lf*) mutations in *goa-1* ($G_o\alpha$) cause hyperactivity, whereas *lf* mutations in *egl-30* ($G_q\alpha$) cause severe lethargy. The *egl-30* $G_q\alpha$ regulates a phospholipase C signaling pathway that facilitates synaptic transmission by body-wall muscle motor neurons and perhaps other neuronal cell types (Brundage *et al.* 1996; Lackner *et al.* 1999; Miller *et al.* 1999; Nurrish *et al.* 1999). The *goa-1* $G_o\alpha$ appears to mediate serotonergic antagonism of the *egl-30* pathway (Hajdu-Cronin *et al.* 1999; Lackner *et al.* 1999; Miller *et al.* 1999; Nurrish *et al.* 1999). *goa-1* and *egl-30* also regulate several other behaviors in addition to locomotion rate, including egg laying (Trent *et al.* 1983; Park and Horvitz 1986; Mendel *et al.* 1995; Reiner *et al.* 1995; Ségalat *et al.* 1995; Brundage *et al.* 1996).

The *C. elegans* Ca^{2+}-calmodulin-dependent serine/threonine protein kinase II (CaMKII) encoded by *unc-43* is also widely expressed in neurons and regulates locomotion rate, as well as other behaviors (Reiner *et al.* 1999; E. Newton and J. H. Thomas, unpublished results). A gain-of-function (*gf*) mutation in *unc-43* causes severe lethargy, as well as body-wall muscle hypercontraction, reduced egg laying, and reduced defecation (Reiner *et al.* 1999). Several of these effects are genetically separable, indicating that *unc-43* regulates defecation, body-wall muscle tone, and locomotion rate through different effectors (Reiner *et al.* 1999). CaMKII has been shown to be an important regulator of synaptic strength by electrophysiological studies in the marine snails Aplysia and Hermissenda (Nelson and Alkon 1997), Drosophila (Griffith *et al.* 1994), and rodents (Malinow *et al.* 1989; Silva *et al.* 1992a; Mayford *et al.* 1995; Rotenberg *et al.* 1996). These and other studies indicate that CaMKII plays a role in sensitization, learning and memory, fear and aggressive responses, and olfactory attenuation (Silva *et al.* 1992b; Griffith *et al.* 1993; Chen *et al.* 1994; Bach *et al.* 1995; Wei *et al.* 1998). CaMKII is unique among Ca^{2+}-responsive proteins because it forms multimers with subunits that interact cooperatively to produce a nonlinear graded response to calcium (Hanson and Schulman 1992; Hanson *et al.* 1994; De Koninck and Schulman 1998). Adjacent subunits with bound Ca^{2+}-calmodulin can phosphorylate each other, resulting in a large increase in Ca^{2+}-calmodulin affinity and partial kinase activity even after Ca^{2+}-calmodulin dissociates. This mechanism of activation enables CaMKII to integrate Ca^{2+} signaling events over time, which may be the basis for the role of CaMKII in regulating synaptic strength (Hanson *et al.* 1994; De Koninck and Schulman 1998).

To understand how *unc-43* controls locomotion rate in *C. elegans*, we performed a genetic suppressor screen with *unc-43*(*gf*) to identify genes that act with *unc-43* to control locomotion rate. From our screen, we recovered multiple alleles of the genes *goa-1*, *dgk-1*, and *eat-16*, all involved in the *goa-1*/*egl-30* G-protein network (Hajdu-Cronin *et al.* 1999; Miller *et al.* 1999; Nurrish *et al.* 1999), and alleles of a fourth gene, *eat-11*, that probably affects this same pathway. Our results indicate that UNC-43 may regulate this G-protein signaling network to control locomotion rate in *C. elegans*.

MATERIALS AND METHODS

Strain maintenance: Worms were cultured using standard methods (Brenner 1974). The standard N2 Bristol strain was used as wild type, and all strains were grown at 20° except those containing *goa-1*(*n363*) and *goa-1*(*sa734*). We found that these strains were healthier when grown at 15°. For behavioral assays, these strains were staged as L4 larvae and grown for 38 hr at 15°, which is equivalent to 24 hr of growth at 20° (J. H. Thomas, unpublished observations). These strains were then placed at the assay temperature for 30 min prior to the start of each assay. Assays with wild type and *unc-43*(*n498*) animals grown under the same conditions were performed and found to be equivalent to assays performed with animals grown for 24 hr at 20°.

Mutations analyzed: The following *C. elegans* mutations were analyzed in this work: *eat-11*(*ad541*, *sa581*, *sa586*, *sa603*, *sa604*, *sa762*, *sa765*, *sa833*) I, *eat-16*(*sa609*, *sa735*, *sa768*, *sa839*, *sy438*) I, *egl-30*(*ad805*) I, *goa-1*(*n363*, *n1134*, *sa585*, *sa734*, *sa837*, *sa841*) I, *glr-1*(*n2461*) III, *unc-93*(*e1500*) III, *unc-103*(*n500*) III, *unc-43*(*n498*, *n1186*) IV, *dgk-1*(*sa605*, *sa748*, *sa760*, *sa766*, *sy428*) X, *unc-110*(*sa859*) X, *nIs51*[*egl-10*(+)], *syIs36*[*egl-30*(+)], and *syIs9*[*goa-1*(*gf*)].

Identification of suppressor mutations: *unc-43*(*n498*) hermaphrodites were treated with ethane methylsulfonate as described (Sulston and Hodgkin 1988) and F_2 self-progeny were screened for suppression of the *unc-43*(*n498*) locomotion defect under a dissecting microscope. Animals that moved well spontaneously or animals that moved well after the plate was tapped were picked as putatively suppressed animals. The broods of these animals were rescored for suppression. In total, ~28,000 haploid genomes were screened. Extragenic suppressors were crossed out of the *unc-43*(*n498*) background based on their hyperactive and reduced egg-retention phenotypes and retested for their ability to suppress the *unc-43*(*n498*) locomotion defect by backcrossing to the unmutagenized *unc-43*(*n498*) parent strain. Suppressor mutations that were closely linked to *unc-43* and caused *unc-43*(*lf*) locomotory phenotypes were considered likely to be intragenic revertants. All mutations analyzed in this study are independent. All mutations were backcrossed to N2 at least twice before analysis.

Mapping to specific chromosomes was performed using *dpy-5*(*e61*) I, *rol-6*(*e187*) II, *unc-32*(*e189*) III, *unc-5*(*e53*) IV, *dpy-11*(*e224*) V, *dpy-3*(*e27*) X, and *lon-2*(*e678*) X. We tested each of our suppressor alleles for complementation of *goa-1*(*n1134*), *dgk-1*(*sy428*), and *eat-16*(*sy438*). For *eat-11* complementation tests, we first tested *sa765* for complementation of *eat-11*(*ad541*), and then subsequently tested all other alleles for complementation of *sa765*. Since *sa604* and *sa833* exhibited decreased egg retention in comparison to other *eat-11* alleles, we tested *sa604* and *sa833* for complementation of *ad541* as well as *sa765*. We scored complementation tests in the *unc-43*(*n498*)/+; *dpy-11*(*e224*)/+ background for all genes. We ruled out second-site noncomplementation by scoring for wild-type and *unc-43*(*n498*) homozygous progeny in the self-

progeny broods of noncomplementing mutant heterozygotes. All noncomplementing mutations were closely linked by this test.

Construction of double mutant strains: For the *egl-30*(*ad805*); *unc-43*(*n1186*) double mutant, *n1186/+* males were mated to *ad805* hermaphrodites and weakly Egl F_1 progeny were picked to individual plates. Plates that segregated convulsive-Unc animals were used to pick convulsive-Unc animals that were weakly Egl to individual plates. Their progeny were examined to confirm that *n1186* was homozygous, and many of these progeny were picked to homozygose *ad805* in the next generation. Animals that produced all Egl progeny were kept. The resulting strains were then tested for homozygosity of both mutations by crossing with N2 males and observing both convulsive-Unc animals and Egl animals segregating from all heterozygotes.

For *syIs9*[*goa-1*(*gf*)]; *unc-43*(*n1186*), the linked double mutant *dpy-20*(*e1282*) *unc-43*(*n1186*) was first constructed. *e1282-n1186/+* males were then crossed to *dpy-20(e1282); syIs9*[*goa-1*(*gf*)] hermaphrodites. F_1 progeny were picked and plates that segregated Dpy, convulsive-Unc animals were used to pick many *syIs9* animals (Unc, Egl, non-Dpy) to individual plates. From the broods of these parents, animals that were Unc, Egl, non-Dpy and had a slightly flaccid body posture, as exhibited by *unc-43*(*n1186*) animals (Reiner *et al.* 1999), were picked. The resulting strains were then tested for homozygosity of *n1186* and *syIs9* by crossing with N2 males and observing both convulsive-Unc animals and Egl animals segregating from all heterozygotes.

Double mutants with *unc-43*(*n498*) and *goa-1*, *dgk-1*, *eat-16*, or *eat-11* mutations were constructed by picking individual *unc-43*(*n498*) homozygous animals (severe Unc) from the broods of double mutant heterozygotes (weaker Unc). Double mutants were picked in the next generation from those broods that segregated one quarter suppressed animals (double mutants) and three quarters severe Unc animals.

