Systematic profiling of *Caenorhabditis elegans* locomotive behaviors reveals additional components in G-protein Gaq signaling

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Contributed by Paul W. Sternberg, June 4, 2013 (sent for review March 8, 2013)

Genetic screens have been widely applied to uncover genetic mechanisms of movement disorders. However, most screens rely on human observations of qualitative differences. Here we demonstrate the application of an automatic imaging system to conduct a quantitative screen for genes regulating the locomotive behavior in *Caenorhabditis elegans*. Two hundred twenty-seven neuronal signaling genes with viable homozygous mutants were selected for this study. We tracked and recorded each animal for 4 min and analyzed over 4,400 animals of 239 genotypes to obtain a quantitative, 10-parameter behavioral profile for each genotype. We discovered 87 genes whose inactivation causes movement defects, including 50 genes that had never been associated with locomotive defects. Computational analysis of the high-content behavioral profiles predicted 370 genetic interactions among these genes. Network partition revealed several functional modules regulating locomotive behaviors, including sensory genes that detect environmental conditions, genes that function in multiple types of excitable cells, and genes in the signaling pathway of the G protein Gaq, a protein that is essential for animal life and behavior. We developed quantitative epistasis analysis methods to analyze the locomotive profiles and validated the prediction of the γ isoform of phospholipase C as a component in the Gaq pathway. These results provided a system-level understanding of how neuronal signaling genes coordinate locomotive behaviors. This study also demonstrated the power of quantitative approaches in genetic studies.

A number of neuronal signaling genes are known to regulate locomotive behaviors of animals. For example, disruption of the heterotrimeric G protein subunit Gaq in neurons causes movement disorders in *Caenorhabditis elegans* and mice (1, 2). The Gaq signaling pathway is composed of proteins and lipids conserved in all animals (3–5). The main target of Gaq is phospholipase C (PLC), which converts phosphatidylinositol 4,5-bisphosphate to the second messengers, diacyl glycerol (DAG) and inositol trisphosphate (3–5). In *C. elegans* excitatory motor neurons, DAG promotes ACh release, necessary for locomotion. Despite the wealth of information on individual signaling genes and pathways, a system-level understanding remains missing on how these genes coordinate animal behavior. For example, among all neuronal signaling genes, which ones are involved in regulating a specific stereotyped behavior? How do these genes interact with each other to form networks that process information? A successful method to uncover large-scale gene networks in metazoans is high-content phenotypic profiling. Using binary parameters to score presence and absence of multiple phenotypic details, this approach enabled computational approaches such as hierarchical clustering to infer interactions among development genes (6–8). Behavioral phenotypes such as movement disorders are intrinsically quantitative. Therefore, a quantitative method is needed to extend such an approach to examine behavioral gene networks.

A quantitative behavioral study will also extend our knowledge on individual genes and pathways. For example, although numerous genetic screens have been performed on Gaq signaling, most of these screens rely on human observations that limit their scope to qualitative differences. Therefore, our knowledge for Gaq, one of the most studied genes, is limited to major pathway components that have drastic effects. A quantitative screen will thus complement this knowledge by detecting pathway components with subtle phenotypic differences.

Here we demonstrate the application of an automated imaging system, WormTracker (9, 10), to conduct quantitative, high-content profiling of *C. elegans* locomotive behaviors. We systematically analyzed 227 neuronal signaling genes to understand the gene networks regulating locomotive behaviors. We identified 87 genes required for locomotion and predicted 370 interactions among the genes. Our results enabled reconstruction of known interactions with Gaq and discovery of others. In particular, we discovered PLCγ as a component in the Gaq pathway that functions in parallel to the known Gaq target, PLCβ. Our data are publicly available at www.WormLoco.org.

Results

Phenotypic Profiling of *C. elegans* Locomotive Behaviors. The WormTracker consists of a digital camera, a microscope with a motorized stage, and a computer controlling the camera and the stage (Fig. 1A). It tracks a worm by automatically recentering the animal when it reaches the border of the field of view. This system records a high-resolution video of the animal, reduces the animal to 13 equally distributed points along the midline, and quantitatively measures multiple parameters of the sinusoidal movement of *C. elegans* (9, 10).

Among all signaling genes with neuronal expression in *C. elegans*, 227 genes have viable homozygous mutants publicly available (WormBase version WS220). These genes encode a broad spectrum of proteins including neuropeptides, neurotransmitter receptors, and protein kinases (Table S1). We obtained 229 loss-of-function alleles of these genes and used the WormTracker to record each animal for 4 min. We examined at least 10 animals for each genotype and analyzed over 4,400 animals.

The WormTracker measures a total of 66 *C. elegans* locomotive parameters (Table S2). We chose 10 representative parameters that are independent of each other and showed low variance among wild-type animals (SI Results and Tables S3 and S4). The parameters are velocity, flex, frequency, amplitude, and...
and wavelength for both forward and backward locomotion. They measure the speed of the animal, the propagation of the sinusoidal wave along the axis of the worm body, and the shape of the wave (Fig. 1A).

Because the parameters are measured in different units, a normalization process is needed to facilitate further analysis. We used wild-type animals tracked on the same day as controls. The parameter values of mutants were divided by the means of control values to obtain a normalized dataset, so that 1 is the wild-type value for each parameter (Fig. 1A). This process also normalized day-to-day environmental variance. Overall, our data collection step produced a quantitative, multiparameter behavioral profile for each of the mutants.

