# System-wide Rewiring Underlies Behavioral Differences in Predatory and Bacterial-Feeding Nematodes

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## SUMMARY

The relationship between neural circuit function and patterns of synaptic connectivity is poorly understood, in part due to a lack of comparative data for larger complete systems. We compare system-wide maps of synaptic connectivity generated from serial transmission electron microscopy for the pharyngeal nervous systems of two nematodes with divergent feeding behavior: the microbivore *Caenorhabditis elegans* and the predatory nematode *Pristionchus pacificus*. We uncover a massive rewiring in a complex system of identified neurons, all of which are homologous based on neurite anatomy and cell body position. Comparative graph theoretical analysis reveals a striking pattern of neuronal wiring with increased connectional complexity in the anterior pharynx correlating with tooth-like denticles, a morphological feature in the mouth of *P. pacificus*. We apply focused centrality methods to identify neurons I1 and I2 as candidates for regulating predatory feeding and predict substantial divergence in the function of pharyngeal glands.

## INTRODUCTION

Despite the central importance of the synapse to neuroscience, the correlation between patterns of synaptic connectivity and nervous system function is poorly understood. The emerging discipline of connectomics seeks to address this fundamental gap in the understanding of nervous systems (Denk et al., 2012; Lichtman and Denk, 2011; Seung, 2012). Recent studies demonstrate that the comprehensive systems approach of synapse-level connectomics can yield insight unobtainable through other methods (Bock et al., 2011; Briggman et al., 2011; Jarrell et al., 2012). These studies are transforming anatomically derived maps of synaptic connectivity into the realm of bioinformatics. A strong comparative component has been incorporated into all modern bioinformatics approaches from their inception (Lander et al., 2001; Pellegrini et al., 1999; Su et al., 2002; Suthram et al., 2005; Venter et al., 2001). In order to emulate the success of other "-omics" approaches, the emerging discipline of synapse-level connectomics must also incorporate the comparative method as a central component of its research agenda.

The nematode pharyngeal nervous system offers many advantages for comparative studies of system-level connectivity. It is a pumping organ organized into four substructures that concentrate food for deposition into the intestine (Figure 1). The pharyngeal nervous system of *C. elegans* is composed of a network of 20 identified neurons of 14 cell types with a nerve ring "brain" largely independent from the much larger somatic nervous system (Figure 1C). Only a bilateral pair of gap junctions connects these two nervous systems. A complete wiring diagram has long been available for the pharynx of *C. elegans* (Albertson and Thomson, 1976), and comprehensive studies provide considerable context for interpreting connectivity data (Franks et al., 2006). Nematode feeding strategies differ greatly, and studies in species other than *C. elegans* have shown corresponding differences in pharyngeal function (Baldwin et al., 2004; Brownlee et al., 2000; Chiang et al., 2006; Rivard et al., 2010; Tahseen et al., 2004; von Lieven, 2003). The pharynx is less than 300 μm long in most nematodes, greatly facilitating reconstruction using electron microscopy techniques.

The diplogastrid nematode *Pristionchus pacificus* was selected as a point of comparison to the *C. elegans* wiring diagram. *P. pacificus* is a satellite model to *C. elegans* with an annotated genome, forward and reverse genetics, transgenics, and a well-resolved phylogeny facilitating studies on the evolution of development and the genetics of ecologically relevant traits (Bento et al., 2010; Dieterich et al., 2008; Hong and Sommer, 2006; Kanzaki et al., 2012; Mayer et al., 2007; Schlager et al., 2009; Sommer, 2006a, 2006b). The ecology and behavior of *P. pacificus* differ substantially from *C. elegans*. *Pristionchus* nematodes have a necromenic association with scarab beetles (Figure 1A). Their dauer larvae rest on the insect and resume development after the beetle's death to feed on microbes on the decaying carcass. Like *C. elegans*, *P. pacificus* can be easily cultured on bacteria. However, *P. pacificus* is also predatory on other nematodes (Figure 1B and Movie S1). Predation in diplogastrid nematodes such as *P. pacificus* is a behavioral mode discrete from bacterial feeding and represents an evolutionary novelty unknown from *C. elegans* or their most recent common ancestor.

*P. pacificus* and its relatives develop teeth-like denticles in their mouth opening and can form two distinct mouth forms.
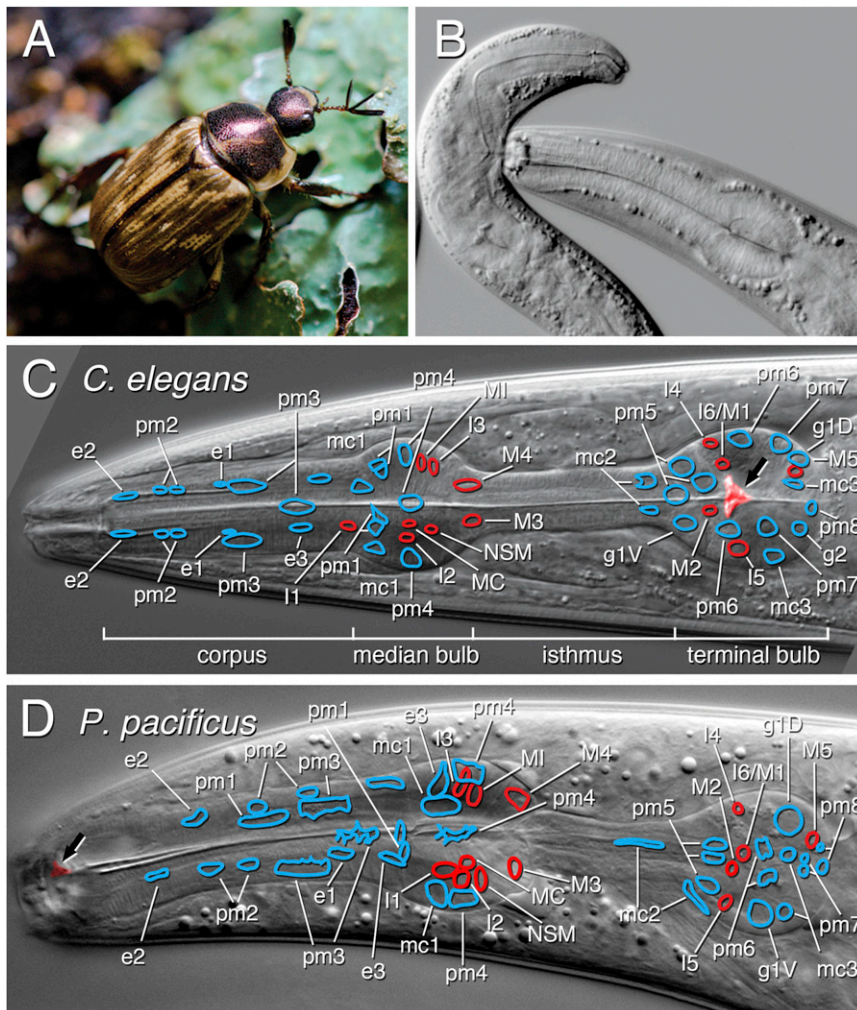
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**Figure 1. Ecological and Behavioral Differences between *P. pacificus* and *C. elegans***

(A) The oriental beetle *Exomala orientalis* is a common scarab beetle host of *P. pacificus*.