Locomotion assays: Radial locomotion assays were performed at 23° on 8.5-cm plates harboring a 1.5-day-old lawn of OP50 bacteria. Animals were picked as L4 larvae and assayed after 24 hr of growth at 20°. Five animals were placed in the center of a plate and radial distance traveled was measured at 5, 10, and 15 min after the start of the assay. Two alleles of each suppressor gene were assayed, with the exception of *egl-10* and *egl-30*, for which only one transgene was available. The assay was performed twice per genotype.

Body-bend assays were performed at 20° on 5-cm plates with a reproducibly thin lawn of OP50 bacteria that had been applied 8 hr prior to the assay. Animals were picked as L4 larvae and assayed after 24 hr of growth at 20° (or the equivalent; see section on strain maintenance). One animal was transferred to the assay plate, left undisturbed for 5 min, and then assayed for 3 min. At least four animals per genotype were assayed. Body bends were counted by observing flexing in the middle of the animal, using the vulva as a reference point. A flex was counted as a body bend when the vulva reached the peak or trough of the sine wave. Strains containing *goa-1*(*null*) or *dgk-1*(*null*) often alternated rapidly between forward and backward movement. Frequently, only partial body bends were completed during this behavior. Since partial body bends were not counted in the assay, our data is an underestimate of the movement rate of strains containing *goa-1*(*null*) and *dgk-1*(*null*). For assays with *unc-93*(*gf*), *unc-110*(*gf*), and *unc-103*(*gf*) mutants, body bends were counted anterior to the vulva since body bends did not always propagate along the entire length of the animal.

Aldicarb assays: Aldicarb assays were performed at 23°. Stock solutions were prepared by dissolving aldicarb (Chem Service, West Chester, PA) in 70% ethanol to a final concentration of 100 mM. Aldicarb plates were prepared by adding the aldicarb stock solution to NG agar to a final concentration of 1 mM. Plates were stored at 4° until used. Twelve hours before an assay, a single drop of OP50 bacterial solution was added to each plate and incubated at 23°. Parallel experiments were performed on plates from the same batch. Aldicarb response was assayed by picking 19–25 animals to a single assay plate and scoring paralysis at 10-min intervals. Animals were scored as paralyzed when no spontaneous movement was exhibited, no movement was elicited by tapping the plate, and no movement was elicited by harsh touch to the anterior or posterior. *unc-43*(*n1186*) animals that initially appeared paralyzed by the above criteria would occasionally resume movement after harsh touch to the anterior or posterior. Therefore, we scored these animals twice at each timepoint and counted an animal as paralyzed when the above criteria for paralysis were met both times. Some strains were scored at 10-min intervals for an entire 120-min period. Strains for which the data exhibited a clear trend at early timepoints were scored at 10-min intervals for the first 60 min and then scored again at the 120-min timepoint.

Egg-staging assays: Suppression of the *unc-43*(*n498*) egg-laying defect was quantified by assaying the stages of eggs laid at 20° on plates harboring a 2-day-old lawn of OP50 bacteria. Animals were picked as L4 larvae and assayed after 24 hr of growth at 20° (or the equivalent; see section on strain maintenance). Two alleles of each suppressor gene were assayed. A total of 10–14 animals were placed on the assay plate and allowed to recover from the transfer for 30 min. Eggs laid during the recovery period were removed, and at 10-min intervals, eggs laid were examined under Nomarski optics to ascertain their developmental stages (Sulston *et al.* 1983; Schierenberg 1986). After examination, eggs were removed from the assay plate before beginning the next 10-min egg-laying period. Animals were assayed for 2–3 hr.

Sequencing of *goa-1*(*sa734*): *goa-1*(*sa734*) was outcrossed three times before sequencing. Sequencing was performed on bulk PCR product generated directly from genomic DNA with Taq and Pfu polymerases in a ratio of 100:1. Sequencing reactions were performed with Taq Dye Terminator reagents (Applied Biosystems, Foster City, CA). Primers for amplification and sequencing were designed using program Primer 3.0 from *goa-1* genomic sequence in the cosmid C26C6 (as reported in GenBank). The *goa-1*(*sa734*) allele is a C to T change at base pair 154 that results in a Q52stop mutation. This mutation was confirmed by sequencing both strands.

RESULTS

Mutations in *goa-1*, *dgk-1*, *eat-16*, and *eat-11* are recovered as suppressors of *unc-43*(*gf*): The *gf* mutation *unc-43*(*n498*) causes pleiotropic defects including reduced egg laying, reduced defecation, and lethargy (Reiner *et al.* 1999). *n498* causes an E108K change in the kinase domain of UNC-43. This change is predicted to result in a partially Ca^{2+}-calmodulin independent kinase by analogy to a rat αCaMKII mutant in the same residue (Reiner *et al.* 1999). Comparison with the CaMKI crystal structure (Goldberg *et al.* 1996) suggests that this mutated residue destabilizes the autoinhibitory loop of the kinase without affecting substrate specificity, since the residue is far from the substrate binding cleft. In strong support of this hypothesis, *lf* mutations in *unc-43* confer phenotypes that are generally reciprocal to the *unc-43*(*gf*) phenotypes, including hyperactivation of the egg-laying muscles and increased frequency of the en-

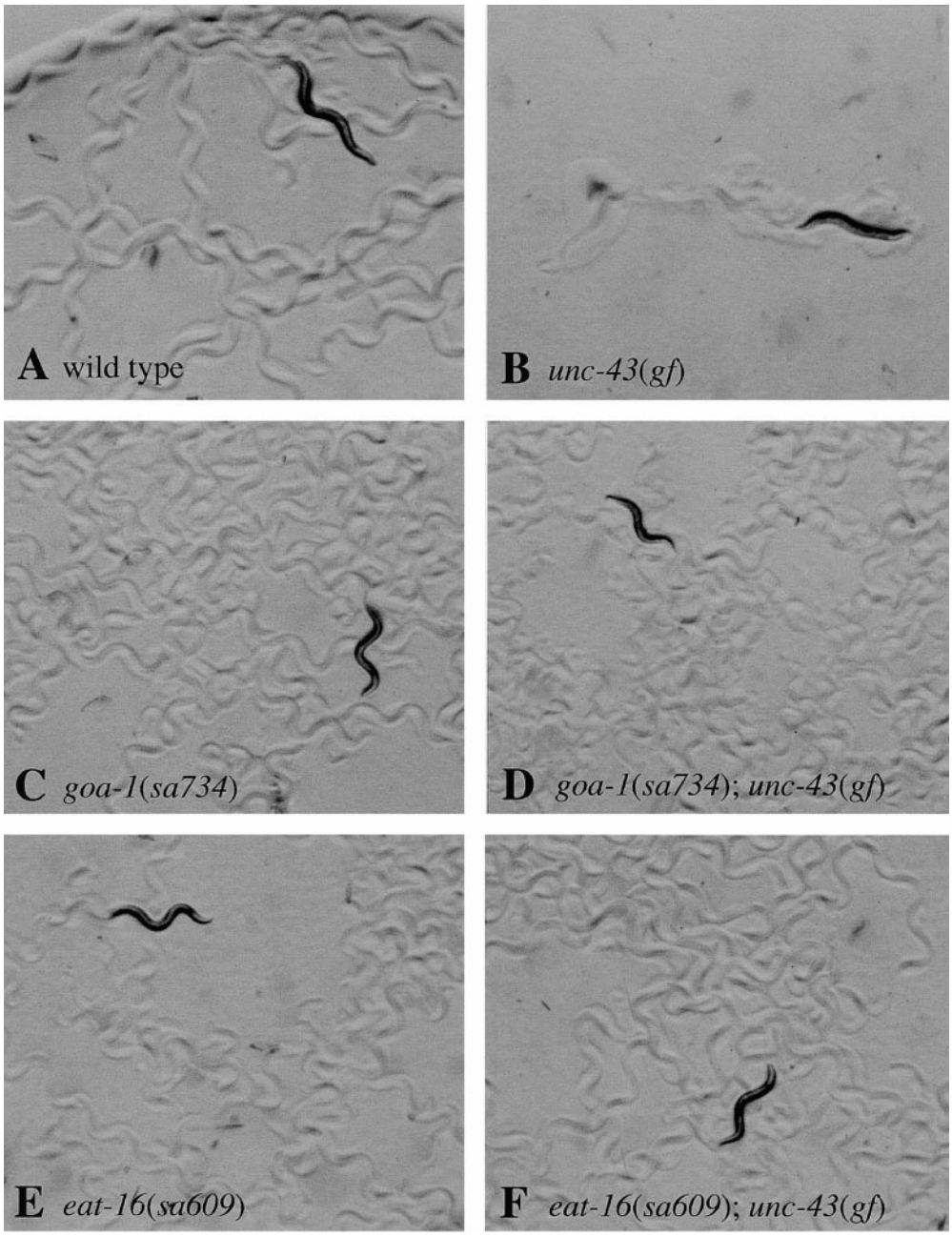

FIGURE 1.—Suppressors of the *unc-43*(*gf*) lethargy. Representative photographs compare (A) wild-type *C. elegans* with (B) *unc-43*(*gf*), (C) *goa-1*(*sa734*), (D) *goa-1*(*sa734*); *unc-43*(*gf*), (E) *eat-16*(*sa609*), and (F) *eat-16*(*sa609*); *unc-43*(*gf*) animals. The *unc-43*(*gf*) allele is *n498*. One animal was placed in the center of a bacterial lawn and photographed 30 min later. All animals were staged as L4 larvae and assayed 24 hr after growth at 20°. All photographs were taken at the same magnification. Note the increase in the number of tracks made in the bacterial lawn by suppressed animals in comparison to *unc-43*(*gf*).

teric muscle contractions required for defecation (REINER *et al.* 1999). The reciprocity of the locomotion rate phenotype is more difficult to assess since *unc-43*(*lf*) disrupts locomotory coordination in a manner that obscures the determination of locomotion rate. The coordination defects exhibited by *unc-43*(*lf*) animals include kinking when moving backward and spontaneous convulsions that involve the simultaneous contraction of dorsal and ventral body-wall muscles (REINER *et al.* 1999). Since these defects severely alter the coordinated pattern of locomotion, the locomotion rate of *unc-43*(*lf*) animals cannot be ascertained readily. However, *unc-43*(*lf*) animals variably exhibit short bursts of rapid forward movement, which may indicate underlying hyperactivity. The spontaneous convulsions exhibited by these animals may also reflect underlying hyperactivity (REINER *et al.* 1999).