Neuronal Signaling Genes Required for Locomotive Behaviors. Among the 239 mutants we analyzed, 119 mutants of 111 genes displayed abnormality in their locomotor behaviors with at least one parameter significantly different from wild-type values \((P < 0.0001, \text{ Student } t \text{ test})\). Among these, 36 strains were unoutsourced. To verify whether the phenotypes of these mutants were due to background mutations, we performed RNAi of these 36 genes on the strain TU3401, a strain that is sensitized to RNAi in neurons, and desensitized to RNAi in other tissues (11). TU3401 animals showed no significant locomotive phenotype. We evaluated RNAi phenotypes by comparing TU3401 animals on RNAi bacteria with those on control bacteria. Twelve genes displayed RNAi phenotypes consistent with those of mutants \((SI \text{ Results})\). Most of the remaining RNAi did not show any apparent locomotive phenotype, possibly owing to low RNAi penetrance. After the genetic screen and RNAi verification process \((\text{Tables S5 and S6})\), we identified 87 neuronal signaling genes involved in regulating locomotive behaviors.

Eight of these 87 locomotive genes have mammalian orthologs, including 37 genes that have implications in human diseases \((\text{Table S7})\). Among the 87 locomotive genes, 50 (57%) have never been associated with locomotive phenotypes \((\text{WormBase WS220})\). The mean mutant parameter values from 27 (54%) of these 50 genes are within 2 SD \((z\text{-score of 2})\) shifts from wild-type values \((\text{Fig. 1B})\). In contrast, only three (8%) of the previously known locomotive genes have mutant phenotypes in this subtle range \((\text{Fig. 1B})\). These data strongly demonstrated that our quantitative approach is highly sensitive in detecting movement disorder genes, particularly those with subtle phenotypes.

Gene Networks Regulating Locomotive Behaviors. To obtain a systematic view of the locomotive gene network, we computed the absolute value of the Pearson correlation coefficient \((\text{PCC})\) for each pair of genes to capture genes with similar and opposite behavioral profiles. A \(\text{PCC}\) value of 0 indicates no correlation between two profiles, and 1 indicates a perfect correlation. Among the 87 locomotive genes, there are 54 known genetic interactions \((\text{WormBase WS220})\). Compared with all 3,741 possible pairs among the 87 genes, the majority of these interacting gene pairs have \(\text{PCC}\) above 0.7 \((67\% \text{ vs. } 42\%, \text{ Fig. 2A})\). This suggested that \(\text{PCC}\) is an effective predictor for genetic interactions. Using \(\text{PCC}\) of 0.7 as a threshold, we obtained 1,574 probable interactions among the locomotive genes.

To further prioritize these probable interactions, we queried \text{www.GeneOrienteer.org}, a database that integrates cross-species functional data to predict genetic interactions (12). GeneOrienteer examines each \(C. \text{ elegans}\) gene pair and its orthologous pairs in eight eukaryote species for features such as physical or genetic interactions, identical expression pattern, related phenotypes, and similar gene ontology annotations. Each feature is assigned a weighted score, and the combined score of all features indicates a likelihood of an interaction. Known interacting locomotive genes are enriched with high GeneOrienteer scores \((\text{Fig. 2A})\), indicating that GeneOrienteer scores are another good predictor for genetic interactions. GeneOrienteer predicted 762 interactions \((\text{score } > 4)\) among the locomotive genes. Three hundred seventy of these pairs also have \(\text{PCC}\) above 0.7, forming a group of high-confidence interactions. Ninety-three percent \((344/370)\) of these high-confidence interactions have not previously been reported.

Sixty-eight of the 87 locomotive genes are connected by the 370 high-confidence predicted interactions. We used the graph partition software METIS (13) to automatically split the genes into five groups based on their connectivity \((\text{Fig. 2B})\). Surprisingly, these groups revealed several distinct classes of locomotive genes. One group \((\text{green in Fig. 2B})\) is enriched with genes that are involved in response to environmental changes. For example, 7 of 14 of these genes \((\text{daf-11, daf-19, egl-2, ins-4, ins-19, tao-2, and tax-6})\) are expressed in sensory neurons and required for normal chemotaxis \((\text{WormBase WS220})\). Another group of 12 genes \((\text{blue in Fig. 2B})\) function in other excitable cells such as muscle and intestine to regulate rhythmic movements such as pharyngeal pumping and defecation. Eight genes in this group \((\text{ace-3, dyh-1, dys-1, eat-2, gph-2, ir-1, rap-1, and unc-44})\) are expressed in muscle or intestine cells in addition to neurons. Five genes \((\text{ace-3, dkq-1, eat-2, gph-2, and ir-1})\) are known to regulate pharyngeal pumping or defecation. As key regulators of locomotive behavior, components in the EGL-30/Goa signaling network \((\text{eat-16, egl-30, egl-8, unc-73, egl-10, and goa-1})\) span two groups \((\text{pink and purple in Fig. 2B})\), suggesting diverse functions of this class. We did not detect a consensus of gene function for only one group of genes \((\text{yellow in Fig. 2B})\).

Predicted Gqa Subnetwork. As an example of these high-confidence interactions, we examined the predicted interactions for Gqa. In \(C. \text{ elegans}\), Gqa is encoded by the gene egl-30. As illustrated in Fig. 3A, EGL-30/Gqa is known to directly act on EGL-8, a \(\beta\) isoform of PLC to produce DAG in motor neurons \((3, 4)\). EGL-8 was argued not to be the only effector of EGL-30/Gqa because egl-30 null mutants arrest as larvae whereas egl-8 null mutants are viable \((14)\). A later study argued that the Rho GEF domain of UNC-73/Trio (referred to as UNC-73 hereafter) was the other EGL-30/Gqa target \((15)\). It was suggested that
UNC-73 functions in parallel or downstream of the DAG kinase DGK-1 and inhibits the conversion of DAG to phosphatidic acid (16, 17). Although this explains how UNC-73 stabilizes DAG once it is produced, it remains unclear how UNC-73 regulates the production of DAG. With EGL-8 being the only known DAG producer, we still cannot explain the phenotypic differences between egl-8 and egl-30 null mutants.

