A functional connectivity atlas of *C. elegans* measured by neural activation

Francesco Randi¹, Anuj K Sharma¹, Sophie Dvali¹, and Andrew M Leifer¹,²,*

¹Princeton University, Department of Physics, Princeton, NJ, 08544, United States of America
²Princeton University, Princeton Neurosciences Institute, Princeton, NJ, 08544, United States of America
¹leifer@princeton.edu

ABSTRACT

Neural processing and dynamics are governed by the details of how neural signals propagate from one neuron to the next through the brain. We systematically measured functional properties of neural connections in the head of the nematode *Caenorhabditis elegans* by direct optogenetic activation and simultaneous calcium imaging of 10,438 neuron pairs. By measuring responses to neural activation, we extracted the strength, sign, temporal properties, and direction of information flow between neurons in order to create an atlas of causal functional connectivity. We find that functional connectivity differs from predictions based on anatomy, in part, because of extrasynaptic signaling. Our measurements show that neurons such as RID signal extrasynaptically to other neurons for which there are no direct wired connections. We show that functional connectivity better predicts spontaneous activity than anatomy, suggesting that functional connectivity captures properties of the network that are critical for interpreting neural function.

Main

Introduction

Brain connectivity mapping is motivated by the claim that “nothing defines the function of a neuron more faithfully than the nature of its inputs and outputs”¹,². One way to measure inputs and outputs is to make a map of the anatomical electrical and chemical synapses of a brain, called a connectome. The connectome of the nematode *C. elegans*³–⁵ has informed our understanding of circuit-level mechanisms underlying mechanosensation⁶, locomotion⁷, chemosensation⁸, and other behaviors and also serves as the basis for connectome-constrained models of neural activity⁹,¹⁰. Connectomes for *Ciona intestinalis* larvae¹¹, *Drosophila* larvae¹², larval zebrafish¹³, and adult *Drosophila*¹⁴,¹⁵ are at various stages of completion, and have already elucidated circuit-level mechanisms, such as those underlying the head-direction system in the adult fly¹⁶,¹⁷. Efforts to measure the mouse connectome are also underway¹⁸.

Yet, an anatomical map of synaptic contacts leaves ambiguous some aspects of neurons’ inputs and outputs. One cannot always infer a neural connection’s strength and sign (excitatory or inhibitory) from anatomy or gene expression. For example, the strength and sign of the *C. elegans* synapses from neurons ASE to AIB are ambiguous because ASE releases glutamate and AIB expresses both excitatory and inhibitory glutamate receptors¹⁹. Anatomy does not provide a clear picture of the timescales of neural transmission because these timescales are governed by molecular properties that are hidden from view. Moreover, not all anatomical neural connections are operational. In the head compass circuit in *Drosophila*, many anatomical connections exist, but plasticity from long-term potentiation and long-term depression selects only a subset to function in order to guide relevant visual inputs²⁰. Similarly functional connections in the central complex appear to be sparser than expected from anatomy²¹. Neuromodulators also adjust the properties of neural connections to strengthen or weaken them or to turn on only a subset of circuits out of a larger menu of possible latent circuits, for example, in the stomatogastric ganglion (STG)²²–²⁴. Additionally, neurons can release many signaling molecules from outside the synapses, to facilitate “wireless” neural connections that are not visible in the anatomical wiring²⁵, as we explore in this work. These additional properties of a neuron’s inputs and outputs pose challenges for accurately predicting a network’s activity or function from anatomy alone.

A more direct way to characterize the nature of a neuron’s inputs and outputs is to measure their functional properties directly, namely by activating a neuron and observing responses in other neurons. We refer to this approach as “measuring functional connectivity,” because our measurements are used to define mathematical functions that describe how neural signals of an upstream neuron drive activity in a downstream neuron. The general approach of activating a neuron and measuring a response has also been described as “influence mapping”²⁶. Functional connectivity measured by neural activation captures the strength and sign of neural connections and reflects plasticity, neuromodulation, and even wireless signaling. Measuring
downstream responses to neural activation provides an unambiguous arrow of causality and temporal dynamics, in contrast to correlative maps of spontaneous activity. Perturbing the network allows us to probe neural connections that might not be active spontaneously, and therefore would be omitted from a correlation map. Previous efforts have employed optogenetic perturbations27,28 and calcium imaging29,30 in vivo to map the functional connectivity of selected circuits in C. elegans31, mouse visual cortex32, mouse hippocampus33, mouse somatosensory cortex34, and zebrafish35, among others. More recently, investigators measured a functional connectivity map of the Drosophila central complex by probing genetically defined cell types in order to relate functional connectivity to anatomy.21

Here we use neural activation to measure functional connectivity of neurons from throughout the head of C. elegans instead of any specific circuit or brain area. We survey 10,438 neuron pairs to present a systematic functional connectivity atlas of neural connections in the head. We show that functional measurements are more suitable for predicting spontaneous activity than anatomy and gene expression, and that the functional description captures instances of wireless signaling that are absent from anatomy.

**Simultaneous population imaging and single-cell activation**

To probe functional connectivity, we systematically activated each neuron, one at a time, while simultaneously recording network activity. We recorded population calcium activity from 43 wild-type (WT) background animals, each one for up to 40 min, while stimulating a randomly selected sequence of neurons one-by-one every 30 s (Fig. 1). To perform these measurements, we combined whole-brain population calcium imaging via spinning disk single-photon confocal microscopy37,38 with two-photon39 targeted optogenetic stimulation40 in an immobilized animal (Fig. 1a). Animals are awake and pharyngeal pumping is visible during recordings. We spatially restricted the optogenetic excitation volume in three dimensions to the typical size of a C. elegans cell soma (Supplementary Fig. S1a) using temporal focusing33,41 to address a single neuron without also activating its neighbors (Supplementary Figs. S2 and S3). To overcome challenges associated with spectral overlap33,34,42–44, we expressed the GUR-3/PRDX-2 purple-light activatable optogenetic system35,46 and a nuclear-localized calcium indicator GCaMP6s47 in each neuron (Fig. 1b). For calcium imaging, we used a wavelength and intensity of excitation light that does not elicit photoactivation of GUR-3/PRDX-2 (Supplementary Fig. S1b)48. We also included additional genetically encoded fluorophores from NeuroPAL36 to identify neurons consistently across animals (Fig. 1c). Many neurons exhibited calcium activity in response to activation of one or more other neurons (Fig. 1d). A downstream neuron's response to a stimulated neuron is evidence of a functional connection between them.

We highlight three examples of excitatory or inhibitory responses that we observed among interneurons in the motor circuit (Fig. 1e-g). Stimulation of the interneuron AVIR evoked activity in AVDR (Fig. 1e). AVJ is thought to coordinate locomotory activity upon egg laying and has been proposed to promote forward locomotion49. AVD activity is associated with sensory-evoked (but not spontaneous) backward locomotion6,7,50,51 and receives chemical and electrical synaptic input from AVJ5,52. Both the wiring and our functional measurements suggest that AVJ may also play a role in coordinating backward locomotion, in addition to its previously described roles related to egg laying and forward locomotion.

The premotor interneurons AVA5,51,53–58 and AVE51,55 both exhibit increased calcium activity during backward movement that is correlated with one another55. AVE has gap junctions and makes many chemical synaptic contacts with AVA5,52. We found that stimulating AVE evoked activity in AVA (Fig. 1f). In contrast, stimulation of AVE inhibited activity in SAAD (Fig. 1g). SAAD is involved in turning behavior and has been proposed to inhibit motor neurons associated with backward locomotion in order to prevent conflict between turning and reversal circuitry51. Our measurements suggest a complementary mechanism in which the backward-associated AVE neuron functionally inhibits the turning machinery via inhibition of SAAD.

**Activation-response map**

We generated a single activation-response map by aggregating downstream responses to stimulation for each neuron pair across recordings from 43 individuals (Fig. 2). We report the average calcium response in a time window $\langle \Delta F/F_0 \rangle$ averaged across trials and animals (Supplementary Fig. S4). We imaged calcium activity in response to stimulation for 10,438 neuron pairs (30% of all possible pairs in the head) at least once, and as many as 27 times (Supplementary Fig. S5a). This includes activity from 162 of 188 neurons in the head (86%, Supplementary Fig. S6). To assess the significance of the average observed calcium transients in each neuron pair, we compared features of the transients to a null distribution of activity recorded from animals in which no neurons had been stimulated. We then accounted for multiple hypotheses and calculated a $q$-value for each pair that reports significance59,60 considering both the magnitude of the response and the number of observations (Supplementary Fig. S5b). Our activation-response map comprises the response amplitude and its associated $q$-value (Fig. 2a, Supplementary Fig. S7).

This functional dataset, overlaid on the anatomical wiring diagram5,52, is browseable through online interactive visualization software (https://funconn.princeton.edu) built on the NemaNode platform5. If we hold the false discovery rate at 5% ($q < 0.05$), we estimate that at least 1,285 of the 10,438 measured neuron pairs in our compiled datasets are functionally connected, or 12% (Fig. 2b,c). Note that these functional connections are “effective connections” because they represent
Figure 1. Measuring causal brain-wide functional connectivity at a cellular resolution. a.) Schematic of instrument that allows sequential stimulation of individual neurons while population calcium activity is simultaneously recorded. b.) A spatially restricted two-photon excitation spot activates GUR-3/PRDX-2 during single-photon GCaMP6s calcium imaging. c.) Additional fluorophores from NeuroPAL are expressed to identify neurons. d.) Example recording from an individual worm. Calcium activity is simultaneously recorded from 74 neurons with unambiguous neural identities. Stimulation events are shown as gray vertical lines, and the identity of the stimulated neuron is listed at top. For visualization, activity is shown as fluorescence intensity normalized by noise \( F/\sigma_F \). e.) Paired activity of AVJR and AVDR in response to AVJR stimulation is shown as the fold change of fluorescence from baseline, \( \Delta F/F_0 \). Top panel shows mean (blue line) and standard deviation (light shading) across trials and recordings. Bottom panel shows simultaneously recorded paired activity for individual trials, including across animals, sorted by mean activity in AVDR. All trials with stimulus events that resulted in activity in the stimulated neuron above a threshold are shown. f.) Same as e for AVER stimulation and AVAR response. g.) Same as e for AVEL stimulation and SAADL response.
Figure 2. Activation-response map of *C. elegans*. a.) Activation-response map showing mean amplitude of neural activity in a post-stimulus time window ($\langle \Delta F/F_0 \rangle_t$) averaged across trials and individuals is shown. The $q$-value reports the false discovery rate and is a metric of significance that accounts for response amplitude and number of observations (more gray is less significant). White indicates no measurement. To be included in the map, a stimulation event must evoke a response in the stimulated neuron. Therefore, the strength of a neuron’s response to its own stimulation is not displayed (black diagonal). N=43 animals. Neurons that were recorded but never stimulated are presented in Supplementary Fig. S7. b.) Network graph showing activation response map of neurons in the head. Neurons are positioned anatomically. Connection width and transparency indicate mean response amplitude (red, excitatory; blue inhibitory). Only $q < 0.05$ connections are shown. c.) A bilaterally symmetric pair is more likely to have a $q < 0.05$ functional connection than a pair chosen at random. d.) Fraction of connections that are inhibitory as a function of the $q$-value threshold. Green indicates $q < 0.05$. e.) Probability of being functionally connected ($q < 0.05$) given the minimum anatomical path length $l$ is shown. f.) Distribution of $l$ is shown for neuron pairs that are functionally connected (blue) compared to all possible neuron pairs (orange).
the propagation of signals over all paths in the network between the stimulated and responding neuron, not just the direct (monosynaptic) connections between them.