To identify genes that act with *unc-43* to control locomotion rate, we used the lethargic phenotype of *unc-43*(*gf*) as the basis for a genetic suppressor screen. *unc-43*(*gf*) animals rarely move if undisturbed (Figure 1B). We reasoned that screening for increased locomotion of *unc-43*(*gf*) animals might identify genes that act with *unc-43* to control locomotion rate. After chemical mutagenesis of *unc-43*(*gf*) animals, we screened F_2 progeny for increased locomotion. We recovered 43 independent revertants from a screen of ~28,000 haploid genomes. Twenty-four of these were closely linked to the

TABLE 1

Summary of genes recovered as suppressors of the *unc-43*(*gf*) lethargy

Gene	Chromosome	Alleles isolated	No. other alleles	References
goa-1	I	*sa585*, *sa734*, *sa837*, *sa841*	4	Mendel *et al.* (1995); Ségalat *et al.* (1995)
dgk-1[a]	X	*sa605*, *sa748*, *sa760*, *sa766*	20	Hajdu-Cronin *et al.* (1999); Miller *et al.* (1999); Nurrish *et al.* (1999)
eat-16	I	*sa609*, *sa735*, *sa768*, *sa838*	2	Avery (1993); Hajdu-Cronin *et al.* (1999)
eat-11	I	*sa581*, *sa586*, *sa603*, *sa604*,[b] *sa762*, *sa765*, *sa833*[c]	1	Avery (1993)

[a] *dgk-1* has also been referred to as *sag-1* (Hajdu-Cronin *et al.* 1999).
[b,c] *eat-11*(*sa604*) and *eat-11*(*sa833*) are associated with decreased retention of eggs in comparison with other *eat-11* alleles.

unc-43 locus and exhibited *unc-43*(*lf*) locomotory characteristics. Therefore, these revertants are likely *lf* alleles of *unc-43*. The remaining 19 suppressor mutations were genetically unlinked to *unc-43* and exhibited recessive inheritance, consistent with *lf* mutations. In addition to increasing *unc-43*(*gf*) locomotion (Figure 1, D and F), these 19 mutations also increased *unc-43*(*gf*) egg laying. We obtained the suppressors as single mutants in an *unc-43*(+) background and found that all 19 exhibited hyperactive locomotion compared to wild type, and most also exhibited decreased retention of eggs. These phenotypes suggested that the suppressors could be allelic to genes in the *goa-1/egl-30* heterotrimeric G-protein signaling network. Complementation tests and genetic mapping showed that we had indeed isolated multiple alleles of *goa-1*, *dgk-1*, *eat-16*, and *eat-11* (Table 1; Figure 1).

goa-1, *dgk-1*, and *eat-16* are expressed throughout the nervous system and are components of the *goa-1/egl-30* network that has been shown to affect locomotion rate and egg-laying activity (Figure 2; Mendel *et al.* 1995; Ségalat *et al.* 1995; Hajdu-Cronin *et al.* 1999; Lackner *et al.* 1999; Miller *et al.* 1999; Nurrish *et al.* 1999). *goa-1*, *dgk-1*, and *eat-16* encode proteins that antagonize the EGL-30 ($G_q\alpha$) signaling pathway, which regulates production of the second messenger diacylglycerol (DAG). *goa-1* encodes a $G_o\alpha$ that may inhibit EGL-30 directly, act on a regulator of the EGL-30 pathway, or function in parallel (Mendel *et al.* 1995; Ségalat *et al.* 1995; Hajdu-Cronin *et al.* 1999; Miller *et al.* 1999; Nurrish *et al.* 1999). *eat-16* encodes a regulator of G-protein signaling (RGS) that appears to regulate EGL-30 activity directly (Hajdu-Cronin *et al.* 1999), and *dgk-1* encodes a diacylglycerol kinase that reduces levels of DAG, a product of phospholipase C activity (Nurrish *et al.* 1999). The effects of *goa-1*, *dgk-1*, and *eat-16* mutations on locomotion rate and egg laying are opposite to those of *egl-30* mutations. *goa-1*(*lf*), *dgk-1*(*lf*), and *eat-16*(*lf*) mutants exhibit hyperactivity and decreased retention of eggs, whereas *egl-30*(*lf*) mutants exhibit severe lethargy and increased retention of eggs (Mendel *et al.* 1995; Ségalat *et al.* 1995; Brundage *et al.* 1996; Hajdu-Cronin *et al.* 1999; Nurrish *et al.* 1999). The *goa-1/egl-30* network has been shown to regulate cholinergic neurotransmission between excitatory motor neurons and body-wall muscle (Lackner *et al.* 1999; Miller *et al.* 1999; Nurrish *et al.* 1999). Although its molecular identity is not known, *eat-11* has been shown to interact genetically with *egl-30*; therefore, *eat-11* is likely another gene that negatively regulates the EGL-30 signaling pathway (Avery 1993; Brundage *et al.* 1996; Lackner *et al.* 1999).

Since our screen recovered multiple alleles of genes in the *goa-1/egl-30* network, we infer that *unc-43* and the *goa-1/egl-30* network act together to control locomotion rate. In addition, since the *unc-43*(*gf*) egg-laying defect was also suppressed by mutations in the *goa-1/egl-30* network, we infer that *unc-43* and this G-protein network also act together in the egg-laying system.

Analysis of locomotory behavior indicates that UNC-43 may regulate the GOA-1/EGL-30 network: If mutations in *goa-1*, *dgk-1*, *eat-16*, and *eat-11* suppress *unc-43*(*gf*) because one or more of the gene products is a direct target of the UNC-43 kinase, then null alleles of these genes may completely suppress the *unc-43*(*gf*) lethargy. To test this, we first measured the *unc-43*(*gf*) suppression using a radial locomotion assay. In this assay, animals are allowed to disperse from the origin of a circular plate. In addition to several of our suppressor alleles, we also assayed *goa-1*(*n1134*), a *lf* allele identified in other work (Ségalat *et al.* 1995). We compared *unc-43*(*gf*) animals, *suppressor* (*sup*); *unc-43*(*gf*) double mutant animals, and *sup* single mutant animals. As expected, we found that *unc-43*(*gf*) animals have a severely reduced dispersal distance compared to wild type (Figure 3A). We found that alleles of *goa-1*, *dgk-1*, *eat-16*, and *eat-11* suppress this locomotion defect significantly in *sup*; *unc-43*(*gf*) double mutant animals (Figure 3, B–E). However, the suppression of *unc-43*(*gf*) by the alleles of *goa-1*, *dgk-1*, and *eat-16* that we examined in this assay is incomplete since the *sup*; *unc-43*(*gf*) double mutants do not disperse as far as the corresponding *sup* single mutants (Figure 3, B–D). In contrast, we found that the *eat-11*; *unc-43*(*gf*) double mutant animals disperse as well as the *eat-11* single mutant animals at early timepoints (Figure 3E). However, even for *eat-11*, the

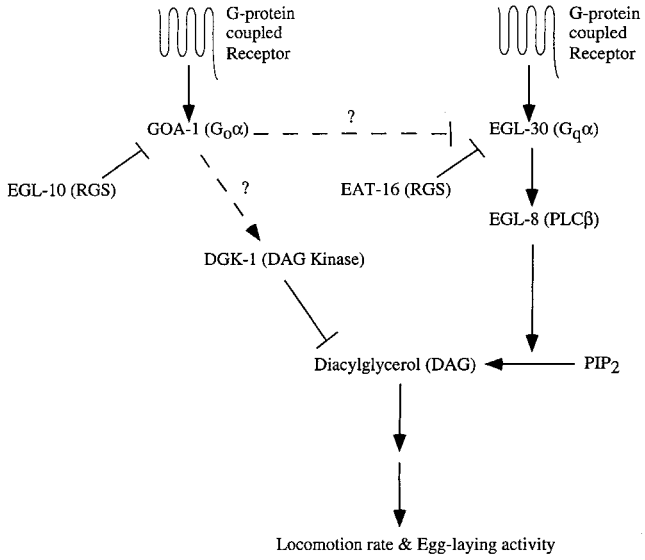

FIGURE 2.—A model for regulation of locomotion rate and egg-laying activity by the GOA-1/EGL-30 network. This model is compiled from the work of several groups (MENDEL *et al.* 1995; SÉGALAT *et al.* 1995; BRUNDAGE *et al.* 1996; HAJDU-CRONIN *et al.* 1999; LACKNER *et al.* 1999; MILLER *et al.* 1999; NURRISH *et al.* 1999). GOA-1($G_o\alpha$) and EGL-30($G_q\alpha$) couple to receptors in the plasma membrane and are each regulated by RGS proteins. EGL-30($G_q\alpha$) activates phospholipase Cβ (PLCβ), encoded by the *egl-8* gene. PLCβ cleaves phosphatidylinositol 4,5-bisphosphate into DAG and inositol-1,4,5-triphosphate (BERRIDGE 1984). GOA-1, DGK-1, and EAT-16 antagonize signaling by the EGL-30 pathway. GOA-1 may inhibit EGL-30 directly, act on a regulator of the EGL-30 pathway such as EAT-16 or DGK-1, or function in parallel to EGL-30.

dispersal of the single mutant exceeds that of the double mutant at later timepoints (data not shown).