Our list of high-confidence interactions suggested 30 genes as egl-30 interaction candidates, including seven genes known to function in the egl-30 pathway (Fig. 3B). These 30 genes and egl-30 form a densely connected subnetwork with 219 high-confidence interactions. Among the most connected genes in this subnetwork is plc-3 (Fig. 3B), a gene that has not been previously associated with egl-30. Further, plc-3 was partitioned into the same group with egl-30 (Fig. 2B), suggesting a close association of plc-3 and egl-30. plc-3 encodes the γ isoform of PLC. This sparks an exciting hypothesis that PLC-3 is the missing PLC in the EGL-30/Gαq pathway that functions in the UNC-73 branch in parallel to EGL-8 to catalyze DAG production (Fig. 3A).

**PLCγ Functions in the Gαq Signaling Pathway.** To test the hypothesis that PLC-3/PLCγ and EGL-8/PLCβ are two Egl-30/Gαq targets, we examined the double mutant lacking both egl-8 and plc-3. If the hypothesis is true, the double mutant should have the larval arrest phenotype resembling that of egl-30 null alleles. This is exactly what we observed. Whereas null alleles of either plc-3 or egl-8 showed no apparent larval arrest, the double mutant plc-3(tm1340);egl-8(n488) displayed complete larval arrest (Fig. 3C). In contrast, double mutants of unc-73 and plc-3 displayed no synthetic effect. Is the larval lethality of plc-3(tm1340);egl-8(n488) similar to that of egl-30 null mutants? Phorbol 12-myristate 13-acetate (PMA), a DAG analog, can rescue the larval arrest phenotype of egl-30 null mutants (18). If EGL-30 acts through PLC-3 and EGL-8 to produce DAG, then PMA should also rescue the larval arrest phenotype of plc-3(tm1340);egl-8(n488) double mutants. Indeed, the double mutant showed a developmental profile similar to that of wild-type animals when exposed to PMA (Fig. 3D). In the control group, when the animals were cultured on the PMA solvent ethanol, all plc-3 (tm1340)egl-8(n488) animals were young larvae when wild-type animals became adults (Fig. 3D). Therefore, egl-8 and plc-3 likely function in parallel as egl-30 targets. It has been reported that the Double mutants of egl-8 and unc-73 also displayed synthetic larval lethality that can be rescued by PMA (15). Such phenotypic similarity between plc-3 and unc-73 is consistent with our model that plc-3 functions in the unc-73 branch parallel to egl-8.

In the C. elegans genome, there are five PLCs in four isozyme families: PLC-2 and EGL-8 (PLCβ), PLC-3 (PLCγ), PLC-4 (PLCδ), and PLC-1 (PLCα). We then asked whether the interaction between EGL-8 and PLC-3 is a specific interaction or a general redundancy among all PLCs. To answer this, we examined null alleles of all PLCs for their locomotion behavior (Fig. 3E). Besides egl-8 and plc-3, only plc-1 displayed locomotive phenotypes (P < 0.0001 for at least one parameter). However, in contrast to

**Fig. 2.** Predicted genetic interactions among locomotive genes. (A) Distribution of PCC values and GeneOrienteer scores: interacting genes have higher PCC values and GeneOrienteer scores. (Upper) A scatterplot with each dot representing a gene pair. (Lower) Percentage of gene pairs in each quadrant. (B) Predicted network of locomotive genes. Genes in different partitions of the graph are labeled in different colors. A dot indicates a predicted interaction.
known interactions solely based on behavioral profile functions displayed similar (positively correlated) locomotive lease. In the other group are EGL-10 and EGL-30, both of which suppress ACh release. We selected two groups of genes with opposite functions from contrasting phenotypes function in a linear pathway, then the model of PLC-3 function in the EGL-30 pathway. Epistasis analysis places genes in pathways by comparing known relationships of such epistatic relationships from our locomotive data, we used the Manhattan distance to quantify the similarity between behavioral profiles. Given two phenotype profiles A and B with parameter values \( A = (A_1, A_2, ..., A_n) \) and \( B = (B_1, B_2, ..., B_n) \), their Manhattan distance is defined as \( d_{\text{Manhattan}} = \sum_{i=1}^{n} |A_i - B_i| \). We designated the single mutant with longer distance to the double mutant as “upstream” and the less divergent single mutant as “downstream.” If we set the distance between the double and the upstream mutant as 1, then the normalized distance between the double and the downstream mutant \( d_{\text{down}} \) is a value between 0 and 1. The \( d_{\text{down}} \) value should be close to 0 if the two genes function in a linear pathway and close to 1 otherwise. We reasoned that a linear relationship is strongly suggested by an upstream distance more than five times the magnitude of the downstream distance, corresponding to \( d_{\text{down}} < 0.2 \). Using \( d_{\text{down}} = 0.2 \) as a cutoff for linear relationship, we carried out the analysis on the locomotive profiles and successfully reconstructed the known relationships of egl-30 being downstream of eat-16, and dgk-1 being downstream of egl-10 (Fig. 4D). We also found that neither egl-8 nor plc-3 showed a linear epistatic relationship to the plc-3 behavioral profile, which is highly similar to that of egl-8 and unc-73 (PCC of 0.94 and 0.94, respectively), the plc-1 profile showed little similarity to those two known egl-30 targets (PCC 0.53 and 0.32). The double mutant of plc-1 and egl-8 also did not display any synthetic larval arrest. Therefore, the interaction between egl-8 and plc-3 is highly specific.

