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System-wide mapping of peptide-GPCR interactions in *C. elegans*

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Abstract

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Author Contributions

I.B., S.Z. and L.S. designed experiments. I.B., S.Z., E.V., J.C., E.B., and I.H. performed GPCR screens. A.C. and L.G. performed experiments in *Xenopus* oocytes. P.E.V. and W.R.S. conducted the bipartite network analysis. O.M. performed the phylogenetic analysis. I.B., S.Z., J.D., and L.S. analyzed and interpreted results. I.B. and L.S. wrote the paper with input from all authors.

Declaration of Interests

The authors declare no competing interests.

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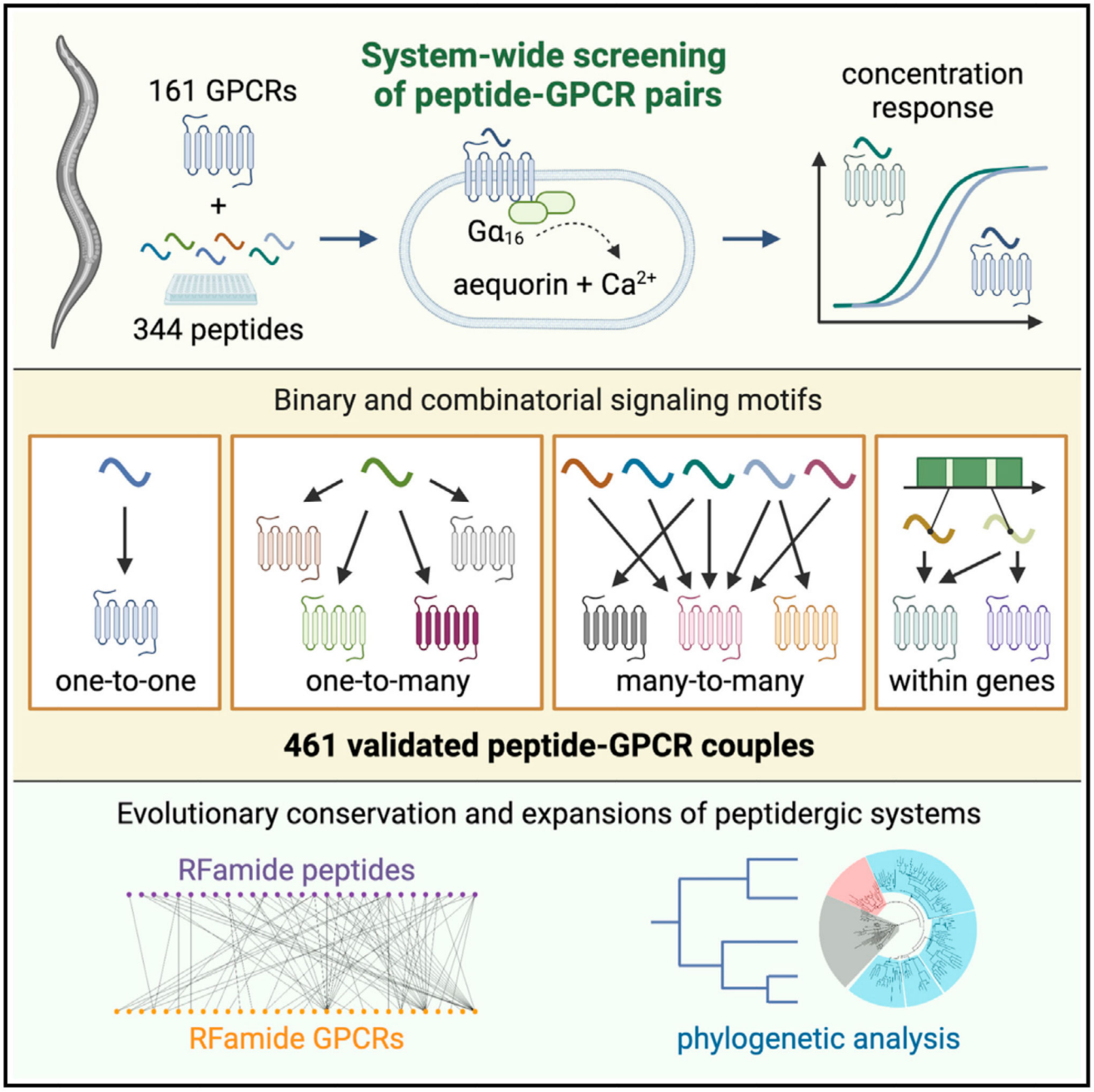

Graphical abstract.

Summary

Neuropeptides and peptide hormones are ancient, widespread signaling molecules that underpin almost all brain functions. They constitute a broad ligand-receptor network, mainly by binding to G protein-coupled receptors (GPCRs). However, the organization of the peptidergic network and roles of many peptides remain elusive, as our insight into peptide-receptor interactions is limited and many peptide GPCRs are still orphan receptors. Here we report a genome-wide peptide-GPCR

interaction map in *Caenorhabditis elegans*. By reverse pharmacology screening of over 55,384 possible interactions, we identify 461 cognate peptide-GPCR couples that uncover a broad signaling network with specific and complex combinatorial interactions encoded across and within single peptidergic genes. These interactions provide insights into peptide functions and evolution. Combining our dataset with phylogenetic analysis supports peptide-receptor co-evolution and conservation of at least 14 bilaterian peptidergic systems in *C. elegans*. This resource lays a foundation for system-wide analysis of the peptidergic network.

Introduction

Peptide hormones and neuropeptides are one of the largest and most widespread classes of signaling molecules that regulate physiology and behavior in all animals. Although bioactive peptides are also secreted from non-neuronal tissues, most are released by the nervous system and signal via G protein-coupled receptors (GPCRs). Peptide messengers are among the most ancient signaling molecules, likely predating the evolution of neurons,[1,2] and many peptidergic systems have broadly conserved functions across bilaterian animals.[3–7] Because of their crucial roles, peptide-GPCR pairs are increasingly gaining traction as potential therapeutic targets for human diseases.[8–11] GPCRs represent over 34% of the targets for prescribed drugs, and abnormalities in peptidergic transmission have been implicated in multiple diseases.[8,10,12] Indeed, these signaling systems are involved in a broad range of biological processes, including development, reproduction, feeding, nociception, learning, and memory, as well as the regulation of behavioral states, such as sleep and arousal.[2,3,7,13–19]

While several efforts have been made to map the brain-wide expression of peptide and GPCR genes,[20–23] our insight into the structure of the peptidergic signaling network is limited, as huge gaps remain in our understanding of peptide-receptor interactions in animals. Neuropeptides mainly bind to GPCRs that activate second-messenger pathways and, because the number of second-messenger pathways is limited, the diversity of peptide-GPCR couples is thought to provide the complexity required for brain function.[24] Most animal genomes harbor around 100 to 150 putative peptide-GPCR genes and encode hundreds of bioactive peptides that are cleaved from prepropeptides, containing one or multiple peptide sequences.[25–31] Some receptor genes also code for multiple GPCR isoforms, which may have different ligand interactions.[25,26] In addition, peptide ligands can bind to multiple receptors and vice versa.[27–29] These complex interactions further diversify the peptidergic network.

Peptide-GPCR couples are challenging to predict from prior knowledge, such as neuronal connectivity or sequence information, as peptide ligands have only short sequences and activate GPCRs that are often distantly located from their release site.[30–32] Reverse pharmacology has proved to be a successful approach for the deorphanization of GPCRs by expressing receptors in heterologous cells and identifying their ligand(s) in compound libraries.[33] This way, peptide ligands have been found for a broad range of GPCRs, including at least 138 receptors in humans, 36 in *Drosophila melanogaster*, and 29 in *Caenorhabditis elegans*.[29,34–50] Nevertheless, many predicted peptide GPCRs remain orphan

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receptors, hampering investigations into their functions.[51] Although some large-scale efforts have been undertaken, such as for *Platynereis* and human peptide GPCRs,[29,52,53] most deorphanization studies have focused on single receptors or a subset of GPCR candidates, which may overlook complex interactions of peptides with multiple receptors.[29,54] To gain a deeper understanding of the peptidergic network, system-wide approaches are required that examine as many interactions as possible between peptides and GPCR isoforms encoded in animal genomes.

Due to its compact and well-defined nervous system, the nematode *C. elegans* represents an attractive model to map and functionally characterize the peptidergic network at an organismal level. The complete anatomy and wiring diagram of its nervous system has been defined, facilitating studies of peptidergic interactions with the wired circuitry.[55–58] The *C. elegans* genome encodes over 300 peptides that are classified as FMRFamide-like (FLP), insulin-like (INS), and other neuropeptide-like (NLP) peptides.[59,60] In addition, its genome is thought to encode around 150 putative peptide GPCRs.[36,61] Many of these peptidergic systems are shared between nematodes and other animals, including humans, and have conserved functions.[6,31,62,63] Transcriptional profiles are available for individual neuron classes among the 302 neurons of the *C. elegans* hermaphrodite nervous system.[21,64] As in other animals, distinct neuron types in *C. elegans* were found to express unique codes of neuropeptide genes and receptors, suggesting different roles for neuron classes in transmitting peptidergic signals.[20,21,23] However, the relationships between sender and receiver cells in the extensive peptide network have not been determined, as over 80% of the peptide-GPCR candidates in *C. elegans* are still orphan, and target receptors for most *C. elegans* peptides remain unknown.[36,61]

Here, we describe a large-scale reverse pharmacology pipeline for pairing *C. elegans* peptides to GPCRs and identify 461 cognate peptide-receptor couples. A system-wide screening approach allows us to discover novel ligands of known and orphan peptide GPCRs and reveals several types of complex ligand-receptor interactions in the peptidergic signaling network, including promiscuous receptors and different interactions encoded within single peptide genes. We combine our dataset with phylogenetic analysis to delineate the evolutionary history of *C. elegans* peptidergic systems. This resource of ligand-receptor pairs, combined with single-cell transcriptome data,[21] also provides a basis for constructing a connectome of neuropeptidergic signaling in *C. elegans*.[65]

Results

Large-scale screening for *C. elegans* peptide-GPCR pairs

The *C. elegans* genome has at least 149 genes for putative peptide GPCRs (Table S2), most of which encode orphan receptors.[36,61,66,67] To map the peptide-GPCR interaction network, we set up a reverse pharmacology platform in which we systematically screened for peptide ligands of these receptors in cultured cells (Figure 1A). We first constructed a GPCR library by cloning receptor cDNA sequences in a pcDNA3.1 vector. Multiple GPCR isoforms were included, as receptor variants may be differentially activated by peptide ligands.[25,26,68] In total, we cloned 161 receptors, covering 87% of peptide-GPCR genes that are known and predicted in *C. elegans* (Table S2; Data S1).

To identify peptide-receptor couples, we screened each GPCR with a comprehensive library of 344 synthetic *C. elegans* peptides, belonging to the FMRFamide-like (FLP) and neuropeptide-like protein (NLP) families, that we compiled from biochemical identifications and bioinformatic predictions (Table S3). Insulin-like peptides were not included in the library, as they mainly activate receptor tyrosine kinases and are structurally less well characterized in *C. elegans*.[69,70] We expressed *C. elegans* GPCRs in Chinese hamster ovary (CHO) cells, which are widely used for heterologous GPCR expression, and screened for ligand-receptor interactions in an established calcium mobilization assay (Figure 1A).[5,6,38,39,44,52,71–75] Each receptor was transiently expressed in CHO cells that stably express the calcium-sensitive bioluminescent protein aequorin together with a promiscuous $G\alpha_{16}$ protein. The $G\alpha_{16}$ subunit allows coupling of many GPCRs to phospholipase Cβ (PLCβ) activity, resulting in calcium release from intracellular storage sites and an increase in luminescence (Figure 1A).[75–77] Using a high-throughput fluorometric imaging plate reader (FLIPR) screening system, we challenged each GPCR with peptides from the synthetic library (at 10 μM), testing 55,384 pairs, and monitored GPCR activation by measuring luminescent responses.

To identify hits in the peptide library, we manually inspected peptide-evoked luminescent responses and determined an activation value for each peptide-GPCR pair based on *Z* scores (see STAR Methods). We calculated *Z* scores for each pair, standardized the scores for each GPCR by dividing by its maximal activation, and log2 transformed this fraction to rank individual peptide-GPCR couples (Table S4). Known peptide-GPCR couples with sub-micromolar half-maximal effective concentrations (EC_{50} < 1 μM) nearly all showed *Z* scores above 20 and log2-standardized values above -1.2 in our assays (Figure S1A; Table S4). Therefore, we considered all peptide-GPCR couples with a strong calcium response over background (*Z* score >20), reaching at least 40% of that of the receptor's maximum *Z* score (log2-standardized *Z* > -1.2), as potential hits in the library. Based on these criteria, we found 416 putative hits for 114 GPCRs derived from 81 peptide- and 95 receptor-encoding genes (Figure S1; Table S4).

Validation of peptide-GPCR interactions

We first screened for peptide-GPCR pairs by testing high peptide concentrations (10 μM). However, peptide GPCRs typically show concentration-dependent activation with EC_{50} values in the nanomolar range.[32] To determine whether the potential peptide hits are cognate GPCR ligands, we quantified GPCR activation for decreasing peptide concentrations (Figure 1B). Prior to concentration-response measurements, synthetic peptides were purified by reversed phase high-performance liquid chromatography (HPLC) and verified by mass spectrometry (see STAR Methods). We then performed concentration-response tests for all putative hits identified in library screens. Because false-positive pairs could bias hit selection, we assigned hits by iterative ranking of peptide-GPCR couples. When the top interaction was not confirmed by concentration-response tests, we restandardized the *Z* scores for that receptor relative to the next top hit in the screen. Concentration-response assays were then repeated until all pairs matching the hit criteria were tested (*Z* score >20, log2-standardized *Z* > -1.2). For each hit, we screened all peptides derived from the same peptide-encoding gene, which may be co-released from peptidergic cells (except for

promiscuous receptors; see STAR Methods). In total, we tested 776 peptide-GPCR couples, of which 459 pairs showed concentration-dependent receptor activation (Figures 1B and S2–S7; Table S4). None of the peptide ligands evoked a calcium response in CHO cells transfected with an empty pcDNA3.1 plasmid (Table S5).

Among the 459 validated pairs, we identified ligands for 66 GPCRs (including isoforms) and receptors for 151 peptides (Table S4). We characterized GPCRs for peptides of all 31 *flp* genes and 22 *nlp* encoding genes (Table S1; Figures S2–S7). These include 39 receptors that are activated only by RFamide peptides, 24 GPCRs that interact with peptides of the NLP family, and three receptors that are activated by peptides from both families. EC_{50} values, as calculated from concentration-response curves, range between 0.1 pM and 22 μM but are in the sub-micromolar range for 73% of the couples (Figure 2A; Table S6), suggesting that the majority of the identified interactions are likely cognate pairs.

To probe whether peptide-GPCR couples are cognate pairs *in vivo*, we set up a collaborative network through which researchers could prioritize GPCRs for ligand screening and functionally test peptide-receptor interactions in *C. elegans* during our study (via https://worm.peptide-gpcr.org). This way, over 10 novel peptide-GPCR couples in our dataset have already been validated in *in vivo* studies.[62,63,78–84] These revealed roles of peptide systems in the regulation of foraging, nociception, locomotory arousal, aversive learning, and other behaviors,[62,63,78–80] and illustrate that our screening approach allows identifying physiologically relevant interactions. Here, we present the complete resource of peptide-GPCR pairs (Figures S2–S7); *in vivo* studies that have validated and reported functional ligand-receptor couples in this dataset are listed in Table S1.

Discovery of novel peptide receptors and additional ligands for peptide GPCRs

Deorphanization efforts previously identified peptide-receptor couples for 16 *nlp*, 19 *flp*, and 29 peptide-GPCR genes in *C. elegans* (Table S1). We compared these reported interactions to the 459 peptide-GPCR couples discovered in our system-wide screen and confirmed known agonists for 23 receptors (Table S1). Our dataset recapitulates the majority of high-potency interactions (EC_{50} < 500 nM) reported previously (Table S1). For eight peptide GPCRs, we discovered novel peptide ligands in addition to known agonists. By systematically screening for peptide-GPCR interactions, we identified 79 additional ligands for DMSR-1, NPR-6, NPR-11, NPR-12, NTR-1, CKR-1, and two EGL-6 receptors (Table S1). This underscores the importance of system-wide screening approaches to map peptide-GPCR interactomes.

In addition, our dataset reveals peptide ligands for 35 orphan GPCRs (including isoforms; Table S1). These include receptors for peptides from 58 peptide-encoding genes (31 *flp* and 27 *nlp* genes). We also asked whether our screen might uncover receptors for predicted peptides that have as yet not been biochemically isolated. To probe this, we compared our dataset of peptide-GPCR couples to the *C. elegans* neuropeptidome, a resource of peptide sequences identified by mass spectrometry.[59] Of the 344 peptide sequences in our synthetic library, 206 peptides were detected in whole-mount peptide extracts using mass spectrometry. Among these, 115 peptides were identified as agonists in our screen (Figure

2B). In addition, we found receptors for 36 peptides that so far have not been biochemically isolated (Figure 2B; Table S7), which suggests bioactive roles for these peptides.

A system-wide screening strategy reveals promiscuous peptide GPCRs and ligands

Peptides of the FLP family share a C-terminal RFamide motif, whereas NLPs form a structurally diverse class of peptides that lack a common consensus sequence. In agreement with these structural differences, most peptide GPCRs in our dataset are activated by either FLP or NLP ligands. Only three receptors (NTR-1, FRPR-16, and NPR-22) have ligands from both peptide families. For NPR-22, NLP and FLP ligands also show clear sequence similarity: NLP-72 peptides have a C-terminal RYamide motif that resembles the RFamide sequence of FLP ligands (Figure S2; Table S3).