The incomplete suppression of *unc-43*(*gf*) could be the result of non-null *sup* alleles. To control for this, we obtained *dgk-1*(*sy428*) and *eat-16*(*sy438*), which were identified as putative null alleles by genetic criteria in other work (HAJDU-CRONIN *et al.* 1999). We also sequenced the entire *goa-1* coding region in *goa-1*(*sa734*) because this allele behaves similarly to *goa-1*(*n363*), a deletion allele that removes a region containing the *goa-1* gene and perhaps other genes (SÉGALAT *et al.* 1995). We found that *sa734* contains an early stop mutation and is thus an excellent candidate for a molecular null. Assaying these alleles, we found that *eat-16*(*sy438*) shows strong but incomplete suppression in the radial assay, similar to the *eat-16* alleles isolated in our screen (data not shown). In addition, our *sa609* allele of *eat-16* was also shown to behave genetically as a null (HAJDU-CRONIN *et al.* 1999). Interestingly, we found that null alleles of *goa-1* and *dgk-1* severely reduce dispersal of both *sup* single mutant and *sup*; *unc-43*(*gf*) double mutant animals. These alleles cause animals to move in a rapid but ineffective manner that results in little dispersal from a point of origin (data not shown). This ineffective mode of locomotion appears to result from an increase in wave amplitude, which has been previously reported for *goa-1* (MENDEL *et al.* 1995; HAJDU-CRONIN *et al.* 1999), and an increase in the frequency of direction reversals. Therefore, we found that radial locomotion rate is a poor measure of the suppression conferred by null alleles of *goa-1* and *dgk-1*.

As an alternative means of measuring the suppression of the *unc-43*(*gf*) lethargy, we measured the body-bend rate of single and double mutant animals. Table 2 shows that *unc-43*(*gf*) animals have markedly fewer body bends per minute than wild-type animals and that *sup*; *unc-43*(*gf*) double mutant animals exhibit a significantly higher body-bend rate than *unc-43*(*gf*) animals. We found that *goa-1*(*sa734*) suppresses *unc-43*(*gf*) to the level of the *goa-1*(*sa734*) single mutant in this assay, suggesting that UNC-43 may regulate GOA-1 activity. In contrast, the suppression by *dgk-1*(*sy428*) is incomplete. For *eat-11*, we analyzed *sa833* since this allele confers greater hyperactivity and egg-laying activity than other *eat-11* alleles (data not shown). We found that the suppression by *eat-11*(*sa833*) is also incomplete. Although the strength of the *sa833* phenotype suggests that this may be a strong loss-of-function allele, rigorous determination of the *eat-11* null phenotype awaits the cloning of the *eat-11* gene. *eat-16*(*sy438*) conferred variable suppression, with some individual *sup*; *unc-43*(*gf*) animals showing complete suppression and others exhibiting weaker suppression. Because of this variability, we also assayed *eat-16*(*sa609*) and obtained similar results (data not shown). Since neither *sy438* nor *sa609* is a clear molecular null by sequence analysis (HAJDU-CRONIN *et al.* 1999), the variability of the *eat-16* suppression could be due to residual EAT-16 activity. However, because *sy438* and *sa609* were shown to behave genetically as null alleles, we suggest that the body-bend assay may be a less sensitive measure of the *eat-16* suppression than the radial assay, in which both *sy438* and *sa609* clearly conferred incomplete suppression. Differences between the radial assay and the body-bend assay are not surprising since these assays measure locomotion rate differently. Although other explanations are possible, a simple interpretation of our analysis of locomotion rate is that UNC-43 may regulate GOA-1 activity.

If the suppression of *unc-43*(*gf*) reflects a direct biochemical interaction between UNC-43 and the GOA-1/EGL-30 network, mutations in this network should suppress *unc-43*(*gf*) specifically and not strongly affect *gf* mutations that reduce locomotion rate by other mechanisms. To test the specificity of the *unc-43*(*gf*) suppression by mutations in the *goa-1/egl-30* network, we examined the effect of *goa-1*(*null*) mutations on *gf* mutations in *unc-93, unc-103*, and *unc-110* (Table 3). Like *unc-43*(*gf*), these *gf* mutants exhibit few body bends/minute. We found that *goa-1*(*null*) mutations increase the body-bend rate of *unc-93*(*gf*), *unc-103*(*gf*), and *unc-110*(*gf*) animals only slightly in comparison to their effect on *unc-43*(*gf*) animals. The weak effect of *goa-*