Epistasis Analysis of Behavioral Profiles with Opposite Phenotypes. A powerful method to infer genetic interactions is epistasis analysis. Epistasis analysis places genes in pathways by comparing double mutant phenotypes to those of single mutants. However, classical epistasis analysis does not readily accommodate our quantitative, multiparameter data. Inspired by the concept of quantitative epistasis analysis applied to yeast metabolic networks and slime mold expression data (19, 20), we sought a new strategy to extract epistasis from the behavioral profiles such as those listed in Fig. 4A. The method should reconstruct known epistatic relationships as well as identify new ones such as the model of PLC-3 function in the EGL-30 pathway.

One principle of epistasis analysis is that if two genes with contrasting phenotypes function in a linear pathway, then the double mutant shows the phenotype of the downstream gene. We selected two groups of genes with opposite functions from the EGL-30/GqG network to test whether we can reconstruct known interactions solely based on behavioral profiles. In one group are EAT-16 and DGK-1, both of which suppress ACh release. In the other group are EGL-10 and EGL-30, both of which promote ACh release. Accordingly, mutants of genes with identical functions displayed similar (positively correlated) locomotive phenotypes, whereas mutants of genes with opposite functions displayed contrasting (negatively correlated) locomotive phenotypes (Fig. 4B). Correlations of phenotypic patterns are, however, not suitable for epistasis analysis because they do not capture phenotype severity. For example, the behavioral profiles showed that eat-16 and egl-10 single mutants have drastically contrasting phenotypes, and that their double mutant has an almost wild-type behavioral profile (Fig. 4A). The additive effects of these two genes on the double mutant suggested that these two genes function in parallel. However, the PCC method misaligned egl-10 to be downstream of eat-16 because the double mutant is positively correlated to egl-10 and negatively correlated to eat-16 (Fig. 4C). It is known that the EAT-16 protein directly inhibits EGL-30, and dgk-1 mutations are epistatic to egl-10 (4). To reconstruct such epistatic relationships from our locomotive data, we used the Manhattan distance to quantify the similarity between behavioral profiles. Given two phenotype profiles A and B with parameter values \( A = (A_1, A_2, ..., A_n) \) and \( B = (B_1, B_2, ..., B_n) \), their Manhattan distance is defined as \( d_{\text{Manhattan}} = \sum_{i=1}^{n} |A_i - B_i| \). We designated the single mutant with longer distance to the double mutant as “upstream” and the less divergent single mutant as “downstream.” If we set the distance between the double and the upstream mutant as 1, then the normalized distance between the double and the downstream mutant \( d_{\text{down}} \) is a value between 0 and 1. The \( d_{\text{down}} \) value should be close to 0 if the two genes function in a linear pathway and close to 1 otherwise. We reasoned that a linear relationship is strongly suggested by an upstream distance more than five times the magnitude of the downstream distance, corresponding to \( d_{\text{down}} < 0.2 \). Using \( d_{\text{down}} = 0.2 \) as a cutoff for linear relationship, we carried out the analysis on the locomotive profiles and successfully reconstructed the known relationships of egl-30 being downstream of eat-16, and dgk-1 being downstream of egl-10 (Fig. 4D). We also found that neither egl-8 nor plc-3 showed a linear epistatic relationship to
Neuronal Signaling Gene Network. A major challenge in studying signaling genes is to identify how these genes interact (Fig. S1). Although epistasis analysis provides direct experimental evidence of genetic interactions, this method requires inactivation of two genes. Constructing double mutants in metazoans is a low-throughput process, and *C. elegans* neuronal genes are resistant to feeding RNAi, unless in a sensitized genetic background (24), therefore, computational predictions became a more practical method to map neuronal signaling gene networks by prioritizing experiments.

By integrating the behavioral profiles, we obtained experimentally and publicly available functional data and we predicted 370 interactions among 68 genes. Partition of this network based solely on connectivity revealed several interesting functional modules regulating locomotion. In addition to the well-established Gq signaling modules, we found two additional classes of genes modulating locomotive behaviors. The first is a class of genes functioning in sensory neurons, presumably coordinating locomotive behavior with environmental changes. The second is a class of genes that function in muscles or intestine cells in addition to neurons. These genes are likely required for basic functions of excitable cells.

PLC-3 as an EGL-30/Gq Target. The finding of PLC-3 as an additional EGL-30/Gq target strongly demonstrated the power of our quantitative approach. The EGL-30/Gq pathway has been studied extensively for decades for its role in regulating locomotion and egg-laying behavior, and has been long hypothesized to have a missing PLC in addition to EGL-8/PLCβ (see refs. 3 and 4 for reviews). plc-3 has likely escaped detection in numerous locomotion-based screens because its locomotion defects are too subtle to be detected by human observation. plc-3 also eluded detection in chemical screens (*SI Results* and Fig. S2) or screens for abnormal egg-laying because of its other phenotypes such as sterility.

Among the five PLCs in the *C. elegans* genome, PLC-3 and EGL-8 may share similar functions in more than one biological process. Consistent with our observation of PLC-3 and EGL-8 functioning together in regulating locomotive behaviors and larval development, it was reported that both PLC-3 and EGL-8 share functional redundancy with PLC-1 in embryogenesis (25), and that PLC-3 and EGL-8 function in parallel to regulate rhythmic Ca2+ oscillations in the intestine (26). Because PLCs are highly pleiotropic, it is possible that PLC-3 and EGL-8 may function together in multiple traits.