*Caenorhabditis elegans* neuron subtypes typically consist of two bilaterally symmetric neurons, often connected by gap junctions, that have similar neural wiring, similar gene expression, and correlated activity. Our measurements show that bilaterally symmetric neurons are four times more likely to be functionally connected than pairs of neurons chosen at random (Fig. 2c).

We observe both excitatory and inhibitory calcium responses in the activation-response map. For example, activation of neuron RIS evokes inhibitory transients in many neurons (Supplementary Fig. S8), consistent with its expected role in inducing quiescence. The balance of excitation and inhibition is important for a network's stability and a lack of balance may lead to dysfunction. In the mammalian cortex, approximately 20% of cells are inhibitory and similar values were predicted for the fraction of synapses in *C. elegans* based on wiring and gene expression patterns, but until now this has not been directly measured in the worm. Our measurements indicate that 33% of functional connections are inhibitory (Fig. 2d), which suggests that there may be commonalities in the levels of excitation and inhibition across animals and across network descriptions.

Neurons with a single-hop anatomical connection were more likely to be functionally connected at *q* < 0.05 compared to neurons with only indirect anatomical connections, and the likelihood decreases with increasing minimal anatomical path length (Fig. 2e). We investigated how far responses to neural stimulation penetrate into the anatomical network. Functionally connected (*q* < 0.05) neurons were on average connected by a minimal anatomical path length of 2.3 hops (Fig. 2e), suggesting that neural perturbations often propagate multiple hops through the anatomical network or that neurons are also signaling directly through non-wired means, as explored later.

We observed two types of variability in neural responses for many neuron pairs across trials and individuals: (1) a downstream neuron responds to stimulation of a given upstream neuron in only some simulations but not others (Fig. S9a) and (2) the amplitude, temporal shape, and sign of the response can vary across trials or individuals. Some variability in the responding neuron’s activity can be attributed to variability of the activity in the upstream neuron. For example, in Fig. 1f, downstream neurons with weak responses occurred when upstream neurons had weak auto-responses to stimulation. (We refer to a neuron’s response to its own stimulation as an auto-response.) To study variability in the strength, temporal properties, and sign of the connection, while excluding variability contributed by the upstream neuron’s activity, we calculated a kernel for each stimulation that caused a response. The kernel gives the activity of the downstream neuron when convolved with the activity of the upstream neuron. The kernel describes how the signal is transformed from the upstream to the downstream neuron for that stimulus event, including the timescales of the signal transfer (Supplementary Fig. S10). We characterized variability of each functional connection by comparing how these kernels transform a standard stimulus (Fig. S9b). Many neuron pairs had collections of kernels with properties that varied across trials. We did not identify the sources of this variability, but they likely include state- and history-dependent effect, including from neuromodulation, habituation and plasticity, and inter-animal variability in wiring and gene expression. Collections of kernels within a neuron pair were more stereotyped than collections of kernels randomly selected from across shuffled pairs (Fig. S9c), as expected.

**Functional connectivity differs from anatomy**

We sought to compare our measured functional connectivity to anatomy. Functional connectivity and anatomical connectivity describe different levels of the network — functional connectivity measures effective connection between two neurons, including contributions from all paths through the network, direct and indirect (Fig. 3). In contrast, anatomical features such as synapse count are properties of only direct connections between two neurons. A bridge is needed between these two levels of description in order to make a like-to-like comparison. Therefore, we used connectome-constrained simulations to derive properties of effective connections from anatomy. We systematically activated neurons in silico and simulated the network’s response, using known synaptic weights from anatomy, synaptic polarities based on expression of three common neurotransmitters and receptors, and common assumptions about timescales and dynamics. We found poor agreement between those response amplitudes derived from anatomy and those functional measurements in our activation-response map (Fig. 3b). Our measurements indicate that functional connectivity differs from what one would expect based on anatomy.

Three hypotheses may explain why functional connectivity disagrees with predictions of neural dynamics derived from anatomy. The first is that the strength or sign of connections may have been misinterpreted or otherwise insufficiently specified from anatomy. A second and not mutually exclusive hypothesis is that additional connections exist that are not present in the anatomical wiring. A third possibility, that we do not explore, is that existing computational models may fail to predict neural responses even when given accurate strength, sign, and connectivity — e.g., because they rely on incorrect assumptions about the biophysical dynamics of synapses. Here, we consider the first two hypotheses: (1) incorrect strengths or signs and (2) missing connections.

There are known instances in which anatomy leads to incorrect predictions about the functional strength or sign of connections. For example, the somatosensory neuron AFD’s primary synaptic partner is AIY. The overall functional connection
Figure 3. Functional connectivity differs from anatomy.  

a.) Functional connectivity describes effective connections. For example, the effective connection between neurons A and B includes contributions from all paths through the network: direct (black), indirect (orange), and recursive (blue). By contrast, anatomical features such as synapse count describe only the direct path (black). To make a like-to-like comparison, connectome-constrained simulations are used to derive properties of the effective connections from anatomy.  

b.) Functional connectivity is compared to anatomy. The confidence of a measured functional connection $q_{i,j}$ is plotted against the in silico amplitude of the corresponding anatomy-derived response $\Delta V_{i,j}$, calculated from a connectome-constrained simulation. Smaller values of $q$ are more confident. Note the reversed $y$-axis.  

c.) Agreement between functional connectivity and the anatomy-derived responses is shown for head neurons using either synaptic weights from anatomy (same as in b), or allowing the synaptic weights to be fitted favorably while preserving network topology (i.e., anatomical strength and sign may change, but no new connections are permitted.) Agreement is reported as a Pearson’s correlation coefficient. Agreement is also reported using anatomical weights for only the subset of neurons in the pharynx.  

d.) Fast functional connections better agree with anatomy than slow connections. Here a $q_{i,j} < 0.05$ functional connection agrees if its corresponding anatomy-derived response has a sufficiently large amplitude $|\Delta V_{i,j}|$, determined as the threshold that would make the matrix of anatomy-derived responses as sparse as the $q_{i,j} < 0.05$ matrix. Speed of a functional connection is classified based on its kernel’s rise-time.
between AFD and AIY had at one time been thought to be inhibitory because AFD releases glutamate and AIY expresses inhibitory metabotropic glutamate receptors. However, our functional measurements (Supplementary Fig. S11) and prior optogenetic and electrophysiology recordings all show that AFD-AIY is in fact functionally excitatory, likely due to peptidergic signaling between the two neurons. In this case, even if anatomy and gene expression correctly predict network topology and transmission across the synaptic cleft, there may be other modes of communication such as extrasynaptic peptidergic signaling, and this might explain why predictions from anatomy disagree with functional measurements.

To investigate whether strengths or signs had been misinterpreted from anatomy, we tested whether adjusting the strengths and signs of neural connections could bring anatomical predictions of neural responses into closer agreement with functional connectivity. We constructed a connectome-constrained model of neural activity in which we allowed the strength and sign of the direct anatomical connections to be fit in order to best reproduce the measured effective functional connections, while preserving the overall network topology (i.e., allowing no new connections). For simplicity during fitting, we assumed a linear network at steady state, but during the comparison we relaxed this assumption and ran full simulations. Even when allowing the anatomical weights and signs to change in the most favorable way – without adding any new connections – the agreement between anatomical wiring and our functional connectivity measurements remained poor (Fig. 3c bar labeled “Head, fitted weights” in ). Our measurements suggest that adjusting anatomical weights and signs alone is insufficient to bring anatomy into agreement with functional connectivity. We therefore explored the hypothesis that anatomy omits connections that are needed to accurately predict neural responses.

Wireless signaling

Neurons can communicate extrasynaptically by releasing transmitter via dense core vesicles from areas of the neuron beyond the synapse that then diffuses through the extracellular milieu to bind to receptors of other neurons. This extrasynaptic communication, sometimes called volume transmission, creates neural connections that are hidden from anatomy. Extrasynaptic signaling can be mediated by several classes of molecules including GABA, NMDA, monoamines, and neuropeptides and these form an additional wireless layer of communication in the C. elegans nervous system. Neuropeptides and neuropeptide receptors in particular are reported to be ubiquitous across the C. elegans nervous system and are one possible substrate for extrasynaptic communication.

Extrasynaptic signaling acts in two ways that are not mutually exclusive: (1) they act as neuromodulators to alter excitability or synaptic properties and change how neurons respond to input and (2) they alter the activity of neurons themselves. While the former might appear as variability in our functional connectivity measurements, the latter should appear as neural responses.

Two observations hinted that missing anatomical connections in the form of extrasynaptic signaling may partly explain why our functional connectivity differs from anatomy. First, we noticed that agreement between functional connectivity and anatomy, while still poor, improved slightly when we considered only those neural connections in the pharyngeal network (Fig. 3c). Neural connections in the pharynx are less likely to be wireless than neural connections in the rest of the brain. Specifically, the fraction of functional connections that is thought to be mediated by monoamines and neuropeptides is only 28% in the pharynx but 58% in the head based on anatomy and gene expression. Therefore, anatomy may better agree with our measured functional connectivity in the pharynx because the anatomical description of the pharynx may omit a smaller proportion of connections.

Second, fast functional connections showed better agreement with anatomy than did slow functional connections (Fig. 3d.) Extrasynaptic signaling is expected to be slower than chemical or electrical synapses, because instead of traveling across the short synaptic cleft, signaling molecules must diffuse further through the extracellular milieu to reach targeted neurons. The slower-timescale connections that we measure may be extrasynaptic and this would explain why they show poorer agreement with anatomy. Our measurements from the pharynx and of fast and slow functional connections both suggest that extrasynaptic signaling in our functional measurements may contribute to discrepancies with anatomy. We therefore chose to more directly investigate extrasynaptic signaling in our measured functional connectivity using mutant animals.

We measured an activation-response map in unc-31-mutant animals that have deficiencies in extrasynaptic signaling (Supplementary Fig. S12, 18 individuals) and compared their functional connectivity to WT animals. This mutant disrupts dense-core vesicle-mediated signaling because it lacks the UNC-31/CAPS protein involved in dense core vesicle fusion but is not expected to disrupt chemical or electrical synapses. An online interactive map allows users to browse the activation-response map of this mutant and compare it to WT, https://funconn.princeton.edu. As expected, some neural responses in the unc-31 mutant background were similar to those in WT, including for example functional connections in a small gap-junction sub-circuit from the pharynx (Supplementary Fig. S13). If extrasynaptic signaling is present in WT animals, it follows that animals lacking extrasynaptic signaling should have faster and fewer connections overall. As expected, functional connections in the unc-31 mutant background were more likely to be fast than in the WT background (Supplementary Fig. S14a), and had a smaller proportion of high-confidence functional connections (Supplementary Fig. S14b), further suggesting that extrasynaptic
Figure 4. Anatomy omits extrasynaptic signaling from neuron RID. a.) ADL, AWB, and URX are among the neurons predicted from anatomy to have no response to RID stimulation because there is no strong anatomical path from RID to those neurons (vertical line at or near 0 volts). Their RID-evoked anatomy-predicted response is shown within the distribution of anatomy-predicted responses for all neuron pairs, as in Fig. 3b. b-d.) Activity of neurons b.) URXL, c.) ADLR, and d.) AWBL are shown in response to RID stimulation in WT and mutant backgrounds. Top panel shows the average across all trials and animals and bottom panel shows individual traces. Here trials are shown even in cases when RID activity was not measured.
signaling is present in WT functional connectivity.