(B) *P. pacificus* shows predatory behavior and can feed on other nematodes. Here, *C. elegans* was used as prey.

(C and D) Nomarski microscopy of the pharynx of *C. elegans* (C) and *P. pacificus* (D), with an overlay indicating the position and shape of cell bodies for neurons (interneuron, I; motorneuron, M), pharyngeal muscles (pm), epidermal (e), marginal (mc), and gland cells (g). Neurons are shown in red, and all other cell types are shown in blue. Note that ventral cells occur in pairs, except for I5. For these cells, only the left side is shown. The pharynx contains four major subdivisions: the corpus, median bulb, isthmus, and the terminal bulb. Neurons and pharyngeal muscle cells are homologous between *P. pacificus* and *C. elegans* based on position and shape. The grinder (arrow) in the terminal bulb of *C. elegans* mechanically breaks up bacteria (C). *P. pacificus* lacks a grinder but possesses a predatory dorsal tooth to break open prey items (arrow) (D).

bacteria in *C. elegans* (Figures 1C and 1D). *Pristionchus* and other diplogastrid nematodes carry living bacteria in the gut, and they gain energy from organismic interactions during bacterial feeding (Rae et al., 2008). Together, the extended feeding repertoire of *P. pacificus* has resulted in a gain of structures in the anterior and in a loss in the posterior pharynx.

In order to introduce the comparative method into system-level studies of synaptic connectivity, we utilized serial thin section transmission electron microscopy to generate anatomically defined and comprehensive maps of chemical synaptic connectivity for the pharynx of *P. pacificus*. We then analyzed the networks of *P. pacificus* and *C. elegans* utilizing a graph theoretical approach. Focused centrality methods were developed in order to ask specific biological questions that relate to behavior. Our analysis demonstrates a massive rewiring of connectivity between the two species and highlights network architecture likely involved in behavioral differences between them.

## RESULTS

### Cell Identity and Synaptic Connectivity

Electron microscopy work yielded serial section data sets through the anterior region of three eurystomatous individuals of *P. pacificus*, each of more than 3,000 sections and containing the entire pharynx (Figure 2A). For two of these, complete networks of connectivity were reconstructed (Figures 2B–2E). Due to missing sections in the pharyngeal nerve ring, the third network was used for homology assessment only. *P. pacificus*

Eurystomatous animals have a broad buccal cavity, a claw-like dorsal tooth, and an additional ventral tooth, whereas stenostomatous have a narrow and deep cavity, a dorsal tooth that is not claw like, and the ventral tooth is absent (Bento et al., 2010). In both mouth forms, the dorsal tooth contains the opening for the gland cell g1D. This gland cell is conserved with *C. elegans*, where it secretes in the absence of teeth-like structures into the mouth opening but is poorly understood in terms of function. The *P. pacificus* mouth-form decision of an individual animal is executed in the J4 stage and is irreversible. It is important to note that both eurystomatous and stenostomatous mouth forms exhibit predatory and bacterial feeding behavior.

Several aspects of predatory versus bacterial feeding in *P. pacificus* are mediated by the pharyngeal nervous system, including modulation of pumping rate as well as the actuation of the tooth-like denticles. The dorsal tooth (Figure 1D) is primarily active during predatory rather than bacterial feeding, functioning to puncture prey items (Bento et al., 2010) (Movie S1). Whereas the tooth-like denticles have been gained in the Diplogastridae, nematodes of this family have lost the grinder, the key pharyngeal structure for mechanically breaking up

contains 20 neurons in its pharyngeal nervous system, the same number as in *C. elegans*. The shape of all neurons and the location of synapses and gap junctions were reconstructed from the serial section data set (Figures 1D and 2B–2G and Data S1 available online). An interactive connectivity map was constructed that contains the targets for all neurons (Figure 3A and http://www.pristionchus.org/connectomics/).

The comparison of the *P. pacificus* data set with the available neural network of *C. elegans* reveals that homology of individual neurons between the two species is unambiguous based on cell body position and neurite branching patterns (Figures 1C, 1D, and 2F). Despite remarkable conservation in cell identity, we demonstrate massive rewiring of synaptic connectivity between the two species (Figures 3A, 3B, and 4A). Fewer than half of the synapse classes identified in *P. pacificus* are found in *C. elegans* (Figure 4A), and *P. pacificus* is more highly connected. In the *C. elegans* pharynx, six out of 14 neuron classes are considered interneurons based on the lack of motor synapses. Surprisingly, in *P. pacificus*, 13 out of 14 neuron classes are presynaptic to muscle cells, including I1, which remains the only connection to the somatic nervous system. Only I4 is anatomically an interneuron in *P. pacificus*. These results suggest fundamental changes in information flow and more complex motor regulation in *P. pacificus* than in *C. elegans*.

### Differences in Synaptic Connectivity Correlate with Feeding Behavior

To determine regulatory linkages in the neural network that are associated with predatory feeding behavior, we analyzed motor synapse patterning in *P. pacificus*. Upon initiation of predation, the pharyngeal corpus (pm3) and dorsal tooth (pm1) muscles contract rhythmically, puncturing its prey (Figures 1B, 1D, and 4B and Movie S1). pm1 usually contracts shortly after pm3 and is typically not active during bacterial feeding. Thus, differential but coordinated regulation of pm1 and pm3 in *P. pacificus* represents motor output that is specific to predatory feeding and does not exist in *C. elegans*. In both species, M1 is presynaptic to the muscle cells pm1, pm2, and pm3 (Figure 4B). However, in *P. pacificus*, pm1 and pm3 receive additional synapses from I1 and I2, respectively. Through the gap junction between I1 and RIP, pm1 can receive direct input from the somatic nervous system. I2 has a feedback loop with M1 and is presynaptic to I1, which is consistent with a role in coordinating corpus and tooth contractions during predation. We conclude that *P. pacificus* has evolved altered connectivity in the pharynx to control motor cells responsible for predatory behavior.