There is no systematic study on *plc-3* expression pattern. Therefore, we cannot pinpoint the PLC-3 site of action. It was reported that UNC-73 has EGL-30-independent functions regulating locomotive behaviors in neurons other than motor neurons (17). It would be interesting to find whether PLC-3 also functions in those cells.

**Quantitative Epistasis.** We developed unique quantitative epistasis analysis methods to extract genetic interactions from these behavioral profiles. Such methods can be extended beyond genetic screens. For example, automatic behavioral profiling enabled a screen of over 5,000 psychoactive drugs for chemicals affecting zebrafish sleep/awake patterns (22). Our method of quantitative epistasis analysis might be applicable to discover gene–drug or drug–drug interactions. This study provides a framework to further explore the potential of such high-throughput, quantitative approaches in addressing basic biological questions.

**Methods**

**Animal Culture.** *C. elegans* strains were cultured on Nematode Growth Medium at 20 °C as described (27, 28). Bristol N2 was used as the wild-type strain.

**Strains.** The strains we tested are listed in Table S1. We obtained gene expression data from WormBase (version WS220) to find neurally expressed genes, gene function annotation from Gene Ontology to find

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eat-16 (Fig. 4D), which is consistent with our model that *plc-3* and *egl-8* are two branched targets of *egl-30* (Fig. 3A).

**Epistasis Analysis of Behavioral Profiles with Similar Phenotypes.** The principle of epistasis analysis for two genes with similar phenotypes is that if two genes function in the same pathway, the double null mutant phenotype should be no more severe than that of the single null mutants. We applied this to examine whether *plc-3* functions with *unc-73* in the same pathway. *plc-3* and *unc-73* have highly similar phenotypes (Fig. 4B). To quantify phenotype severity, we computed the distance between a mutant and wild-type (wt) profiles. For example, the phenotype severity of a mutant profile (\(A_1, A_2, \ldots, A_n\) is \(d(A, wt) = \sum_{i=1}^{n} |A_i - 1|\). Our data showed that *unc-73* (ev802), a null loss-of-function allele of the Rho GEF domain, has a more severe locomotion phenotype than *unc-73* (ce362), a nonnull reduction-of-function allele (Fig. 4E), validating the sensitivity of our assay in detecting phenotypic differences. *plc-3* null allele, *plc-3* (tm1340), enhanced the phenotype severity of *unc-73* (ce362) but not *unc-73* (ev802) (Fig. 4E), suggesting that *plc-3* and *unc-73* function in the same pathway.

**Open-Access Data Resource.** All our data, including over 300 h of video, 60 parameter measurements for each animal, and statistics for each experimental group, are publicly available at www.WormLoco.org. In addition, one can also query and download similar or opposite phenotypic patterns and predicted interaction scores.

**Discussion**

**Quantitative Profiling of Locomotive Behaviors.** Compared with classic genetic screens, our quantitative approach showed several advantages. It is more sensitive in detecting subtle phenotypes, enabling us to discover more genes regulating motor behavior. In addition, it provides higher data content with multiple parameters, allowing us to conduct bioinformatic analysis to deduce the interaction network of these signaling genes. Finally, combined with a large number of animals surveyed, it provides insights on the intrinsic nature (e.g., variation and dependency) of phenotypic parameters.

Our results revealed that genetic screens are far from saturation in exploring locomotive defects, particularly in the quantitative domain. We were able to discover 50 additional neuronal signaling genes that affect locomotion and 23 additional components in the extensively studied Gq network. When we visually examined mutants of those genes, we were unable to detect any locomotive phenotype in many mutants including *plc-3*. The WormTracker or similar imaging systems are crucial for capturing those subtle phenotypes.

Several imaging systems have been developed for animal behavior analysis. A low-resolution approach reduces the animal to one point and analyzes velocity and other position-based parameters (21, 22). This method is efficient for simultaneous recording of multiple animals. The WormTracker took a different approach and used a high resolution to represent a worm with 13 points. This enabled measurement of parameters such as body shape. However, the high-resolution approach also limits the throughput to one animal at a time. Therefore, we used a short recording time (4 min) to accommodate a large-scale screen. This short recording time makes our analysis focused on acute locomotive defects. A longer recording time will enable analysis of other phenotypes such as sleep/awake patterns.

Whereas 10 parameters were used in this study, our data can be used to derive more locomotive parameters. For example, we can normalize wavelength and amplitude by body length. Further, animal-to-animal variation of some parameters can also provide new measurements (*SI Results*). In addition to the 66 locomotive parameters measured in this analysis, the WormTracker can be programmed to measure more complicated patterns of behaviors (23).
cell signaling genes, and strain information from the Caenorhabditis Genetics Center (CGC) to find viable homozygous mutants. We selected mutants from the intersection of the three datasets and obtained them from the CGC.

When there were multiple alleles for a gene, we chose the allele based on the following criteria (ranked in preference):

(i) An allele that is well documented and has been referenced by multiple publications;
(ii) An allele that has been well characterized and sequenced;
(iii) A null allele with a stop codon or a deletion mutant;
(iv) A strain that has been outcrossed; and
(v) A mutant with a simple genetic background and no secondary mutations in other genes. We avoided mutants with a high Tc1 copy number.

The RNAi-sensitized strain TU3401 [sid-1(pk3221) V; unc-69 V] (11) was used in RNAi experiments. The genotype for unc-69 is [pcF790(myo-2p::mCherry) + urA19;pk3221].