We sought to investigate specific extrasynaptic connections that exhibit discrepancies between an anatomical and functional description. We inspected responses to the neuron RID, a neuroendocrine-like cell that is thought to predominantly signal to other neurons extrasynaptically via neuropeptides such as FLP-14, PDF-1, and INS-1.

RID has many potential extrasynaptic signaling partners but only very few and weak outgoing wired connections (Supplementary Fig. S15a,b) making it a good candidate in which to observe extrasynaptic signaling. In our imaging strain, RID exhibited only dim tagRFP-T expression, which prevented us from consistently recording its own calcium activity. We nonetheless stimulated RID and observed other neurons’ responses (Supplementary Fig. S16).

We inspected the activity of three neuron subtypes, URX, ADL, and AWB, that were predicted to have little or no response to RID stimulation based on anatomy (Fig. 4a) but showed notably strong responses to RID stimulation when measured in WT background (Fig. 4b-d). Several lines of evidence led us to conclude that RID predominantly sends signals to URX, ADL and AWB extrasynaptically. (1) When RID was stimulated in the unc-31 background, these three neurons all exhibited reduced amplitude or were less likely to respond at all, suggesting that these neurons’ connections to RID are dense-core-vesicle-dependent and likely extrasynaptic. (2) All three neuronal subtypes express receptors for peptides produced by RID (NPR-4 and NPR-11 for FLP-14 and PDFR-1 for PDF-1). And (3) there are no direct connections through the anatomical network from RID to URX, ADL, or AWB. The shortest paths from RID to each of the neurons show in (Fig. 4b-d) requires two hops (l = 2) for URXL and AWBL and three hops for ADLR (l = 3) and in each case relies on fragile single-contact synapses that appear in only one out of the four individual connectomes. Taken together, RID is a striking example where functional measurements capture neural responses that are not predicted from anatomy, due to the lack of information about wireless signaling in anatomy.

**Functional connectivity better predicts spontaneous activity than anatomy**

A key motivation for mapping neural networks is to understand how they give rise to neural dynamics. We therefore compared how well functional and anatomical descriptions of the network predict spontaneous neural activity. We measured the spontaneous network activity of immobilized worms without any optogenetic activators under bright imaging conditions. Activity correlations derived from functional connectivity were notably more predictive than either of the anatomy-based approaches (Fig. 5). We compared spontaneous activity to anatomy in two ways. First, we compared the bare anatomical weight, or synapse count between two neurons, to the correlation of their spontaneous activity (Fig. 5a). Previous reports had shown that the anatomical weight between neurons is a particularly poor predictor of correlations in activity patterns and our measurements further support this conclusion. Comparing anatomical weights to spontaneous activity correlations, however, likely overstates the discrepancy because synapse counts are properties of only the direct connection between two neurons, while activity correlations instead are influenced by all possible paths through the network. We therefore also predicted activity correlations derived from anatomy in a second way – by using the connectome-constrained simulations from Fig. 3. We drove activity in all neurons in the network in silico and measured correlations from the resulting simulated activity. These anatomy-derived correlations showed marginally better but still poor agreement with measured spontaneous activity (Fig. 5b, “All driven”).

We also predicted network activity from our functional measurements. Functionally-derived correlations better predicted spontaneous activity than either of the anatomy-based approaches (Fig. 5c). To derive correlations from our measured functional connectivity we drove all neurons in silico, propagated their activity through our measured kernels, and calculated the correlation matrix from the resulting activity. An interactive version of the simulations we used to derive activity from measured kernels is available at [https://funsim.princeton.edu](https://funsim.princeton.edu). All of the parameters in the equations governing neural dynamics, including the timescales, weights, signs, and connectivity, are extracted directly from the measurements; specifically, they are captured by the measured kernels. This is in contrast to simulations constrained only by anatomy, which rely on assumptions about timescales and dynamics. This difference may contribute to why functional connectivity outperforms anatomy at predicting spontaneous correlations.

We sought to further improve our predictions of spontaneous activity by inspecting other assumptions in our simulations. When we derive activity correlations via simulations we face a choice about which neurons should be driven, corresponding to our estimate of the neurons that drive spontaneous activity in the network. For the analysis above, we drove activity in all neurons. However, it may be that only a subset of neurons drives the spontaneous activity observed in our recordings. We therefore tested whether a more optimal subset of neurons improved predictions of spontaneous activity. We rank-ordered all the neurons based on how well their individual in silico stimulation reproduced spontaneous network activity and selected the set of top-n neurons that collectively had the best agreement when driven. For activity derived from measured kernels, we found that driving activity in the top 20 neurons in silico generated activity patterns with correlations that more closely matched those of spontaneous recordings (Fig. 5c, “top-n”).

We next investigated whether selecting an optimal subset of neurons also improved the predictive performance of the
Figure 5. **Functional connectivity better predicts spontaneous activity than anatomy.** Spontaneous activity correlations are compared to predictions from anatomy and functional connectivity. In each case, agreement is reported as a Pearson’s correlation coefficient across neuron pairs. Schematic illustrates the measurements used to predict spontaneous activity correlations. 

a.) Anatomical weights from the synapse count are directly compared to the correlation matrix of spontaneous activity. 

b.) Correlations in the anatomy-derived activity are compared to correlations of spontaneous activity. A connectome-constrained simulation is used to derive activity correlations from anatomy by driving neurons *in silico*. Two variations of anatomy-derived activity are shown: when all neurons are driven (dark blue) or when only an optimal subset of top-n neurons is driven (light blue). For anatomy, the optimal set is the set of all neurons so the two bars are the same. 

c.) Measured functional connectivity kernels are used to simulate activity. Correlations of functionally-derived activity are compared to those of spontaneous activity. Agreement to spontaneous activity is shown for two variations of functionally-derived activity: when all neurons are driven (dark blue) or when only an optimal subset is driven, in this case the top 20 (light blue).
anatomy. Unlike for functional connectivity, for anatomy-derived activity we did not find any top-n subset that significantly improved performance. The best performing set of top-n neurons was the set of all neurons (Fig. 5b, “top-n”). Thus, in all cases, functionally derived activity outperformed anatomy-derived activity, further indicating that measurements of functional connectivity are more effective than anatomy at predicting properties of real neural activity.

Discussion

A key finding from this work is that the functional connectivity of C. elegans differs from predictions based on anatomy and gene expression. Why such a discrepancy? One answer is that anatomy fails to account for wireless connections. For example, we show specific functional connections in which RID signals extrasynaptically via peptides and this type of connection is not visible in anatomy. This explanation is consistent with recent gene expression profiling results showing that the molecular machinery for neuropeptide signaling is ubiquitous in the C. elegans nervous system. Here, we present evidence that wireless signaling is present in the functional connectome and provides a meaningful contribution to neural dynamics.

What are the implications of wireless signaling for interpreting connectivity in other organisms? In mammals, hypothalamus and midbrain exhibit peptidergic signaling and even mouse cortex widely expresses molecular machinery for neuropeptide signaling. Diffusion would appear to place strict limits on the speed, strength, and spatial extent of extrasynaptic signaling in the brain. Therefore, the small size of the worm’s head (50x50x150 microns) may make it particularly amenable to extrasynaptic signaling. However, in larger vertebrate brains, signaling molecules may circumvent limits of diffusion by traveling through the vasculature, as is the case for hormones in the neuroendocrine system. More research is needed to determine the role of wireless signaling in generating neural dynamics in vertebrates. If studies show that wireless signaling is important, functional measurements will be needed to complement anatomy for accurately predicting neural dynamics.

Other factors likely also contribute to the discrepancies between the anatomical and functional descriptions of this network. Researchers have suggested that anatomy may represent a superset of possible connections in the brain, but that only a subset of connections are active while the rest are dormant, waiting to be activated by either neuromodulators or plasticity (e.g., as in the Drosophila head direction circuit). An important future study on functional connectivity will be to map these latent circuits and determine when they turn on or off.

To map the functional connectivity presented here, we measured activity in response to the stimulation of 133 identified neurons, corresponding to 71% of all 188 neurons in the head. We found 1,285 functional connections, with a false discovery rate of q < 0.05. This result likely underestimates the true number of effective connections for several reasons. Our analysis omits some neurons visible in our recordings because they are either too dim or too difficult to unambiguously identify, even with color information provided by NeuroPAL fluorophores. For example, the neuron AVB is consistently very dim, and identifying neurons in the ventral ganglion is challenging. Other neurons such as RID are bright enough to be stimulated, but not bright enough to always track and observe calcium responses, and so their functional connections are undercounted in the maps of Fig. 2. We also do not expect to detect neural responses that lack calcium activity in the nucleus, such as compartmentalized calcium dynamics. More generally, the lack of an observed response does not conclusively indicate that no functional connection exists. As discussed in, because we are limited by the efficiency of stimulation and the sensitivity of the GCaMP6s reporter, our results may not capture weak or variable effective connections. Future work with voltage indicators may provide better sensitivity.

Conversely, we are unlikely to overestimate the prevalence of functional connections. We exclude apparent responses that would have likely arisen without stimulation by comparing response amplitudes to recordings in which no stimulation was delivered. We also control the false discovery rate in the face of multiple hypothesis testing. Therefore, the functional connectivity map presented here represents a lower bound on the complete set of functional connections in the worm.

The measured functional connectivity map reports effective connections, not direct connections. Effective connections are the most relevant and useful for answering the circuit-level questions that motivate our work. These connections describe how a stimulus or perturbation to a neuron in one part of the network drives activity in another. Effective connections are a natural framework for determining how signals propagate through the brain or how neural computations are performed. By contrast, the properties of a direct connection, treated in isolation from the rest of the network, can be misleading when considering network function. For example, a direct connection between two neurons may be slow or weak, but may overlook a fast and strong effective connection via other paths through the network.

Direct connections, by contrast, are more naturally suited for probing questions of anatomy, gene expression, and development. In this work, we compared different network descriptions at the level of effective connections. For example, we used connectome-constrained simulations to derive effective connections from anatomy. A limitation of this approach, and of interpreting anatomy generally, is that it relies on assumptions about the timescales, nonlinearities, and other properties of signal propagation. An alternative approach would be to infer properties of direct connections from the measured effective connections (e.g., by), but solving this inverse problem is non-trivial and may require a higher signal-to-noise ratio than our.
current measurements. Future efforts to infer functional properties of direct connections are needed to explore, for example, how gene expression relates to synaptic strength.

We investigated functional connections on timescales of less than 30 s (set by our inter-stimulus interval). However, the brain surely has longer response timescales. Functional connections are likely history-dependent and undergo modulation over longer timescales based on the animal’s internal state, or by learning. Neuromodulators or internal states are thought to functionally reconfigure neural circuits and alter information processing. Future measurements of functional connectivity across longer timescales will be needed to reveal how functional connectivity changes upon learning or in different internal, sensory, or neuromodulatory states.

The neural dynamics we observe are slow but importantly they are no slower than typical calcium responses observed in *C. elegans* to natural sensory stimuli, such as odor delivery. Several factors likely contribute to the slow speed of the activity responses. Graded potentials in *C. elegans* are slower than typical vertebrate action potentials, even when measured via electrophysiology. GCaMP6s also has a slow rise time, and we expect to see further slower dynamics because we measure calcium from the nucleus and because the GUR-3/PRDX-2 system also appears to have a slow rise time compared with traditional opsins. Even though *C. elegans* neural dynamics may be slow, they still can propagate signals very quickly through the nervous system. We identify fast signal transmission, even in slow dynamics, by fitting kernels to relate the dynamics of upstream and downstream neurons.

Slow dynamics do pose a technical challenge in one respect: As the timescale of signal transmission decreases, our signal-to-noise ratio, combined with the slow neural dynamics, makes us less confident in our ability to discern small differences in fast timescales. This limitation prevents us from confidently solving the inverse problem of inferring properties of direct connections from measured effective connections.