To further probe the diversity of ligands for individual GPCRs, we generated a heatmap of EC_{50} values for the identified peptide-receptor pairs (Figure 2C). The majority of receptors (48 out of 66 GPCRs) were activated by ligands from a single peptide-encoding gene, showing specific interactions. However, we also identified six GPCRs with many ligands, from at least eight peptide-encoding genes (Figure 2C). These promiscuous receptors are encoded by *dmsr-1*, *dmsr-7*, *frpr-8*, and *egl-6* genes and are all activated by diverse RFamide (FLP) peptides. Most FLPs also activate multiple GPCRs encoded by different receptor genes (Figure 2C). FLP-1, FLP-8, and FLP-14 are among the most versatile peptides and interact with 8 to 12 GPCRs. By contrast, peptides of the NLP family activate only a few (one to three) receptors (Figure 2C).

We aimed at further validating the promiscuity of peptide GPCRs and asked which second-messenger pathways might be activated in response to different ligands. We first focused on DMSR-7 that shows the highest promiscuity. Previous studies suggested that DMSR-7 has inhibitory effects *in vivo*.[82,85] To validate its ligand interactions and determine its cellular response, we measured DMSR-7 activation in different heterologous expression systems in the absence of the promiscuous $G\alpha_{16}$ subunit. First, we expressed DMSR-7 in *Xenopus laevis* oocytes and measured responses to multiple FLP ligands, with EC_{50} values in the nanomolar to micromolar range, by means of two-electrode voltage-clamp (TEVC) recording. DMSR-7 strongly responded to FLP-1, its most potent ligand, causing robust stimulation of K^+ current through G protein inward rectifying potassium (GIRK) channels (Figures 3A and 3B). Treatment with pertussis toxin (PTX) inhibited the activation of DMSR-7 to the same extent as the known $G_{i/o}$-coupled muscarinic M2 receptor (Figures 3A and 3B), suggesting that DMSR-7 is also a $G_{i/o}$-coupled GPCR. In the oocyte system, DMSR-7 was also activated by multiple RFamide peptides, while none of them elicited a response in oocytes that lacked the receptor (Figures 3C and 3D). Using a luminescence-based cyclic AMP (cAMP) reporter in HEK cells, we found that DMSR-7 inhibits cAMP signaling in response to 10 different FLP ligands (Figures 3E and 3F). These peptides did not evoke a calcium response in cells expressing DMSR-7 in the absence of $G\alpha_{16}$ (Figures 3G and 3H). Our results thus suggest that DMSR-7 responds to diverse RFamide peptides by activating the $G_{i/o}$ pathway, not the calcium pathway, which is in agreement with functional studies.[82,85] We also investigated the second-messenger responses of two other promiscuous FLP receptors, FRPR-8 and EGL-6 (Figure 2C). Activation of EGL-6 inhibited

cAMP signaling in response to all RFamide ligands (Figures S8A, S8B, S8E, and S8G). By contrast, activation of FRPR-8 increased cAMP levels, although these results were less conclusive, because three ligands evoked a significant cAMP response in control cells and three other peptides did not activate the receptor in the absence of $G\alpha_{16}$ (Figures S8C–S8G). Together, these results suggest that promiscuous peptide GPCRs stimulate or inhibit cAMP signaling and that the same second-messenger pathway is induced by different FLP ligands. We conclude that promiscuous FLP receptors are activated by multiple diverse RFamide peptides, which may result in similar cellular responses.

The RFamide peptide-receptor network is characterized by peptidergic crosstalk

Our results suggested that the RFamide signaling network shows a higher level of ligand-receptor crosstalk in comparison to non-RFamide (NLP) peptide systems. RFamide peptides also interact with a receptor network that is largely distinct from that of other peptides. To further investigate the structure of FLP and NLP ligand-receptor networks, we used a bipartite network representation and generated its projections. We focused our analysis on interactions with sub-micromolar EC_{50} values (<1 μM), comprising most pairs in our dataset. Bipartite graphs of both networks show different topologies and more crosstalk for ligands and receptors of the RFamide family than for other peptidergic systems (Figure 4A). To investigate the relationships among FLP and NLP peptides, we projected the bipartite networks into simple, monopartite ligand networks in which the nodes (peptides) are connected only if they have at least one common receptor. Nearly all clusters in the NLP network include peptides from a single precursor, which often have similar sequences and activate the same receptor(s) (Figure 4B). The only two exceptions are the *C. elegans* RPamide (NLP-2, NLP-22, and NLP-23) and PDF-like peptides (PDF-1 and NLP-37). Both are families of homologous peptide genes that likely arose through gene duplication and share significant sequence similarity.[83,86] By contrast, the monopartite projection of the FLP network shows a higher number of edges, consistent with the receptor promiscuity that we observed for RFamide peptides (Figure 4C).

Many complex networks have a modular structure, whereby they contain subsets of nodes—called modules—that are more densely interconnected with each other than with the rest of the network. We further analyzed the modularity of the FLP ligand network and identified several modules, two of which contained the majority of RFamide peptides (red and dark blue in Figure 4C). Peptides in these two modules interact with one or multiple receptors. However, they all activate the promiscuous receptors DMSR-1 and/or DMSR-7 (Figure 2C). One module comprises all peptides that activate DMSR-7 but not DMSR-1 (dark blue in Figure 4C). The second module groups all ligands of DMSR-1 (red in Figure 4C), of which some—closest to the DMSR-7 module—also activate DMSR-7. The peptide agonists of DMSR-1 and DMSR-7 thus only partially overlap, although many of them also activate other receptors (Table S6). Besides the two DMSR modules, we found eight smaller groups of FLPs that constitute separate modules in the ligand network, such as FLP-2, FLP-3, FLP-15, FLP-20, FLP-21, and FLP-34 peptides (Figure 4C). These include several peptides of the evolutionarily conserved short neuropeptide F (sNPF) and neuropeptide Y/F (NPY/F) families (e.g., FLP-15, FLP-21, and FLP-34),[87] which arose in a common ancestor of

bilaterian animals and, through ligand-receptor co-evolution, may have evolved interactions with different receptor targets than those of other FLPs.[31,88]

We found similar modules in a receptor-focused projection of the bipartite FLP network, in which nodes (receptors) are connected when they share at least one peptide ligand. Two modules are centered on DMSR-1 and DMSR-7 (red and dark blue in Figure 4D). Other receptors that share multiple ligands with these promiscuous GPCRs, such as DMSR-5 and DMSR-7, cluster in the same modules, while seven modules are distinct from the DMSR-1 and DMSR-7 clusters (Figure 4D). Consistent with the ligand projection, receptors of the sNPF receptor family (NPR-1, NPR-2, NPR-3, NPR-4, NPR-5, NPR-6, and NPR-10)[87] cluster in separate modules, as they share none or only a few ligands with other GPCRs. In addition, the FLP-receptor projection revealed a novel module in the FLP signaling network, consisting of seven GPCRs that are uniquely activated by FLP-8 and FLP-14 peptides (light blue in Figure 4D).

Taken together, our results indicate three main types of ligand-receptor interactions in the peptide-GPCR network (Figure 4E): (1) complex “many-to-many” interactions mediated by promiscuous receptors (e.g., DMSR-1 and DMSR-7), which have diverse RFamide peptide ligands that often activate other GPCRs as well; (2) divergent “one-to-many” interactions by RFamide peptides (e.g., FLP-1, FLP-8 and FLP-14) that activate multiple specific receptors; and (3) specific “one-to-one” interactions between peptides and receptors from single-peptide- and GPCR-encoding genes. Most non-RFamide systems belong to this category, whereas the majority of RFamide peptides cross-interact with many GPCRs.

Different interactions encoded within single peptidergic genes

Peptide-encoding genes often code for multiple peptide sequences that may be co-released.[32,89] To probe how this multi-peptide signaling affects the peptide-receptor network, we compared sequences and receptor interactions for peptides derived from the same precursor. Most peptides from a single gene show clear sequence similarity and activate the same receptor(s) (Figures S9–S11). However, we also found several genes, such as *flp-1*, *flp-11*, *nlp-2*, and *nlp-23*, of which individual peptides differ in their potency to activate specific GPCRs (Figures S10 and S11). For example, FLP-1 peptides with the PNFLRF-NH_2 motif (FLP-1-1 to -7 and FLP-1-10) activate DMSR-5, DMSR-6, and FRPR-7, while these receptors are not activated by the two PNFMRY-NH_2 FLP-1 peptides (FLP-1-8 and FLP-1-9) that interact with NPR-22 (Figure S10; Table S3).

Next, we asked whether ligand interactions differ between GPCR isoforms encoded by the same gene. Our dataset includes isoforms of 11 GPCR genes that differ at N-terminal, C-terminal, or intertransmembrane regions (Figure S12A). Most receptor variants display similar ligand interactions (Figure S12B). However, two N-terminal variants of the NLP-17 receptor NPR-37 showed a 1,000-fold difference in the respective EC_{50} values for their ligands (Figure S12B). Two variants of the *Drosophila* myosuppressin receptor ortholog DMSR-1, which differ at their intracellular C terminus, also displayed different interactions (Figures S12A and S12B). FLP-25 peptides potently activate DMSR-1B (DMSR-1-2), with picomolar EC_{50} values, whereas these peptides activate DMSR-1A (DMSR-1-1) only at high micromolar concentrations (Figure S12B). Because EC_{50} values can differ depending

on the heterologous system,[90–92] we compared the concentration-dependent activation of the two DMSR-1 variants in *Xenopus* oocytes. In this system, FLP-25 potently activated both DMSR-1 isoforms with similar EC_{50} values (Figure S12C). EC_{50} values were also comparable for most other DMSR-1 agonists in our calcium mobilization assay (Figure S12B), suggesting that both isoforms show similar ligand interactions.

Characterization of ancestral bilaterian peptidergic systems in *C. elegans*

Many peptidergic systems show long-range evolutionary conservation. Comparative genomic analyses of these systems across widely divergent animal phyla revealed at least 31 peptidergic systems that are ancestral to bilaterian animals, such as oxytocin and vasopressin, cholecystokinin, neuropeptide Y (NPY), and gonadotropin-releasing hormone (GnRH) systems.[31,93–95] Several bilaterian systems are also thought to be conserved in *C. elegans*, but not all predicted peptide-receptor couples have been experimentally demonstrated. To further clarify the conservation of peptidergic systems in *C. elegans*, we performed a phylogenetic analysis of nematode GPCRs in our screen, identified receptor orthologs for bilaterian peptide systems, and compared their ligands to peptides that activate known representatives of the same receptor family.

To reconstruct phylogenetic relationships, we first used the sequences of all rhodopsin and secretin-type peptide GPCRs from species in eight bilaterian phyla as bait to identify potential homologs in transcriptomic and expressed sequence tag (EST) datasets of 34 phylogenetically dispersed nematode species (Table S8). We then constructed a maximum-likelihood tree, which revealed 31 bilaterian receptor clusters that contain a diversified set of protostome and deuterostome GPCRs (Figures 5A and 5B; Data S1). Representative *C. elegans* receptors were present in 17 of the 31 ancestral receptor families (Figure 5C; Table S9). These GPCR families were well conserved in nematode species across different clades (Figure 5C).

We identified ligand-receptor pairs for 12 of the 17 bilaterian families of peptide GPCRs that are conserved in *C. elegans* (Figure 5C; Table S9). In most cases, the *C. elegans* receptor was activated by a peptide that was a previously recognized ortholog of the peptide ligands of related GPCRs.[31,93,94] Consistent with previous reports, our data support the conservation of vasopressin/oxytocin (NTC-1/ NTR-1), tachykinin (NLP-58/TKR-1), GnRH (NLP-47/GNRR-1), cholecystokinin (NLP-12/ CKR-1/ CKR-2), neuromedin U (CAPA-1/NMUR-1/NMUR-2), NPY (FLP-34/NPR-11), thyrotropin-releasing hormone (TRH-1/ TRHR-1), luqin (LURY-1/NPR-22), pigment-dispersing factor (PDF-1/PDF-2/PDFR-1), and myoinhibitory peptide (MIP-1/ SPRR-2) systems in *C. elegans*.[5,6,41,46,49,50,62,63,74,79,86,96–98] We also identified peptide ligands for four *C. elegans* GPCRs related to neuropeptide FF/SIFamide (NPR-35), tachykinin (TKR-2), NPY (NPR-12), and myoinhibitory peptide (SPRR-1) receptors (Table S9). Our results provide biochemical evidence for the predicted interactions of these receptors with orthologs of neuropeptide FF/SIFamide (NLP-10), tachykinin (NLP-58), NPY (FLP-33), and myoinhibitory peptides (NLP-42).[31,46,49,99] In addition, we identified a ligand for SEB-3, a GPCR that belongs to the same bilaterian secretin superfamily of receptors as bilaterian CRH/DH44 and calcitonin/DH31 receptors, vertebrate VIP/ PACAP receptors, and

invertebrate PDF receptors.[31,78,93,94] SEB-3 is most closely related to PDF receptors (Figure 5B) and orthologous to orphan GPCRs in many other species.[31,94] The identification of a peptide ligand for SEB-3 may help to characterize ligands of other receptors within this family. A functional study of this peptide-receptor pair validated its interaction *in vivo* and characterized its role in the regulation of locomotion, arousal, and reproduction.[78]

C. elegans receptors in five bilaterian GPCR families remain orphan. For the *C. elegans* galanin/allatostatin A (NPR-9) and leucokinin (TKR-3) receptor orthologs,[31] peptides related to allatostatin A (NLP-6) and leucokinin (NLP-43) were included in our screen but did not activate their predicted GPCR. Reasons for this lack of interaction include (1) the functional expression of GPCRs in CHO cells may have failed, or (2) in the case of NPR-9, the predicted receptor cDNA sequence could not be cloned (Data S1). For *C. elegans* orexin/allatotropin (NPR-14), elevenin (NPR-34), and calcitonin/DH31 (SEB-2) receptor orthologs, the predicted ligands were initially not included in our peptide library. *C. elegans* homologs of orexin/allatotropin (NLP-59) and elevenin (SNET-1) were identified after constructing the library,[94,100] whereas a calcitonin-like peptide (NLP-73) was included but lacked a disulfide bridge that may be critical for receptor activation (Table S3). We therefore retested these receptors with their predicted ligands and found that SNET-1 and NLP-73 activate NPR-34 and SEB-2, respectively (Figure 5D; Table S5). We did not observe activation of NPR-14 with NLP-59, which may be because this receptor did not express well or is activated by another, unidentified orexin/allatotropin-like peptide. Taken together, our results support the conservation of at least 14 ancestral bilaterian peptide systems in *C. elegans* (Figure 5C). Based on these findings, we suggest naming these peptide and receptor-encoding genes in accordance with their evolutionary relationship to bilaterian peptidergic systems (Table S9).

Nematode expansions of evolutionarily conserved peptidergic systems

Besides the conservation of bilaterian peptide systems, phylogenetic analysis revealed expansions of peptide-GPCR families in nematodes, including GnRH, somatostatin, sNPF, myosuppressin, and FMRFamide-related receptors (Figure 5A; Table S9; Data S1). We find that several *C. elegans* receptors of these expanded families are activated by peptides of the corresponding related peptide family, which supports the conservation of these peptidergic systems in nematodes. For example, one nematode-expanded receptor family clustered near the bilaterian family of somatostatin (SST), allatostatin C (AstC), and opioid (Op) receptors (cluster 37; Figure 5A; Data S1). This group of 12 *C. elegans* GPCRs (Table S9) includes NPR-17, which was previously shown to interact with the opioid-like peptide NLP-24.[37] We found two other receptors in this family, NPR-24 and NPR-32, to interact with orthologs of the SST/AstC family, NLP-62 and NLP-64 (Figure S2), supporting the conservation of SST/AstC-like receptors in *C. elegans*.[31,101] A second expansion occurred for GnRH-related receptors (cluster 38; Figure 5A; Data S1). *C. elegans* GNRR-1 clusters most closely to GnRH-like receptors in other animals and is specifically activated by NLP-47 (Figure S3; Data S1), which is orthologous to GnRH and the related insect adipokinetic hormone (AKH).[97] Seven additional GnRH-like receptors (GNRR-2 to GNRR-8) form a paralogous group of which two are activated by RPamide peptides (Figure S3), a nematode family of peptides that shows sequence similarity to GnRH/AKH peptides.[83]

In addition, three protostomian families of peptide GPCRs diversified in nematodes. The largest expansion occurred in the FMRFamide-like receptor family, related to the *Drosophila* FMRFamide receptor (FMRFaR), including 37 GPCRs in *C. elegans* (cluster 33; Figure 5A; Table S9; Data S1). As expected, nearly all GPCRs in this cluster interact with FLP peptides (Table S9). In addition, the protostomian sNPF and myosuppressin receptor families expanded in nematodes (clusters 32 and 34; Figure 5A; Data S1). Protostomian sNPF receptors are orthologous to deuterostomian prolactin-releasing peptide (PrRP) receptors, although protostomian sNPF peptides and deuterostomian PrRP-type peptides share low levels of sequence similarity.[88,93] In *C. elegans*, the sNPF receptor family includes nine GPCRs, of which eight have sNPF-like peptide ligands with the canonical motif XLRFamide (FLP-15, FLP-18, FLP-21, and FLP-26)[87] (Table S9; Data S1). The myosuppressin-like receptor family includes 16 *C. elegans* receptors (Table S9; Data S1), three of which are activated by FLP-1 peptides that show sequence similarity to insect myosuppressins (Figure S6).[102] Interestingly, these FLP-1 receptors—DMSR-5 to–DMSR-7—all cluster together in the phylogenetic tree along with several orphan GPCRs (DMSR-4, DMSR-9, and DMSR-11 to 16), whereas other DMSRs form separate sub-groups and are activated by different, more divergent peptide ligands (Table S9; Data S1). In addition, we found that some RFamide peptides, such as FLP-3 and FLP-14, activate multiple receptors that belong to different expanded peptide-GPCR families (Table S9). Because the FLP system has largely diversified in nematode evolution, it is conceivable that some receptors from phylogenetically distinct clusters evolved the ability to interact with similar peptides, possibly at different evolutionary times. Promiscuous FLP receptors also belong to these expanded GPCR families (Table S9), which may explain ligand promiscuity. Taken together, the biochemical interactions identified for all these receptors provide general insights into the evolutionary history and diversification of peptidergic systems in nematodes.