The following alleles were used for the epistasis analysis in Fig. 4: egl-8 (n488) (the canonical null allele, or a possible nonmorphorphic allele that better represents the null phenotype than other alleles in behavioral assays) (14), egl-30(ad809) (the strongest viable allele) (1), dgk-1(is428) (a putative null allele) (29, 30), eat-16(is3448) (a putative null allele) (29), plc-3(tm1340) (a null allele), unc-73(ev802) [a RhD guanine nucleotide exchange factor (GEF) domain null allele] (31), unc-73(ec362) (a strong reduction of function allele for the GEF activity) (15). Although egl-30(ad809) is not a null allele, its locomotive phenotypes are stronger than those of egl-8 (n488) (distance to wild-type 4.52 ± 0.13 vs. 3.25 ± 0.11, respectively, P < 0.001, Student t test). This was consistent with the knowledge that egl-8 is not the only target of egl-30 (15) and suggested that the egl-30 allele is strong enough for epistasis analysis of locomotive behaviors.

Worm Tracking. We applied the WormTracker to track C. elegans locomotion following a protocol described previously (9) (SI Methods). First-day adult animals were analyzed on a fresh bacterial lawn at 20 °C.

RNAi. RNAi was performed as described (32, 33) (SI Methods). Animals at larval stage L4 were placed on RNAi bacteria and cultured at 20 °C. Their progeny were analyzed at the stage of first-day adults.

Phorbol Ester Rescue Experiment. PMA (5 μM) was administered as described (18, 34) (SI Methods).

ACKNOWLEDGMENTS. We thank the Caenorhabditis Genetics Center (CGC), Erin Cram, and Shohel Mitani for strains, Iylla Hicks for helpful discussions, Joaquina Nunez, Barbara Perry, and Julie Nguyen for technical assistance, Ranjan Kishore for a critical reading of the manuscript, and WormBase. This work was supported by National Institutes of Health Grant HG004724 and a Searle Scholar grant from the Kinship Foundation (to W.Z.), by National Institutes of Health Grant DA018341 (to P.W.S. and W.Z.), and by the Howard Hughes Medical Institute, with which P.W.S. is an investigator. CGC is funded by National Institutes of Health Research Infrastructure Programs Grant P40 OD010440.

We applied the WormTracker to track Caenorhabditis elegans locomotion following a protocol described previously (1). To prepare recording plates, 10-cm Nematode Growth Medium (NGM) agar plates were equilibrated to 20 °C for 18–20 h and all subsequent procedures were conducted at 20 °C. Approximately 1 h before tracking, 1 mL of fresh overnight culture of the bacteria OP50 in L-broth was added to each recording plates and spread to cover the entire agar surface by fast swirling. Excess liquid was then withdrawn and discarded. The plates were covered with a sheet of Kimwipe to prevent dust accumulation and let dry. The recording plates had an even, thin lawn of bacteria on the entire surface.

Hermaphrodites were picked at the L4 larval stage, 18–20 h before tracking to control the age of the animals tracked. At the time of tracking, these animals became young adults. Individual animals were placed on recording plates. The recording plate was placed on a WormTracker. The worm was located under the microscope and recorded for 4 min. The entire process, including animal culture and recording, was performed at 20 °C.

To use the parallel worm tracker (2) for population assays, about 150 first-day adult worms were placed on each plate. Each plate was recorded at three nonoverlapping areas on the bacterial lawn. Each area was recorded for 30 s. The recording was performed at 20 °C. The average velocity of each area is used as a data point. Thus, one plate generates three data points.

**RNAi.** RNAi was performed as described (3). We used RNAi clones from the Vidal library (3), and from the Ahringer library (4) when the former was not available. Five to 10 L4 animals were placed on RNAi plates. They were transferred to another RNAi plate the next day and removed after 4 h when there were about 150 eggs on the plate. These eggs were cultured at 20 °C until they become first-day adults. The plates were then directly used for the parallel worm tracker analysis. For each gene, two trials of RNAi were performed and duplicates were used in each trial. For the WormTracker analysis of individual worms, RNAi animals were picked at L4 stage, moved to a new RNAi plate, and analyzed the next day following the WormTracker procedure.

**Clustering.** We performed hierarchical clustering on genes with locomotive phenotypes (Fig. S1). Average linkage hierarchical clustering was performed using uncentered correlations as a measure of distance between clusters. This method uses an agglomerative procedure to identify the most similar locomotion profiles and group them into clusters (for reviews see refs. 5 and 6). We used the software Cluster 3.0 (7) and Java Treeview (8) for calculation and visualization of the clusters.

**Phorbol Ester Rescue Experiment.** Phorbol 12-myristate 13-acetate (PMA, 5 μM) plates were prepared as described (9, 10). Briefly, 5 mM PMA stock (dissolved in 100% ethanol) was added to 55 °C molten NGM medium to a final concentration of 5 μM. The medium was poured into six-well plates in a fume hood, and allowed to solidify for 16 h. The control plates contained ethanol (EtOH) only. The plates were then seeded with 100 μL of fresh OP50 culture and kept at room temperature for 24 h.

First-day adults of wild-type N2 or plc-3(tm1340)/mIn1; egl-8 (n488) were picked to NGM plates seeded with OP50 to lay eggs overnight. On the next day (day 1), hatched young-stage-L1 larvae were transferred to 5 μM PMA plates or EtOH control plates. The homozygous plc-3(m1340); egl-8(n488) double-mutant larvae were picked under a SteREO Discovery V20 stereomicroscope (Carl Zeiss) by selecting against the myo-2::GFP carried by mIn1. We placed 50 stage-L1 animals in each well and used triplicates for each condition. Animal growth was monitored daily and the distribution of developmental stages was recorded when N2 animals started to reach adulthood. Specifically, the animals were recorded after 48 h (day 3) on EtOH control plates and 120 h (day 6) on PMA plates because animals grow slower on PMA plates (11). Missing and dead animals (due to early larval death or crawling off the media) were excluded in the final numbers.