In this work, we measured functional connectivity by stimulating one neuron at a time, with an approximate delta function, usually at a constant amplitude. To better probe nonlinearities in the network, future measurements that explore a larger stimulation space are needed, including variable-amplitude stimulations and simultaneous stimulation of multiple neurons.

The functional connectivity measured here is a powerful tool for more accurately simulating neural activity. Unlike simulations derived from anatomy, simulations based on functional connectivity rely entirely on measured properties of neural connections and do not require assumptions about timescales or synaptic weights.

**Methods**

**Worm Maintenance**

*C. elegans* were maintained and handled in the dark. Strains generated in this study (Supplementary Table S1) are being deposited in the Caenorhabditis Genetics Center, University of Minnesota, for public distribution.

**Transgenics**

To measure functional connectivity, we generated the transgenic strain AML462. This strain expresses the calcium indicator GCaMP6s in the nucleus of each neuron, a purple light-sensitive optogenetic protein system (i.e., GUR-3 and PRDX-2) in each neuron, and multiple fluorophores of various colors from the NeuroPAL system also in the nucleus of neurons. We also used a QF-GR drug-inducible gene expression strategy to turn on gene expression of optogenetic actuators only later in development. To create this strain, we first generated an intermediate strain, AML456, by injecting a plasmid mix into *C. elegans* using electroporation. This also resulted in the knock-in of the *pAS3-5xQUAS::l pes-10P::AI::gur-3G::unc-54 + 75 ng/µl pAS3-5xQUAS::l pes-10P::AI::prdx-2G::unc-54 + 35 ng/µl pAS3-5xQUAS::l pes-10P::AI::QF+GR::unc-54 + 100 ng/µl unc-122::GFP) into CZ20310 worms followed by UV integration and 6 outcrosses.

The intermediate strain, AML456, was then crossed into the pan-neuronal GCaMP6s calcium imaging strain, with NeuroPAL, AML320.

An *unc-31* mutant background with defects in the dense-core vesicle release pathway was used to diminish wireless signaling. We created an *unc-31* knockout version of our functional connectivity strain by performing CRISPR/Cas9-mediated genome editing on AML462 using a single-strand oligodeoxynucleotide (ssODN)-based homology-dependent repair strategy. This approach resulted in strain AML508 (unc-31 [wtf502] IV; otIs669 [NeuroPAL] V 14x; wtfls145 [pBX + rab-3::his-24::GCaMP6s::unc-54]; wtfls348 [75 ng/µl pAS3-5xQUAS::l pes-10P::AI::gur-3G::unc-54 + 75 ng/µl pAS3-5xQUAS::l pes-10P::AI::prdx-2G::unc-54 + 35 ng/µl pAS3-5xQUAS::l pes-10P::AI::QF+GR::unc-54 + 100 ng/µl unc-122::GFP]) into CZ20310 worms followed by UV integration and 6 outcrosses.

**CRISPR/Cas-9 editing was carried out as follows.** Protospacer adjacent motif (PAM) sites were selected in the first intron (gagcuucgcaauguugaccgagcccagcgg) and the last intron (augucaauugguccguggcgg) of the *unc-31* gene (ZK897.1a.1) to delete 12,476 out of 13,169 bp (including the 5' and 3' untranslated regions [UTRs]) and 18 out of 20 exons from the genomic locus, while adding 6 bp (GGTACC) for the Kpn-I restriction site (Supplementary Figure S17). Alt-R S.p. Cas9 Nuclease V3, Alt-R-single guide RNA (sgRNA), and Alt-R homology-directed repair (HDR)-ODN were used (IDT, USA). We introduced the Kpn-I restriction site (gaccccgccgagccagatatgaaacataagtacccttggttgttgtgtGTTACCCca cggaccaatgtcataatatatgccgagaaatttatatatggtcag) into our repair oligo to screen and confirm the deletion
Worms were individually mounted on 10% agarose pads prepared with M9 buffer and immobilized using 2 µL of 1M KCL, 0.375 µL of 200 mM HEPES (pH 7.4). sgRNAs for unc-31 [1 µL each for both sites], and 0.75 µL for dpy-10 from a stock of 100 µM, ssODNs [1 µL for unc-31 and 0.5 µL for dpy-10 from a stock of 25 µM], and nuclease-free water to a final volume of 10 µL in a PCR tube, kept on ice. The injection mix was then incubated at 37 °C for 15 min before it was injected into the germline of AML462 worms. Progenies from plates showing roller or dumpy phenotypes in the F1 generation post-injection were individually propagated and PCR to Kpn-I digestion screened to confirm deletion. Single-worm PCR was carried out using GXL-PRIME STAR taq-Polymerase (Takara Bio, USA) and the Kpn-1-HF restriction enzyme (NEB, USA). Worms without a roller or dumpy phenotype and homozygous for deletion were confirmed by Sanger sequencing fragment analysis.

**Dexamethasone treatment**

To increase expression of optogenetic proteins while avoiding toxicity during the animals’ early life development, a drug-inducible gene expression strategy was used. Dexamethasone (dex; cataloge Number: D1756, Sigma Life Science) activates QF-GR to temporally control the expression of downstream targets, in this case the optogenetic proteins in the functional connectivity imaging strains AML462 and AML508. Dex-NGM plates were prepared by adding 200 µM of dex in DMSO just before pouring the plate. For dex treatment, L2/L3 worms were transferred to overnight-seeded dex-NGM plates and further grown until worms were ready for imaging.

**Preparation of worms for imaging**

Worms were individually mounted on 10% agarose pads prepared with M9 buffer and immobilized using 2 µL of 100 nm polystyrene beads solution and 2 µL of levamisole (500 µM stock). This concentration of levamisole, after dilution in the polystyrene bead solution and the agarose pad water, largely immobilized the worm while still allowing the worm to slightly move, especially before placing the coverslip. Pharyngeal pumping was observed during imaging.

**Multi-channel imaging and neural identification**

Volumetric, multi-channel imaging was performed to capture images of the following fluorophores in the Neuropal transgene: mtagBFP2, CyOFP1.5, tagRFP-T, and mNeptune2.5. Light downstream of the same spinning disk unit used for calcium imaging traveled on an alternative light path through channel-specific filters mounted on a mechanical filter wheel, while mechanical shutters alternated illumination with the respective lasers, similar to in. Channels were as follows: mtagBFP2 was imaged using a 405 nm laser and a Semrock FF01-440/40 emission filter; CyOFP1.5 was imaged using a 505 nm laser and a Semrock 609/54 emission filter; tagRFP-T was imaged using a 561 nm laser and a Semrock 732/68 nm emission filter; and mNeptune2.5 was imaged using a 561 nm laser and a Semrock 732/68 nm emission filter.

After the functional connectivity recording was complete, a human manually assigned neuron identities by comparing each neuron’s color, position, and size to a known atlas. Some neurons are particularly hard to identify in NeuroPAL and are therefore absent or less frequently identified in our recordings. Some neurons have dim tagRFP-T expression, which makes it difficult for the neuron segmentation algorithm to find them and, therefore, to extract their calcium activity. These neurons include, for example, AVB, ADF, and RID. RID’s distinctive position and its expression of CyOFP allowed us nevertheless to manually target it optogenetically. Neurons in the ventral ganglion are hard to identify because they appear as very crowded when viewed in the most common orientation that worms assume when mounted on a microscope slide. Neurons in the ventral ganglion are therefore sometimes difficult to distinguish one from another, especially for dimmer neurons such as the SIA, SIB, and RMF neurons. In our strain, the neurons AWCon and AWCoiff were difficult to tell apart based on color information.

**Volumetric image acquisition**

Neural activity was recorded at whole-brain scale and cellular resolution via continuous acquisition of volumetric images in red and green channels with a spinning disk confocal unit and via LabView software, similarly to, with a few upgrades. The imaging focal plane was scanned through the brain of the worm remotely using an electrically tunable lens (Optotune EL-16-40-TC) instead of moving the objective. The use of remote focusing allowed us to decouple the z-position of the imaging focal plane and that of the optogenetics 2-photon spot (described below).

Images were acquired by an sCMOS camera, and each acquired image frame was associated to the focal length of the tunable lens (z-position in the sample) at which it was acquired. To ensure the correct association between frames and z-position, we recorded the analog signal describing the focal length of the tunable lens at time points synchronous with a trigger pulse output by the camera. By counting the camera triggers from the start of the recording, the z-positions could be associated to the correct frame, bypassing unknown operating-system-mediated latencies between the binary image stream from the camera and acquisition of analog signals.
Additionally, real-time “pseudo”-segmentation of the neurons (described below) required the ability to separate frames into corresponding volumetric images in real-time. Because the z-position was acquired at low sample rate, splitting of volumes based on finite differences between successive z-positions could lead to errors in assignment at the edge of the z-scan. An analog OP-AMP-based differentiator was used to independently detect the direction of the z-scan in hardware.

Calcium imaging
Calcium imaging was performed in single-photon regime with a 505 nm excitation laser via spinning disk confocal microscopy, at 2 vol/s. For functional connectivity experiments, an intensity of 1.4 mW/mm² at the sample plane was used to image GCaMP6s, well below the threshold needed to excite the GUR-3/PRDX-2 optogenetic system. We note that at this wavelength and intensity animals exhibited very little spontaneous calcium activity.

For certain analyses (Fig. 5), recordings with ample spontaneous activity were desired. In those cases, we increased the 505 nm intensity seven-fold to approximately 10 mW/mm² and recorded from AML320 strains that lacked exogenous GUR-3/PRDX-2 to avoid potential widespread neural activation. Under these imaging conditions, we observed population-wide slow stereotyped spontaneous oscillatory calcium dynamics, as previously reported.

Extraction of calcium activity from the images
The extraction of calcium activity from the raw images was performed using Python libraries implementing optimized versions of the algorithm described in, available at https://www.github.com/leiferlab/pumpprobe, https://www.github.com/leiferlab/wormdatamodel, https://www.github.com/leiferlab/wormneuronsegmentation-c and https://www.github.com/leiferlab/wormbrain.

The positions of neurons in each acquired volume was determined by computer vision software implemented in C++. This software was greatly optimized to identify neurons in real-time in order to also enable closed-loop targeting and stimulus delivery (as described in the section Stimulus delivery and pulsed laser). Two design choices made this algorithm dramatically faster than previous approaches. First, a local maxima search was used instead of a slower watershed-type segmentation. The nuclei of C. elegans neurons are approximately spheres and so they can be identified and separated by a simple local maxima search. Second, we factorized the 3D local maxima search into multiple 2D local maxima searches. In fact, any local maximum in a 3D image is also a local maximum in the 2D image in which it is located. Local maxima were therefore first found in each 2D image separately, and then candidate local maxima were discarded or retained by comparing them to their immediate surroundings in the other planes. This makes the algorithm less computationally intensive and fast enough to be used also in real time. We refer to this type of algorithm as “pseudo”-segmentation because it finds the center of neurons without fully describing the extent and boundaries of each neuron.

After neural locations were found in each of the volumetric images, a nonrigid pointset registration algorithm was used to track their locations across time. Even worms that are mechanically immobilized still move slightly and contract their pharynx, thereby deforming their brain and requiring the tracking of neurons. We implemented in C++ a fast and optimized version of the Dirichelet-Student-t Mixture Model (DSMM).