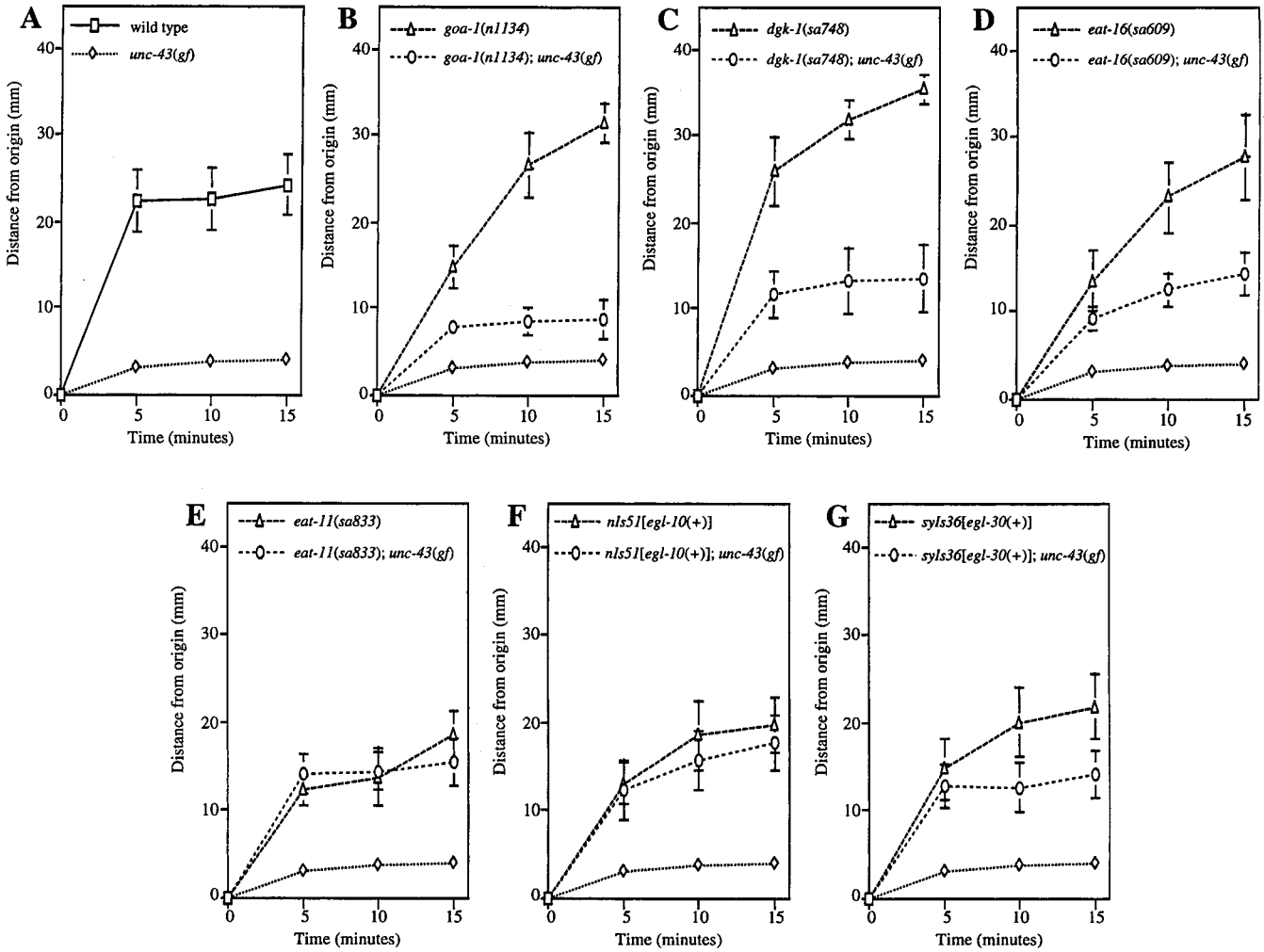

FIGURE 3.—Radial locomotion of *unc-43(gf)* suppressors. (A) Wild-type animals disperse rapidly and evenly across the assay plate. *unc-43(gf)* animals are strikingly defective in dispersal compared with wild-type animals ($P < 0.0001$). (B–G) *sup; unc-43(gf)* strains disperse farther than *unc-43(gf)* [$P = 0.02$ for *unc-43(gf)* *vs. goa-1(n1134); unc-43(gf)*, $P = 0.003$ for *unc-43(gf)* *vs. dgk-1(sa748); unc-43(gf)*, and $P < 0.0001$ for all others]. The *unc-43(gf)* allele is *n498*. Each data point represents the combined measurements from 10 animals. The same *unc-43(gf)* curve is included in each panel for comparison. Error bars represent standard error of the mean. Differences between genotypes were analyzed for significance at the 15-min timepoint using Student's *t*-test. Other alleles of each suppressor gene were analyzed and the same trend as shown was observed. *n1134* was identified in other work as a *lf* allele of *goa-1* (SÉGALAT *et al.* 1995). The curves resulting from the *goa-1*, *dgk-1*, and *eat-16* single mutant data extend above the curve for wild-type because these single mutant animals tended to remain at the edge of the assay plate, rather than continuing to disperse evenly over the plate like wild-type animals.

1(null) on *unc-110(gf)* and *unc-93(gf)* mutants must be indirect and nonspecific since *unc-93* and *unc-110* function in body-wall muscle (LEVIN and HORVITZ 1992; D. JOHNSTONE and J. H. THOMAS, unpublished results), whereas *goa-1* acts neuronally to control locomotion (MENDEL *et al.* 1995; SÉGALAT *et al.* 1995; NURRISH *et al.* 1999). In contrast, *unc-103* may function in some of the same neurons as the *goa-1/egl-30* network since *unc-103* probably acts in excitatory motor neurons (D. REINER and J. H. THOMAS, unpublished results). However, since *goa-1(null)* has only a weak effect on *unc-103(gf)*, we conclude that this interaction is also indirect and nonspecific. Thus, *gf* mutations in *unc-43*, *unc-93*, *unc103*, and *unc-110* reduce locomotion rate to a similar degree, but *goa-1* mutations suppress *unc-43(gf)* significantly better. These results suggest that *unc-43* and the *goa-1/egl-30* pathway regulate locomotion rate via the same mechanism, supporting a model in which UNC-43 regulates GOA-1 activity.

Since the *goa-1/egl-30* network has been shown to regulate synaptic transmission at cholinergic synapses (LACKNER *et al.* 1999; MILLER *et al.* 1999; NURRISH *et al.* 1999), we examined the interaction between *unc-43* and the *goa-1/egl-30* network by assaying the response of animals to the acetylcholinesterase inhibitor aldicarb. Loss-of-function mutations in *goa-1* or *dgk-1* confer hypersensitivity to the paralytic effects of aldicarb (NURRISH *et al.* 1999), whereas *lf* mutations in *egl-30* or *egl-8*

TABLE 2

Body-bend rate of *unc-43*(*gf*) suppressors

Genotype	Body bends/minute
N2 (wild type)	37.7 ± 2.0
unc-43(*gf*)	3.2 ± 1.2
goa-1(*sa734*)	35.1 ± 2.4
goa-1(*sa734*); *unc-43*(*gf*)	37.1 ± 2.6
dgk-1(*sy428*)	32.8 ± 2.4
unc-43(*gf*); *dgk-1*(*sy428*)	16.6 ± 3.8
eat-16(*sy438*)	50.5 ± 2.7
eat-16(*sy438*); *unc-43*(*gf*)	42.3 ± 5.2
eat-11(*sa833*)	49.7 ± 2.4
eat-11(*sa833*); *unc-43*(*gf*)	20.4 ± 2.2

Body bends/minute data are the mean ± standard error from 4–11 animals. *goa-1*, *dgk-1*, and *eat-16* alleles shown are putative null alleles (see text). The *unc-43*(*gf*) allele is *n498*. *eat-16*(*sa609*) gave results similar to *eat-16*(*sy438*). Strains containing *goa-1*(*sa734*) and *dgk-1*(*sy428*) reversed direction frequently, often exhibiting only partial body bends, which were not counted in this assay (see MATERIALS AND METHODS). Therefore, this assay underestimates the movement rate of strains containing these mutations. Differences between genotypes were analyzed using Student's *t*-test. Differences from *unc-43*(*gf*) include: *goa-1*(*sa734*); *unc-43*(*gf*) ($P < 0.0001$), *dgk-1*(*sy428*); *unc-43*(*gf*) ($P = 0.02$), *eat-16*(*sy438*); *unc-43*(*gf*) ($P < 0.0001$), and *eat-11*(*sa833*); *unc-43*(*gf*) ($P = 0.0001$). Other differences include: *dgk-1*(*sy428*) from *dgk-1*(*sy428*); *unc-43*(*gf*) ($P = 0.005$) and *eat-11*(*sa833*) from *eat-11*(*sa833*); *unc-43*(*gf*) ($P < 0.0001$). *goa-1*(*sa734*) is not different from *goa-1*(*sa734*); *unc-43*(*gf*) ($P = 0.6$), and *eat-16*(*sy438*) is not different from *eat-16*(*sy438*); *unc-43*(*gf*) ($P = 0.3$).

TABLE 3

Effect of *goa-1*(*null*) on the body-bend rate of other mutants with reduced locomotion

Genotype	Body bends/minute	Fold increase over *unc*(*gf*) single mutant
N2 (wild type)	37.7 ± 2.0	
goa-1(*sa734*)	35.1 ± 2.4	
goa-1(*n363*)	31.9 ± 1.6	
unc-43(*gf*)	3.2 ± 1.2	
unc-103(*gf*)	1.5 ± 0.8	
unc-93(*gf*)	3.6 ± 0.4	
unc-110(*gf*)	8.7 ± 0.7	
goa-1(*sa734*); *unc-43*(*gf*)	37.1 ± 2.6	11.5
goa-1(*n363*); *unc-43*(*gf*)	26.7 ± 3.1	8.3
goa-1(*sa734*); *unc-103*(*gf*)	2.7 ± 1.4	1.8
goa-1(*n363*); *unc-103*(*gf*)	5.5 ± 1.0	3.7
goa-1(*sa734*); *unc-93*(*gf*)	7.3 ± 1.3	2.0
goa-1(*n363*); *unc-93*(*gf*)	9.1 ± 1.3	2.5
goa-1(*sa734*); *unc-110*(*gf*)	12.5 ± 1.9	1.4

Body bends/minute data are the mean ± standard error from five or six animals. *gf* alleles used are: *unc-43*(*n498*), *unc-93*(*e1500*), *unc-103*(*n500*), and *unc-110*(*sa859*). *goa-1*(*sa734*) is a putative null allele (see text), and *goa-1*(*n363*) is a deletion allele that removes a region containing the *goa-1* gene (SÉGALAT *et al.* 1995). Strains containing *goa-1*(*sa734*) and *goa-1*(*n363*) reversed direction frequently, often exhibiting only partial body bends, which were not counted in this assay (see MATERIALS AND METHODS). Therefore, this assay underestimates the movement rate of strains containing these mutations. Data from *goa-1*(*sa734*) and *goa-1*(*sa734*); *unc-43*(*gf*) are the same as the data shown in Table 2. Differences between genotypes were analyzed using Student's *t*-test. The following differences were obtained: *unc-43*(*gf*) from *goa-1*(*sa734*); *unc-43*(*gf*) ($P < 0.0001$) and from *goa-1*(*n363*); *unc-43*(*gf*) ($P = 0.0001$), *unc-103*(*gf*) from *goa-1*(*n363*); *unc-103*(*gf*) ($P = 0.01$), *unc-93*(*gf*) from *goa-1*(*sa734*); *unc-93*(*gf*) ($P = 0.03$) and from *goa-1*(*n363*); *unc-93*(*gf*) ($P = 0.004$). There was no significant difference between *unc-103*(*gf*) and *goa-1*(*sa734*); *unc-103*(*gf*) ($P = 0.5$) or between *unc-110*(*gf*) and *goa-1*(*sa734*); *unc-110*(*gf*) ($P = 0.1$). *goa-1*(*n363*) is a slightly weaker suppressor of *unc-43*(*gf*) than *goa-1*(*sa734*). Since *goa-1*(*n363*) is a deletion that may affect genes other than *goa-1* (SÉGALAT *et al.* 1995), the slight difference in suppression by the two alleles is likely due to genetic background differences.

confer resistance to aldicarb-induced paralysis (LACKNER *et al.* 1999; MILLER *et al.* 1999). If *unc-43* regulates the *goa-1/egl-30* pathway, mutations in *unc-43* should confer an altered response to aldicarb. To test this, we subjected wild-type, *unc-43*(*gf*), and *unc-43*(*null*) animals to 1 mM aldicarb and scored paralysis over time. We found that the *unc-43* mutants show strikingly altered responses to aldicarb-induced paralysis in comparison to wild type: *unc-43*(*gf*) confers resistance to aldicarb-induced paralysis, whereas the putative null allele *unc-43*(*n1186*) (REINER *et al.* 1999) confers hypersensitivity (Figure 4A). In agreement with our measurements of locomotion rate, *goa-1*(*null*) suppresses *unc-43*(*gf*) completely: *goa-1* (*null*); *unc-43*(*gf*) animals are as hypersensitive to aldicarb-induced paralysis as the *goa-1*(*null*) single mutant (Figure 4B). In contrast, the other suppressors confer incomplete suppression (Figure 4, C–E). These results indicate that *unc-43* and the *goa-1/egl-30* pathway regulate cholinergic synaptic transmission similarly and further support a model in which UNC-43 regulates GOA-1 activity.