Discussion

Bioactive peptides and peptide GPCRs constitute the most diverse ligand-receptor signaling network in animals. However, many GPCRs are still orphan receptors, precluding the construction of peptidergic signaling maps at organismal scale. By system-wide screening of peptide-receptor interactions, we have generated a resource of 461 peptide-GPCR couples in *C. elegans* that, along with the synaptic connectome, single-cell transcriptome data and other resources in this model,[21,55–57] lays a foundation for functional studies of the peptidergic signaling network at system level.

A peptide-GPCR interaction resource for mapping peptidergic circuits and functions in *C. elegans*

Reverse pharmacology strategies based on $G\alpha_{16}$-mediated calcium signaling have been widely used for characterizing GPCR ligands.[6,52,103,104] Using this strategy, we identify 68 peptide GPCRs (including isoforms) that interact with 153 peptides of 60 peptide-encoding genes (31 *flp* and 29 *nlp* genes; Table S1). This dataset expands the number of known peptide GPCRs in *C. elegans* from 29 to 60 receptor genes. Many of the interactions that we uncover have been validated in other *in vitro* systems and in *in vivo* studies (Table

S1), illustrating that this resource can guide targeted research into peptidergic pathways and functions. Furthermore, we identify additional ligands for peptide GPCRs that were previously deorphanized.

This peptide-GPCR interaction dataset combined with single-cell transcriptome data[21] provides a basis for mapping the peptide signaling network in *C. elegans*. Peptide-receptor interactions are one of the most ancient types of signaling pathways that link up cells into complex chemical networks.[1,105] While synaptically wired circuits can be anatomically mapped, the brain's chemo-connectome constituted by numerous neuromodulators, such as neuropeptides, monoamines, and their receptors, cannot be resolved solely from anatomical expression, as this extensive "wireless" network relies on chemical messengers that often act remotely from their release site.[30,106,107] Knowledge of ligand-receptor interactions is therefore crucial to delineate which nodes or cells in the network can communicate with each other. In a complementary study, we have generated a draft connectome of neuropeptidergic signaling in the *C. elegans* nervous system by integrating this interaction resource with gene expression and anatomical datasets.[65] The development of novel tools to delineate peptidergic circuits, such as genetically encoded sensors for peptide-GPCR signaling, provides a promising avenue to further understand the functional organization of this broad signaling network and to validate peptide-receptor interactions *in vivo*.[108–110] In addition, the ligands identified in this resource allow further investigation of the cellular responses of peptidergic signaling by studying the second-messenger pathways and G protein coupling of peptide GPCRs.

System-wide screening of peptide-GPCR pairs reveals specific and combinatorial signaling motifs in the peptide network

Our dataset reveals three main types of peptide-GPCR interactions that show different levels of complexity: specific, one-to-many, and many-to-many interactions. Many peptide GPCRs interact with ligands derived from only a single peptide-encoding gene, generating a large diversity of specific ligand-receptor pairs for information coding. By contrast, we also identify peptides that interact with a broad range of specific GPCRs as well as promiscuous peptide receptors. This suggests that the peptidergic system forms a complex interaction network in which information is coded not only through a binary mechanism of specific ligand-receptor couples but also through complex combinatorial peptide-receptor signaling.

We find that peptidergic crosstalk is a feature characteristic of the *C. elegans* FLP-receptor network, as all promiscuous receptors interact with diverse FLP peptides, typified by a carboxyterminal RFamide sequence motif. A few FLPs (e.g., FLP-1, FLP-14) activate multiple closely related as well as phylogenetically more distant receptors. Since the number of RFamide peptide and receptor genes has diversified extensively in nematode evolution, the complex cross-interactions in the RFamide network may be explained by the large expansion of the FLP system in *C. elegans*.[31,94,111] However, ligand and receptor promiscuity has been demonstrated for peptidergic systems that did not undergo large expansions in other species as well.[29,54,112] For example, human neuropeptide FF receptors are activated by all five groups of mammalian RFamide peptides.[113–116] Peptides that activate multiple unrelated GPCRs have also been described in other animals.[117–120]

Receptor promiscuity and peptidergic crosstalk thus appear to be general features of the peptidergic system, especially of RFamide peptides and their receptors.

Our results indicate that promiscuous peptide GPCRs (DMSR-7, FRPR-8, and EGL-6) can stimulate or inhibit cAMP signaling in response to diverse ligands. This finding is in agreement with functional genetic studies showing that DMSR-7 and EGL-6 function as inhibitory receptors *in vivo*.[82,85,121] The promiscuous receptor DMSR-1 also inhibits foraging and sleep-like behavior by interacting with multiple RFamide peptides. Stress-induced sleep is regulated by FLP-13/DMSR-1 signaling,[39] whereas FLP-5 peptides—which activate the same receptor with a higher EC_{50} value than FLP-13 (Table S6)—regu-late temperature- and food-dependent behavioral states through DMSR-1.[82] FLP-6 and its receptor EGL-6 were also shown to regulate these behavioral states, while activation of the same EGL-6 receptor by other FLP peptides (FLP-10 and FLP-17) inhibits egg-laying behavior.[82,121] These findings suggest that promiscuous peptide GPCRs can be involved in the regulation of different behaviors *in vivo* by interacting with different ligands. RFamide peptides that activate many specific GPCRs, such as FLP-1 and several of its receptors, are also involved in multiple biological processes.[45,85,122,123] In nematodes, these are among the most highly conserved RFamide peptide genes (e.g., *flp-1* and *flp-14*),[111] which during evolution may have acquired multiple functions mediated by different receptors. Although more functional studies of peptide-GPCR couples are needed, distinct types of interactions in the peptidergic network may serve specialized functions. We speculate that peptide-GPCR couples with complex or promiscuous interactions may be important for orchestrating global states and cellular activity by integrating or broadcasting peptidergic signals. Unique peptide-receptor couples, on the other hand, may have important roles in mediating specific local or long-range signaling.

Finally, multiple bioactive peptides are often encoded within the same peptide precursor. These peptides can activate different receptors, as has been described for the mammalian pro-opiomelanocortin (POMC) precursor.[124] In *C. elegans*, we find that different peptides from a single peptide-encoding gene often interact with the same receptor(s). As peptidergic communication is limited by diffusion, encoding multiple ligands of a single receptor may amplify signaling by increasing the concentration of secreted peptide ligands.[105] However, we also identify peptide genes encoding ligands for different receptors, which may diversify signaling.

Evolution of peptidergic signaling systems

Because biologically active peptides typically have short sequences and co-evolve with their receptors, identifying orthology between peptidergic systems from distant animals remains a challenge. Annotations of the first invertebrate genome sequences revealed a large number of GPCRs that resembled vertebrate peptide receptors but relatively few vertebrate-like peptides.[125–127] Initially, this led to the assumption that vertebrate peptidergic systems are not well conserved in *C. elegans* and other invertebrates. The increasing number of available genome sequences, however, has enhanced our knowledge on the long-range evolution of peptidergic systems,[1,5,31,88,93,94] revealing deep conservation of these systems across bilaterian animals, which in some cases has been confirmed by

biochemical interactions.[31,52,88] Our dataset provides further evidence for orthologs of at least 14 bilaterian peptidergic systems in *C. elegans* (Table S9). In addition, we clarify the diversification of specific peptide-receptor families in nematodes. Indeed, many peptide receptors that we characterized belong to expanded GPCR families, for which specific ligands could not have been predicted based on available data, as the corresponding peptide families (e.g., RFamide, sNPF, and somatostatin-like peptides) also diversified in nematodes.[31,101]

Our phylogenetic analysis suggests that additional peptide ligands remain to be discovered in *C. elegans*. For example, we identified a large family of orphan nematode GPCRs that may be activated by potentially unknown peptides (cluster 39, Table S9). Likewise, we discovered two orphan receptor families related to the *Drosophila* trissin and CNMamide receptors (clusters 35 and 36, Table S9) but orthologous peptides have not been identified in nematodes so far.[128,129] Peptidomics and comparative genomic studies also identified novel peptides in *C. elegans*.[59,130] Peptide discovery by these and other approaches, such as machine learning methods,[29,131] will facilitate future deorphanizations.

In addition, this resource can facilitate predictions of peptide-GPCR couples in other species, such as parasitic nematodes. Conservation of peptide-receptor interactions has been shown between *C. elegans* and nematode parasites,[132,133] and peptidergic signaling regulates key aspects of nematode biology, such as reproduction, development, and locomotion.[36,130,134,135] The discovery of peptide-GPCR pairs in parasitic species may thus provide promising leads for the development of novel anthelminthics. Comparisons of peptide-GPCR pairs between nematode species may also further clarify the diversification and ligand promiscuity of GPCRs.

Limitations of the study

Although our system-wide screen more than doubles the number of characterized peptide GPCRs in *C. elegans*, this peptide-receptor interaction map is incomplete for several reasons. First, we did not identify ligands for all known and predicted peptide receptors, likely because not all GPCRs are functionally expressed in the heterologous system that we used. There are several possible explanations for this lack of functionality, including improper folding or trafficking, insufficient membrane expression, or the requirement of specific co-factors, such as receptor-activity modifying proteins (RAMPs).[136,137] We tested untagged receptors, as tagging might interfere with ligand-GPCR signaling. However, GPCR tagging strategies could be used in future studies to verify membrane expression in heterologous cells. Alternatively, the remaining orphan receptors may be activated by other ligands that were not included in our library or remain to be identified. Likewise, some peptides may interact with other GPCRs than those in our screen or with other types of receptors, such as ion channels.[120] Second, the promiscuous $G\alpha_{16}$ subunit couples many but not all GPCRs to the PLCβ pathway and is less effective in linking G_i-coupled receptors to the release of intracellular calcium than, for example, chimeric Gα proteins.[76,138] Co-expressed Ga proteins may also influence the EC_{50} values of ligand-GPCR interactions,[138] which potentially differ from those *in vivo*. Finally, GPCRs can show constitutive, ligand-independent activity or signal via G protein-independent pathways,[136,139] which are not

captured by our readout. Different heterologous systems and screening strategies could be combined in future studies, for example, using chimeric G proteins and other readouts of key signaling events in the receptor activation cascade,[46,104,138,140,141] to further complete the peptide-receptor network.

Star⋆Methods

Key Resources Table

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Bacterial and virus strains		
E. coli: Strain OP50	*Caenorhabditis* Genetics Center	WB OP50; RRID:WB-STRAIN:OP50
Chemicals, peptides, and recombinant proteins		
See Table S3 for a list of peptides	GL Biochem Ltd. and ThermoFisher Scientific	N/A
1% Penicilin/Streptomycin Mixture	Gibco	Cat#15140-122
Zeocin selection reagent	Invitrogen	Cat#R25001
Puromycin	Sigma-Aldrich	Cat#P8833
Fetal Bovine Serum	Sigma-Aldrich	Cat#F7524
Lipofectamine LTX, Plus Reagent	Invitrogen	Cat#15338–500, Cat#11514015
Coelenterazin H	Invitrogen	Cat#C6780
Critical commercial assays		
MycoAlert Mycoplasma Detection Kit	Lonza	Cat#LT07-418
Steady Plus Reporter Gene Assay System	PerkinElmer	Cat#6066751
T3 mMessage mMachine transcription kit	Thermo Fisher Scientific	Cat#AM1348
GeneJET RNA purification kit	Thermo Fisher Scientific	Cat#K0731
Experimental models: Cell lines		
CHO/mtAEQ/Gα_{16}	PerkinElmer	ES-000-A24
CHO/mtAEQ	PerkinElmer	ES-000-A12
HEK 293T	ATCC	Cat#CRL-11268
Experimental models: Organisms/strains		
C. elegans: N2 Bristol wild type	*Caenorhabditis* Genetics Center	N2 Bristol
Oligonucleotides		
See Table S2 for a list of primers	Integrated DNA Technologies	N/A
Software and algorithms		
Prism	Graphpad	https://www.graphpad.com/scientific-software/prism/
ScreenWorks System control software	Molecular Devices	FLIPR Tetra control program
MicroBeta control software	PerkinElmer	Microbeta control program
RoboCyte2 control software	Multichannel systems	Roboocyte2 control program
RoboCyte2+ analysis software	Multichannel systems	Roboocyte2+ analysis program
Other		
Xenopus laevis oocytes	EcoCyte Bioscience	https://ecocyte-us.com/products/xenopus-oocyte-delivery-service/

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Resource Availability

Lead contact—Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Isabel Beets (isabel.beets@kuleuven.be).

Materials availability—The HEK 293T and CHO-K1 cell lines stably expressing mitochondrial-targeted apo-aequorin are under MTA and cannot be freely distributed.

Experimental Model and Subject Details

***C. elegans* maintenance—**The wild-type *C. elegans* (N2 Bristol) strain was obtained from the *Caenorhabditis* Genetics Center (University of Minnesota). Worms were cultivated at 20°C on nematode growth medium (NGM) plates seeded with *E. coli* OP50 bacteria and used for mRNA extraction and subsequent cDNA synthesis. cDNA prepared from mixed-stage culture plates was used as template for PCR of peptide GPCR coding sequences.

Microbe strains—The *Escherichia coli* OP50 strain was used as a food source for *C. elegans*.

Cell lines—CHO-K1 cells stably expressing mitochondrial-targeted apo-aequorin and a promiscuous human $G\alpha_{16}$ protein were used for reverse pharmacology screening (CHO/mtAEQ/$G\alpha_{16}$, ES-000-A24, PerkinElmer). Calcium measurements in the absence of $G\alpha_{16}$ were done in CHO cells that stably express only apo-aequorin (CHO/mtAEQ, ES-000-A12, PerkinElmer). Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM)/Nutrient Mixture F-12 Ham (Sigma-Aldrich), supplemented with 10% fetal bovine serum (FBS, Sigma-Aldrich) and 1% penicillin/streptomycin mixture (10.000 units/ml penicillin and 10 mg/mL streptomycin, Gibco). Growth medium was supplemented with 250 μg/mL zeocin or 5 μg/mL puromycin, which serves as a selection reagent for CHO cells with or without the $G\alpha_{16}$ subunit, respectively. For cAMP-based receptor activation assays, GPCRs were transiently expressed in HEK 293T cells cultured in DMEM/high glucose supplemented with 10% FBS and 1% penicillin/streptomycin. All cells were grown in stable conditions of 37°C, 5% CO_2 and high relative humidity, unless otherwise indicated. Mycoplasma tests were performed using the MycoAlert Mycoplasma Detection Kit (Lonza) to verify that cells were free of mycoplasma contamination.

***Xenopus laevis* oocytes—**Defolliculated *Xenopus Laevis* oocytes were sourced from EcoCyte Bioscience and maintained in ND96 solution (in mM: 96 NaCl, 1 MgCl2, 5 HEPES, 1.8 CaCl2, 2 KCl) at 16°C until RNA injection.

Method Details

Cloning of peptide GPCR candidates—A list of peptide GPCR candidates (Table S2) was compiled from *in silico* searches for putative peptide GPCR genes in the *C. elegans* genome.[36,61,66,67] Oligonucleotide primers for amplifying receptor cDNAs (Table S2) were designed based on gene models in Wormbase (http://wormbase.org, version WS240). Only receptors with a seven transmembrane topology, as predicted from the translated cDNA sequence (http://www.cbs.dtu.dk/services/TMHMM), were considered as peptide GPCR candidates. Forward primers included a 'CACC' sequence at the 5′ end

that introduces a partial Kozak sequence for increased translation efficiency in mammalian cells. Receptor cDNAs were amplified by PCR with Q5 High-Fidelity DNA Polymerase (New England Biolabs) from cDNA of mixed-stage populations of wild-type *C. elegans*, and were cloned in a pcDNA3.1 vector (ThermoFisher Scientific). Plasmids for C16D6.2, T14B1.2,[42] F59D12.2, and C54A12.2[40] were kind gifts from Dr. Derek Sieburth (University of Southern California, Los Angeles, USA), Dr. Rachel McMullan (The Open University, Milton Keynes, UK), and Dr. David Raizen (University of Pennsylvania, Philadelphia, USA). All plasmids were verified by sequencing of the full receptor cDNA and purified with an EndoFree Plasmid Maxi Kit (Qiagen). cDNA sequences of all GPCR variants cloned in pcDNA3.1 are summarized in Data S1.

Peptide library composition and synthesis—A library of 344 synthetic peptides was generated for reverse pharmacology screening of *C. elegans* peptide GPCRs. For this, we compiled a list of known *C. elegans* peptide precursors of the FLP and NLP families by assembling peptide sequences from pepti-domics studies and *in silico* searches in the *C. elegans* genome.[5,6,31,60,86,96,97,99,102,127,135,142–155] We assembled 94 precursors and searched each of them for peptide sequences identified by peptidomics or flanked by mono- or dibasic cleavage sites for proprotein convertases (RK, RR, KR, KK or [RK]-$X_{2/4/6/8}$-[RK]).[99] This search yielded 344 mature peptide sequences. Replicates of the identified peptides were synthesized as crude peptides (40%–70% purity) by GL Biochem Ltd. and ThermoFisher Scientific, and were chemically modified to match post-translational modifications (e.g., disulfide bridge formation, C-terminal amidation or N-terminal pyro-glutamation) that are commonly found in bioactive peptides.[59] Peptides carrying a disulfide bridge were synthesized at >95% purity. Peptide stocks were reconstituted in 80% acetonitrile and vortexed. If needed, peptide solutions were sonicated to enhance solubilization, and hydrophobic peptides were first dissolved in small amounts of solvent and then diluted using water. Sequences of all synthetic peptides in the library are listed in Table S3.

Transfection of CHO and HEK cells—GPCRs were heterologously expressed in CHO or HEK cells by transient transfection. CHO cells, used in aequorin-based GPCR activation assays, were transfected with GPCR/pcDNA3.1 plasmids when cells reached 60–70% confluency, using Lipofectamine LTX with Plus Reagent (Invitrogen) in serum-free medium. Shifting the cultivation temperature from 37°C to 28°C, at 24h after transfection, was shown to increase the response of *C. elegans* peptide GPCRs in CHO cells.[92,156,157] Transfected cells were therefore supplemented with complete growth medium the day after transfection and transferred to an incubator at 28°C 16 h prior to cell harvesting for the GPCR activation assay.