Calcium pre-processing
The GCaMP6s intensity extracted from the images undergoes the following pre-processing steps. (1) Missing values are interpolated based on neighboring time points. Missing values can occur when a neuron cannot be identified in a given volumetric image. (2) Photobleaching is removed by fitting a double exponential to the baseline signal. (3) Outliers more than 5 standard deviations away from the average are removed from each trace. (4) Traces are smoothed via a causal polynomial filtering with a window size of 6.5 s and polynomial order of 1 [Savitzky–Golay filters with windows completely “in the past”, e.g., obtained with scipy.signal.savgol_coeffs(window_length=13, polyorder=1, pos=12)]. This type of filter with the chosen parameters is able to remove noise without smearing the traces in time. Note that when fits are performed (e.g., to calculate kernels), they are always performed on the original, non-smoothed traces. (5) Where ∆F/F₀ of responses is used, F₀ is defined as the value of F in a short interval before the stimulation time.

Stimulus delivery and pulsed laser
For two-photon optogenetic targeting, we used an optical parametric amplifier (OPA, Light Conversion ORPHEUS) pumped by a femtosecond amplified laser (Light Conversion PHAROS). The output of the OPA was tuned to a wavelength of 850 nm, at a 500 kHz repetition rate. We used temporal focusing to spatially restrict the size of the 2-photon excitation spot along the microscope axis. A motorized iris was used to set its lateral size. For temporal focusing, the first-order diffraction from a reflective grating, oriented orthogonally to the microscope axis, was collected (as in) and traveled through the motorized iris, placed on a plane conjugate to the grating. To arbitrarily position the 2-photon excitation spot in the sample volume, the beam then traveled through an electrically tunable lens (Optotune EL-16-40-TC, on a plane conjugate to the objective), to set its
position along the microscope axis, and finally was reflected by two galvo-mirrors to set its lateral position. The pulsed beam was then combined with the imaging light path by a dichroic mirror immediately before entering the back of the objective.

The majority of the stimuli were delivered automatically by computer control. Real-time computer vision software found the position of the neurons for each volumetric image acquired, using the tagRFP-T channel. To find neural positions, we used the same “pseudo”-segmentation algorithm described above. The algorithm found neurons in each 2D frame in ~500 µs as the frames arrived from the camera. In this way locations for all neurons in a volume were found within a few milliseconds of acquiring the last frame of that volume.

Every 30 s, a random neuron was selected among the neurons found in the current volumetric image. After galvo-mirrors and tunable lens set the position of the 2-photon spot on that neuron, a 500 ms (300 ms for the unc-31 mutant strain) train of light pulses was used to optogenetically stimulate that neuron. The output of the laser was controlled via the external interface to its built-in pulse picker, and the power of the laser at the sample was 1.2 mW at 500 kHz. Neuron identities were assigned to stimulated neurons after the completion of experiments using NeuroPAL. To probe the AFD-AIY neural connection, a small set of stimuli used variable pulse durations from 100 ms to 500 ms in steps of 50 ms selected randomly to vary the amount of optogenetic activation of AFD.

In some cases, neurons of interest were too dim to be detected by the real-time software. For those neurons of interest, additional recordings were performed in which a human manually selected the neuron to be stimulated based on its color, size, and position. This was the case for certain stimulations of neurons RID and AFD.

Characterization of the 2-photon excitation spot size
The lateral (xy) size of the 2-photon excitation spot was measured with a fluorescent microscope slide, while the axial (z) size was measured using 0.2 nm fluorescent beads (Suncoast Yellow, Bangs Laboratories), by scanning the z-position of the optogenetic spot while maintaining the imaging focal plane fixed (Fig. S1a).

We further tested our targeted stimulation in two ways: selective photobleaching and neuronal activation. First, we targeted individual neurons at various depth in the worm’s brain, and we illuminated them with the pulsed laser to induce selective photobleaching of tagRFP-T. Supplementary Fig. S2 shows how our 2-photon excitation spot selectively targets individual neurons, because it photobleaches tagRFP-T only in the neuron that we decide to target, and not in nearby neurons. To faithfully characterize the spot size, we set the laser power such that the 2-photon interaction probability profile of the excitation spot would not saturate the 2-photon absorption probability of tagRFP-T. Second, we showed that our excitation spot is restricted along the z-axis by targeting a neuron and observing its calcium activity. When the excitation was directed at the neuron but shifted by 4 µm along z, the neuron showed no activation. In contrast, the neuron showed activation when the spot was correctly positioned on the neuron (Supplementary Fig. S3).

Inclusion criteria
Stimulation events were included for further analysis if they evoked a detectable calcium response in the stimulated neuron. A classifier determined whether the response was detected by inspecting whether the amplitude of the ΔF/F₀ transient and its second derivative exceeded hand-tuned thresholds. Stimulation events that did not meet this threshold were excluded. RID responses shown in Fig. 4 and Supplementary Fig. S16 are an exception to this policy. RID is visible based on its CyOFP expression, but its tagRFP-T expression is too dim to consistently extract calcium signals. Therefore in Fig. 4 and Supplementary Fig. S16 (but not Fig. 2) responses to RID stimulation were included even in cases where it was not possible to extract a calcium-activity trace in RID.

Neuron traces were excluded from analysis if a human was unable to assign an identity or if 30% or more of the imaging time points were absent due to imaging artifacts or tracking errors.

Kernels were computed only for stimulation-response events for which the automatic classifier detected responses in both the stimulated and downstream neurons. If the downstream neuron did not show a response, we considered the downstream response to be below the noise level and the kernel to be zero.

Statistical analysis
To assess the relative significance of a functional connection between a target and putative responding neuron, we calculated the probability of observing the measured calcium response given no neural stimulation. We used a Kolmogorov–Smirnov test to compare the distributions of ΔF/F₀ and its temporal second derivative from all observations of that neuron pair to the empirical null distributions from control recordings lacking stimulation. p-values were calculated separately for ΔF/F₀ and its temporal second derivative, and then combined using Fischer’s method to report a single fused p-value for each neuron pair. Finally, to account for the large number of hypotheses tested, a false discovery rate was estimated. From the list of p-values, each neuron was assigned a q-value via the Storey–Tibshirani method. q-values are interpreted as follows: when considering an ensemble of putative functional connections of q-values all less than or equal to qₓ, approximately qₓ of those connections would have appeared in a recording that lacked any stimulation.
Measuring path length through the synaptic network

To find the minimum path length between neurons in the anatomical network topology, we proceeded iteratively. We started from the original binary connectome and computed the map of strictly 2-hop connections by looking for pairs of neurons that are not connected in the starting connectome (the actual anatomical connectome at the first step) but that are connected through a single intermediate neuron. To generate the strictly 3-hop connectome, we repeated this procedure using the binary connectome including direct and 2-hop connections, as the starting connectome. This process continued iteratively to generate the strictly n-hop connectome.

In the anatomical connectome (the starting connectome for the first step in the procedure above), a neuron was considered to be directly anatomically connected if the connectomes of any of the four L4 or adult individuals in and contained at least one synaptic contact between them. Note that this is a permissive description of anatomical connections as it considers even neurons with only a single synaptic contact in only one individual to be connected.

Fitting kernels

Kernels $k_{ij}(t)$ were defined as the functions to be convolved with the activity $\Delta F_i$ of the stimulated neuron to obtain the activity $\Delta F_i(t) = (k_{ij} * \Delta F_j)(t)$. To fit kernels, each kernel $k(t)$ was parametrized as a sum of convolutions of decaying exponentials

$$k(t) = \sum_m c_m \left( \theta(t)e^{-\gamma_m t} \right) * \left( \theta(t)e^{-\gamma_m t} \right) * \ldots,$$

where the indices $i, j$ are omitted for clarity and $\theta$ is the Heaviside function. This parametrization is exact for linear systems, and works as a description of causal signal transmission also in nonlinear systems. Note that increasing the number of terms in the successive convolutions does not lead to overfitting, as would occur by increasing the degree of a polynomial. Overfitting could occur by increasing the number of terms in the sum, which in our fitting is constrained to be a maximum of 2. The presence of two terms in the sum allows the kernels to represent signal transmission with saturation (with $c_0$ and $c_1$ of opposite signs) and assume a fractional-derivative-like shape.

The convolutions are performed symbolically. The construction of kernels as in Eq. (1) starts from a symbolically stored, normalized decaying exponential kernel with a factor $A_i \gamma_i \theta(t)e^{-\gamma_i t}$. Convolutions with normalized exponentials $\gamma_i \theta(t)e^{-\gamma_i t}$ are performed sequentially and symbolically, taking advantage of the fact that successive convolutions of exponentials always produce a sum of functions in the form $\kappa(t)e^{-\gamma t}$. Once rules are found to convolve an additional exponential with a function in that form, any number of successive convolution can be performed. These rules are as follows:

1. If the initial term is a simple exponential with a given factor (not necessarily just the normalization $\gamma$) $c_i \theta(t)e^{-\gamma_i t}$ and $\gamma_i \neq \gamma_n$, then the convolution is

$$c_i \theta(t)e^{-\gamma_i t} * \gamma_n \theta(t)e^{-\gamma_n t} = c_i \mu \theta(t)e^{-\gamma_n t} + c_v \theta(t)e^{-\gamma_v t},$$

with $c_i = \frac{c_i \gamma_n}{\gamma_n - \gamma}$, $c_v = -\frac{c_i \gamma_n}{\gamma_n - \gamma}$, and $\gamma_i = \gamma_n, \gamma_v = \gamma_v$.

2. If the initial term is a simple exponential and $\gamma = \gamma_n$, then

$$c_i \theta(t)e^{-\gamma_i t} + \gamma_n \theta(t)e^{-\gamma_n t} = c_i \mu \theta(t)e^{-\gamma_n t},$$

with $c_i = c_i \gamma$ and $\gamma_i = \gamma_n$.

3. If the initial term is a $c_i \theta(t)t^n e^{-\gamma_i t}$ term and $\gamma = \gamma_n$, then

$$c_i \theta(t)t^n e^{-\gamma_i t} * \gamma_n \theta(t)e^{-\gamma_n t} = c_i \mu \theta(t)t^{n+1} e^{-\gamma_n t},$$

with $c_i = \frac{c_i \gamma_n}{n+1}$ and $\gamma_i = \gamma_n$.

4. If the initial term is a $c_i \theta(t)t^n e^{-\gamma_i t}$ term and $\gamma \neq \gamma_n$, then

$$c_i \theta(t)t^n e^{-\gamma_i t} * \gamma_n \theta(t)e^{-\gamma_n t} = c_i \mu \theta(t)t^n e^{-\gamma_n t} + c_v \left( \theta(t)t^{n-1} e^{-\gamma_i t} * \theta(t)e^{-\gamma_n t} \right),$$

where $c_i = \frac{c_i \gamma_n}{\gamma - \gamma_n}, \gamma_i = \gamma_n$, and $c_v = -n\frac{c_i \gamma_n}{\gamma - \gamma_n}$.

Additional terms in the sum in Eq. (1) can be introduced by keeping track of the index $m$ of the summation for every term and selectively convolving new exponentials only with the corresponding terms.
Kernel-based simulations of activity

Using the kernels fitted from our functional data, we can simulate neural activity without making any further assumptions about the dynamical equations of the network of neurons. To compute the response of a neuron $i$ to the stimulation of a neuron $j$, we simply convolve the kernel $k_{i,j}(t)$ with the activity $\Delta F_j(t)$ induced by the stimulation in neuron $j$. The activity of the stimulated neuron can be either the experimentally observed activity or an arbitrarily shaped activity introduced for the purposes of simulation.