**Aldicarb and Levamisole Sensitivity.** Aldicarb and levamisole acute paralysis assays were performed as previously described (10–12). Staged 1-d-old adult animals were used in both assays. For the aldicarb assay, NGM plates containing 2 mM aldicarb were made and seeded with bacteria the day before the assay. For each strain, 20 animals were placed on the plate and monitored every 10 min for 2 h. Animals were considered paralyzed if they show no movement after a gentle tap on the head using a worm pick. Three trials were performed for this assay. For the levamisole assay, 10 animals of each genotype were placed in a well of a 96-well microtiter plate containing 50 μL of 100 μM levamisole in M9 buffer. The number of paralyzed (not thrashing) animals was inspected every 10 min over a 60-min period. We performed six trials for this assay.

**Bleach Sensitivity.** The bleach sensitivity protocol was modified from ref. 13. A diluted alkaline hypochlorite solution was used in our assay to allow more accurate timing. The solution was freshly prepared with 0.05 M NaOH and 2% (vol/vol) household bleach. Staged 1-d-old adult animals were used. Each animal was placed in a well of a 96-well microtiter plate containing 50 μL of alkaline hypochlorite solution. Both the time taken for the animal to stop moving and to start to break-up were recorded. Ten to 20 animals for each strain were tested.

**SI Methods**

**Worm Tracking.** We applied the WormTracker to track Caenorhabditis elegans locomotion following a protocol described previously (1). To prepare recording plates, 10-cm Nematode Growth Medium (NGM) agar plates were equilibrated to 20 °C for 18–20 h and all subsequent procedures were conducted at 20 °C. Approximately 1 h before tracking, 1 mL of fresh overnight culture of the bacteria OP50 in L-broth was added to each recording plates and spread to cover the entire agar surface by fast swirling. Excess liquid was then withdrawn and discarded. The plates were covered with a sheet of Kimwipe to prevent dust accumulation and let dry. The recording plates had an even, thin lawn of bacteria on the entire surface.

Hermaphrodites were picked at the L4 larval stage, 18–20 h before tracking to control the age of the animals tracked. At the time of tracking, these animals became young adults. Individual animals were placed on recording plates. The recording plate was placed on a WormTracker. The worm was located under the microscope and recorded for 4 min. The entire process, including animal culture and recording, was performed at 20 °C.

To use the parallel worm tracker (2) for population assays, about 150 first-day adult worms were placed on each plate. Each plate was recorded at three nonoverlapping areas on the bacterial lawn. Each area was recorded for 30 s. The recording was performed at 20 °C. The average velocity of each area is used as a data point. Thus, one plate generates three data points.

**DNA Extraction.** DNA extraction from wild-type and RNAi animals was performed as described (6). Brie
intrinsically random in C. elegans locomotion, or that our experimental conditions were not optimal for such measurements.

We then used mutant profiles to compute the Pearson correlation coefficient (PCC), a standard measurement of correlation, among all parameters (Table S4). There are three cases of highly correlated (PCC >0.7) parameters. (i) When the same parameter (e.g., flex) is measured for each of the 13 articulation points along the animal midline, the values for all points are highly correlated. We thus chose the parameter for the middle point to represent the entire group. (ii) The values for all parameters in forward movement showed strong correlations with those in backward movement (Table S4). Nevertheless, it is known that some mutants (e.g., unc-4 mutants) have defects in only one type of movement (14). Therefore, we kept both forward and backward measurements. (iii) Velocity, centroid velocity, and frequency are highly correlated. We kept velocity and frequency because they measure two different aspects of worm movement.

After applying these two criteria, the remaining 10 locomotion parameters are velocity, flex, frequency, amplitude, and wavelength for both forward and backward locomotion.

**Reproducibility of Locomotive Phenotypes.** To evaluate the reliability of our genetic screen, we asked two questions: Can a phenotypic pattern detected in our screen be reproduced with a different set of animals? Can a phenotypic pattern be reproduced with different alleles?

We chose 30 strains with locomotive phenotypes and repeated the assay at least once with another set of animals on a different date. For each strain, the animals were split into two groups based on experimental dates. Both groups had at least five animals and were similar in size. A phenotypic profile was derived from each group and the PCC value between the two profiles was calculated. If the phenotypes are reproducible, the profile derived from earlier experiments should be highly correlated to that from later experiments. Indeed, the two phenotypic profiles are strongly correlated (PCC above 0.6) for 25 of the 30 strains (83%) and mildly correlated (PCC between 0.3 and 0.6) for three strains (10%) (Table S5). Only two strains showed inconsistent phenotypic patterns (PCC between –0.3 and 0.3) (Table S5). Because both of these strains were unoutcrossed, it is likely that the phenotypes were affected by unstable background mutations.

To evaluate the effects of background mutations, we chose 11 genes with locomotive phenotypes and tested them with at least two alleles. Comparing the locomotive profiles from different alleles of the same gene, we found that the phenotypic profiles are highly correlated (PCC above 0.6) in 10 out 12 cases (Table S6). Even the phenotypes of three out of four unoutcrossed strains were confirmed by another strain (Table S6). Altogether, these data showed that our screen results are reliable.