HEK 293T cells, used for cAMP-based GPCR activation assays, were co-transfected with the cAMP indicator CRE(6x)-luciferase and GPCR/pcDNA3.1 plasmids in a 1:1 ratio when cells reached 60–70% confluency. Transfection medium contained Opti-MEM (Gibco), CRE(6x)-luciferase and receptor plasmid DNA, Plus Reagent (Invitrogen), and Lipofectamine LTX (Invitrogen). One day post-transfection, fresh culture medium was

added and cells were kept at 37°C. Two days post-transfection, the cAMP reporter assay was performed.

Aequorin-based GPCR activation assay—We used an aequorin-based calcium mobilization assay to identify and validate peptide-GPCR couples in the synthetic *C. elegans* peptide library (in CHO/mtAEQ/Gα_{16} cells). Two days after transfection, CHO cells were detached and collected in phosphate buffered saline (PBS) containing 0.2% EDTA. Viable cells were quantified using a Biorad TC20 Cell counter. Cells were pelleted by 5 min centrifugation at 800 rpm at room temperature and resuspended in DMEM/BSA (DMEM/F-12 without phenol red, with L-glutamine and 10 mM HEPES, 0.1% bovine serum albumin; Gibco, Thermo Fisher Scientific) at a concentration of 5 3 10^6 cells/ml. Coelenterazine H (Invitrogen) was added at a final concentration of 2.5 μM, and cells were gently shaken at room temperature for 4 h in the dark, allowing the aequorin holoenzyme to be reconstituted. After a 10-fold dilution in DMEM/BSA, cells were incubated for 30 min before starting the assay.

Library screening was performed on an FLIPR Tetra High-Throughput Cellular Screening System with ScreenWorks software (Molecular Devices). Using this system, cells were transferred to 96-well plates (25,000 cells/well) containing 50 μL of synthetic peptide, dissolved in DMEM/BSA, and calcium responses were simultaneously monitored for 36 s. Peptides were individually tested in black 96-well plates with clear bottom (Greiner Bio One) at a final concentration of 10 μM. Wells containing 50 μL DMEM/BSA were used as a negative control. ATP (1.5 μM in DMEM/BSA), which activates an endogenous receptor in CHO cells, was used asa positive control. After measuring peptide-evoked responses, 50 μL of Triton X-100 (0.2% in DMEM/BSA) was added to lyse the cells and obtain a measure of the maximum calcium response.

Selection of putative peptide hits—Peptide-GPCR interactions were initially tested at high peptide concentrations (10 μM) in luminescence-based GPCR activation screens with a synthetic peptide library. From this data, we selected putative receptor ligands by manually inspecting relative light unit (RLU) plots of calcium responses and by ranking peptides based on standardized Z scores. We integrated peptide-evoked calcium responses over time and normalized the signals per assay plate and subsequently per receptor to compute an activation value for each peptide-GPCR pair. Specifically, we performed Z score normalization on the integrated RLU signal using the mean and variance of the on-plate negative controls to obtain an activation value for each peptide-GPCR pair. Next, we standardized the Z scores for each GPCR by dividing by its maximal activation Z score, and log2-transformed this fraction to rank individual peptide-GPCR couples. Based on the scores obtained for known peptide-GPCR interactions, we considered all peptide-GPCR couples with a strong calcium response over background (Z score >20), reaching at least 40% of that receptor's maximum activation (log2-standardized Z > -1/2), as potential hits in the peptide library. When the top hit (log2-standardized Z =0) could not be validated in dose-response tests, Z scores for that receptor were restandardized to the next top hit in the library screen and dose-response assays were performed for all peptides matching the selection criteria above. The NLP-2-1 synthetic peptide was excluded from the standardization procedure

(log2-standardized Z was set to NA), as it produced a signal (Z score ≥5) in 136 out of 176 assays and was therefore considered to be a likely false positive.

When concentration-response measurements validated a peptide-receptor pair, all peptides derived from the same peptide precursor were systematically tested in concentration-response assays. An exception was made for GPCRs with peptide hits originating from more than 10 different FLP precursor proteins (DMSR-1, DMSR-7, and FRPR-8). For these promiscuous receptors, at least one FLP peptide from each FLP precursor was tested for concentration-dependent receptor activation.

Concentration-response measurements—All putative peptide hits identified in library screens were further tested in concentration-response assays (in CHO/mtAEQ/Gα_{16} cells). Crude peptides were first purified by reversed phase HPLC on a Symmetry-C8 Prep Column (7.83300 mm HPLC cartridge with pore size of 100 Å and particle size of 7 μM), typically yielding peptide stocks of >95% purity. Peptide masses of purified peptides were verified by MALDI-TOF/TOF mass spectrometry on a rapifleX MALDI Tissuetyper (Bruker Daltonic) and peptide concentrations were measured using the bicinchoninic acid (BCA) protein assay.

Purified peptides were tested in concentration series using the aequorin-based GPCR activation assay on a MicroBeta2 LumiJET luminometer with MicroBeta control software (PerkinElmer). Positive and negative controls were similar to those used in library screens. Calcium responses were measured for 30 s after adding cells to the compound plate. After the addition of Triton X-100 (0.2% in DMEM/BSA), a maximum response per well was measured for 30s, which was used for normalization of peptide-evoked calcium responses.

Concentration-response measurements were performed in triplicate on at least two independent days. For each peptide concentration and replicate, calcium responses were first normalized to the maximum calcium response (sum of peptide- and Triton-evoked response) and subtracted with the negative control (BSA) value. Next, a relative calcium response (%) compared to the maximum peptide-evoked response (100% activation) was calculated. Concentration-response data were fitted in function of log[peptide] using GraphPad Prism 5 or 7. EC_{50} values were calculated from concentration-response curves by fitting a 3- or 4-parameter concentration-response curve.

Calcium-based assay in the absence of Gα_{16}—Calcium signaling in the absence of Gα_{16} was measured in CHO/mtAEQ cells using the aequorin-based GPCR activation assay. Peptides were dissolved in DMEM/BSA and 50 μL of the peptide solution was added to the wells of a white flat bottom 96-well plate. Wells containing DMEM/BSA were used as a negative control, while wells containing 1.5 μM ATP were used as a positive control. Incubated cells were added to the wells at a density of 25,000 cells/well and luminescence was monitored for 30 s on a MicroBeta2 LumiJET luminometer (PerkinElmer). After 30 s, 0.2% Triton X-100 dissolved in DMEM/BSA was added to lyse the cells and light emission was recorded for another 30 s. Light emission from each well was calculated relative to the total calcium response (ligand + Triton X-100). Assays were done on at least two independent days in triplicate.

cAMP-based GPCR activation assay—On the day of the assay, cells were detached, counted, pelleted, and resuspended in 200 μM 3-isobutyl-1-methylxanthine (IBMX) medium. Peptides were also dissolved in IBMX medium and 50 μL of the peptide solution was added to the wells of a white flat bottom 96-well plate. Wells containing 50 μL IBMX medium, without peptide, were used as negative control. Each well was supplemented with 50 μL of the cell suspension (50,000 cells/well) and the plate was incubated at 37°C for 3.5 h. Cells were then loaded with 100 μL SteadyLitePlus substrate (PerkinElmer) and incubated on a shaking plate for 15 min under dark conditions at RT. Finally, luminescence was measured twice for 5s (at 0s and 5s) per well at 469 nm on a Mithras LB940 luminometer (Berthold Technologies). Measurements were performed in triplicate on at least two independent days.

Two-electrode voltage clamp (TEVC) recording—Plasmids for expressing *C. elegans* GPCRs in *Xenopus laevis* oocytes were generated using the KSM backbone. cDNA sequences of *dmsr-1a* (DMSR-1-1), *dmsr-1b* (DMSR-1-2), and *dmsr-7* (DMSR-7-1) were inserted into the KSM vector, which contains *Xenopus* β-globin UTR regions and a T3 promoter, using HiFi assembly. Mouse G protein inward rectifying potassium channels, mGIRK1 (NM_001355118.1) and mGIRK2 (NM_001025584.2), and the mouse muscarinic GPCR M2 (NM_001411688.1) were prepared from mouse cDNA as previously described.[158]

Prior to RNA injection, KSM plasmids were linearised using NotI and 5′-capped cRNA was synthesized *in vitro* using the T3 mMessage mMachine transcription kit (Thermo Fischer Scientific). RNA was then purified using the GeneJET RNA purification kit (Thermo Fischer Scientific). The defolliculated *Xenopus* oocytes were placed individually into V-bottom 96 well plates and injected with RNA using the Roboinject system (Multi Channel Systems GmbH). For each oocyte a total of 10 ng RNA was injected for the GPCR and 7.5 ng RNA for mGIRK1 and mGIRK2 each. All constructs were injected in one mix and the expression of the GIRKs was used to determine if the injection had been successful. After injection, the oocytes were incubated for 2 days in ND96 at 16°C until recording began.

Two-electrode voltage-clamp recordings were performed using the Robocyte2 recording system (Multi Channel Systems). Recording electrodes were purchased from Multichannel systems and typically had a resistance of 0.7–2 MΩ. The pipettes were filled with 1.5 M KCl and 1 M acetic acid. Oocytes were clamped at -80 mV and continuous recordings were done at 500 Hz. All recordings were collected using the Robocyte2 control software and the data was extracted using the Robocyte2+ analysis software.

DMSR-7 promiscuity analysis was performed by first perfusing the oocytes with ND96 to estimate resting potential of the oocytes. The high K^+ solution (96 mM KaCl, 1 mM MgCl2, 5 mM HEPES, 1.8 mM CaCl2, 2 mM NaCl) was then applied for 40s to open the GIRK channels and get their baseline current. Peptide solutions were prepared in the High K^+ solution and then subsequently applied for 20s. The ratio between the baseline High K^+ current and the Peptide/High K^+ current was used to determine the '% GIRK activation'. The oocytes were then perfused with ND96 again to bring the oocyte back to

resting potential. Each application of the peptide was performed on an individual oocyte to ensure desensitisation of the GPCR did not affect the results.

Concentration-response analysis of DMSR-1 isoforms was performed in a similar manner with an initial baseline High K^+ application for 40s, and then increasing concentrations of the peptide in High K^+ was applied stepwise for 20s each, without a wash period in between. The protocol ended with a 30s ND96 washout to bring the oocyte back to resting potential. Data was normalised by calculating the I/Imax for each oocyte. Normalized mean, SD and n numbers were imported into GraphPad where data was plotted and EC_{50} values were calculated by fitting to the Hill equation using three parameter slopes to obtain the highest degree of fit.

To determine the G protein coupling of DMSR-7, on the recording day half the oocytes were injected with 50 pg pertussis toxin (PTX) and incubated at room temperature for 6 h. The TEVC recordings were performed and analyzed as described above for the promiscuity experiments using FLP-1-6. M2 is a known $G_{i/o}$-coupled GPCR and was used as a control.

Bipartite network analysis—Bipartite graphs were constructed for all FLP and NLP peptide-GPCR couples with sub-micromolar EC_{50} values (<1 μM) identified in this resource (Table S6). Promiscuous receptors were assumed to interact with all peptides from the same peptide precursor unless our results indicate otherwise. Monopartite network projections were generated and analyzed using custom scripts in MATLAB; scripts are available upon request, MATLAB version 9.10.0.1602886 (R2021a). Modules were defined using a popular consensus clustering approach,[159] with the Louvain community detection algorithm as implemented in the Brain Connectivity Toolbox.[160,161] The Fruchterman-Reingold force-directed layout algorithm was used to layout graphs in 2D space for visualisation.[162]

Peptide sequence alignments—Pairwise peptide sequence alignment scores in Figure S9 were generated using the *stringDist* method from the R/Bioconducor package Biostrings. The BLOSUM50 substitution matrix was used with gap opening and gap extension penalties set to 13 and 2, respectively.

Phylogenetic analysis—We first drew a list of 150 *C elegans* sequences predicted to be peptide GPCRs (Table S8B). We then added to this list the predicted peptide GPCRs from non-nematode bilaterian genomes of *Homo sapiens* (Vertebrata), *Takifugu rubripes* (Vertebrata), *Branchiostoma floridae* (Cephalochordata), *Saccoglossus kowalevskii* (Hemichordata), *Strongylocentrotus purpuratus* (Echinodermata), *Capitella teleta* (Annelida), *Lottia gigantea* (Mollusca), *Dapnia pulex* (Arthropoda) and *Drosophila melanogaster* (Arthropoda) and those from the nematode species *Strongyloides ratti*, *Trichinella spiralis*, *Brugia malayi*, *Pristionchus pacificus, Loa loa* and *Onchocercus volvulus* downloaded from the Ensembl *metazoan* database, using the strategy described in Mirabeau and Joly, 2013.[31] We complemented these sequences with the predicted peptide GPCRs from the complete set of nematode ESTs available from NCBI (https://www.ncbi.nlm.nih.gov/nucest). All nematode species used in the construction of the phylogenetic tree are listed in Table S8A.

We retrieved 150 secretin-like and 1963 rhodopsin-like GPCRs for this analysis (Tables S8C and S8D) and annotated each bilaterian sequence that was included in Mirabeau and Joly, 2013 for its membership in a conserved bilaterian group (for example, the *Capitella* sequence jgi|Capca1|219484|estExt_fgenesh1_pg.C_20057 was annotated as jgi|Capca1|219484|estExt_fgenesh1_ pg.C_20057_Calc/DH31). We then constructed two separate phylogenetic trees with the SATé -II maximum-likelihood algorithm,[163,164] with default settings (Aligner:MAFFT,[165] Merger:MUSCLE,[166] Tree Estimator:FASTTREE,[164] substitution model:GTR+G20, Maximum size subproblem:50%, Decomposition:Centroid, Stop after Last Improvement). Branch support values were generated using the implementation of the Shimodaira-Hasegawa test of FASTTREE, displayed as “label” in supplemental tree data.

Quantification and Statistical Analysis

Tests used to determine statistical significance were one-way ANOVA with Dunnett’s multiple comparisons test (cAMP-based reporter assays), the Kruskal-Wallis test with Dunn’s test for multiple comparisons (calcium-based reporter and oocyte assays), the Pearson’s Chi-squared test (GPCR activation probability), two-way ANOVA with Tukey post-hoc test (empty vector experiments), and the Mann-Whitney test (PTX-assays in oocytes). Statistical information for each experiment can be found in the corresponding figure legend.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Data and code availability

- All data generated in this study is reported in this paper and will be shared by the lead contact upon request.
- This paper does not report original code.
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

References

1. Yañez-Guerra LA, Thiel D, Jékely G. Premetazoan origin of neuropeptide signaling. Mol Biol Evol. 2022; 39 msac051 doi: 10.1093/molbev/msac051 [PubMed: 35277960]

2. Schoofs L, De Loof A, Van Hiel MB. Neuropeptides as regulators of behavior in insects. Annu Rev Entomol. 2017; 62: 35–52. DOI: 10.1146/annurev-ento-031616-035500 [PubMed: 27813667]
3. Van Damme S, De Fruyt N, Watteyne J, Kenis S, Peymen K, Schoofs L, Beets I. Neuromodulatory pathways in learning and memory: Lessons from invertebrates. J Neuroendocrinol. 2021; 33 e12911 doi: 10.1111/jne.12911 [PubMed: 33350018]
4. Nässel DR, Zandawala M. Endocrine cybernetics: neuropeptides as molecular switches in behavioural decisions. Open Biol. 2022; 12 220174 doi: 10.1098/rsob.220174 [PubMed: 35892199]
5. Van Sinay E, Mirabeau O, Depuydt G, Van Hiel MB, Peymen K, Watteyne J, Zels S, Schoofs L, Beets I. Evolutionarily conserved TRH neuropeptide pathway regulates growth in *Caenorhabditis elegans*. Proc Natl Acad Sci USA. 2017; 114: E4065–E4074. DOI: 10.1073/pnas.1617392114 [PubMed: 28461507]
6. Beets I, Janssen T, Meelkop E, Temmerman L, Suetens N, Rademakers S, Jansen G, Schoofs L. Vasopressin/oxytocin-related signaling regulates gustatory associative learning in *C. elegans*. Science. 2012; 338: 543–545. DOI: 10.1126/science.1226860 [PubMed: 23112336]
7. Taghert PH, Nitabach MN. Peptide neuromodulation in invertebrate model systems. Neuron. 2012; 76: 82–97. DOI: 10.1016/j.neuron.2012.08.035 [PubMed: 23040808]
8. Hauser AS, Attwood MM, Rask-Andersen M, Schiöth HB, Gloriam DE. Trends in GPCR drug discovery: new agents, targets and indications. Nat Rev Drug Discov. 2017; 16: 829–842. DOI: 10.1038/nrd.2017.178 [PubMed: 29075003]
9. Edvinsson L, Haanes KA, Warfvinge K, Krause DN. CGRP as the target of new migraine therapies — successful translation from bench to clinic. Nat Rev Neurol. 2018; 14: 338–350. DOI: 10.1038/s41582-018-0003-1 [PubMed: 29691490]
10. Azam S, Haque ME, Jakaria M, Jo SH, Kim IS, Choi DK. G-protein-coupled receptors in CNS: A potential therapeutic target for intervention in neurodegenerative disorders and associated cognitive deficits. Cells. 2020; 9: 506. doi: 10.3390/cells9020506 [PubMed: 32102186]
11. Doijen J, Van Loy T, Landuyt B, Luyten W, Schols D, Schoofs L. Advantages and shortcomings of cell-based electrical impedance measurements as a GPCR drug discovery tool. Biosens Bioelectron. 2019; 137: 33–44. DOI: 10.1016/j.bios.2019.04.041 [PubMed: 31077988]
12. Li C, Wu X, Liu S, Zhao Y, Zhu J, Liu K. Roles of neuropeptide Y in neurodegenerative and neuroimmune diseases. Front Neurosci. 2019; 13: 869. doi: 10.3389/fnins.2019.00869 [PubMed: 31481869]
13. Shen YC, Sun X, Li L, Zhang HY, Huang ZL, Wang YQ. Roles of neuropeptides in sleep-wake regulation. Int J Mol Sci. 2022; 23: 4599. doi: 10.3390/ijms23094599 [PubMed: 35562990]
14. Neugebauer V, Mazzitelli M, Cragg B, Ji G, Navratilova E, Por-reca F. Amygdala, neuropeptides, and chronic pain-related affective behaviors. Neuropharmacology. 2020; 170 108052 doi: 10.1016/j.neuropharm.2020.108052 [PubMed: 32188569]
15. Melzer S, Newmark ER, Mizuno GO, Hyun M, Philson AC, Quiroli E, Righetti B, Gregory MR, Huang KW, Levasseur J, et al. Bombesin-like peptide recruits disinhibitory cortical circuits and enhances fear memories. Cell. 2021; 184: 5622–5634. e25 doi: 10.1016/j.cell.2021.09.013 [PubMed: 34610277]
16. Lin HH, Kuang MC, Hossain I, Xuan Y, Beebe L, Shepherd AK, Rolandi M, Wang JW. A nutrient-specific gut hormone arbitrates between courtship and feeding. Nature. 2022; 602: 632–638. DOI: 10.1038/s41586-022-04408-7 [PubMed: 35140404]
17. Zitnik GA. Control of arousal through neuropeptide afferents of the locus coeruleus. Brain Res. 2016; 1641: 338–350. DOI: 10.1016/j.brainres.2015.12.010 [PubMed: 26688115]
18. Tinoco AB, Barreiro-Iglesias A, Yañez Guerra LA, Delroisse J, Zhang Y, Gunner EF, Zampronio CG, Jones AM, Egertová M, Elphick MR. Ancient role of sulfakinin/cholecystokinin-type signalling in inhibitory regulation of feeding processes revealed in an echinoderm. Elife. 2021; 10 e65667 doi: 10.7554/elife.65667 [PubMed: 34488941]
19. Gäde G, Hoffmann KH. Neuropeptides regulating development and reproduction in insects. Physiol Entomol. 2005; 30: 103–121. DOI: 10.1111/j.1365-3032.2005.00442.x
20. Smith SJ, Sümbül U, Graybuck LT, Collman F, Seshamani S, Gala R, Gliko O, Elabbady L, Miller JA, Bakken TE, et al. Single-cell transcriptomic evidence for dense intracortical neuropeptide networks. Elife. 2019; 8 e47889 doi: 10.7554/elife.47889 [PubMed: 31710287]