To compute kernel-derived neural activity correlations (Fig. 5), we completed the following steps. (1) We computed the responses of all the neurons $i$ to the stimulation of a neuron $j$ chosen to drive activity in the network. To compute the responses, for each pair $i, j$, we used the kernel $\langle k_{i,j}(t) \rangle_{\text{trials}}$ averaged over multiple trials. For kernel-based analysis, pairs with connections of $q > 0.05$ were considered not connected. We set the activity $\Delta F_j(t)$ in the driving neuron to mimic an empirically observed representative activity transient. (2) We computed the correlation coefficient of the resulting activities. (3) We repeated steps 1 and 2 for a set of driving neurons (all or top $n$ neurons, as in Fig. 5). (4) For each pair $k, l$, we took the average of the correlations obtained by driving the set of neurons $j$ in step 3.

For comparisons with predictions derived from anatomy, bilateral pairs of neurons were merged (e.g., AVAL and AVAR were merged into a single class AVA).

Anatomy-derived simulations of activity

Anatomy-derived simulations were performed as described in\textsuperscript{72}. Briefly this simulation approach uses differential equations to model signal transmission via electrical and chemical synapses and includes a nonlinear equation for synaptic activation variables. We injected current \textit{in silico} into individual neurons and simulated the responses of all the other neurons. Anatomy-derived effective weights (Fig. 3) of the connection from neuron $j$ to neuron $i$ were computed as the peak of the response of neuron $i$ to the stimulation of $j$. Anatomy-based predictions of spontaneous correlations were calculated analogously to kernel-based predictions.

Synapse polarities were assigned based on a gene expression analysis of ligand-gated ionotropic synaptic connections that considered glutamate, acetylcholine, and GABA neurotransmitter and receptor expression\textsuperscript{68} that were taken from CeNGeN\textsuperscript{61} and other sources. Specifically, we used a published dataset \url{https://doi.org/10.1371/journal.pcbi.1007974.s003} and aggregated polarities across all members of a cellular subtype (e.g., polarities from source AVAL and AVAR were combined). In cases of ambiguous polarities, connections were assumed to be excitatory.

Characterizing stereotypy of functional connections

To characterize the stereotypy of a neuron pair’s functional connection, its kernels were inspected. A kernel was calculated for each stimulus-response event in which both the upstream and downstream neuron exhibited activity that exceeded a threshold. At least two stimulus-response events that exceeded this threshold were required in order to calculate their stereotypy. The general strategy for calculating stereotypy was to convolve different kernels with the same stimulus inputs and compare the resulting outputs. Similarity of two outputs is reported as a Pearson’s correlation coefficient. Kernels corresponding to different stimulus-response events of the same pair of neurons were compared with one another round-robin style, one round-robin each for a given input stimuli. For inputs we chose the set of all stimuli delivered to the upstream neuron. The neuron-pairs stereotypy is reported as the average Pearson’s correlation coefficient across all round-robin kernel pairings and across all stimuli.

Classifying connections as fast

Connections between neurons were classified as fast based on the rise time of their kernels. The rise time was defined as the interval between the earliest time at which the value of the kernel was $1/e$ its peak value and the time of its peak (whether positive or negative). The rise time was zero if the peak of the kernel was at time $t=0$. However, saturation of the signal transmission can make kernels appear slower than the connection actually is. For example, the simplest instantaneous connection would be represented by a single decaying exponential in Eq. 1, which would have its peak at time $t = 0$. However, if that connection is saturating, a second, opposite-sign term in the sum is needed to fit the kernel. This second term would make the kernel have a later peak, thereby masking the instantaneous nature of the connection. To account for this effect of saturation, in classifying connections as fast or slow, we removed terms representing saturation from the kernels and found the rise time of these “non-saturating” kernels. In Fig. 3 and Supplementary Fig. S14, connections are classified as fast if their rise time is smaller than 50 ms.

References

1. Mesulam, M. Imaging connectivity in the human cerebral cortex: The next frontier? Annals Neurol. 57, 5–7, DOI: 10.1002/ana.20368 (2005). eprint: https://onlinelibrary.wiley.com/doi/pdf/10.1002/ana.20368.
2. Seung, H. S. Towards functional connectomics. *Nature* **471**, 171–172, DOI: 10.1038/471170a (2011). Number: 7337 Publisher: Nature Publishing Group.

3. White, J. G., Southgate, E., Thomson, J. N. & Brenner, S. The Structure of the Ventral Nerve Cord of Caenorhabditis elegans. *Philos. Transactions Royal Soc. London. B, Biol. Sci.* **275**, 327–348, DOI: 10.1098/rstb.1976.0086 (1976).

4. Cook, S. J. *et al.* Whole-animal connectomes of both Caenorhabditis elegans sexes. *Nature* **571**, 63–71, DOI: 10.1038/s41586-019-1352-7 (2019).

5. Witvliet, D. *et al.* Connectomes across development reveal principles of brain maturation. *Nature* **596**, 257–261, DOI: 10.1038/s41586-021-03778-8 (2021). Bandiera_abtest: a Cg_type: Nature Research Journals Number: 7871 Primary_atype: Research Publisher: Nature Publishing Group Subject_term: Development of the nervous system;Developmental biology;Neural circuits;Neuroscience Subject_term_id: development-of-the-nervous-system;developmental-biology;neural-circuit;neuroscience.

6. Chalfie, M. *et al.* The neural circuit for touch sensitivity in Caenorhabditis elegans. *The J. Neurosci. The Off. J. Soc. for Neurosci.* **5**, 956–64, DOI: 3981252 (1985).

7. Gray, J. M., Hill, J. J. & Bargmann, C. I. A circuit for navigation in Caenorhabditis elegans. *Proc. Natl. Acad. Sci. United States Am.* **102**, 3184–3191, DOI: 10.1073/pnas.0409009101 (2005). PMC546636 PMID: 15689400.

8. Perkins, L. A., Hedgecock, E. M., Thomson, J. N. & Culotti, J. G. Mutant sensory cilia in the nematode Caenorhabditis elegans. *Dev. Biol.* **117**, 456–487, DOI: 10.1016/0012-1606(86)90314-3 (1986).

9. Kunert-Graf, J. M., Shlizerman, E., Walker, A. & Kutz, J. N. Multistability and Long-Timescale Transients Encoded by Network Structure in a Model of C. elegans Connectome Dynamics. *Front. Comput. Neurosci.* **11** (2017).

10. Mi, L. *et al.* Connectome-constrained latent variable models of whole-brain neural activity (2022).

11. Ryan, K., Lu, Z. & Meinertzhagen, I. A. The CNS connectome of a tadpole larva of Ciona intestinalis (L.) highlights sidedness in the brain of a chordate sibling. *eLife* **5**, e16962, DOI: 10.7554/eLife.16962 (2016). Publisher: eLife Sciences Publications, Ltd.

12. Schneider-Mizell, C. M. *et al.* Quantitative neuroanatomy for connectomics in Drosophila. *eLife* **5**, e12059, DOI: 10.7554/eLife.12059 (2016). Publisher: eLife Sciences Publications, Ltd.

13. Hildebrand, D. G. C. *et al.* Whole-brain serial-section electron microscopy in larval zebrafish. *Nature* **545**, 345–349, DOI: 10.1038/nature22356 (2017). Number: 7654 Publisher: Nature Publishing Group.

14. Scheffer, L. K. *et al.* A connectome and analysis of the adult Drosophila central brain. *eLife* **9**, e57443, DOI: 10.7554/eLife.57443 (2020). Publisher: eLife Sciences Publications, Ltd.

15. Dorkenwald, S. *et al.* FlyWire: online community for whole-brain connectomics. *Nat. Methods* **19**, 119–128, DOI: 10.1038/s41592-021-01330-0 (2022). Number: 1 Publisher: Nature Publishing Group.

16. Hulse, B. K. *et al.* A connectome of the Drosophila central complex reveals network motifs suitable for flexible navigation and context-dependent action selection. *eLife* **10**, e66039, DOI: 10.7554/eLife.66039 (2021). Publisher: eLife Sciences Publications, Ltd.

17. Kim, S. S., Rouault, H., Druckmann, S. & Jayaraman, V. Ring attractor dynamics in the Drosophila central brain. *Science* eaal4835, DOI: 10.1126/science.aal4835 (2017).

18. Abbott, L. F. *et al.* The Mind of a Mouse. *Cell* **182**, 1372–1376, DOI: 10.1016/j.cell.2020.08.010 (2020).

19. Kuramochi, M. & Doi, M. An Excitatory/Inhibitory Switch From Asymmetric Sensory Neurons Defines Postsynaptic Tuning for a Rapid Response to NaCl in Caenorhabditis elegans. *Front. Mol. Neurosci.* **11** (2019).

20. Fisher, Y. E., Lu, J., D’Alessandro, I. & Wilson, R. I. Sensorimotor experience remaps visual input to a heading-direction network. *Nature* **576**, 121–125, DOI: 10.1038/s41586-019-1772-4 (2019). Number: 7785 Publisher: Nature Publishing Group.

21. Franconville, R., Beron, C. & Jayaraman, V. Building a functional connectome of the Drosophila central complex. *eLife* **7**, e37017, DOI: 10.7554/eLife.37017 (2018). Publisher: eLife Sciences Publications, Ltd.

22. Harris-Warrick, R. M. & Marder, E. Modulation of Neural Networks for Behavior. *Annu. Rev. Neurosci.* **14**, 39–57, DOI: 10.1146/annurev.ne.14.030191.000351 (1991). _eprint: https://doi.org/10.1146/annurev.ne.14.030191.000351.