In the posterior pharynx of *C. elegans*, the grinder is actuated by the pm6 and pm7 muscle cells, which receive synaptic input from the M5 neuron (Figure 4B). In *P. pacificus*, which lacks a grinder, pm7 was found to be greatly reduced in size, contain no myofilaments, and receive no synaptic input. Nonetheless, the homology of pm7 remains clear in light of the otherwise highly conserved cell identity as all surrounding cell bodies are accounted for (Figure 1D). The motorneuron M5 is only presynaptic to pm5, whereas M4, a cell that controls isthmus peristalsis in *C. elegans*, is presynaptic to pm5 and pm6 in *P. pacificus*. This is consistent with the coupled activity of pm5 and pm6 in *P. pacificus*, but not *C. elegans* (Chiang et al., 2006). Thus,

gain (teeth) and loss (grinder) of function in the *P. pacificus* pharynx is reflected in motor synapse patterning and in the evolution of synaptic connectivity.

### Network Centrality Measures Predict System-Level Functional Differences

To explore system-level functional differences between both species, we analyzed network centrality. Degree centrality counts the number of connections associated with a particular node. I1, I2, and M5 were identified as being the most different in degree (Table 1). I1 and I2 are more highly connected in *P. pacificus* and may function as network hubs. All unique connections of I1 and most of I2 are focused on the corpus and median bulb (Figure 4B), where behavior must be modulated according to food type. Thus, nodality correlates with behavioral complexity and has increased in *P. pacificus* with the acquisition of predatory behavior.

Closeness centrality reflects how many shortest-path steps exist between a node and all possible targets (Freeman, 1979). Despite the massive system rewiring, three neurons (M3, MC, and M4) have the maximum closeness of 1 in both species (Table 1), due to having only motor synapses and lacking any that feed back into the system. Interestingly, these three neurons yield strongest cell ablation phenotypes in *C. elegans*. M4 ablations also yield a strong phenotype in *P. pacificus* (Chiang et al., 2006). M2 has a closeness of 1 in *C. elegans*, but not *P. pacificus*, and M5 has a closeness value of 1 in *P. pacificus*, but not *C. elegans*, indicating probable functional shifts for these neurons between the two species.

Betweenness centrality defines how much shortest-path information flows through a node in the network (Freeman, 1977). There are large differences in betweenness centrality for most neurons between *C. elegans* and *P. pacificus* (Table 1). One of the most different is I4, the only anatomical interneuron in *P. pacificus*, which appears to mediate more shortest-path information flow in *P. pacificus* than in *C. elegans*. M1 has a higher betweenness in *C. elegans* due to substantial information flow being diverted through I1 and I2 in *P. pacificus*.

PageRank centrality simulates a diffusion process through a network and describes the probability of stopping at a node (Page et al., 1999). It differs from shortest-path measures in being able to rank output nodes, such as muscle and gland cells. The muscle cells pm4, pm5, and the gland cell g1D are the output cells with the highest PageRank in both species (Table 2). An unexpected pattern in PageRank centrality shows a shift in network focus, with cells connected to anterior network outputs of the pharynx generally having higher PageRank in *P. pacificus* and cells connected to outputs in the posterior pharynx having higher PageRank in *C. elegans* (Figure 3C).

### Focused Centrality Reveals Neuronal Correlates of Predation

Centrality analysis as described above defines the global importance of nodes for a network. Here, we introduce "focused centrality analysis" to instead define which parts of a network are most important for a node. Although it also relies on a quantitative model of information flow and an importance criterion (Borgatti, 2005), focused centrality analysis considers only
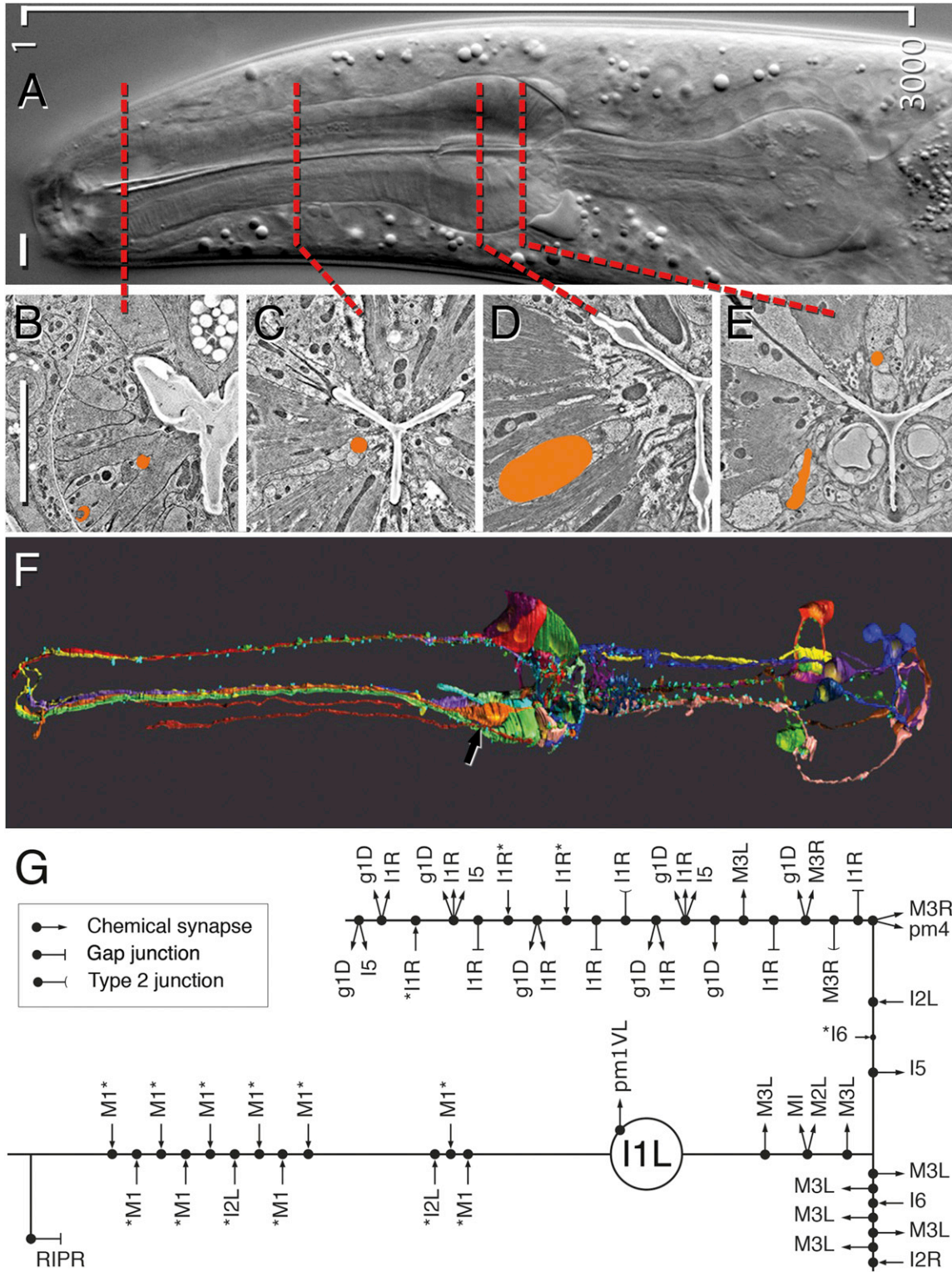
**Figure 2. Reconstruction of the *P. pacificus* Pharyngeal Nervous System**