21. Taylor SR, Santpere G, Weinreb A, Barrett A, Reilly MB, Xu C, Varol E, Oikonomou P, Glenwinkel L, McWhirter R, et al. Molecular topography of an entire nervous system. Cell. 2021; 184: 4329–4347. e23 doi: 10.1016/j.cell.2021.06.023 [PubMed: 34237253]
22. Zhong W, Barde S, Mitsios N, Adori C, Oksvold P, Feilitzen Kv, O'Leary L, Csiba L, Hortobágyi T, Szocsics P, et al. The neuropeptide landscape of human prefrontal cortex. Proc Natl Acad Sci USA. 2022; 119 e2123146119 doi: 10.1073/pnas.2123146119 [PubMed: 35947618]
23. Williams EA, Verasztó C, Jasek S, Conzelmann M, Shahidi R, Bauknecht P, Mirabeau O, Jékely G. Synaptic and peptidergic connectome of a neurosecretory center in the annelid brain. Elife. 2017; 6: 503. doi: 10.7554/elife.26349
24. Civelli O. Orphan GPCRs and neuromodulation. Neuron. 2012; 76: 12–21. DOI: 10.1016/j.neuron.2012.09.009 [PubMed: 23040803]
25. Lai JP, Lai S, Tuluc F, Tansky MF, Kilpatrick LE, Leeman SE, Douglas SD. Differences in the length of the carboxyl terminus mediate functional properties of neurokinin-1 receptor. Proc Natl Acad Sci USA. 2008; 105: 12605–12610. DOI: 10.1073/pnas.0806632105 [PubMed: 18713853]
26. Marti-Solano M, Crilly SE, Malinverni D, Munk C, Harris M, Pearce A, Quon T, Mackenzie AE, Wang X, Peng J, et al. Combinatorial expression of GPCR isoforms affects signalling and drug responses. Nature. 2020; 587: 650–656. DOI: 10.1038/s41586-020-2888-2 [PubMed: 33149304]
27. Martelli C, Pech U, Kobbenbring S, Pauls D, Bahl B, Sommer MV, Pooryasin A, Barth J, Arias CWP, Vassiliou C, et al. SIFamide translates hunger signals into appetitive and feeding behavior in *Drosophila*. Cell Rep. 2017; 20: 464–478. DOI: 10.1016/j.celrep.2017.06.043 [PubMed: 28700946]
28. Mena W, Diegelmann S, Wegener C, Ewer J. Stereotyped responses of *Drosophila* peptidergic neuronal ensemble depend on downstream neuromodulators. Elife. 2016; 5 e19686 doi: 10.7554/elife.19686 [PubMed: 27976997]
29. Foster SR, Hauser AS, Vedel L, Strachan RT, Huang XP, Gavin AC, Shah SD, Nayak AP, Haugaard-Kedström LM, Penn RB, et al. Discovery of human signaling systems: Pairing peptides to G protein-coupled receptors. Cell. 2019; 179: 895–908. e21 doi: 10.1016/j.cell.2019.10.010 [PubMed: 31675498]
30. Bentley B, Branicky R, Barnes CL, Chew YL, Yemini E, Bullmore ET, Vértes PE, Schafer WR. The multilayer connectome of *Caenorhabditis elegans*. PLoS Comput Biol. 2016; 12 e1005283 doi: 10.1371/journal.pcbi.1005283 [PubMed: 27984591]
31. Mirabeau O, Joly JS. Molecular evolution of peptidergic signaling systems in bilaterians. Proc Natl Acad Sci USA. 2013; 110: E2028–E2037. DOI: 10.1073/pnas.1219956110 [PubMed: 23671109]
32. vanden Pol AN. Neuropeptide transmission in brain circuits. Neuron. 2012; 76: 98–115. DOI: 10.1016/j.neuron.2012.09.014 [PubMed: 23040809]
33. Civelli O, Reinscheid RK, Zhang Y, Wang Z, Fredriksson R, Schioth HB. G protein-coupled receptor deorphanizations. Annu Rev Pharmacol Toxicol. 2013; 53: 127–146. DOI: 10.1146/annurev-pharmtox-010611-134548 [PubMed: 23020293]
34. Caers J, Verlinden H, Zels S, Vandersmissen HP, Vuerinckx K, Schoofs L. More than two decades of research on insect neuropeptide GPCRs: an overview. Front Endocrinol. 2012; 3: 151. doi: 10.3389/fendo.2012.00151
35. Jiang H, Lkhagva A, Daubnerová I, Chae H-S, Šimo L, Jung SH, Yoon YK, Lee NR, Seong JY, Žitňan D, et al. Natalisin, a tachykinin-like signaling system, regulates sexual activity and fecundity in insects. Proc Natl Acad Sci USA. 2013; 110: E3526–E3534. DOI: 10.1073/pnas.1310676110 [PubMed: 23980168]
36. Frooninckx L, Van Rompay L, Temmerman L, Van Sinay E, Beets I, Janssen T, Husson SJ, Schoofs L. Neuropeptide GPCRs in *C. elegans*. Front Endocrinol. 2012; 3: 167. doi: 10.3389/fendo.2012.00167
37. Cheong MC, Artyukhin AB, You YJ, Avery L. An opioidlike system regulating feeding behavior in *C. elegans*. Elife. 2015; 4 e06683 doi: 10.7554/elife.06683 [PubMed: 25898004]
38. Ezcurra M, Walker DS, Beets I, Swoboda P, Schafer WR. Neuropeptidergic signaling and active feeding state inhibit nociception in *Caenorhabditis elegans*. J Neurosci. 2016; 36: 3157–3169. DOI: 10.1523/jneurosci.1128-15.2016 [PubMed: 26985027]

39. Iannacone MJ, Beets I, Lopes LE, Churgin MA, Fang-Yen C, Nelson MD, Schoofs L, Raizen DM. The RFamide receptor DMSR-1 regulates stress-induced sleep in *C. elegans*. Elife. 2017; 6 e19837 doi: 10.7554/elife.19837 [PubMed: 28094002]
40. Nelson MD, Janssen T, York N, Lee KH, Schoofs L, Raizen DM. FRPR-4 is a G-protein coupled neuropeptide receptor that regulates behavioral quiescence and posture in *Caenorhabditis elegans*. PLoS One. 2015; 10 e0142938 doi: 10.1371/journal.pone.0142938 [PubMed: 26571132]
41. Ohno H, Yoshida M, Sato T, Kato J, Miyazato M, Kojima M, Ida T, Iino Y. Luqin-like RYamide peptides regulate food-evoked responses in *C. elegans*. Elife. 2017; 6 e28877 doi: 10.7554/elife.28877 [PubMed: 28847365]
42. Wang H, Girskis K, Janssen T, Chan JP, Dasgupta K, Knowles JA, Schoofs L, Sieburth D. Neuropeptide secreted from a pacemaker activates neurons to control a rhythmic behavior. Curr Biol. 2013; 23: 746–754. DOI: 10.1016/j.cub.2013.03.049 [PubMed: 23583549]
43. Chen L, Liu Y, Su P, Hung W, Li H, Wang Y, Yue Z, Ge MH, Wu ZX, Zhang Y, et al. Escape steering by cholecystokinin peptidergic signaling. Cell Rep. 2022; 38 110330 doi: 10.1016/j.celrep.2022.110330 [PubMed: 35139370]
44. Chew YL, Tanizawa Y, Cho Y, Zhao B, Yu AJ, Ardiel EL, Rabi-nowitch I, Bai J, Rankin CH, Lu H, et al. An afferent neuropeptide system transmits mechanosensory signals triggering sensitization and arousal in *C. elegans*. Neuron. 2018; 99: 1233–1246. e6 doi: 10.1016/j.neuron.2018.08.003 [PubMed: 30146306]
45. Oranth A, Schultheis C, Tolstenkov O, Erbguth K, Nagpal J, Hain D, Brauner M, Wabnig S, Steuer Costa W, McWhirter RD, et al. Food sensation modulates locomotion by dopamine and neuropeptide signaling in a distributed neuronal network. Neuron. 2018; 100: 1414–1428. e10 doi: 10.1016/j.neuron.2018.10.024 [PubMed: 30392795]
46. Gershkovich MM, Groß VE, Kaiser A, Prömel S. Pharmacological and functional similarities of the human neuropeptide Y system in *C. elegans* challenges phylogenetic views on the FLP/NPR system. Cell Commun Signal. 2019; 17: 123. doi: 10.1186/s12964-019-0436-1 [PubMed: 31533726]
47. E L, Zhou T, Koh S, Chuang M, Sharma R, Pujol N, Chisholm AD, Eroglu C, Matsunami H, Yan D. An antimicrobial peptide and its neuronal receptor regulate dendrite degeneration in aging and infection. Neuron. 2018; 97: 125–138. e5 doi: 10.1016/j.neuron.2017.12.001 [PubMed: 29301098]
48. Yin JA, Gao G, Liu XJ, Hao ZQ, Li K, Kang XL, Li H, Shan YH, Hu WL, Li HP, Cai SQ. Genetic variation in glia-neuron signalling modulates ageing rate. Nature. 2017; 551: 198–203. DOI: 10.1038/nature24463 [PubMed: 29120414]
49. Sakai N, Ohno H, Yoshida M, Iwamoto E, Kurogi A, Jiang D, Sato T, Miyazato M, Kojima M, Kato J, Ida T. Characterization of putative tachykinin peptides in *Caenorhabditis elegans*. Biochem Bio-ph Res Co. 2021; 559: 197–202. DOI: 10.1016/j.bbrc.2021.04.063
50. Peymen K, Watteyne J, Borghgraef C, Van Sinay E, Beets I, Schoofs L. Myoinhibitory peptide signaling modulates aversive gustatory learning in *Caenorhabditis elegans*. PLoS Genet. 2019; 15 e1007945 doi: 10.1371/journal.pgen.1007945 [PubMed: 30779740]
51. Wacker D, Stevens RC, Roth BL. How ligands illuminate GPCR molecular pharmacology. Cell. 2017; 170: 414–427. DOI: 10.1016/j.cell.2017.07.009 [PubMed: 28753422]
52. Bauknecht P, Jékely G. Large-scale combinatorial deor-phanization of *Platynereis* neuropeptide GPCRs. Cell Rep. 2015; 12: 684–693. DOI: 10.1016/j.celrep.2015.06.052 [PubMed: 26190115]
53. Roth BL, Kroeze WK. Integrated approaches for genomewide interrogation of the druggable non-olfactory G protein-coupled receptor superfamily. J Biol Chem. 2015; 290: 19471–19477. DOI: 10.1074/jbc.r115.654764 [PubMed: 26100629]
54. Abid MSR, Mousavi S, Checco JW. Identifying receptors for neuropeptides and peptide hormones: Challenges and recent progress. ACS Chem Biol. 2021; 16: 251–263. DOI: 10.1021/acschembio.0c00950 [PubMed: 33539706]
55. White JG, Southgate E, Thomson JN, Brenner S. The structure of the nervous system of the nematode *Caenorhabditis elegans*. Philos Trans R Soc Lond B Biol Sci. 1986; 314: 1–340. [PubMed: 22462104]

56. Jarrell TA, Wang Y, Bloniarz AE, Brittin CA, Xu M, Thomson JN, Albertson DG, Hall DH, Emmons SW. The connectome of a decision-making neural network. Science. 2012; 337: 437–444. DOI: 10.1126/science.1221762 [PubMed: 22837521]
57. Cook SJ, Jarrell TA, Brittin CA, Wang Y, Bloniarz AE, Yakovlev MA, Nguyen KCQ, Tang LT-H, Bayer EA, Duerr JS, et al. Whole-animal connectomes of both *Caenorhabditis elegans* sexes. Nature. 2019; 571: 63–71. DOI: 10.1038/s41586-019-1352-7 [PubMed: 31270481]
58. Witvliet D, Mulcahy B, Mitchell JK, Meirovitch Y, Berger DR, Wu Y, Liu Y, Koh WX, Parvathala R, Holmyard D, et al. Connec-tomes across development reveal principles of brain maturation. Nature. 2021; 596: 257–261. DOI: 10.1038/s41586-021-03778-8 [PubMed: 34349261]
59. Van Bael S, Zels S, Boonen K, Beets I, Schoofs L, Temmerman L. A *Caenorhabditis elegans* mass spectrometric resource for neuropeptidomics. J Am Soc Mass Spectrom. 2018; 29: 879–889. DOI: 10.1007/s13361-017-1856-z [PubMed: 29299835]
60. Li C, Kim K. Neuropeptides. WormBook : The Online Review of C. elegans Biology. 2008; 1–36. DOI: 10.1895/worm-book.1.142.1
61. Hobert O. The Neuronal Genome of *Caenorhabditis elegans*. WormBook : The Online Review of *C. elegans*. Biology. 2013; 1–106. DOI: 10.1895/wormbook.1.161.1
62. Fadda M, De Fruyt N, Borghgraef C, Watteyne J, Peymen K, Van-dewyer E, Naranjo Galindo FJ, Kieswetter A, Mirabeau O, Chew YL, et al. NPY/NPF-related neuropeptide FLP-34 signals from serotonergic neurons to modulate aversive olfactory learning in *Caenorhabditis elegans*. J Neurosci. 2020; 40: 6018–6034. DOI: 10.1523/jneurosci.2674-19.2020 [PubMed: 32576621]
63. Watteyne J, Peymen K, Vander Auwera P, Borghgraef C, Vandew-yer E, Van Damme S, Rutten I, Lammertyn J, Jelier R, Schoofs L, Beets I. Neuromedin U signaling regulates retrieval of learned salt avoidance in a *C. elegans* gustatory circuit. Nat Commun. 2020; 11 2076 doi: 10.1038/s41467-020-15964-9 [PubMed: 32350283]
64. Cao J, Packer JS, Ramani V, Cusanovich DA, Huynh C, Daza R, Qiu X, Lee C, Furlan SN, Steemers FJ, et al. Comprehensive single-cell transcriptional profiling of a multicellular organism. Science. 2017; 357: 661–667. DOI: 10.1126/science.aam8940 [PubMed: 28818938]
65. Ripoll-Sánchez L, Watteyne J, Sun H, Fernandez R, Taylor SR, Weinreb A, Hammarlund M, Miller DM, Hobert O, Beets I, et al. The neuropeptidergic connectome of *C. elegans*. Preprint at bio-Rxiv. 2022; doi: 10.1101/2022.10.30.514396
66. Janssen T, Lindemans M, Meelkop E, Temmerman L, Schoofs L. Coevolution of neuropeptidergic signaling systems: from worm to man. Ann N Y Acad Sci. 2010; 1200: 1–14. DOI: 10.1111/j.1749-6632.2010.05506.x [PubMed: 20633129]
67. Keating CD, Kriek N, Daniels M, Ashcroft NR, Hopper NA, Siney EJ, Holden-Dye L, Burke JF. Whole-genome analysis of 60 G protein-coupled receptors in *Caenorhabditis elegans* by gene knockout with RNAi. Curr Biol. 2003; 13: 1715–1720. [PubMed: 14521838]
68. Janssen T, Husson SJ, Lindemans M, Mertens I, Rademakers S, Ver Donck K, Geysen J, Jansen G, Schoofs L. Functional characterization of three G protein-coupled receptors for pigment dispersing factors in *Caenorhabditis elegans*. J Biol Chem. 2008; 283: 15241–15249. DOI: 10.1074/jbc.m709060200 [PubMed: 18390545]
69. Tatulian SA. Structural dynamics of insulin receptor and transmembrane signaling. Biochemistry. 2015; 54: 5523–5532. DOI: 10.1021/acs.biochem.5b00805 [PubMed: 26322622]
70. Pierce SB, Costa M, Wisotzkey R, Devadhar S, Homburger SA, Buchman AR, Ferguson KC, Heller J, Platt DM, Pasquinelli AA, et al. Regulation of DAF-2 receptor signaling by human insulin and ins-1, a member of the unusually large and diverse *C. elegans* insulin gene family. Genes Dev. 2001; 15: 672–686. DOI: 10.1101/gad.867301 [PubMed: 11274053]
71. Meeusen T, Mertens I, Clynen E, Baggerman G, Nichols R, Nachman RJ, Huybrechts R, De Loof A, Schoofs L. Identification in *Drosophila melanogaster* of the invertebrate G protein-coupled FMRFamide receptor. Proc Natl Acad Sci USA. 2002; 99: 15363–15368. DOI: 10.1073/pnas.252339599 [PubMed: 12438685]
72. Mertens I, Meeusen T, Huybrechts R, De Loof A, Schoofs L. Characterization of the short neuropeptide F receptor from *Drosophila melanogaster*. Biochem Biophys Res Commun. 2002; 297: 1140–1148. DOI: 10.1016/s0006-291x(02)02351-3 [PubMed: 12372405]