23. Marder, E. Neuromodulation of Neuronal Circuits: Back to the Future. *Neuron* **76**, 1–11, DOI: 10.1016/j.neuron.2012.09.010 (2012).
24. Bargmann, C. I. Beyond the connectome: how neuromodulators shape neural circuits. *BioEssays: News Rev. Mol. Cell. Dev. Biol.* **34**, 458–465, DOI: 10.1002/bies.201100185 (2012).
25. Bentley, B. *et al.* The Multilayer Connectome of Caenorhabditis elegans. *PLOS Comput. Biol.* **12**, e1005283, DOI: 10.1371/journal.pcbi.1005283 (2016). Publisher: Public Library of Science.
26. Panzeri, S., Moroni, M., Safaai, H. & Harvey, C. D. The structures and functions of correlations in neural population codes. *Nat. Rev. Neurosci.* 1–17, DOI: 10.1038/s41583-022-00606-4 (2022). Publisher: Nature Publishing Group.
27. Petreanu, L., Huber, D., Sobczyk, A. & Svoboda, K. Channelrhodopsin-2–assisted circuit mapping of long-range callosal projections. *Nat. Neurosci.* **10**, 663–668, DOI: 10.1038/nn1891 (2007). Number: 5 Publisher: Nature Publishing Group.
28. Huber, D. *et al.* Sparse optical microstimulation in barrel cortex drives learned behaviour in freely moving mice. *Nature* **451**, 61–64, DOI: 10.1038/nature06445 (2008). Number: 7174 Publisher: Nature Publishing Group.
29. Zhang, Y.-P. & Oertner, T. G. Optical induction of synaptic plasticity using a light-sensitive channel. *Nat. Methods* **4**, 139–141, DOI: 10.1038/nmeth988 (2007). Number: 2 Publisher: Nature Publishing Group.
30. Emiliani, V., Cohen, A. E., Deisseroth, K. & Haeusser, M. All-Optical Interrogation of Neural Circuits. *J. Neurosci.* **35**, 13917–13926, DOI: 10.1523/JNEUROSCI.2916-15.2015 (2015). WOS:000366051800013.
31. Guo, Z. V., Hart, A. C. & Ramanathan, S. Optical interrogation of neural circuits in Caenorhabditis elegans. *Nat. methods* **6**, 891–896, DOI: 10.1038/nmeth1397 (2009).
32. Wilson, N. R., Runyan, C. A., Wang, F. L. & Sur, M. Division and subtraction by distinct cortical inhibitory networks in vivo. *Nature* **488**, 343–348, DOI: 10.1038/nature11347 (2012). Number: 7411 Publisher: Nature Publishing Group.
33. Rickgauer, J. P., Deisseroth, K. & Tank, D. W. Simultaneous cellular-resolution optical perturbation and imaging of place cell firing fields. *Nat. Neurosci.* **17**, 1816–1824, DOI: 10.1038/nn.3866 (2014). Tex.pmcid: PMC4459599.
34. Packer, A. M., Russell, L. E., Dalgleish, H. W. P. & Häusser, M. Simultaneous all-optical manipulation and recording of neural circuit activity with cellular resolution in vivo. *Nat. Methods* **12**, 140–146, DOI: 10.1038/nmeth.3217 (2015). Bandiera_abtest: a Cg_type: Nature Research Journals Number: 2 Primary_atype: Research Publisher: Nature Publishing Group Subject_term: Fluorescence imaging;Neuroscience;Optogenetics Subject_term_id: fluorescence-imaging;neuroscience;optogenetics.
35. McRaven, C. *et al.* High-throughput cellular-resolution synaptic connectivity mapping in vivo with concurrent two-photon optogenetics and volumetric Ca2+ imaging. *bioRxiv* 2020.02.21.959650, DOI: 10.1101/2020.02.21.959650 (2020). Publisher: Cold Spring Harbor Laboratory Section: New Results.
36. Yemini, E. *et al.* NeuroPAL: A Multicolor Atlas for Whole-Brain Neuronal Identification in C. elegans. *Cell* **184**, 272–288.e11, DOI: 10.1016/j.cell.2020.12.012 (2021).
37. Nguyen, J. P. *et al.* Whole-brain calcium imaging with cellular resolution in freely behaving Caenorhabditis elegans. *Proc. Natl. Acad. Sci.* **113**, E1074–E1081, DOI: 10.1073/pnas.1507110112 (2016).
38. Venkatachalam, V. *et al.* Pan-neuronal imaging in roaming Caenorhabditis elegans. *Proc. Natl. Acad. Sci. United States Am.* **113**, E1082–E1088, DOI: 10.1073/pnas.1507109113 (2016).
39. Denk, W., Strickler, J. H. & Webb, W. W. Two-photon laser scanning fluorescence microscopy. *Sci. (New York, N.Y.)* **248**, 73–76 (1990).
40. Rickgauer, J. P. & Tank, D. W. Two-photon excitation of channelrhodopsin-2 at saturation. *Proc. Natl. Acad. Sci. United States Am.* **106**, 15025–15030, DOI: 10.1073/pnas.0907084106 (2009). Publisher: Proceedings of the National Academy of Sciences.
41. Andrasfalvy, B. K., Zemelman, B. V., Tang, J. & Vaziri, A. Two-photon single-cell optogenetic control of neuronal activity by sculpted light. *Proc. Natl. Acad. Sci.* **107**, 11981–11986, DOI: 10.1073/pnas.1006620107 (2010). Publisher: Proceedings of the National Academy of Sciences.
42. Yang, W., Carrillo-Reid, L., Bando, Y., Peterka, D. S. & Yuste, R. Simultaneous two-photon imaging and two-photon optogenetics of cortical circuits in three dimensions. *eLife* **7**, DOI: 10.7554/eLife.32671 (2018).
43. Zhang, Z., Russell, L. E., Packer, A. M., Gauld, O. M. & Häusser, M. Closed-loop all-optical interrogation of neural circuits in vivo. *Nat. Methods* **15**, 1037, DOI: 10.1038/s41592-018-0183-z (2018).
44. Russell, L. E. *et al.* All-optical interrogation of neural circuits in behaving mice. *Nat. Protoc.* 1–42, DOI: 10.1038/s41596-022-00691-w (2022). Publisher: Nature Publishing Group.
45. Bhatla, N. & Horvitz, H. R. Light and hydrogen peroxide inhibit C. elegans Feeding through gustatory receptor orthologs and pharyngeal neurons. *Neuron* **85**, 804–818, DOI: 10.1016/j.neuron.2014.12.061 (2015).
69. Gordus, A., Pokala, N., Levy, S., Flavell, S. W. & Bargmann, C. I. Feedback from network states generates variability in a probabilistic olfactory circuit. *Cell* **161**, 215–227, DOI: 10.1016/j.cell.2015.02.018 (2015).

70. Stern, S., Kirst, C. & Bargmann, C. I. Neuromodulatory Control of Long-Term Behavioral Patterns and Individuality across Development. *Cell* **171**, 1649–1662.e10, DOI: 10.1016/j.cell.2017.10.041 (2017).

71. Varshney, L. R., Chen, B. L., Paniagua, E., Hall, D. H. & Chklovskii, D. B. Structural Properties of the Caenorhabditis elegans Neuronal Network. *PLoS Comput. Biol* **7**, e1001066, DOI: 10.1371/journal.pcbi.1001066 (2011).

72. Kunert, J., Shlizerman, E. & Kutz, J. N. Low-dimensional functionality of complex network dynamics: Neurosensory integration in the Caenorhabditis connectome. *Phys. Rev. E* **89**, 052805, DOI: 10.1103/PhysRevE.89.052805 (2014).

73. Narayan, A., Laurent, G. & Sternberg, P. W. Transfer characteristics of a thermosensory synapse in Caenorhabditis elegans. *Proc. Natl. Acad. Sci. United States Am.* **108**, 9667–9672, DOI: 10.1073/pnas.1106617108 (2011).

74. Agnati, L. F., Zoli, M., Strömberg, I. & Fuxe, K. Intercellular communication in the brain: wiring versus volume transmission. *Neuroscience* **69**, 711–726, DOI: 10.1016-0306-4522(95)00308-6 (1995).

75. Brickley, S. G. & Mody, I. Extrasynaptic GABAA Receptors: Their Function in the CNS and Implications for Disease. *Neuron* **73**, 23–34, DOI: 10.1016/j.neuron.2012.08.015 (2012).

76. Shen, Y. et al. An extrasynaptic GABAergic signal modulates a pattern of forward movement in Caenorhabditis elegans. *eLife* **5**, e14197, DOI: 10.7554/eLife.14197 (2016). Publisher: eLife Sciences Publications, Ltd.

77. Hardingham, G. E. & Bading, H. Somatic versus extrasynaptic NMDA receptor signalling: implications for neurodegenerative disorders. *Nat. Rev. Neurosci.* **11**, 682–696, DOI: 10.1038/nrn2911 (2010). Number: 10 Publisher: Nature Publishing Group.

78. Taghert, P. H. & Nita-Bach, M. N. Peptide neuromodulation in invertebrate model systems. *Neuron* **76**, 82–97, DOI: 10.1016/j.neuron.2012.08.035 (2012).

79. Speese, S. et al. UNC-31 (CAPS) Is Required for Dense-Core Vesicle But Not Synaptic Vesicle Exocytosis in Caenorhabditis elegans. *The J. Neurosci.* **27**, 6150–6162, DOI: 10.1523/JNEUROSCI.1466-07.2007 (2007).

80. Lim, M. A. et al. Neuroendocrine modulation sustains the C. elegans forward motor state. *eLife* **5**, e19887, DOI: 10.7554/eLife.19887 (2016).

81. Smith, S. J. et al. Single-cell transcriptomic evidence for dense intracortical neuropeptide networks. *eLife* **8**, e47889, DOI: 10.7554/eLife.47889 (2019). Publisher: eLife Sciences Publications, Ltd.

82. Hendricks, M., Ha, H., Maffey, N. & Zhang, Y. Compartmentalized calcium dynamics in a C. elegans interneuron encoding head movement. *Nature* **487**, 99–103, DOI: 10.1038/nature11081 (2012).

83. Randi, F. & Leifer, A. M. Nonequilibrium Green’s Functions for Functional Connectivity in the Brain. *Phys. Rev. Lett.* **126**, 118102, DOI: 10.1103/PhysRevLett.126.118102 (2021).

84. Lin, A. et al. Functional imaging and quantification of multi-neuronal olfactory responses in C. elegans, DOI: 10.1101/2022.05.27.493772 (2022). Pages: 2022.05.27.493772 Section: New Results.

85. Lindsay, T. H., Thiele, T. R. & Lockery, S. R. Optogenetic analysis of synaptic transmission in the central nervous system of the nematode Caenorhabditis elegans. *Nat. Commun.* **2**, 306, DOI: 10.1038/ncomms1304 (2011).

86. Noma, K. & Jin, Y. Rapid Integration of Multi-copy Transgenes Using Optogenetic Mutagenesis in Caenorhabditis elegans. *G3 Genes–Genomes–Genetics* **8**, 2091–2097, DOI: 10.1534/g3.118.200158 (2018).

87. Evans, T. Transformation and microinjection. In *WormBook* (2006). Doi/10.1895/wormbook.1.108.1.

88. Yu, X. et al. Fast deep learning correspondence for neuron tracking and identification in C.elegans using synthetic training. DOI: 10.17605/OSF.IO/T7DZU (2021).

89. Monsalve, G. C., Yamamoto, K. R. & Ward, J. D. A New Tool for Inducible Gene Expression in Caenorhabditis elegans. *Genetics* **211**, 419–430, DOI: 10.1534/genetics.118.301705 (2019).

90. Nguyen, J. P. et al. Whole-brain calcium imaging with cellular resolution in freely behaving Caenorhabditis elegans. *Proc. Natl. Acad. Sci. United States Am.* **112**, 10534–10539, DOI: 10.1073/pnas.1507110112 (2015).

91. Hallinen, K. M. et al. Decoding locomotion from population neural activity in moving C. elegans. *bioRxiv* 445643, DOI: 10.1101/445643 (2021). Publisher: Cold Spring Harbor Laboratory Section: New Results.
93. Nguyen, J. P., Linder, A. N., Plummer, G. S., Shaevitz, J. W. & Leifer, A. M. Automatically tracking neurons in a moving and deforming brain. *PLOS Comput. Biol.* **13**, e1005517, DOI: 10.1371/journal.pcbi.1005517 (2017).

94. Zhou, Z. *et al.* Accurate and Robust Non-rigid Point Set Registration using Student’s-t Mixture Model with Prior Probability Modeling. *Sci. Reports* **8**, 8742, DOI: 10.1038/s41598-018-26288-6 (2018). Number: 1 Publisher: Nature Publishing Group.

95. Papagiakoumou, E., Sars, V. d., Oron, D. & Emiliani, V. Patterned two-photon illumination by spatiotemporal shaping of ultrashort pulses. *Opt. Express* **16**, 22039–22047, DOI: 10.1364/OE.16.022039 (2008). Publisher: Optica Publishing Group.