The interaction between *unc-43* and the *goa-1/egl-30* network also occurs in the egg-laying system: Although we isolated mutations in the *goa-1/egl-30* network as suppressors of the *unc-43*(*gf*) lethargic phenotype, we found that these same mutations also suppressed the *unc-43*(*gf*) egg-laying defect. However, since the targets of *unc-43* may vary in different tissues, the interaction between *unc-43* and the *goa-1/egl-30* pathway in the egg-laying system need not be the same as the interaction in the locomotory system. To test whether or not the gene products of the *goa-1/egl-30* network might be targets of the UNC-43 kinase in the egg-laying system, we compared the egg-laying behavior of *unc-43*(*gf*), *sup*; *unc-43*(*gf*), and *sup* single mutant animals. To assess the activity of the egg-laying muscles, we scored the developmental stages of eggs laid (Table 4). Wild-type animals lay eggs at about the gastrulation stage of embryogenesis and do not accumulate excess eggs in their gonad. *unc-43*(*gf*) animals lay later-staged eggs and be-

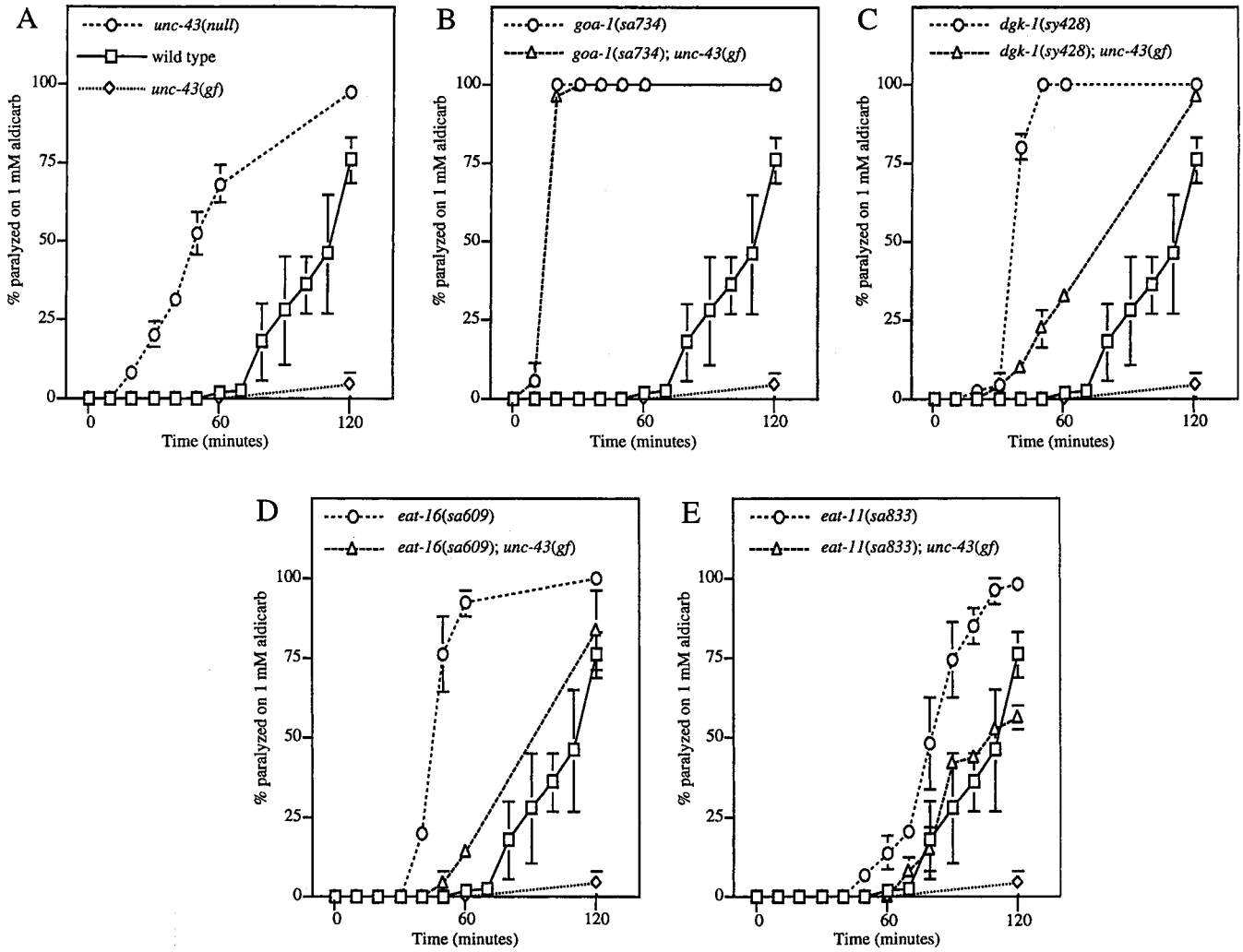

FIGURE 4.—Effect of aldicarb on *unc-43* mutants and *unc-43*(*gf*) suppressed strains. (A) Comparison between the response of wild-type, *unc-43*(*gf*), and *unc-43*(*null*) animals to 1 mM aldicarb exposure over 2 hr. *unc-43*(*gf*) animals show a decreased rate of paralysis compared with wild type ($P = 0.002$ for data at the 120-min timepoint), and *unc-43*(*null*) animals show an increased rate of paralysis in comparison with wild type ($P < 0.0001$ for data at the 60-min timepoint). (B) The *goa-1*(*sa734*); *unc-43*(*gf*) double mutant shows the same rapid rate of paralysis as the *goa-1*(*sa734*) single mutant ($P = 0.25$ for data at the 20-min timepoint). *goa-1*(*sa734*) is a putative null allele (see text). (C–E) *sup*; *unc-43*(*gf*) strains with putative null alleles of *dgk-1* and *eat-16* and with *eat-11*(*sa833*) exhibit paralysis rates that are intermediate. *eat-16*(*sy438*) gave similar results to *eat-16*(*sa609*). The *unc-43*(*gf*) allele is *n498*. *unc-43*(*null*) is the putative null allele *n1186* (REINER *et al.* 1999). Each data point represents data combined from at least two independent experiments. Strains were analyzed in parallel for each experiment. Error bars represent standard error of the mean. The same wild-type and *unc-43*(*gf*) curves were included in each panel for comparison. Differences between *unc-43* mutants and wild type were analyzed using Student's *t*-test. *goa-1*(*sa734*) *vs. goa-1*(*sa734*); *unc-43*(*gf*) was analyzed using Fisher's exact test applied to raw data since the *goa-1*(*sa734*) data at the 20-min timepoint had a standard deviation of zero. Data were not collected at the 70- to 110-min timepoints for some of the strains because their response trend became apparent at earlier timepoints.

come bloated with retained eggs, indicating reduced activity of the egg-laying muscles. For *goa-1*, *dgk-1*, *eat-16*, and *eat-11*, the *sup*; *unc-43*(*gf*) strains lay eggs at significantly earlier stages than *unc-43*(*gf*) and retain fewer eggs in their gonad than *unc-43*(*gf*). The suppression by *goa-1*(*sa734*) is the strongest: *goa-1*(*sa734*); *unc-43*(*gf*) animals lay eggs as early as *goa-1*(*sa734*) single mutant animals, indicating complete suppression. In contrast, the *dgk-1*, *eat-16*, and *eat-11 sup*; *unc-43*(*gf*) strains show strong but incomplete suppression. In support of the specificity of the interaction between *unc-43* and the *goa-1*/*egl-30* network in the egg-laying system, *unc-93*(*gf*) and *unc-103*(*gf*), which cause animals to become bloated with retained eggs in addition to their effect on locomotion (GREENWALD and HORVITZ 1980; PARK and HORVITZ 1986), were not noticeably suppressed for egg laying by *goa-1*(*sa734*) (data not shown). These results are similar to the results we obtained for the suppression of the *unc-43*(*gf*) lethargy and indicate that UNC-43 may regulate GOA-1 activity in the egg-laying system.