(A) Nomarski representation of the pharynx of a eurystomatous animal. The entire pharynx of three individuals was sectioned, resulting in more than 3,000 consecutive sections each.

(B–E) Representative low magnification electron micrographs of cross-sections through the anterior corpus (B), median corpus (C), central median bulb (D), and the pharyngeal nerve ring (E). The interneuron I1 is indicated in orange as example for one of the 20 neurons.

*(legend continued on next page)*

portions of information flow at one time in order to reveal patterns hidden in global metrics. We model information flow with random walks (Noh and Rieger, 2004). We define indirect information flow as edges along random walks that are not along shortest paths between specified nodes. For each focus node, we separated walks into upstream and downstream portions and calculated the proportion of indirect (not along shortest paths) information between each node and the focus node. Indirect information indicates the presence of feedback and feed-forward motifs, which are statistically overrepresented and functionally important in nervous systems (Milo et al., 2002) and have been shown to reveal additional patterns of centrality (da Fontoura Costa et al., 2011).

I1 and I2 are the primary candidates for regulating pharynx muscles during bacterial versus predatory feeding in *P. pacificus.* Both are unusual in that they do not receive input from within the pharynx in *C. elegans* but have multiple inputs in *P. pacificus* (Figures 5A and 5B). The most important input in *P. pacificus* for both neurons is M1. Close to 40% of random walk information entering I1 from M1 and I2 is indirect. Similarly, information output differs significantly for I1 and I2 between *P. pacificus* and *C. elegans* (Figures 5A and 5B). pm5 is an important output for I1 only in *C. elegans*, whereas the dorsal gland is important for I1 only in *P. pacificus*. The most important outputs of I2 in *C. elegans* are the isthmus muscle cell (pm5) and synapses directed at the basement membrane that function in serotonin volume transmission (Harris et al., 2011). In *P. pacificus*, I2 outputs broadly, yet basement membrane synapses are absent.

Connectivity also differs between species with regard to shortest path versus indirect information flow. Most network flow from I1 to pm4 in *C. elegans* is along shortest paths, whereas nearly all network flow from I1 to pm4 in *P. pacificus* is indirect, despite a direct connection between I1 and pm4 not seen in *C. elegans*. A surprising 73% of information between I2 and pm4 is indirect in *P. pacificus*, compared to just 9% in *C. elegans* (Figures 5C and 5D). Higher indirect information flow in the *P. pacificus* anterior pharynx hints at complex processing that correlates with multiple behavioral modes and may explain unexpected centrality patterns. The broad range of potential I2 outputs and the inclusion of I1 as an important downstream neuron points toward a role for I2 in switching between behavioral modes.

Global centrality identified the dorsal gland as an important output in both species, yet we know little about its function. It opens in the mouth of *C. elegans* and directly through the predatory dorsal tooth in *P. pacificus*. Surprisingly, there is no overlap in nodes found to be important by focused centrality (Figures 5E and 5F). The two cells important for *C. elegans* (M4 and I5) are presynaptic to pm5. All information between M4 and the dorsal gland is along shortest paths, but close to 40% from I5

is indirect. The three cells identified as most important for the dorsal gland in *P. pacificus* (I1, M1, and I2) are candidates for differentially regulating the tooth during predatory versus bacterial feeding. More than half of the input from M1 to the dorsal gland is indirect.

## DISCUSSION

The detailed and comparative anatomical descriptions of neurons by Ramon y Cajal have firmly planted the metaphor of synaptic wiring into neuroscience and have been the seed of inspiration for constructing maps of connectivity in nervous systems for generations of scientists (De Carlos and Borrell, 2007). The practical application of electron microscopy, now more than 70 years ago, seemed to finally provide the tools for massive reconstructions of large systems. This resulted in the significant success of a full wiring diagram for the nematode *C. elegans*, which began with a description of the pharyngeal nervous system (Albertson and Thomson, 1976) and culminated with whole-animal description of synaptic wiring (Hall and Russell, 1991; Varshney et al., 2011; White et al., 1986). Until recently (Bock et al., 2011; Briggman and Denk, 2006; Jarrell et al., 2012), however, practical and technological limitations have prevented further successes. Significant advances in software (Anderson et al., 2011; Cardona et al., 2010, 2012; Chklovskii et al., 2010; Jurrus et al., 2012) and instrumentation (Denk and Horstmann, 2004; Hayworth et al., 2006; Heymann et al., 2006; Lander et al., 2001) are increasing the throughput and decreasing the cost of such work, with the hope of bringing anatomically derived connectivity data into the realm of bioinformatics in the guise of synapse-level connectomics.

The comparative method has been central to the development of both neuroscience—most notably in the form of comparative physiology—and bioinformatics. Work presented here attempts to infuse comparative techniques into connectomics. This comparative analysis of neural networks in the nematode pharynx, which examines wiring of 20 clearly homologous identified neurons, is to our knowledge the largest and most complete study of its kind.

The most significant finding of our study is also conceptually the simplest: the identification of a massive rewiring of synaptic connectivity between two related species in a system in which cell identity is otherwise extremely conserved (Figures 3A, 3B, and 4A). This was not the expectation (Katz and Harris-Warrick, 1999). Most explanations for how differences in neural circuits composed of homologous identified neurons can generate different patterns of behavior are derived from detailed physiological studies of small circuits containing between two and four neuron classes (Baltzley et al., 2010; Katz and Harris-Warrick, 1999; Newcomb and Katz, 2009; Sakurai et al., 2011). They have repeatedly and conclusively

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(F) Three-dimensional reconstruction of the pharyngeal nervous system in *P. pacificus*. Neuron arborization shows a high degree of conservation with *C. elegans*. The pharyngeal nerve ring "brain" is located in the posterior end of the medium bulb. Neuron nuclei are identical in number and relative position to those in *C. elegans*. I1 is rendered in orange (see also B–E), and the cell body position is indicated by an arrow.