**Data availability statement**

Data is available in an interactive and browseable format at https://funconn.princeton.edu and http://funsim.princeton.edu. Machine readable datasets are publicly accessible through on Open Science Foundation repository at https://osf.io/e2syt/

**Code availability statement**

All analysis code is publicly available at https://github.com/leiferlab/pumpprobe, https://github.com/leiferlab/wormdatamodel, https://github.com/leiferlab/wormneuronsegmentation, and https://github.com/leiferlab/wormbrain. Hardware acquisition code is available at https://github.com/leiferlab/pump-probe-acquisition

**Acknowledgements**

We thank Annegret Falkner, Mala Murthy, Eva Naumann, H. Sebastian Seung, and Josh Shaevitz for comments on the manuscript. Online visualization software was created by Research Computing staff in the Lewis-Sigler Institute for Integrative Genomics and the Princeton Neuroscience Institute with special thanks to Fan Kang, Robert Leach, Ben Singer, and Lance Parsons. Research reported in this work was supported by the National Institutes of Health National Institute of Neurological Disorders and Stroke under New Innovator award number DP2-NS116768 to AML; the Simons Foundation under award SCGB #543003 to A.M.L.; by the Swartz Foundation through the Swartz Fellowship for Theoretical Neuroscience to F.R.; by the National Science Foundation, through the Center for the Physics of Biological Function (PHY-1734030); and by the Boehringer Ingelheim Fonds to S.D. Strains from this work are being distributed by the CGC, which is funded by the NIH Office of Research Infrastructure Programs (P40 OD010440).

**Author contributions statement**

A.M.L. and F.R. conceived the experiments, F.R. and S.D. conducted the experiments, A.K.S. designed and performed all transgenics, F.R. designed and built the instrument and the analysis framework and pipeline, F.R. and S.D. performed the bulk of the analysis with additional contributions from A.M.L. and A.K.S. All authors wrote and reviewed the manuscript.

**Supplementary Information**
Figure S1. a) Two-photon stimulation spot size. b) The imaging excitation wavelength and intensity were chosen to avoid GUR-3/PRDX-2 activation. Previously reported activity of a neuron expressing both GCaMP and GUR-3/PRDX-2 is shown in response to 500 nm light illuminated at varying intensities, as reported in\textsuperscript{45}. Vertical gray line indicates light intensity typically used for calcium imaging in the present work. Inset shows previously reported GCaMP6 excitation spectra\textsuperscript{47}. Vertical blue line indicates excitation wavelength used in the present work.

Figure S2. Selected neurons are photobleached to demonstrate targeted illumination across the brain. tagRFP-T expressed in a neuron is photobleached by a two-photon excitation spot that is spatially restricted in three dimensions (20 s illumination, 200 µW at 500 kHz repetition rate, 3.1 µm diameter FWHM spot). Examples are shown for two targeted neurons: a.) one located on the near side of brain with respect to the objective and b.) one on the far side of the brain (8.5 µm deeper in the sample). Fluorescence difference image shows tagRFP-T expression merged with a false-color blue-green image that shows the change in intensity before and after targeted illumination. All neurons express tagRFP-T, but only the targeted neuron and not nearby neurons appear photobleached (visible as white). Inset shows zoomed-in image of the targeted neuron’s original tagRFP-T intensity (left) and difference image (right). Laser power for this experiment was chosen such that the spot’s 2-photon interaction probability profile was non-saturating with respect to tagRFP-T absorption probability.
Figure S3. *In vivo* demonstration of the two-photon effective spot size. Calcium activity is recorded from a neuron expressing GUR-3/PRDX-2 and GCaMP6s and reported as fold change above baseline. 300 ms of two-photon excitation is first (t=11s) delivered to a point that is located 4 µm along the optical axis (z) beyond the center of an approximately 3.5 µm diameter neuron soma. Another 300 ms of two-photon excitation is delivered to the center of the soma (t = 35s). Only the on-target stimulation evokes a calcium transient. Note the stimulation artifact at t = 35 s is due to increased fluorescence of GCaMP6s induced by the 2-photon absorption, and is visible because no smoothing or filtering is applied to this trace. Schematic generated with BioRender.
Figure S4. Activation-response map. Mean amplitude of neural activity in a post-stimulus time window \( \langle \Delta F / F_0 \rangle_t \) averaged across trials and individuals is shown for WT-background. White indicates no measurement. For inclusion in the map, a stimulation event is required to evoke a response in the stimulated neuron. Therefore the strength of neuron’s response to its own stimulation is not displayed (black diagonal). \((N=43\) animals\).
Figure S5. Observations and false discovery rate of neuron pairs in the activation-response map. a.) Number of observations made of each neuron pair for WT-background animals. To be considered an observation, the upstream neuron must have been stimulated, calcium imaging of both the upstream and downstream neuron must have been recorded, and the upstream neuron must have exhibited an auto-response. Sorted as in Supplementary Fig. S7. Reverse cumulative distribution is also shown (bottom panel) and reports the fraction of pairs (number of observed pairs divided by the total number of possible pairs of neurons in the head). b.) $q$-values are shown for each neuron pair. $q$-values report false discovery rate and provide a metric of significance for assessing whether a neuron pair is connected based on the number of observations and the magnitude of the response transients. Cumulative distribution is also shown (bottom panel).
Figure S6. Number of stimulation events (orange) and number of recordings in which the neuron was observed (blue). For the WT activation-response map. Neurons are sorted left to right by number of recordings for which that neuron appears.
Figure S7. Activation-response map showing $q$-values. WT background. Same as Fig. 2 except here neurons that are observed but not stimulated are also included. Mean amplitude of neural activity in a post-stimulus time window ($\langle \Delta F/F_0 \rangle$) averaged across trials and individuals is shown. $q$-value reports false discovery rate and is a metric of significance (more gray is less significant). White indicates no measurement. For inclusion in the map, a stimulation event is required to evoke a response in the stimulated neuron. Therefore the strength of neuron's response to its own stimulation is not displayed (black diagonal). ($N=43$ animals).
Figure S8. Number of downstream neurons that exhibit excitatory (red) and inhibitory (blue) transients. Neurons are sorted from overall excitatory (left) to overall inhibitory (right). Neuron RIS (black arrow) is one of the most inhibitory, consistent with its expected role in inducing quiescence. In this analysis, all responses are included irrespective of \( q \)-value. \( \langle \Delta F / F_0 \rangle > 0.1 \) is classified as excitatory while \( \langle \Delta F / F_0 \rangle < -0.1 \) is classified as inhibitory.

Table S1. List of strains.
Figure S9. Variability of measured functional connectivity in WT background. a.) A downstream neuron’s activity transient was classified as either responding or non-responding based on its amplitude and other criteria described in methods. For each neuron pair, the fraction of observations that were classified as responses is shown. To be included in this analysis, the stimulated neuron is required to exhibit a response, and therefore the fraction responding is not reported for the diagonal, shown in black. b.) Kernels were fit for each stimulus resulting in a response to describe the transfer of signals from upstream to downstream neuron (Supplementary Fig. S10), thereby capturing variability of the connection independent of variability in the upstream neuron’s auto-response. The stereotypy of kernels is reported by inspecting the collection of kernels for each neuron pair. Only neuron pairs with at least two kernels are considered. The average correlation coefficient of the convolution of the kernels to the same stimuli is reported, as described in methods. c. Distribution of the correlation-coefficients of convolved kernels, as calculated in b, for only within each pair of neurons (blue, n=6,574), and across all kernels measured regardless of neuron pair (orange, n = 11,279,522).
Figure S10. Kernels describe how signals are transferred from an upstream neuron to a downstream neuron. a.) Variability in the downstream neuron’s activity can arise in part from variability in the upstream neuron’s activity. Paired activity from URXL and AWBL are shown in response to URXL stimulation. Recordings are sorted according to URXL’s average activity and show instances across trials and animals. b). Kernels are functions that when convolved with the upstream neuron’s activity return the downstream neuron’s activity. Kernels are only derived for instances in which both the upstream and downstream neuron’s activity were of sufficient amplitude to be classified as “responding,” as described in methods, and are here plotted as normalized. Kernels are shown for AWBL responses to URXL stimulation (Orange vertical bar in a). d). Average rise time of kernels for each measured neuron pair.
**Figure S11.** Measured calcium response of neuron AIY to optogenetic stimulation of AFD. Compare to Figure 4b in\textsuperscript{73}. 
Figure S12. Activation-response map of *unc-31* background, with defects in dense-core vesicle-mediated extrasynaptic signaling. Same format as Supplementary Fig. S5. Mean amplitude of neural activity in a post-stimulus time window \((\Delta F/F_0)_t\) averaged across trials and individuals is shown. \(q\)-value reports false discovery rate and is a metric of significance (more gray is less significant). White indicates no measurement. For inclusion in the map, a stimulation event is required to evoke a response in the stimulated neuron. Therefore the strength of neuron’s response to its own stimulation is not displayed (black diagonal). \((N=18\) animals).
Figure S13. Neural responses in a small gap junction sub-circuit are similar in wild-type and unc-31 mutant animals. a.) Anatomical connections among pharyngeal neurons M1, M3, MI, I3 and I4 are shown from NemaNode\textsuperscript{5}. b.) Paired activity traces of M3L and MI in response to M3L stimulation. Paired traces are sorted across trials and animals by MI’s activity. Traces are shown for WT background animals (left) and unc-31 mutant background (right). Only stimulation events resulting in a response in M3L’s activity are shown. c.) I3 responses to M3L stimulation in WT and mutant background. Paired traces are sorted across trials and animals by I3’s response. d.) Same for I4’s response and e.) M1’s response.
Figure S14. Functional connectivity of *unc-31* mutants is faster and has less connections than WT-background animals. **a.)** Functional connectivity is measured for *unc-31* mutants deficient in extrasynaptic signaling. A higher proportion of effective connections are fast in *unc-31* mutants compared to WT animals. **b.)** *unc-31* mutants had a smaller proportion of measured pairwise neurons that were functionally connected ($q < 0.05$) than WT.

![Graph showing functional connectivity](image)

**Figure S14.** Functional connectivity of *unc-31* mutants is faster and has less connections than WT-background animals. **a.)** Functional connectivity is measured for *unc-31* mutants deficient in extrasynaptic signaling. A higher proportion of effective connections are fast in *unc-31* mutants compared to WT animals. **b.)** *unc-31* mutants had a smaller proportion of measured pairwise neurons that were functionally connected ($q < 0.05$) than WT.

Figure S15. Properties of connections from RID. **a.)** The majority of RID’s outgoing connections to other neurons are predicted to be extrasynaptic. Number of wired and wireless postsynaptic partners predicted from anatomy, and gene expression\(^{61, 80}\) is shown for neuron RID. Peptides considered are FLP-14 and PDF-1. **b.)** RID has relatively few outgoing anatomical synaptic contacts compared to other neurons. Distribution of total number of anatomical synaptic contacts across all post-synaptic partners for each neuron is shown compared to RID, as tabulated from\(^3, 52\).

![Graph showing predicted connections](image)

**Figure S15.** Properties of connections from RID. **a.)** The majority of RID’s outgoing connections to other neurons are predicted to be extrasynaptic. Number of wired and wireless postsynaptic partners predicted from anatomy, and gene expression\(^{61, 80}\) is shown for neuron RID. Peptides considered are FLP-14 and PDF-1. **b.)** RID has relatively few outgoing anatomical synaptic contacts compared to other neurons. Distribution of total number of anatomical synaptic contacts across all post-synaptic partners for each neuron is shown compared to RID, as tabulated from\(^3, 52\).

Figure S16. Responses to RID stimulation of additional neurons in wild-type (WT) and *unc-31* backgrounds. Responses to RID stimulation are shown for WT (blue) and *unc-31* (orange). Individual points show mean calcium activity in a time window for each response. Bar shows mean across trials and across animals. Vertical axis is linear until the break, and then logarithmic after the break. Only neurons with a mean WT response above an absolute value threshold are shown. Corresponding traces for the responses of ADLR, AWBL and URXL are shown in Fig. 4. As in that figure, responses here are shown even for those cases when RID’s calcium activity was not measured.
CRISPR knockout of *unc-31* gene

**Figure S17.** a) Schematic of CRISPR knockout of *unc-31*. 