Other genes in the *goa-1*/*egl-30* network can suppress

TABLE 4

Stages of eggs laid by *unc-43*(*gf*) suppressed strains

Genotype	Developmental stages of eggs laid (% total no. eggs laid)			No. of eggs laid
	One to four cells	Five cells to gastrulation	After gastrulation	
N2 (wild type)	2	98	0	167
unc-43(*gf*)	0	0	100	114
goa-1(*sa734*)	90	10	0	70
goa-1(*n363*)	85	15	0	80
dgk-1(*sy428*)	94	6	0	72
eat-16(*sy438*)	81	19	0	130
eat-11(*sa833*)	80	20	0	64
nIs51[*egl-10*(+)]	91	9	0	93
syIs36[*egl-30*(+)]	63	37	0	49
goa-1(*sa734*); *unc-43*(*gf*)	81	18	1	98
goa-1(*n363*); *unc-43*(*gf*)	69	29	2	93
dgk-1(*sy428*); *unc-43*(*gf*)	0	83	17	45
eat-16(*sy438*); *unc-43*(*gf*)	3	97	0	86
eat-11(*sa833*); *unc-43*(*gf*)	0	100	0	54
nIs51[*egl-10*(+)]; *unc-43*(*gf*)	0	100	0	57
syIs36[*egl-30*(+)]; *unc-43*(*gf*)	34	64	2	47

The *goa-1*, *dgk-1*, and *eat-16* alleles shown are putative nulls (see text). The *unc-43*(*gf*) allele is *n498*. *eat-16*(*sa609*) gave results similar to *eat-16*(*sy438*). By grouping the raw data into eggs laid at stages through gastrulation and eggs laid at stages after gastrulation and applying Fisher's exact test, there is a significant difference between each suppressed strain and the *unc-43*(*gf*) single mutant ($P < 0.0001$ for all comparisons). To compare *goa-1*(*null*) and *goa-1*(*null*); *unc-43*(*gf*), data were grouped into eggs laid at one to four cells and eggs laid at stages later than four cells, and Fisher's exact test was applied. There is no significant difference between *goa-1*(*sa734*) and *goa-1*(*sa734*); *unc-43*(*gf*) ($P = 0.1$). *goa-1*(*n363*) is a slightly weaker suppressor than *goa-1*(*sa734*) (see Table 3 legend): *goa-1*(*n363*) may be different from *goa-1*(*n363*); *unc-43*(*gf*), $P = 0.02$.

***unc-43*(*gf*):** Since we found that several genes in the *goa-1/egl-30* network suppress *unc-43*(*gf*), we expected that other genes in this network would show a similar interaction. The suppressor alleles that we had isolated were *lf* mutations in genes that normally antagonize EGL-30 signaling. Therefore, we predicted that *gf* mutations in genes that positively regulate EGL-30 signaling would also suppress *unc-43*(*gf*). Such mutations were probably not isolated in our screen because *gf* mutations are rare. To test our prediction, we combined *unc-43*(*gf*) with transgenes that overexpress either EGL-30 or EGL-10, an RGS protein that is thought to inhibit *goa-1* (Figure 2; Koelle and Horvitz 1996). The integrated transgenes *syIs36*[*egl-30*(+)] and *nIs51*[*egl-10*(+)] overexpress wild-type protein and confer phenotypes that are opposite to the *lf* phenotypes of these genes: animals carrying either transgene alone exhibit hyperactive locomotion and decreased retention of eggs (Brundage *et al.* 1996; Koelle and Horvitz 1996). Both transgenes strongly suppress the *unc-43*(*gf*) lethargy and egg-laying defects (Figure 3, F and G; Table 4). These results are consistent with UNC-43 regulation of the GOA-1/EGl-30 network in the locomotory and egg-laying systems. However, since the strength of this suppression is presumably dependent upon the amount of EGL-10 or EGL-30 that is expressed from the transgenes, we focused our analysis and conclusions on the *lf* mutations that suppress *unc-43*(*gf*).

UNC-43 may act through GOA-1 or EGL-30: Since *unc-43*(*gf*) is likely to encode a kinase with Ca^{2+}-independent activity, UNC-43(gf) may be largely independent of upstream regulators. Therefore, we expected that our screen would preferentially recover genes that act downstream or in parallel to *unc-43*. To further test whether the *goa-1/egl-30* network acts downstream of *unc-43*, we made double mutants with *unc-43*(*null*) and either *egl-30*(*lf*) or *syIs9*[*goa-1*(*gf*)], an integrated transgene that overexpresses an activated form of GOA-1 (Mendel *et al.* 1995). *syIs9*[*goa-1*(*gf*)] and *egl-30*(*lf*) animals lay eggs of later stages, become bloated with retained eggs, and are lethargic (Mendel *et al.* 1995; Brundage *et al.* 1996; Hajdu-Cronin *et al.* 1999). *unc-43*(*null*) animals lay eggs of earlier stages and exhibit complex locomotion phenotypes including spontaneous convulsions (Reiner *et al.* 1999). If *goa-1* and *egl-30* act downstream of *unc-43*, we would expect the double mutants to exhibit the phenotypes of *syIs9*[*goa-1*(*gf*)] and *egl-30*(*lf*) single mutants. To compare the mutants we measured the egg-laying phenotype rather than locomotion rate since *unc-43*(*null*) severely disrupts coordinated movement. We found that the double mutants lay late-staged eggs like the respective *syIs9*[*goa-1*(*gf*)]

TABLE 5

Stages of eggs laid by *unc-43*(*null*) double mutants

Genotype	Developmental stages of eggs laid (% total no. eggs laid)			No. of eggs laid
	One to four cells	Five cells to gastrulation	After gastrulation	
N2 (wild type)	0	100	0	35
unc-43(*null*)	44	56	0	52
syIs9[*goa-1*(*gf*)]	0	45	55	29
syIs9[*goa-1*(*gf*)]; *unc-43*(*null*)	0	33	67	48
egl-30(*lf*)	0	0	100	39
egl-30(*lf*); *unc-43*(*null*)	0	0	100	45

Genotypes are: *unc-43*(*n1186*), *syIs9*[*goa-1*(*gf*)], an integrated transgene that overexpresses an activated form of GOA-1 (Mendel *et al.* 1995), and *egl-30*(*ad805*), a strong *lf* mutation (Brundage *et al.* 1996). *unc-43*(*n1186*) is a putative null allele (Reiner *et al.* 1999). We used *egl-30*(*ad805*) in this analysis because stronger *egl-30*(*lf*) alleles severely reduce viability (Brundage *et al.* 1996). By grouping the raw data into eggs laid at stages through gastrulation and eggs laid at stages after gastrulation and applying Fisher's exact test, we found no significant difference between *syIs9*[*goa-1*(*gf*)] and *syIs9*[*goa-1*(*gf*)]; *unc-43*(*null*) ($P = 0.3$). *unc-43*(*null*) is different from *syIs9*[*goa-1*(*gf*)]; *unc-43*(*null*) ($P < 0.0001$) and from *egl-30*(*lf*); *unc-43*(*null*) ($P < 0.0001$).

or *egl-30*(*lf*) single mutants (Table 5). Furthermore, the double mutants become as bloated with retained eggs as the *syIs9*[*goa-1*(*gf*)] and *egl-30*(*lf*) single mutants (data not shown). Though we did not measure it, the locomotion phenotype of the double mutants appears similar to the respective *syIs9*[*goa-1*(*gf*)] or *egl-30*(*lf*) single mutants, rather than like *unc-43*(*null*): the double mutants are about as lethargic as the *syIs9*[*goa-1*(*gf*)] and *egl-30*(*lf*) single mutants, and we never observed spontaneous convulsions or bursts of rapid forward movement. Occasionally we observed double mutant animals that exhibited a very slight increase in locomotory activity over the respective *syIs9*[*goa-1*(*gf*)] or *egl-30*(*lf*) single mutant. The slight increase in activity may be due to residual EGL-30 activity from the *ad805* allele and from transgene mosaicism in *syIs9* [*goa-1*(*gf*)]; *unc-43*(*null*) animals. The results of this epistasis analysis are consistent with *goa-1* and *egl-30* acting downstream of *unc-43* to control egg-laying activity and locomotion rate. This genetic relationship indicates that UNC-43 may regulate the GOA-1/EGL-30 network in the locomotory and egg-laying systems via the regulation of GOA-1 or EGL-30 activity.

DISCUSSION

The genetic interaction we have described between *unc-43* and the *goa-1/egl-30* network indicates that UNC-43 may directly regulate this network. Such a regulator is expected to act upstream of the *goa-1/egl-30* network and, when activated, should be strongly suppressed by mutations in this network. Our screen with *unc-43*(*gf*) and our double mutant analysis with *unc-43*(*null*) are consistent with *unc-43* acting upstream of *goa-1* and *egl-30*. Quantitative analysis of the *unc-43*(*gf*) suppression demonstrates that mutations in the *goa-1/egl-30* network suppress *unc-43*(*gf*) strongly and specifically. Since previous genetic analysis indicates that *goa-1* may act upstream of *egl-30* (Hajdu-Cronin *et al.* 1999; Miller *et al.* 1999), GOA-1 is a logical candidate for regulation by UNC-43. Our quantitative analysis of the *unc-43*(*gf*) suppression is consistent with this model since our data show that *goa-1*(*null*) suppresses the lethargy, aldicarb resistance, and egg-laying defects of *unc-43*(*gf*) to the level of the *goa-1*(*null*) single mutant. This is the expected result from a single target of UNC-43 regulation; therefore a simple model is that the effect of *unc-43*(*gf*) on locomotion rate and egg-laying activity is caused by inappropriate activation of GOA-1.