73. Mertens I, Meeusen T, Janssen T, Nachman R, Schoofs L. Molecular characterization of two G protein-coupled receptor splice variants as FLP2 receptors in *Caenorhabditis elegans*. Biochem Biophys Res Commun. 2005; 330: 967–974. DOI: 10.1016/j.bbrc.2005.03.071 [PubMed: 15809090]
74. Janssen T, Meelkop E, Lindemans M, Verstraelen K, Husson SJ, Temmerman L, Nachman RJ, Schoofs L. Discovery of a cholecystokinin-gastrin-like signaling system in nematodes. Endocrinology. 2008; 149: 2826–2839. DOI: 10.1210/en.2007-1772 [PubMed: 18339709]
75. Stables J, Green A, Marshall F, Fraser N, Knight E, Sautel M, Milligan G, Lee M, Rees S. A bioluminescent assay for agonist activity at potentially any G-protein-coupled receptor. Anal Biochem. 1997; 252: 115–126. DOI: 10.1006/abio.1997.2308 [PubMed: 9324949]
76. Offermanns S, Simon MI. G alpha 15 and G alpha 16 couple a wide variety of receptors to phospholipase C. J Biol Chem. 1995; 270: 15175–15180. [PubMed: 7797501]
77. Rizzuto R, Simpson AW, Brini M, Pozzan T. Rapid changes of mitochondrial Ca^{2+} revealed by specifically targeted recombinant aequorin. Nature. 1992; 358: 325–327. DOI: 10.1038/358325a0 [PubMed: 1322496]
78. Chew YL, Grundy LJ, Brown AEX, Beets I, Schafer WR. Neuropeptides encoded by nlp-49 modulate locomotion, arousal and egg-laying behaviours in *Caenorhabditis elegans* via the receptor SEB-3. Philos. Trans R Soc Lond B Biol Sci. 2018; 373 20170368 doi: 10.1098/rstb.2017.0368
79. Ramachandran S, Banerjee N, Bhattacharya R, Lemons ML, Florman J, Lambert CM, Touroutine D, Alexander K, Schoofs L, Alkema MJ, et al. A conserved neuropeptide system links head and body motor circuits to enable adaptive behavior. Elife. 2021; 10 e71747 doi: 10.7554/elife.71747 [PubMed: 34766905]
80. Marques F, Falquet L, Vandewyer E, Beets I, Glauser DA. Signaling via the FLP-14/FRPR-19 neuropeptide pathway sustains nociceptive response to repeated noxious stimuli in *C. elegans*. PLoS Genet. 2021; 17 e1009880 doi: 10.1371/journal.pgen.1009880 [PubMed: 34748554]
81. Gadenne MJ, Hardege I, Yemini E, Suleski D, Jaggers P, Beets I, Schafer WR, Chew YL. Neuropeptide signalling shapes feeding and reproductive behaviours in male *Caenorhabditis elegans*. Life Sci Alliance. 2022; 5 e202201420 doi: 10.26508/lsa.202201420 [PubMed: 35738805]
82. Thapliyal S, Beets I, Glauser DA. Multisite regulation integrates multimodal context in sensory circuits to control persistent behavioral states in *C. elegans*. Nat Commun. 2023; 14 3052 doi: 10.1038/s41467-023-38685-1 [PubMed: 37236963]
83. Vander Auwera P, Frooninckx L, Buscemi K, Vance RT, Watteyne J, Mirabeau O, Temmerman L, De Haes W, Fancsalszky L, Gottschalk A, et al. RPamide neuropeptides NLP-22 and NLP-2 act through GnRH-like receptors to promote sleep and wakefulness in *C. elegans*. Sci Rep. 2020; 10 9929 doi: 10.1038/s41598-020-66536-2 [PubMed: 32555288]
84. Wibisono P, Wibisono S, Watteyne J, Chen CH, Sellegounder D, Beets I, Liu Y, Sun J. Neuronal GPCR NMUR-1 regulates distinct immune responses to different pathogens. Cell Rep. 2022; 38 110321 doi: 10.1016/j.celrep.2022.110321 [PubMed: 35139379]
85. Marquina-Solis J, Vandewyer E, Hawk J, Colón-Ramos DA, Beets I, Bargmann CI. Peptidergic signaling controls the dynamics of sickness behavior in *Caenorhabditis elegans*. Preprint at bioRxiv. 2022; doi: 10.1101/2022.04.16.488560
86. Janssen T, Husson SJ, Meelkop E, Temmerman L, Lindemans M, Verstraelen K, Rademakers S, Mertens I, Nitabach M, Jansen G, Schoofs L. Discovery and characterization of a conserved pigment dispersing factor-like neuropeptide pathway in *Caenorhabditis elegans*. J Neurochem. 2009; 111: 228–241. DOI: 10.1111/j.1471-4159.2009.06323.x [PubMed: 19686386]
87. Fadda M, Hasakiogullari I, Temmerman L, Beets I, Zels S, Schoofs L. Regulation of feeding and metabolism by neuropeptide F and short neuropeptide F in invertebrates. Front Endocrinol. 2019; 10: 64. doi: 10.3389/fendo.2019.00064
88. Yañez-Guerra LA, Zhong X, Moghul I, Butts T, Zampronio CG, Jones AM, Mirabeau O, Elphick MR. Echinoderms provide missing link in the evolution of PrRP/sNPF-type neuropeptide signalling. Elife. 2020; 9: 584. doi: 10.7554/elife.57640

89. Jékely G, Melzer S, Beets I, Kadow ICG, Koene J, Haddad S, Holden-Dye L. The long and the short of it – a perspective on peptidergic regulation of circuits and behaviour. J Exp Biol. 2018; 221 jeb166710 doi: 10.1242/jeb.166710 [PubMed: 29439060]

90. Larsen MJ, Lancheros ER, Williams T, Lowery DE, Geary TG, Kubiak TM. Functional expression and characterization of the *C. elegans* G-protein-coupled FLP-2 Receptor (T19F4.1) in mammalian cells and yeast. Int J Parasitol Drugs Drug Resist. 2013; 3: 1–7. DOI: 10.1016/j.ijpddr.2012.10.002 [PubMed: 24533288]

91. Cohen M, Reale V, Olofsson B, Knights A, Evans P, de Bono M. Coordinated regulation of foraging and metabolism in *C. elegans* by RFamide neuropeptide signaling. Cell Metabol. 2009; 9: 375–385. DOI: 10.1016/j.cmet.2009.02.003

92. Kubiak TM, Larsen MJ, Bowman JW, Geary TG, Lowery DE. FMRFamide-like peptides encoded on the flp-18 precursor gene activate two isoforms of the orphan *Caenorhabditis elegans* G-protein-coupled receptor Y58G8A.4 heterologously expressed in mammalian cells. Biopolymers. 2008; 90: 339–348. DOI: 10.1002/bip.20850 [PubMed: 17879267]

93. Jékely G. Global view of the evolution and diversity of metazoan neuropeptide signaling. Proc Natl Acad Sci USA. 2013; 110: 8702–8707. DOI: 10.1073/pnas.1221833110 [PubMed: 23637342]

94. Elphick MR, Mirabeau O, Larhammar D. Evolution of neuropeptide signalling systems. J Exp Biol. 2018; 221 jeb151092 doi: 10.1242/jeb.151092 [PubMed: 29440283]

95. Zhang Y, Yañez Guerra LA, Egertová M, Zampronio CG, Jones AM, Elphick MR. Molecular and functional characterization of somatostatin-type signalling in a deuterostome invertebrate. Open Biol. 2020; 10 200172 doi: 10.1098/rsob.200172 [PubMed: 32898470]

96. Garrison JL, Macosko EZ, Bernstein S, Pokala N, Albrecht DR, Bargmann CI. Oxytocin/vasopressin-related peptides have an ancient role in reproductive behavior. Science. 2012; 338: 540–543. DOI: 10.1126/science.1226201 [PubMed: 23112335]

97. Lindemans M, Liu F, Janssen T, Husson SJ, Mertens I, Gäde G, Schoofs L. Adipokinetic hormone signaling through the gonadotropin-releasing hormone receptor modulates egg-laying in *Caenorhabditis elegans*. Proc Natl Acad Sci USA. 2009; 106: 1642–1647. DOI: 10.1073/pnas.0809881106 [PubMed: 19164555]

98. Lindemans M, Janssen T, Husson SJ, Meelkop E, Temmerman L, Clynen E, Mertens I, Schoofs L. A neuromedin-pyrokininlike neuropeptide signaling system in *Caenorhabditis elegans*. Biochem Biophys Res Commun. 2009; 379: 760–764. DOI: 10.1016/j.bbrc.2008.12.121 [PubMed: 19133232]

99. Husson SJ, Mertens I, Janssen T, Lindemans M, Schoofs L. Neuropeptidergic signaling in the nematode Caenorhabditis ele-gans. Prog Neurobiol. 2007; 82: 33–55. DOI: 10.1016/j.pneurobio.2007.01.006 [PubMed: 17383075]

100. Koziol U, Koziol M, Preza M, Costábile A, Brehm K, Castillo E. *De novo* discovery of neuropeptides in the genomes of parasitic flatworms using a novel comparative approach. Int J Parasitol. 2016; 46: 709–721. DOI: 10.1016/j.ijpara.2016.05.007 [PubMed: 27388856]

101. Zhang Y, Yañez-Guerra LA, Tinoco AB, Escudero Castelán N, Egertová M, Elphick MR. Somatostatin-type and allatostatin-C-type neuropeptides are paralogous and have opposing myoregulatory roles in an echinoderm. Proc Natl Acad Sci USA. 2022; 119 e2113589119 doi: 10.1073/pnas.2113589119 [PubMed: 35145030]

102. Husson SJ, Clynen E, Baggerman G, De Loof A, Schoofs L. Discovering neuropeptides in *Caenorhabditis elegans* by two dimensional liquid chromatography and mass spectrometry. Biochem Biophys Res Commun. 2005; 335: 76–86. DOI: 10.1016/j.bbrc.2005.07.044 [PubMed: 16061202]

103. Saberi A, Jamal A, Beets I, Schoofs L, Newmark PA. GPCRs direct germline development and somatic gonad function in Pla-narians. PLoS Biol. 2016; 14 e1002457 doi: 10.1371/journal.pbio.1002457 [PubMed: 27163480]

104. Szekeres PG. Functional assays for identifying ligands at orphan G protein-coupled receptors. Recept Channel. 2002; 8: 297–308.

105. Jékely G. The chemical brain hypothesis for the origin of nervous systems. Philos Trans R Soc Lond B Biol Sci. 2021; 376 20190761 doi: 10.1098/rstb.2019.0761 [PubMed: 33550946]

106. Deng B, Li Q, Liu X, Cao Y, Li B, Qian Y, Xu R, Mao R, Zhou E, Zhang W, et al. Chemoconnectomics: Mapping chemical transmission in *Drosophila*. Neuron. 2019; 101: 876–893. e4 doi: 10.1016/j.neuron.2019.01.045 [PubMed: 30799021]

107. Bargmann CI. Beyond the connectome: How neuromodulators shape neural circuits. Bioessays. 2012; 34: 458–465. DOI: 10.1002/bies.201100185 [PubMed: 22396302]

108. Wang H, Jing M, Li Y. Lighting up the brain: genetically encoded fluorescent sensors for imaging neurotransmitters and neuromodulators. Curr Opin Neurobiol. 2018; 50: 171–178. DOI: 10.1016/j.conb.2018.03.010 [PubMed: 29627516]

109. Kim MW, Wang W, Sanchez MI, Coukos R, von Zastrow M, Ting AY. Time-gated detection of protein-protein interactions with transcriptional readout. Elife. 2017; 6 e30233 doi: 10.7554/elife.30233 [PubMed: 29189201]

110. Duffet L, Kosar S, Panniello M, Viberti B, Bracey E, Zych AD, Radoux-Mergault A, Zhou X, Dernic J, Ravotto L, et al. A genetically encoded sensor for *in vivo* imaging of orexin neuropeptides. Nat Methods. 2022; 19: 231–241. DOI: 10.1038/s41592-021-01390-2 [PubMed: 35145320]

111. McCoy CJ, Atkinson LE, Zamanian M, McVeigh P, Day TA, Kimber MJ, Marks NJ, Maule AG, Mousley A. New insights into the FLPergic complements of parasitic nematodes: Informing deor-phanisation approaches. EUPROT. 2014; 3: 262–272. DOI: 10.1016/j.euprot.2014.04.002

112. Kim YJ, Bartalska K, Audsley N, Yamanaka N, Yapici N, Lee JY, Kim YC, Markovic M, Isaac E, Tanaka Y, Dickson BJ. MIPs are ancestral ligands for the sex peptide receptor. Proc Natl Acad Sci USA. 2010; 107: 6520–6525. DOI: 10.1073/pnas.0914764107 [PubMed: 20308537]

113. Elhabazi K, Humbert J-P, Bertin I, Schmitt M, Bihel F, Bourguignon JJ, Bucher B, Becker JAJ, Sorg T, Meziane H, et al. Endogenous mammalian RF-amide peptides, including PrRP, kisspeptin and 26RFa, modulate nociception and morphine analgesia via NPFF receptors. Neuropharmacology. 2013; 75: 164–171. DOI: 10.1016/j.neuropharm.2013.07.012 [PubMed: 23911743]

114. Liu X, Herbison AE. Kisspeptin regulation of neuronal activity throughout the central nervous system. Endocrinol Metab. 2016; 31: 193–205. DOI: 10.3803/enm.2016.31.2.193

115. Lyubimov Y, Engstrom M, Wurster S, Savola JM, Korpi ER, Panula P. Human kisspeptins activate neuropeptide FF2 receptor. Neuroscience. 2010; 170: 117–122. DOI: 10.1016/j.neuroscience.2010.06.058 [PubMed: 20600636]

116. Oishi S, Misu R, Tomita K, Setsuda S, Masuda R, Ohno H, Naniwa Y, Ieda N, Inoue N, Ohkura S, et al. Activation of neuropeptide FF receptors by kisspeptin receptor ligands. ACS Med Chem Lett. 2011; 2: 53–57. DOI: 10.1021/ml1002053 [PubMed: 24900254]

117. Sudo S, Kumagai J, Nishi S, Layfield S, Ferraro T, Bathgate RAD, Hsueh AJW. H3 relaxin is a specific ligand for LGR7 and activates the receptor by interacting with both the ectodomain and the exoloop 2. J Biol Chem. 2003; 278: 7855–7862. DOI: 10.1074/jbc.m212457200 [PubMed: 12506116]

118. Liu C, Chen J, Sutton S, Roland B, Kuei C, Farmer N, Sillard R, Lovenberg TW. Identification of relaxin-3/INSL7 as a ligand for GPCR142. J Biol Chem. 2003; 278: 50765–50770. DOI: 10.1074/jbc.m308996200 [PubMed: 14522967]

119. Liu C, Eriste E, Sutton S, Chen J, Roland B, Kuei C, Farmer N, Jornvall H, Sillard R, Lovenberg TW. Identification of relaxin-3/INSL7 as an endogenous ligand for the orphan G-protein-coupled receptor GPCR135. J Biol Chem. 2003; 278: 50754–50764. DOI: 10.1074/jbc.m308995200 [PubMed: 14522968]

120. Schmidt A, Bauknecht P, Williams EA, Augustinowski K, Gründer S, Jékely G. Dual signaling of Wamide myoinhibitory peptides through a peptide-gated channel and a GPCR in *Platynereis*. Faseb J. 2018; 32: 5338–5349. DOI: 10.1096/fj.201800274r [PubMed: 29688813]

121. Ringstad N, Horvitz HR. FMRFamide neuropeptides and acetylcholine synergistically inhibit egg-laying by *C. elegans*. Nat Neurosci. 2008; 11: 1168–1176. DOI: 10.1038/nn.2186 [PubMed: 18806786]

122. Jia Q, Sieburth D. Mitochondrial hydrogen peroxide positively regulates neuropeptide secretion during diet-induced activation of the oxidative stress response. Nat Commun. 2021; 12 2304 doi: 10.1038/s41467-021-22561-x [PubMed: 33863916]

123. Waggoner LE, Hardaker LA, Golik S, Schafer WR. Effect of a neuropeptide gene on behavioral states in Caenorhabditis ele-gans egg-laying. Genetics. 2000; 154: 1181–1192. [PubMed: 10757762]

124. Cawley NX, Li Z, Loh YP. 60 YEARS OF POMC: Biosynthesis, trafficking, and secretion of pro-opiomelanocortin-derived peptides. J Mol Endocrinol. 2016; 56: T77–T97. DOI: 10.1530/jme-15-0323 [PubMed: 26880796]

125. Hewes RS, Taghert PH. Neuropeptides and neuropeptide receptors in the *Drosophila melanogaster* genome. Genome Res. 2001; 11: 1126–1142. DOI: 10.1101/gr.169901 [PubMed: 11381038]

126. Bargmann CI. Neurobiology of the *Caenorhabditis elegans* genome. Science. 1998; 282: 2028–2033. [PubMed: 9851919]

127. Nathoo AN, Moeller RA, Westlund BA, Hart AC. Identification of neuropeptide-like protein gene families in Caenorhabditis el-egans and other species. Proc Natl Acad Sci USA. 2001; 98: 14000–14005. DOI: 10.1073/pnas.241231298 [PubMed: 11717458]

128. Jung SH, Lee JH, Chae HS, Seong JY, Park Y, Park ZY, Kim YJ. Identification of a novel insect neuropeptide, CNMa and its receptor. FEBS Lett. 2014; 588: 2037–2041. DOI: 10.1016/j.febslet.2014.04.028 [PubMed: 24796791]