(G) Reconstruction of the I1 interneuron in *P. pacificus*. The positions of the chemical synapses and different types of gap junctions are indicated. See legend inlay for details.

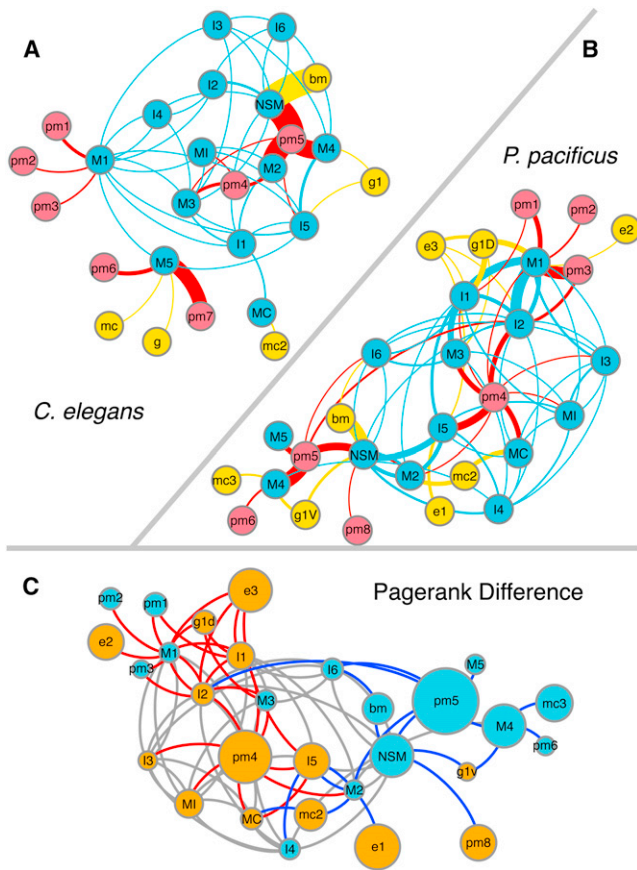Scale bars in (A) and (B) represent 5 μm. The scale bar in (B) applies also to (C)–(E). See also Data S1.

**Figure 3. Synaptic Connectivity and PageRank Centrality**

(A) Two-dimensional representation of the synaptic connectivity in *C. elegans* based on Albertson and Thomson (1976). Nodes indicating neurons (blue), muscle cells (red), and other network outputs (yellow) such as epithelial or gland cells are shown. Edges curve clockwise from the presynaptic to the postsynaptic node. Edges are colored the same as their postsynaptic partner, and their width indicates weight according to multiplicity of synapses. Nodes labeled bm are targeted at the basement membrane of the pharynx and not to a specific cell.

(B) Similar layout of synaptic connectivity for *P. pacificus* based on this study. An online network browser is available at http://www.Pristionchus.org/connectomics.

(C) Differences in PageRank centrality mapped onto the *P. pacificus* network. Node size is proportional to magnitude of the difference in PageRank between *C. elegans* and *P. pacificus*. Orange nodes have a higher centrality in *P. pacificus*, whereas blue nodes have a higher centrality in *C. elegans*. Edges connected to anterior pharynx output nodes (pm1–pm4, g1d, e1–e3, and mc1) are colored red. Edges connected to posterior pharynx outputs (pm5–pm8, g1V, g2, mc2–mc3, and bm) are colored blue. Edges between neurons are colored gray. Nodes with connections to anterior pharynx output cells, including those nodes proposed to control predatory feeding, have a higher PageRank in *P. pacificus* than in *C. elegans*. Nodes with connections to posterior pharynx outputs have a higher PageRank in *C. elegans* than in *P. pacificus*.

demonstrated that even very large and discrete changes in behavior can be sufficiently explained by the physiological properties of neurons and modulatory properties of neurotransmitters rather than the presence or absence of synaptic connections. Genetic studies of larger circuits in model organisms, including
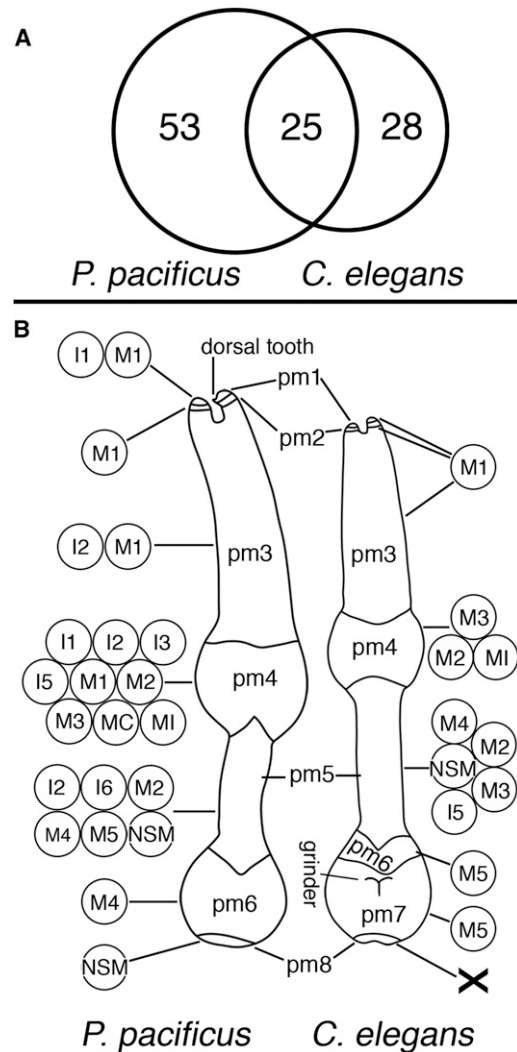


**Figure 4. Global Assessment of Synaptic Connectivity Differences between *P. pacificus* and *C. elegans***

(A) Proportional Venn diagram showing the number of pharynx synapse classes found in *P. pacificus* and *C. elegans*. Only about half the synapse classes in *C. elegans* can be found in *P. pacificus*, and *P. pacificus* has more synaptic complexity than *C. elegans*.

(B) Line drawings of the pharynx in *C. elegans* (right) and *P. pacificus* (left). Classes of muscle cells (pm1 through pm8) are indicated for both species. Black lines connect each muscle cell class to its corresponding motorneurons (circles). No motorneurons are presynaptic to pm8 in *C. elegans* (indicated by a black X). The grinder of *C. elegans* and the dorsal tooth of *P. pacificus* represent anatomical specifications associated with exclusive bacterial and predatory feeding, respectively.