To further eliminate possible noise from background mutations in unoutcrossed strains, we verified the phenotypes of all unoutcrossed strains with RNAi. Among the 119 mutants that showed significant locomotive phenotypes, 36 strains were unoutcrossed. To perform RNAi on these 36 genes, we used the strain TU3401, a strain that is sensitized to RNAi in neurons and desensitized to RNAi in other tissues (15). We first used a different system, the parallel worm tracker (2), to examine whether RNAi can reproduce the velocity phenotype of the mutants. The parallel worm tracker analyzes a population of worms simultaneously by reducing each worm into one dot and measuring its velocity. We eliminated 13 genes that showed significant velocity phenotypes in mutants but normal velocity in RNAi animals. We then used the WormTracker to analyze at least 10 RNAi animals for each of the remaining 23 genes and derived the locomotive profiles from the RNAi data. Twelve genes showed positively correlated (PCC above 0.3) RNAi and mutant locomotive profiles (Table S6) and were kept in our final list. Among them was nlp-17, where the two unoutcrossed alleles showed different phenotypes. RNAi confirmed the phenotype of one allele (ok1469), suggesting that the phenotypes in the other allele (ok1470) are affected by background mutations. Among the remaining 11 genes whose RNAi locomotive profiles are inconsistent with mutant profiles, most of them showed no RNAi phenotype, with the only exception of the gene nlp-17. nlp-17 RNAi showed significant phenotypes with locomotive parameter values more than 10% deviant from wild-type values. The discrepancy between nlp-17 RNAi and mutant phenotypes might be caused by background mutations in the mutant strain. It should be noted that genes with no RNAi phenotypes may still be locomotive genes because of low RNAi penetrance.

After the genetic screen and RNAi verification process, we concluded that 87 neuronal signaling genes are involved in regulating locomotive behaviors. This is based on phenotypic profiles from 94 mutants. The phenotypic profiles from 19 genes have been validated by additional alleles or RNAi results, including all 14 unoutcrossed strains.

**Clustering of Locomotive Behavioral Profiles.** We first attempted to identify interacting genes by clustering their behavioral profiles. Hierarchical clustering of the 94 mutant behavioral profiles revealed a couple of functional groups corresponding to known genetic pathways (Fig. S1). For example, five of six genes (dop-3, dgk-1, eat-16, gpb-2, and goa-1) in one cluster (Fig. S1) encode proteins that inhibit ACh release (16, 17). Another cluster of eight genes (Fig. S1) has five genes (egl-8, unc-2, unc-13, unc-31, and unc-73) that function at least in part, in the Guo–Goq network that promotes ACh release (16, 17). We were, however, unable to recognize more functional modules from the cluster data. Therefore, we sought a new computational approach to construct the locomotive gene networks by using [PCC] values and GeneOrienteer scores.

**Drug Sensitivity of plc-3 Mutants.** Two drugs, the acetocholines-terase inhibitor aldicarb and the ACh receptor agonist levamisole, have been commonly used to examine defects in ACh signaling. Previous findings have shown that egl-30, egl-8 mutants are highly resistant to aldicarb, no different from wild-type in response to levamisole, and that unc-73 mutants are mildly resistant to aldicarb and hypersensitive to levamisole (11, 18, 19). We found that plc-3 mutant animals showed hypersensitivity to both aldicarb and levamisole (Fig. S2 A and B). However, the plc-3 drug sensitivity might be caused by defects in cuticle integrity instead of ACh signaling, because plc-3 mutants displayed increased sensitivity to alkaline hypochlorite treatment (Fig. S2C). When exposed to alkaline hypochlorite, plc-3 mutant animals stopped moving and started to disintegrate significantly faster than wild-type worms (Fig. S2C). Because egl-8 mutants showed normal bleach sensitivity (Fig. S2C), the cuticle defect is likely plc-3 specific. The complication of plc-3 cuticle defects makes drug assay results less conclusive.


Fig. S1. Hierarchical clustering of behavioral profiles. Two clusters labeled in red highlight two opposite functional groups in the G\(\alpha_{o}\) and G\(\alpha_{q}\) signaling network.
Fig. S2. Drug sensitivity of *plc-3* animals. *plc-3* mutant animals are hypersensitive to aldicarb (A), levamisole (B), and alkaline hypochlorite (C). Data are means and SE. *P < 0.01 compared with wild-type N2 animals in Student t test.

Table S1. Mutants of neuronal signaling genes tested

<table>
<thead>
<tr>
<th></th>
<th>N2</th>
<th>egl-8(n488)</th>
<th>plc-3(tm1340)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Time (min) stop moving</td>
<td>7.0 ± 0.2</td>
<td>5.2 ± 0.3</td>
<td>4.8 ± 0.2</td>
</tr>
<tr>
<td>Time (min) start disintegrating</td>
<td>14.0 ± 1.2</td>
<td>16.5 ± 1.8</td>
<td>17.0 ± 1.5</td>
</tr>
</tbody>
</table>

*P < 0.01 compared with wild-type N2 animals in Student t test.
Table S2. Locomotion parameters

Table S3. Coefficient of variance (CV) of parameters (green indicates CV less than 0.5)

Table S4. PCC of parameters (green indicates PCC less than 0.7)

Table S5. PCC of locomotive profiles derived from different animals (red indicates PCC less than 0.3)

Table S6. PCC of locomotive profiles derived from different alleles (red indicates PCC less than 0.3)

Table S7. Worm genes with locomotive phenotypes [ortholog data were obtained from InParanoid and WormBase (WS230); human disease information was obtained from www.omim.org]