In addition to indicating that UNC-43 may regulate GOA-1 activity, our data indicate that GOA-1, in turn, may regulate EGL-30 activity rather than DGK-1 activity (see Figure 2). Since the suppression of *unc-43*(*gf*) by a putative null allele of *dgk-1* is significantly weaker than the suppression by *goa-1*(*null*) in both the locomotory and egg-laying systems, DGK-1 may act partly or fully in parallel to GOA-1 rather than as an effector of GOA-1. This model for GOA-1 activity has been proposed by others (Hajdu-Cronin *et al.* 1999; Miller *et al.* 1999; Nurrish *et al.* 1999). It has also been proposed that GOA-1 regulates EGL-30 activity by modulating EAT-16 (Hajdu-Cronin *et al.* 1999). This model predicts that null alleles of *eat-16* should suppress *unc-43*(*gf*) as strongly as *goa-1*(*null*). However, we found that the suppression by putative null alleles of *eat-16* was clearly incomplete in the radial locomotion assay, the aldicarb response assay, and the egg-laying assay. These results suggest that *eat-16* regulates *egl-30* in parallel to *goa-1*, supporting the model in which GOA-1 regulates EGL-30 directly (Hajdu-Cronin *et al.* 1999; Miller *et al.*

1999). However, since the results of genetic analysis depend on the nature of the mutations analyzed, confirmation of these models awaits the identification of clear molecular null alleles of *dgk-1* and *eat-16*, as well as biochemical analysis.

The genetic interaction between *unc-43* and *goa-1* suggests that UNC-43 could directly activate GOA-1 by phosphorylation or could indirectly activate GOA-1 by interacting with a GOA-1 regulator. EGL-10, the RGS protein that is thought to regulate GOA-1 activity, is an obvious candidate for such an interaction. Since RGS proteins decrease Gα activity by increasing their rate of GTP hydrolysis (Hunt *et al.* 1996), inhibition of EGL-10 (by UNC-43 phosphorylation) would increase GOA-1 activity. The amino acid sequences of both GOA-1 and EGL-10 contain CaMKII consensus phosphorylation sites (RXXS/T). Although such sites are not necessarily required or predictive of CaMKII phosphorylation (Kennelly and Krebs 1991), one consensus site in the N terminus of EGL-10 has been perfectly conserved in the N terminus of human RGS7. Human RGS7 is a proposed functional homolog of EGL-10, sharing 75% amino acid identity in the N terminus and 53% overall amino acid identity (Koelle and Horvitz 1996). However, since *egl-10*(*null*) mutants are not as severely lethargic as *unc-43*(*gf*) (data not shown), a second UNC-43 target that also regulates GOA-1 activity is required to explain the entire *unc-43*(*gf*) effect on locomotion rate. Since CaMKII is a multifunctional CaM kinase able to phosphorylate many substrates *in vitro* (reviewed in Hanson and Schulman 1992), models in which UNC-43 regulates more than one component of the GOA-1/EGL-30 network are plausible. However, we favor the simpler model in which UNC-43 regulates GOA-1 directly. Additional biochemical analysis will be required to examine these possibilities.

Previous identification of putative CaMKII phosphorylation targets has relied almost exclusively on candidate gene approaches and *in vitro* phosphorylation assays (Hanson and Schulman 1992). Such approaches have surely missed some targets and implicated other, nonphysiological targets. Gα subunits have not been previously implicated as CaMKII phosphorylation targets, though $G_i\alpha$ and $G_t\alpha$ (transducin) have been shown to be phosphorylated *in vitro* by protein kinase C, and $G_q\alpha$ and $G_s\alpha$ subunits have been shown to undergo tyrosine phosphorylation (Katada *et al.* 1985; Zick *et al.* 1986; Moyers *et al.* 1995; Umemori *et al.* 1997). RGS proteins belong to a relatively new protein family and their phosphorylation status has not been reported. Interestingly, a member of a different family of GTPase-activating proteins that regulates the small G protein Ras has been shown to be phosphorylated in a CaMKII-dependent manner in rat (Chen *et al.* 1998).

Our genetic analysis does not exclude the possibility that UNC-43 acts in parallel to the GOA-1/EGL-30 network. CaMKII has been shown to regulate neuronal activity by several different mechanisms, including interactions with adenylyl cyclase, α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA)-type glutamate receptors, and Eag-related K^+ channels (McGlade-McCulloh *et al.* 1993; Griffith *et al.* 1994; Barria *et al.* 1997; Wei *et al.* 1998; Reiner *et al.* 1999). However, if the *unc-43*(*gf*) lethargy were due to inappropriate regulation of these genes, we would have expected to recover them in our screen. To examine the possibility that some genes were missed in our screen, we tested whether *lf* mutations in *glr-1*, a conserved AMPA-type glutamate receptor (Hart *et al.* 1995; Maricq *et al.* 1995), would suppress *unc-43*(*gf*). We found that *glr-1*(*lf*) suppressed none of the *unc-43*(*gf*) phenotypes (data not shown). Therefore, since we recovered multiple alleles of *goa-1*, *dgk-1*, *eat-16*, and *eat-11*, and the suppression by these alleles is striking, we propose that CaMKII also regulates neuronal activity by controlling G_o/G_q pathways.

unc-43 and the *goa-1/egl-30* network are widely expressed throughout the nervous system (Mendel *et al.* 1995; Ségalat *et al.* 1995; Koelle and Horvitz 1996; Hajdu-Cronin *et al.* 1999; Lackner *et al.* 1999; Miller *et al.* 1999; Nurrish *et al.* 1999; Reiner *et al.* 1999; E. Newton and J. H. Thomas, unpublished results). This coexpression makes a direct interaction between UNC-43 and the GOA-1/EGL-30 network plausible. Work by other groups indicates that the *goa-1/egl-30* network regulates synaptic transmission in body-wall muscle motor neurons and perhaps other cell types (Lackner *et al.* 1999; Miller *et al.* 1999; Nurrish *et al.* 1999). Specifically, *goa-1* and *egl-30* are thought to mediate presynaptic modulation of motor neuron synaptic transmission, in part, by effecting changes in localization of UNC-13, a DAG-binding protein predicted to regulate synaptic vesicles (Maruyama and Brenner 1991; Betz *et al.* 1997). *goa-1* is thought to mediate the effect of humorally acting serotonin and perhaps other neuromodulators (Nurrish *et al.* 1999). An interaction between *unc-43* and the *goa-1/egl-30* network in motor neurons could explain the effect of these genes on locomotion rate; however, other neurons are also implicated in controlling locomotion rate. For example, disruption of the mechanosensory neurons that mediate the response to gentle body touch also results in lethargy (reviewed in Driscoll and Kaplan 1997). Since the neuronal circuitry controlling locomotion rate has not been fully defined, and *unc-43* and the *goa-1/egl-30* network have broad neuronal expression, experiments with mosaic animals will be required to determine where these genes are acting to control locomotion rate.

unc-43 and members of the *goa-1/egl-30* network are also coexpressed in the egg-laying system. *goa-1*, *egl-10*, and *eat-16* have been shown to be expressed in the hermaphrodite-specific neuron (HSN) motor neurons that control egg laying, and *goa-1* and *eat-16* have also been shown to be expressed in the egg-laying muscles

(Mendel *et al.* 1995; Ségalat *et al.* 1995; Koelle and Horvitz 1996; Hajdu-Cronin *et al.* 1999). Since UNC-43 is also present in both the HSN motor neurons and the egg-laying muscles (E. Newton and J. H. Thomas, unpublished results), an interaction between *unc-43* and the *goa-1/egl-30* network could occur in either cell type. The mechanism by which the *goa-1/egl-30* network controls egg-laying behavior has not been well defined. Heterotrimeric G proteins are activated by ligand-bound seven-pass transmembrane receptors (Simon *et al.* 1991). *goa-1* does not appear to be an effector of serotonin in the egg-laying system since exogenous serotonin stimulates egg laying (Horvitz *et al.* 1982), whereas *goa-1* activity inhibits egg laying (Mendel *et al.* 1995; Ségalat *et al.* 1995). This observation has led to the suggestion that *goa-1* couples to a different neurotransmitter in the egg-laying system (Nurrish *et al.* 1999). The control of egg laying by *unc-43* and the *goa-1/egl-30* network is probably complex since these genes may function both presynaptically (in the HSN neurons) and postsynaptically (in the egg-laying muscles). However, despite this complexity, our genetic analysis indicates that *unc-43* and the *goa-1/egl-30* network function similarly in the egg-laying system.

An interaction between CaMKII, G_o, and G_q pathways could be relevant to mammalian behavior since there is a high degree of conservation between these *C. elegans* proteins and their mammalian counterparts. In particular, GOA-1, EGL-30, and UNC-43 share 70–80% overall amino acid identity with mammalian $G_o\alpha$, $G_q\alpha$, and CaMKII, respectively (Lochrie *et al.* 1991; Brundage *et al.* 1996; Reiner *et al.* 1999). $G_o\alpha$ subunits, several RGS proteins, and CaMKII are highly expressed in the mammalian brain (Sternweis and Robishaw 1984; Erondu and Kennedy 1985; Koelle and Horvitz 1996; Gold *et al.* 1997), indicating that an interaction between the mammalian proteins is plausible. Strikingly, mice lacking either $G_o\alpha$ or αCaMKII exhibit increased locomotory activity (Silva *et al.* 1992a,b; Jiang *et al.* 1998). The striking similarity at the behavioral level of perturbation of these genes in mice and *C. elegans* indicates that the gene interactions we have described for *C. elegans* may be relevant to mammalian behavior.

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