129. Ida T, Takahashi T, Tominaga H, Sato T, Kume K, Yoshizawa-Kumagaye K, Nishio H, Kato J, Murakami N, Miyazato M, et al. Identification of the endogenous cysteine-rich peptide trissin, a ligand for an orphan G protein-coupled receptor in *Drosophila*. Biochem Bioph Res Co. 2011; 414: 44–48. DOI: 10.1016/j.bbrc.2011.09.018

130. McKay FM, McCoy CJ, Crooks B, Marks NJ, Maule AG, Atkinson LE, Mousley A. *In silico* analyses of neuropeptidelike protein (NLP) profiles in parasitic nematodes. Int J Parasitol. 2022; 52: 77–85. DOI: 10.1016/j.ijpara.2021.07.002 [PubMed: 34450132]

131. Shiraishi A, Okuda T, Miyasaka N, Osugi T, Okuno Y, Inoue J, Satake H. Repertoires of G protein-coupled receptors for Ciona-specific neuropeptides. Proc Natl Acad Sci USA. 2019; 116: 7847–7856. DOI: 10.1073/pnas.1816640116 [PubMed: 30936317]

132. Atkinson LE, Liu Y, McKay F, Vandewyer E, Viau C, Irvine A, Rosa BA, Li Z, Liang Q, Marks NJ, et al. *Ascaris suum* informs extrasynaptic volume transmission in nematodes. ACS Chem Neurosci. 2021; 12: 3176–3188. DOI: 10.1021/acschemneuro.1c00281 [PubMed: 34347433]

133. Peeters L, Janssen T, De Haes W, Beets I, Meelkop E, Grant W, Schoofs L. A pharmacological study of NLP-12 neuropeptide signaling in free-living and parasitic nematodes. Peptides. 2012; 34: 82–87. DOI: 10.1016/j.peptides.2011.10.014 [PubMed: 22019590]

134. Bhat US, Shahi N, Surendran S, Babu K. Neuropeptides and behaviors: How small peptides regulate nervous system function and behavioral outputs. Front Mol Neurosci. 2021; 14 786471 doi: 10.3389/fnmol.2021.786471 [PubMed: 34924955]

135. Peymen K, Watteyne J, Frooninckx L, Schoofs L, Beets I. The FMRFamide-like peptide family in nematodes. Front Endocrinol. 2014; 5: 90. doi: 10.3389/fendo.2014.00090

136. Maudsley S, Martin B, Luttrell LM. The origins of diversity and specificity in G protein-coupled receptor signaling. J Pharmacol Exp Therapeut. 2005; 314: 485–494. DOI: 10.1124/jpet.105.083121

137. Mertens I, Vandingenen A, Johnson EC, Shafer OT, Li W, Trigg JS, De Loof A, Schoofs L, Taghert PH. PDF receptor signaling in *Drosophila* contributes to both circadian and geotactic behaviors. Neuron. 2005; 48: 213–219. DOI: 10.1016/j.neuron.2005.09.009 [PubMed: 16242402]

138. Kostenis E. Is Galpha16 the optimal tool for fishing ligands of orphan G-protein-coupled receptors? Trends Pharmacol Sci. 2001; 22: 560–564. [PubMed: 11698099]

139. Hilger D, Masureel M, Kobilka BK. Structure and dynamics of GPCR signaling complexes. Nat Struct Mol Biol. 2018; 25: 4–12. DOI: 10.1038/s41594-017-0011-7 [PubMed: 29323277]

140. Hauser AS, Gloriam DE, Bräuner-Osborne H, Foster SR. Novel approaches leading towards peptide GPCR de-orphanisation. Br J Pharmacol. 2020; 177: 961–968. DOI: 10.1111/bph.14950 [PubMed: 31863461]

141. Hatcher-Solis C, Fribourg M, Spyridaki K, Younkin J, Ellaithy A, Xiang G, Liapakis G, Gonzalez-Maeso J, Zhang H, Cui M, Logothetis DE. G protein-coupled receptor signaling

to Kir channels in *Xenopus* oocytes. Curr Pharmaceut Biotechnol. 2014; 15: 987–995. DOI: 10.2174/1389201015666141031111916

142. Clynen E, Husson SJ, Schoofs L. Identification of new members of the (short) neuropeptide F family in locusts and Caenorhab-ditis elegans. Ann N Y Acad Sci. 2009; 1163: 60–74. DOI: 10.1111/j.1749-6632.2008.03624.x [PubMed: 19456328]
143. Couillault C, Pujol N, Reboul J, Sabatier L, Guichou J-F, Kohara Y, Ewbank JJ. TLR-independent control of innate immunity in *Caenorhabditis elegans* by the TIR domain adaptor protein TIR-1, an ortholog of human SARM. Nat Immunol. 2004; 5: 488–494. DOI: 10.1038/ni1060 [PubMed: 15048112]
144. Husson SJ, Schoofs L. Altered neuropeptide profile of *Caenorhabditis elegans* lacking the chaperone protein 7B2 as analyzed by mass spectrometry. FEBS Lett. 2007; 581: 4288–4292. DOI: 10.1016/j.febslet.2007.08.003 [PubMed: 17707816]
145. Husson SJ, Reumer A, Temmerman L, De Haes W, Schoofs L, Mertens I, Baggerman G. Worm peptidomics. EUPROT. 2014; 3: 280–290. DOI: 10.1016/j.euprot.2014.04.005
146. Husson SJ, Janssen T, Baggerman G, Bogert B, Kahn-Kirby AH, Ashrafi K, Schoofs L. Impaired processing of FLP and NLP peptides in carboxypeptidase E (EGL-21)-deficient Caenorhabditis ele-gans as analyzed by mass spectrometry. J Neurochem. 2007; 102: 246–260. DOI: 10.1111/j.1471-4159.2007.04474.x [PubMed: 17564681]
147. Husson SJ, Clynen E, Baggerman G, Janssen T, Schoofs L. Defective processing of neuropeptide precursors in Caenorhab-ditis elegans lacking proprotein convertase 2 (KPC-2/EGL-3): mutant analysis by mass spectrometry. J Neurochem. 2006; 98: 1999–2012. DOI: 10.1111/j.1471-4159.2006.04014.x [PubMed: 16945111]
148. Husson SJ, Landuyt B, Nys T, Baggerman G, Boonen K, Clynen E, Lindemans M, Janssen T, Schoofs L. Comparative peptidomics of *Caenorhabditis elegans* versus *C. briggsae* by LC-MALDI-TOF MS. Peptides. 2009; 30: 449–457. DOI: 10.1016/j.pep-tides.2008.07.021 [PubMed: 18760316]
149. Kim K, Li C. Expression and regulation of an FMRFamide-related neuropeptide gene family in *Caenorhabditis elegans*. J Comp Neurol. 2004; 475: 540–550. DOI: 10.1002/cne.20189 [PubMed: 15236235]
150. Li C, Kim K, Nelson LS. FMRFamide-related neuropeptide gene family in *Caenorhabditis elegans*. Brain Res. 1999; 848: 26–34. [PubMed: 10612695]
151. Li C, Nelson LS, Kim K, Nathoo A, Hart AC. Neuropeptide gene families in the nematode *Caenorhabditis elegans*. Ann N Y Acad Sci. 1999; 897: 239–252. [PubMed: 10676452]
152. McVeigh P, Alexander-Bowman S, Veal E, Mousley A, Marks NJ, Maule AG. Neuropeptide-like protein diversity in phylum Nematoda. Int J Parasitol. 2008; 38: 1493–1503. DOI: 10.1016/j.ij-para.2008.05.006 [PubMed: 18602104]
153. McVeigh P, Leech S, Mair GR, Marks NJ, Geary TG, Maule AG. Analysis of FMRFamide-like peptide (FLP) diversity in phylum Nematoda. Int J Parasitol. 2005; 35: 1043–1060. DOI: 10.1016/j.ijpara.2005.05.010 [PubMed: 16076468]
154. Nelson LS, Kim K, Memmott JE, Li C. FMRFamide-related gene family in the nematode, *Caenorhabditis elegans*. Brain research. Brain Res Mol Brain Res. 1998; 58: 103–111. [PubMed: 9685599]
155. Rosoff ML, Bürglin TR, Li C. Alternatively spliced transcripts of the flp-1 gene encode distinct FMRFamide-like peptides in *Caenorhabditis elegans*. J Neurosci. 1992; 12: 2356–2361. [PubMed: 1607945]
156. Kubiak TM, Larsen MJ, Nulf SC, Zantello MR, Burton KJ, Bowman JW, Modric T, Lowery DE. Differential activation of "social" and "solitary" variants of the *Caenorhabditis elegans* G protein-coupled receptor NPR-1 by its cognate ligand AF9. J Biol Chem. 2003; 278: 33724–33729. DOI: 10.1074/jbc.m304861200 [PubMed: 12821653]
157. Kubiak TM, Larsen MJ, Zantello MR, Bowman JW, Nulf SC, Lowery DE. Functional annotation of the putative orphan Caeno-rhabditis elegans G-protein-coupled receptor C10C6.2 as a FLP15 peptide receptor. J Biol Chem. 2003; 278: 42115–42120. DOI: 10.1074/jbc.m304056200 [PubMed: 12937167]

158. Özbey NP, Imanikia S, Krueger C, Hardege I, Morud J, Sheng M, Schafer WR, Casanueva MO, Taylor RC. Tyramine acts downstream of neuronal XBP-1s to coordinate inter-tissue UPRER activation and behavior in *C. elegans*. Dev Cell. 2020; 55: 754–770. e6 doi: 10.1016/j.devcel.2020.10.024 [PubMed: 33232669]

159. Lancichinetti A, Fortunato S. Consensus clustering in complex networks. Preprint at Arxiv. 2012; doi: 10.1038/srep00336

160. Rubinov M, Sporns O. Complex network measures of brain connectivity: Uses and interpretations. Neuroimage. 2010; 52: 1059–1069. DOI: 10.1016/j.neuroimage.2009.10.003 [PubMed: 19819337]

161. Blondel VD, Guillaume JL, Lambiotte R, Lefebvre E. Fast unfolding of communities in large networks. Arxiv. 2008; doi: 10.1088/1742-5468/2008/10/p10008

162. Fruchterman TMJ, Reingold EM. Graph drawing by force-directed placement. Software Pract Ex. 1991; 21: 1129–1164. DOI: 10.1002/spe.4380211102

163. Liu K, Warnow TJ, Holder MT, Nelesen SM, Yu J, Stamatakis AP, Linder CR. SATe'-II: Very fast and accurate simultaneous estimation of multiple sequence alignments and phylogenetic trees. Syst Biol. 2012; 61: 90–106. DOI: 10.1093/sysbio/syr095 [PubMed: 22139466]

164. Price MN, Dehal PS, Arkin AP. FastTree 2-Approximately maximum-likelihood trees for large alignments. PLoS One. 2010; 5 e9490 doi: 10.1371/journal.pone.0009490 [PubMed: 20224823]

165. Katoh K, Standley DM. MAFFT multiple sequence alignment software version 7: Improvements in performance and usability. Mol Biol Evol. 2013; 30: 772–780. DOI: 10.1093/molbev/mst010 [PubMed: 23329690]

166. Edgar RC. MUSCLE: multiple sequence alignment with high accuracy and high throughput. Nucleic Acids Res. 2004; 32: 1792–1797. DOI: 10.1093/nar/gkh340 [PubMed: 15034147]

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Highlights

- System-wide reverse pharmacology deorphanizes 68 *C. elegans* peptide GPCRs
- Discovery of 461 peptide-GPCR pairs and additional ligands for characterized GPCRs
- Peptide ligands show specific and complex combinatorial receptor interactions
- Peptide-GPCR pairs support long-range conservation and expansion of peptide systems

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In brief

Beets et al. present a system-wide resource of 461 peptide-GPCR couples in *C. elegans*, revealing binary and complex combinatorial signaling motifs in the peptide-receptor network, which provide insight into the evolution of peptidergic systems and lay a foundation for studying the functional organization of the peptide signaling network at system level.

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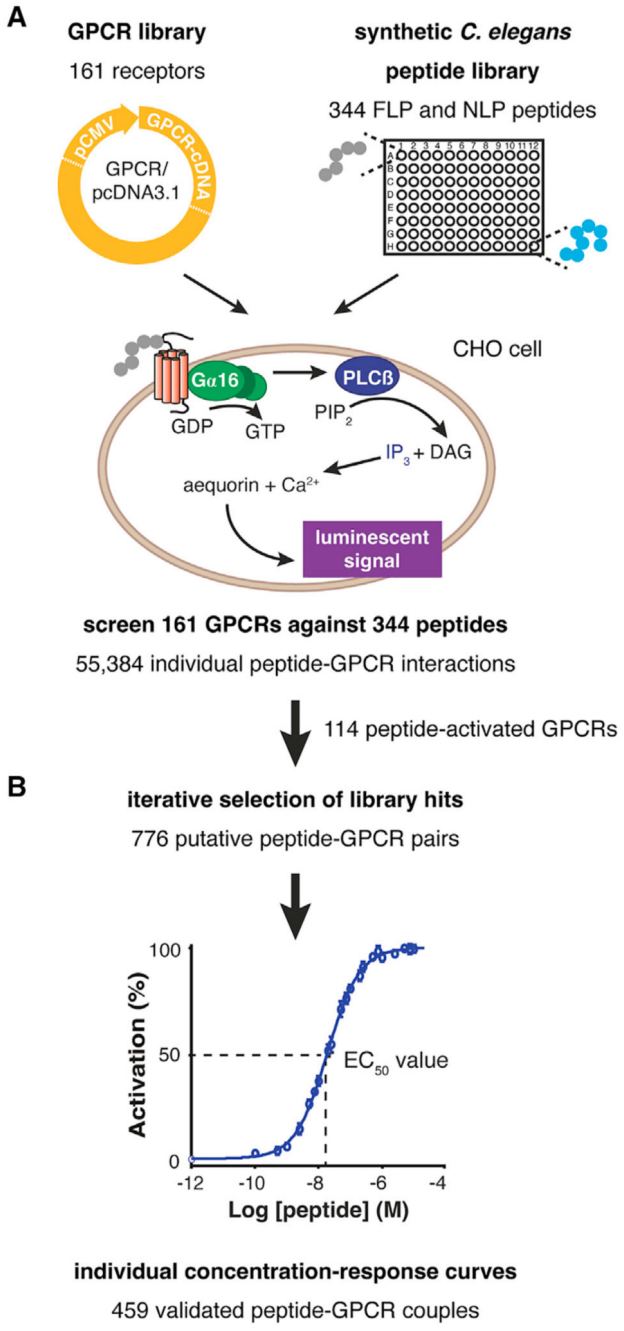

Figure 1. Systematic ligand screening of peptide-GPCR candidates in *C. elegans*

(A) Platform for identifying peptide ligands of *C. elegans* GPCRs. Candidate receptors are cloned under control of the cytomegalovirus immediate-early promoter (pCMV) for heterologous expression in CHO cells. Each GPCR is expressed together with the calcium-activated photoprotein aequorin and the promiscuous human $G\alpha_{16}$ subunit and is screened with a synthetic library of *C. elegans* FLP and NLP peptides. Upon GPCR activation, phospholipase Cβ (PLCβ) is activated and hydrolyzes phosphatidylinositol bisphosphate (PIP_2) into diacylglycerol (DAG) and inositol trisphosphate (IP_3), which activates IP_3-

dependent calcium channels, resulting in calcium release from intracellular storage sites and increased luminescence. Screening of 55,384 individual peptide-GPCR interactions identified putative ligands for 114 out of 161 GPCRs. See also Figure S1, Tables S1–S4, and Data S1.

(B) For each GPCR, putative hits are iteratively selected and each interaction is validated by measuring concentration-response curves, from which half-maximal effective concentrations (EC_{50}) values are calculated. Out of 776 putative hits, 459 pairs show concentration-dependent GPCR activation. See Figures S2–S7 and Tables S4–S6 for tested and validated couples.

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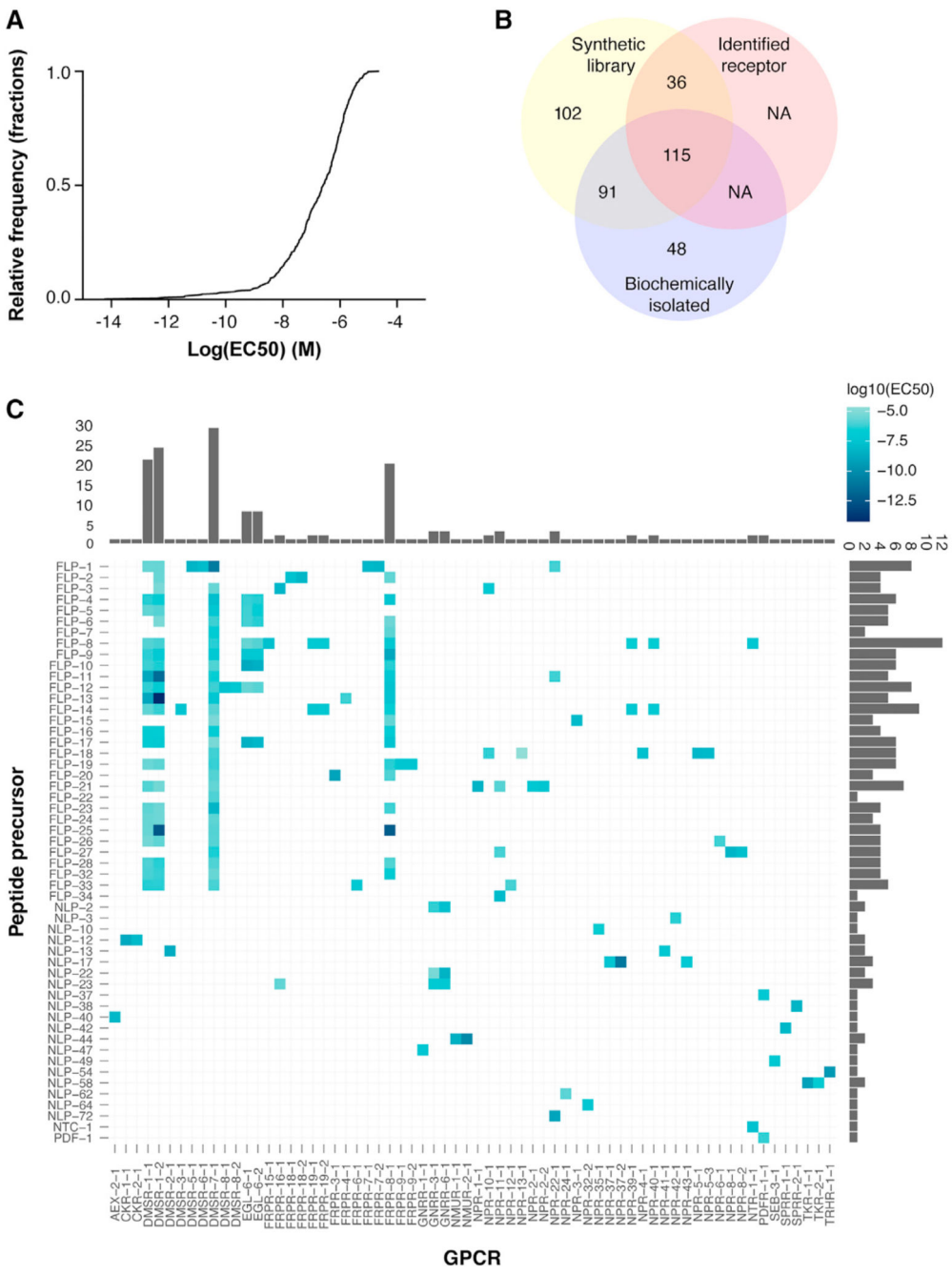

Figure 2. System-wide characterization of peptide GPCRs identifies a complex network of ligand-receptor interactions

(A) Cumulative frequency plot for log(EC_{50}) values of 459 validated peptide-GPCR couples.