*C. elegans*, have also demonstrated a remarkable capacity for behavioral change through neuromodulation (Bargmann, 2012). Thus, the removal or formation of synaptic connections is not required to modify behavior. Contrasting the ability for modulation to promote change, physiological (Saideman et al., 2007) and computational (Prinz et al., 2004) studies of large networks in the stomatogastric ganglion in crabs have shown that network function can be conserved in the presence of major

**Table 1. Degree, Closeness, and Betweenness Centrality for Pharyngeal Neuron Cell Classes of *P. pacificus* and *C. elegans***

| Cell Class | Degree | | | Closeness | | | Betweenness | | |
|---|---|---|---|---|---|---|---|---|---|
| | *P. pacificus* | *C. elegans* | Difference | *P. pacificus* | *C. elegans* | Difference | *P. pacificus* | *C. elegans* | Difference |
| I1 | 11 | 6 | 5 | 0.43 | 0.52 | −0.09 | 0.031 | 0.00 | 0.031 |
| I2 | 13 | 4 | 9 | 0.54 | 0.43 | 0.1144681 | 0.047 | 0.00 | 0.047 |
| I3 | 9 | 5 | 4 | 0.44 | 0.67 | −0.2240437 | 0.020 | 0.02 | 0.005 |
| I4 | 8 | 5 | 3 | 0.51 | 0.41 | 0.0963905 | 0.056 | 0.01 | 0.048 |
| I5 | 9 | 9 | 0 | 0.83 | 0.53 | 0.3055556 | 0.020 | 0.06 | −0.039 |
| I6 | 8 | 5 | 3 | 0.41 | 0.54 | −0.1293706 | 0.024 | 0.01 | 0.017 |
| M1 | 13 | 13 | 0 | 0.47 | 0.53 | −0.0622605 | 0.043 | 0.10 | −0.054 |
| M2 | 7 | 4 | 3 | 0.37 | 1.00 | −0.630137 | 0.018 | 0.00 | 0.016 |
| M3 | 7 | 9 | −2 | 1.00 | 1.00 | 0 | 0.006 | 0.02 | −0.012 |
| M4 | 5 | 5 | 0 | 1.00 | 1.00 | 0 | 0.026 | 0.01 | 0.019 |
| M5 | 1 | 6 | −5 | 1.00 | 0.46 | 0.5365854 | 0.000 | 0.04 | −0.040 |
| MC | 7 | 2 | 5 | 1.00 | 1.00 | 0 | 0.008 | 0.00 | 0.006 |
| MI | 9 | 5 | 4 | 0.39 | 0.47 | −0.0753623 | 0.022 | 0.00 | 0.022 |
| NSM | 11 | 9 | 2 | 0.48 | 0.64 | −0.1542208 | 0.087 | 0.03 | 0.059 |

differences in the physiological properties of connections. The lack of anatomically defined maps of connectivity may bias against explanations based on connectivity differences. Indeed, there are additional indications that large-scale rewiring of homologous neurons may be a more general phenomenon, as partial reconstructions of visual neuropil in several diptera have found 50% of connections to be species specific (Shaw and Moore, 1989), a level of difference similar to what has been identified in this study.

Our analysis utilizes a graph theoretical approach, which, rather than utilizing highly mechanistic models, abstracts neuron interactions to the level of information theory. Nervous systems are easily represented as graphs in which the nodes are neurons and the synaptic connections between them are depicted as edges. This type of abstraction does not easily account for interactions resulting from compartmentalization within neurites, which occurs in animals including vertebrates and nematodes (Euler et al., 2002; Katsuki et al., 2011), as the spatial relationships between individual synapses are not incorporated into the graph. This applies especially in nematodes, where the distinction between an axon and dendrite is seldom clear (White et al., 1986), and interactions within individual neurites can be surprisingly complex (Hendricks et al., 2012). It is, however, a powerful tool for revealing unexpected system-level patterns. This approach was first meaningfully applied to the *C. elegans* nervous system in a paper that revolutionized modern graph theory with the starting conclusion that the statistical properties of most encountered networks, including nervous systems, are nonrandom (Watts and Strogatz, 1998). The implication is that there may be hints of function or network growth processes hidden in both the statistical and specific properties of connectivity. One widely used graph theoretical tool is centrality analysis, which seeks to define the importance of a node in a network based on specified criteria (Rubinov and Sporns, 2010). Network centrality of the whole *C. elegans* nervous system has been examined in detail (Varshney et al., 2011). One of the surprising

conclusions in their study, as well as the work presented here, is that these simple metrics do correlate with certain aspects of network function. Varshney et al. (2011) found an association between closeness centrality and known command interneurons, and our own analysis finds that those neurons that yield strong laser ablation phenotypes in *C. elegans* (M3, M4, and MC) have the maximum closeness in both species (Table 1). This evolutionary conservation in closeness centrality is surprising in light of our findings of significant system-level rewiring.

The most striking pattern identified through our centrality analysis was for PageRank. As for closeness centrality, the nodes with the highest values are the same in both species (pm4, pm5, and g1D) (Table 1), despite massive shifts in individual connections. Even more intriguing is a finding that would have gone unnoticed without a system-level approach. Information flow, as determined by PageRank, is much higher in the anterior pharynx of *P. pacificus* than in *C. elegans* and is much higher in the isthmus of *C. elegans* relative to *P. pacificus* (Figure 3C). This indicates that the increased behavioral complexity of the anterior pharynx in the predatory nematode *P. pacificus* may be correlated with an increase in the complexity of synaptic connectivity and certainly justifies the additional comparative work required to more conclusively demonstrate this correlation.

Though difficult, this hypothesis of a system-wide shift in the focus of network activity could be addressed through systematic network manipulation using either laser microsurgery, as has been done for *C. elegans* (Avery and Horvitz, 1989), or optogenetic tools. Unfortunately, our attempts to ablate I1 and I2, two of the most prominent candidates for novel functions in *P. pacificus*, failed due to rupturing of the wall of the pharynx in most animals. Although the ablation of some of the neurons that form left/right pairs will be technically challenging, work by Chiang et al. (2006) has already demonstrated the power of cell ablation studies in the nematode pharynx when applied in a comparative context.

**Table 2. PageRank for Pharynx Cell Types of *P. pacificus* and *C. elegans***

| PageRank | | | |
| --- | --- | --- | --- |
| Cell Class | *P. pacificus* | *C. elegans* | Difference |
| I1 | 0.032 | 0.023 | 0.009 |
| I2 | 0.032 | 0.023 | 0.009 |
| I3 | 0.032 | 0.024 | 0.007 |
| I4 | 0.025 | 0.029 | −0.004 |
| I5 | 0.041 | 0.029 | 0.011 |
| I6 | 0.032 | 0.032 | 0.000 |
| M1 | 0.033 | 0.039 | −0.006 |
| M2 | 0.028 | 0.030 | −0.002 |
| M3 | 0.040 | 0.053 | −0.013 |
| M4 | 0.024 | 0.052 | −0.028 |
| M5 | 0.021 | 0.026 | −0.005 |
| MC | 0.045 | 0.028 | 0.017 |
| MI | 0.032 | 0.023 | 0.009 |
| NSM | 0.034 | 0.061 | −0.027 |
| bm | 0.030 | 0.048 | −0.018 |
| e1 | 0.030 | X | 0.032 |
| e2 | 0.024 | X | 0.022 |
| e3 | 0.039 | X | 0.030 |
| g2 | X | 0.024 | −0.024 |
| g1D | 0.045 | 0.029 | 0.016 |
| g1V | 0.030 | 0.029 | 0.001 |
| mc2 | 0.045 | 0.024 | 0.021 |
| mc3 | 0.026 | 0.046 | −0.020 |
| pm1 | 0.027 | 0.035 | −0.008 |
| pm2 | 0.024 | 0.029 | −0.005 |
| pm3 | 0.027 | 0.029 | −0.002 |
| pm4 | 0.089 | 0.075 | 0.013 |
| pm5 | 0.061 | 0.121 | −0.060 |
| pm6 | 0.026 | 0.027 | −0.001 |
| pm7 | X | 0.038 | −0.038 |
| pm8 | 0.025 | X | 0.022 |

An X indicates a cell type for which there were no connections in that species.

It has been proposed that biological networks evolve through preferential attachment of new connections to already highly connected nodes (Eisenberg and Levanon, 2003; Light et al., 2005; Teichmann and Babu, 2004). Models of preferential attachment seem to adequately describe the degree distribution of networks of neurons (Barabasi and Albert, 1999). In other biological networks, particularly protein interaction networks, the nodes with a relatively high degree remain so over evolutionary time, consistent with the idea of preferential attachment. However, substantial differences in node degree centrality exist between *P. pacificus* and *C. elegans*, suggesting that preferential attachment may not be sufficient to explain evolutionary patterns in networks of neurons.

Centrality metrics ask how important a particular node is for a network, but some biological problems require the inverse
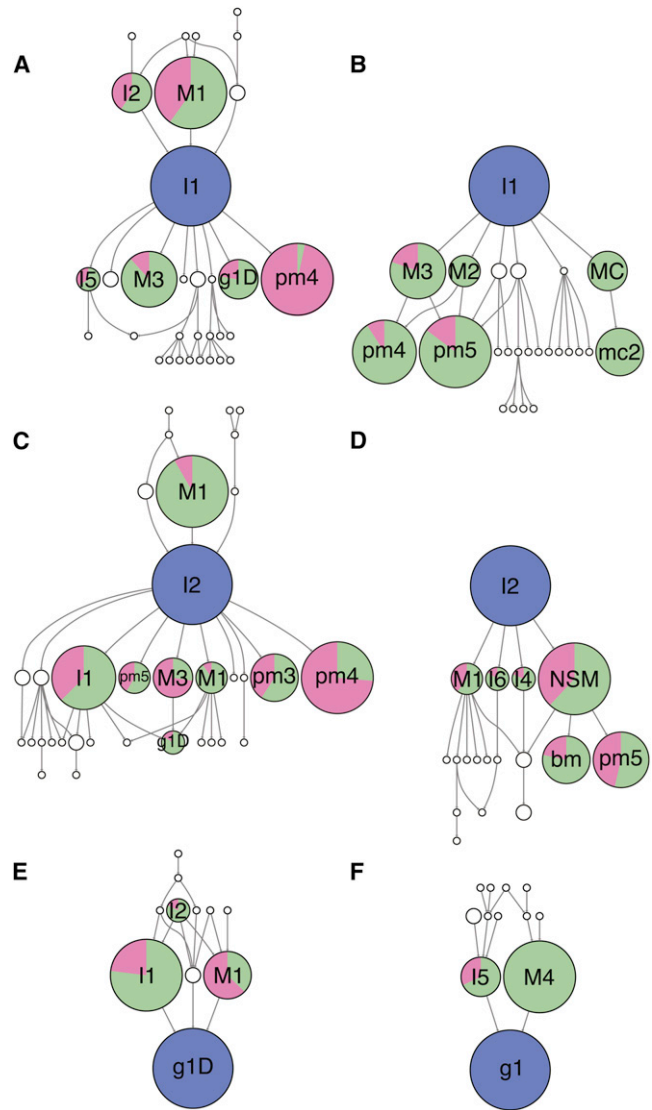


**Figure 5. Focused Centrality and Indirect Information Flow**
(A–F) For all graphs, nodes drawn above the focus node (blue) are upstream, and nodes below are downstream. For visualization, more important nodes are larger, and node size was adjusted according to the corresponding decile of the cumulative targeted information flow distribution. For the larger nodes, the proportion of indirect (pink) versus shortest path (green) edges along random walks between a node and the focus node are indicated as a pie chart. For simplification, only shortest-path edges with respect to the focus node are indicated, and labels are only provided for more important nodes. Cell classes from *P. pacificus* (A, C, and E) are on the left, and for *C. elegans* (B, D, and F), they are on the right.

question: how important are parts of a network for a particular node? To address this, we developed the method of focused centrality. In addition, we distinguished between information flow that is either indirect or along shortest paths. The justification for doing so is that many known functional network motifs, such as feed-forward loops, do not carry information exclusively along shortest paths. One resulting observation is that, generally, there is more indirect information flow in *P. pacificus* than in

*C. elegans* (Figure 5), which may again correlate with a more complex regulation of the system. Indirect information flow in *P. pacificus* is particularly striking between the two primary candidates for mediating predatory feeding behavior, I1 and I2, and their median bulb muscle target (pm4).