(B) Venn diagram of peptides in the synthetic library that have been biochemically isolated[59] and/ or for which a receptor has been identified.

(C) Heatmap of log10(EC_{50}) values for validated peptide-GPCR pairs. When a GPCR interacts with multiple peptides from a single peptide-encoding gene, only the log(EC_{50}) value of the most potent ligand is included. Histograms indicate the number of interactions

for each GPCR (top) or peptide precursor (right). See also Figures S2–S8 and Tables S1, S6, and S7.

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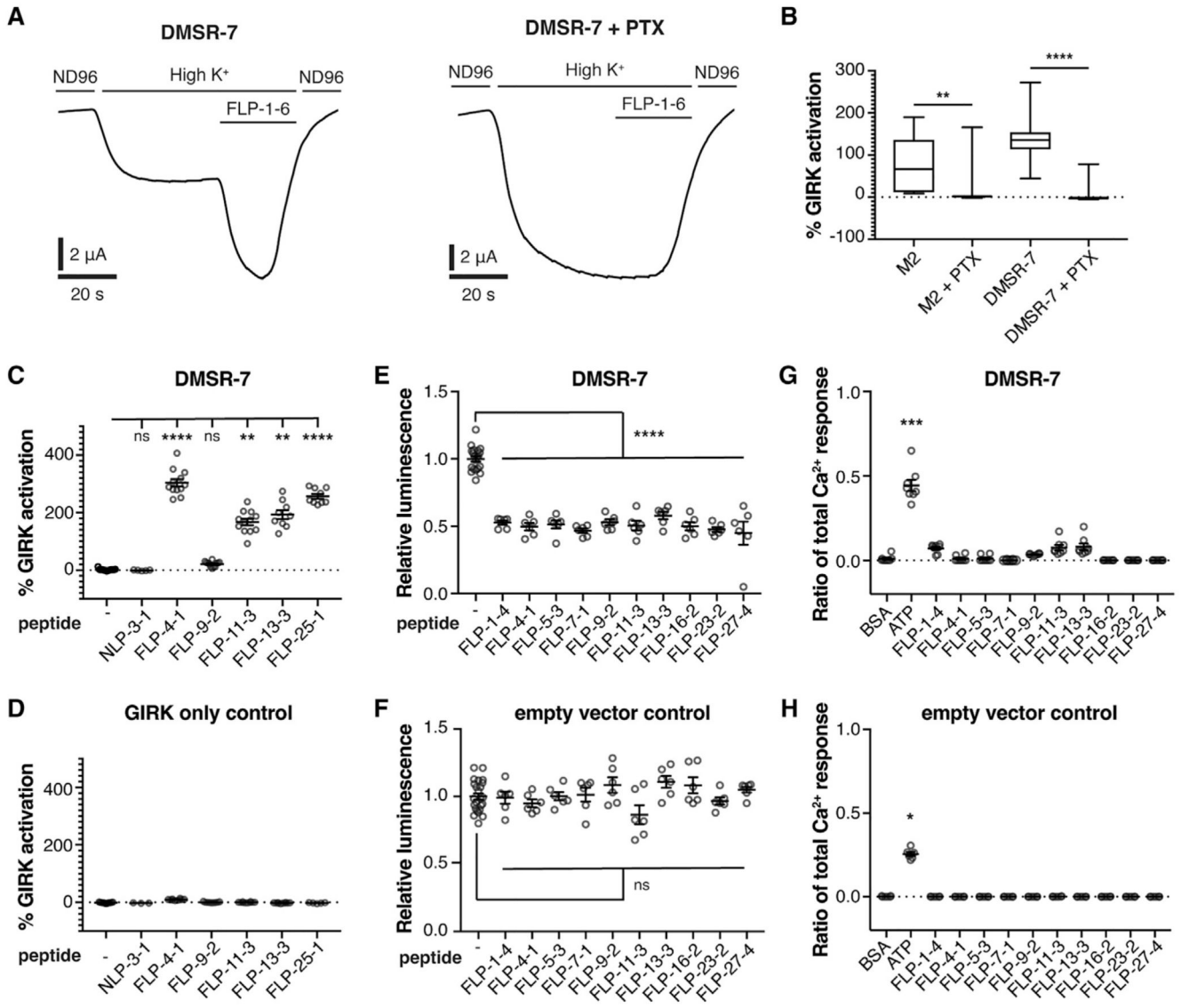

Figure 3. The promiscuous receptor DMSR-7 inhibits cAMP signaling in response to diverse RFamide peptides

(A) Continuous TEVC recordings from untreated (left) and PTX-injected (right) *Xenopus laevis* oocytes expressing DMSR-7, mGIRK1, and mGIRK2 and treated with 100 nM FLP-1-6 peptide. FLP-1-6 activates DMSR-7, resulting in a robust stimulation of K^+ current, which is blocked in PTX-injected oocytes.

(B) The percentage GIRK activation for at least seven oocytes expressing DMSR-7 or the $G_{i/o}$-coupled muscarinic M2 receptor, mGIRK1, and mGIRK2 with or without PTX treatment. M2 was exposed to acetylcholine and DMSR-7 to FLP-1-6 (100 nM). Boxplots indicate 25th (lower boundary), 50th (central line), and 75th (upper boundary) percentiles. Whiskers show minimum and maximum values.

(C and D) Mean percentage GIRK activation for oocytes injected with DMSR-7, mGIRK1, and mGIRK2 (C), or with mGIRK1/2 alone (D), in response to different peptides at 1 µM($n \geq 5$). NLP-3-1 does not activate DMSR-7 (Table S6) and was included as a negative control.

(E and F) Activation of DMSR-7 by diverse FLP ligands (10 μM) significantly decreases cAMP levels in HEK cells (E). None of the peptides affect cAMP signaling in cells transfected with a control vector (F). Data are shown as relative luminescence after normalization to the ligand-free control ($n \geq 6$).

(G and H) Activation of DMSR-7 does not affect calcium levels in CHO cells in the absence of the $G\alpha_{16}$ protein. Values are reported as the ratio of the total calcium response in cells transfected with DMSR-7 (G) or empty vector (H), and challenged with peptides (10 μM), BSA (negative control), or ATP (positive control) ($n \geq 6$). Error bars indicate SEM. Significance was assessed by Mann-Whitney test (A), Kruskal-Wallis with Dunn's test (C, D, G, and H), and one-way ANOVA with Dunnett's test (E and F). ****$p < 0.0001$, ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$; ns, not significant. See also Figure S8.

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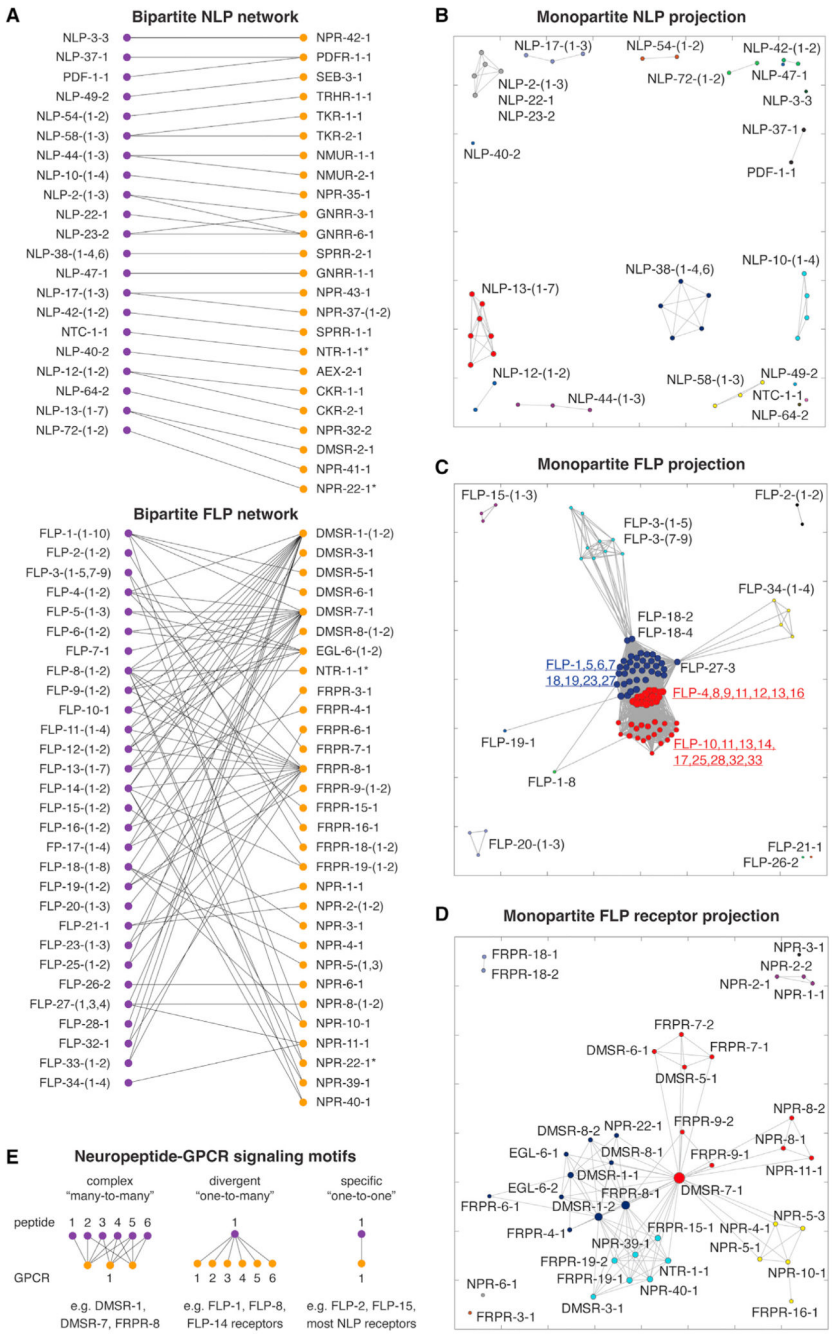

Figure 4. Specific and combinatorial interactions in the peptidergic network

(A) Bipartite graphs of NLP and FLP peptide-GPCR networks. Nodes represent peptides (purple) and receptors (orange). Edges between two nodes depict peptide-GPCR couples with sub-micromolar EC_{50} values (EC_{50} < 1 μM) identified in this resource. Asterisks indicate receptors in both NLP and FLP networks.

(B and C) Monopartite peptide projections of the NLP (B) and FLP (C) bipartite peptide-GPCR networks. Nodes are sized by the total number of connections (degree) and colored by module.

(D) Monopartite receptor projection of the FLP bipartite network. Nodes are sized by degree and colored by module.
(E) Different types of peptide-receptor interaction motifs in the peptidergic network. See also Figures S9–S12.

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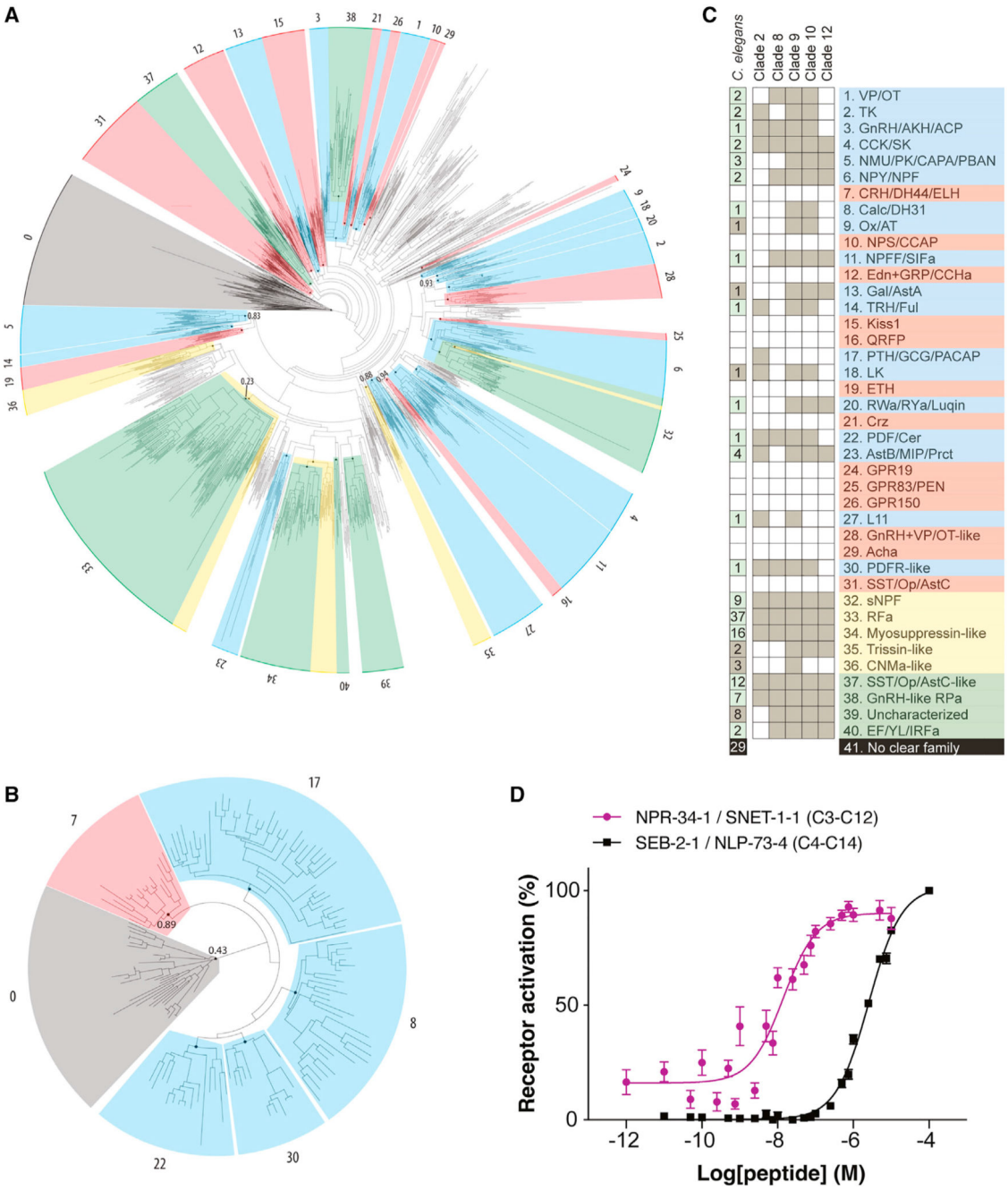

Figure 5. Peptide-GPCR pairs support conservation of bilaterian peptidergic systems and expansions of peptide-GPCR families in nematodes

(A) Maximum-likelihood tree of bilaterian rhodopsin peptide GPCRs. Subtrees are numbered according to the receptor families in (C). Subtrees comprising bilaterian families with nematode representatives are indicated in blue; those without nematode representatives are in red. Subtrees containing only protostomian sequences are colored yellow and nematode-specific subtrees are green. Node support values at the root of all delineated subtrees are above 0.95 unless depicted otherwise. Atypical peptide receptors (angiotensin, bradykinin, and chemokine receptors) were used as outgroup (gray).

(B) Maximum-likelihood tree of bilaterian secretin receptors. Color-coding, numbering, and node support values as in (A). Adhesion and cadherin receptors were used as outgroup.
(C) Inferred evolutionary relationships for nematode peptide-receptor systems. Names of peptidergic systems are assigned according to the classification by Mirabeau and Joly[31] and Elphick et al.[94] and color-coded as in (A). Gray squares mark the presence of a receptor ortholog. A receptor is considered present in a nematode clade when it is positioned inside a well-supported subtree (branch support value >0.95). The number of *C. elegans* receptors in each family is depicted in the left column and squares are colored green if at least one receptor is paired with a peptide ligand.
(D) Elevenin-like SNET-1 (LDCRKFSFAPACRGIML; C3–C12) and calcitonin/DH31-like NLP-73 (NRQCLLNAGLSQGCDFSDLLHAQTQARKFMSFAGPamide; C4–C14) peptides activate the elevenin and calcitonin/DH31 receptor orthologs NPR-34 and SEB-2. Calcium responses of CHO cells expressing NPR-34 or SEB-2 are shown relative (%) to the highest value (100% activation) after normalization to the total calcium response. Error bars represent SEM ($n \geq 6$). See also Data S1 and Tables S6, S8, and S9.

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