As with other bioinformatics approaches, one goal of comparative connectomics must be to generate interesting hypotheses that are experimentally testable. Aside from the more abstract hypothesis that complexity in function correlates with complexity in connectivity, our analytical methods can be used to generate hypotheses related to specific network function. For example, the dorsal gland is known to have a very different function in the plant parasitic Tylenchid nematodes than in the bacterial feeding *C. elegans*. Has dorsal gland function also shifted to accommodate novel predatory feeding behavior in Diplogastrids such as *P. pacificus*? Focused centrality demonstrates that network flow entering the dorsal gland in *P. pacificus* is primarily associated with the corpus, whereas information flow entering the same cell in *C. elegans* is associated with the isthmus, which is consistent with a dramatic shift in regulation (Figures 5E and 5F). As with genomics, transcriptomics, and proteomics, the value of the data and the ability to use them as a tool for generating new biological questions will increase dramatically as new data sets become available.

Although the comparative analysis presented in this study focuses on the connectivity through chemical synapses, gap junctions are also functionally important for communication in the nervous system, including for that of *C. elegans* (Altun et al., 2009; Chalfie et al., 1985; Keane and Avery, 2003; Macosko et al., 2009). In general, we noticed differences in the morphology of gap junctions in and between tissues in *P. pacificus*, an observation that is similar to previous findings in *C. elegans* (Hall, 1987). Whereas some of the junctions observed in the pharynx and other tissues look like bona fide gap junctions, other "gap-junction-like" structures have previously undocumented morphologies (Figure S1). The *P. pacificus* gap junction data provide an important resource, but functional characterization awaits further analysis.

We have focused our attention on the eurystomatous mouth form of *P. pacificus*. Although we cannot rule out that the connectivity map might be different in stenostomatous individuals, hundreds of millions of years of evolutionary distance between *P. pacificus* and *C. elegans* (Dieterich et al., 2008), as well as predatory and bacterial feeding modes in both mouth forms, make it reasonable to assume that most of the differences between species in our comparison are explainable by species-specific variation rather than by the mouth-form dimorphism. Future studies on this dimorphism, however, could provide further insight into the plasticity of neural connectivity.

The integration of neuroanatomy with neural circuits remains key for the understanding of animal behavior and the evolution of behavioral novelty (Lu et al., 2009; Sporns, 2010). That synaptic connectivity evolves faster than the cellular composition of these networks places special emphasis on systems neurobiology. The relative simplicity of nematode nervous systems and their evolutionary diversification represent a unique opportunity to study the neural correlates of behavior with a comparative and integrative connectomics approach.

## EXPERIMENTAL PROCEDURES

### Transmission Electron Microscopy

Specimens of *P. pacificus* strain PS312 were high-pressure frozen with a Bal-tec HPM-10 high-pressure freezer. Prior to freezing, they were placed in 100-μm-deep specimen carriers that were placed on an aluminum plate previously chilled in ice water in order to relax and straighten the worms. Frozen worms in specimen carriers were placed in custom chambers and processed through freeze substitution using a Leica EM AFS2 freeze substitution unit. The substitution was done as follows: 48 hr at −90°C in 5% gallic acid. Temperature was increased at a rate of 4°C per hr to −60°C. Specimens were rinsed 5% in acetone and transferred into 1% osmium tetroxide, 0.1% uranyl acetate, and 2% water in acetone. Temperature was held at −60°C for 24 hr and then raised at 4°C per hr to 0°C. The specimens were rinsed 5× in acetone and transferred though a series to 100% epon, where they remained for 24 hr prior to being embedded in slide-shaped molds. It should be noted that the power of high-pressure freezing as used in this study on *P. pacificus* was not available at the time when the *C. elegans* data were obtained, and it remains unknown whether some of the observed connectivity results from higher resolution.

The resulting slides were examined under a Nomarski microscope to choose young adult hermaphrodite specimens that appeared to have no freezing damage in the anterior portion of the worm. Blocks were sectioned with a Reichert Ultracut S microtome at either 70 nm (specimen 24) or 50 nm (specimens 107 and 148). Sections were picked up on empty slot grids, which were placed on pioloform films cast onto metal plates with holes to allow for grid placement. Excess moisture was wicked from underneath with a wide-slot grid, and then the grid was dried in an oven at 70°C. Sectioned were post stained with an RMC QG-3000EC auto stainer with aqueous uranyl acetate and Sato lead citrate and then coated with a thin layer of carbon to stabilize the films. Sections were then imaged with a Gatan Ultrascan 4K camera mounted on a Tecnai T12 transmission electron microscope. The resulting images were cataloged in a custom MySQL database. Images were aligned and manually segmented using either IMOD (specimen 24) or TrakEM2 (Cardona et al., 2012; Kremer et al., 1996) (specimens 107 and 148). Fully segmented volumes were visualized using Blender.

### Comparative Graph Theoretical Analysis

Networks of synaptic connectivity, weighted by the number of synapses for each synapse class, were reconstructed for specimens 107 and 148 and are included as Data S1. Individual synapse IDs are provided, which can be traced back to the original images of that synapse. A consensus network was constructed with only the synapse classes found in both animals and where neuron classes rather than individual neurons are represented so as to be directly comparable to *C. elegans*. The network of nonvariable synapses for *C. elegans* that was used as a comparison is included in Table S3. Networks of synaptic connectivity were visualized using Gephi (Bastian et al., 2009). Centrality measures were calculated treating the edges as directed using either Gephi or the Network Analyzer plugin for Cytoscape (Smoot et al., 2011). A damping factor of 0.85 was used for PageRank calculations.

Calculations for the targeted centrality analysis were done by modeling information flow through the *C. elegans* and *P. pacificus* pharyngeal nervous system as a first-order Markov process in which cells correspond to states and state transitions are defined by the synaptic connectivity. The probability of a signal starting at a particular neuron is proportional to the number of synapses originating at it, and transition rates from neuron i to j are equal to the number of outgoing synapses from neuron i to j divided by the number of outgoing synapses of neuron i. Based on this model, we simulated $10^7$ random walks for each network that start at a neuron and continue until they reach an output cell.

To investigate information input and output separately, we partitioned all walks containing a predefined focus node and counted occurrences of nodes from the start to the first occurrence of the focus node as information input and occurrences of nodes from the last occurrence of the focus node to the end of the walk as information output. All counts were normalized by dividing by the total number of traversed nodes. The proportion of indirect input information was defined as the fraction of nodes along the information input partition of

all walks that are not on shortest paths. Similarly, indirect output information is the fraction of nodes along the output partition not along shortest paths.

An online network browser tool was developed to compare network connectivity, centrality, and focused centrality between *P. pacificus* and *C. elegans*. This can be accessed at http://www.pristionchus.org/connectomics.

## SUPPLEMENTAL INFORMATION

Supplemental Information includes one data file, one figure, three tables, and one movie and can be found with this article online at http://dx.doi.org/10.1016/j.cell.2012.12.013.

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