1   # The neuropeptidergic connectome of *C. elegans*

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Summary

Efforts are currently ongoing to map synaptic wiring diagrams or connectomes in order to understand the neural basis of brain function. However, chemical synapses represent only one type of functionally important neuronal connection; in particular, extrasynaptic, "wireless" signaling by neuropeptides is widespread and plays essential roles in all nervous systems. By integrating single-cell anatomical and gene expression datasets with a biochemical analysis of receptor-ligand interactions, we have generated a draft connectome of neuropeptide signaling in the *C. elegans* nervous system. This connectome is characterized by a high connection density, extended signaling cascades, autocrine foci, and a decentralized topology, with a large, highly interconnected core containing three constituent communities sharing similar patterns of input connectivity. Intriguingly, several of the most important nodes in this connectome are little-studied neurons that are specialized for peptidergic neuromodulation. We anticipate that the *C. elegans* neuropeptidergic connectome will serve as a prototype to understand basic organizational principles of neuroendocrine signaling networks.

Introduction

Understanding how behavior arises from neuronal interactions in the brain is one of the great challenges of modern neuroscience. In recent years, many projects have started to map the synaptic wiring diagrams (or synaptic connectomes) of simple and complex nervous systems to define their architecture at the cellular level[1–4]. Specifically, connectomics has aimed to identify all the individual units of the nervous system (i.e., its neurons) as well as all the connections between these units. In practice this involves reconstructing volumes of brain tissue using electron microscopy (EM), and then analyzing these images to trace neuronal processes and identify pre- and post-synaptic hallmarks of chemical synapses between identified neurons. Currently there are several endeavors of this type that are compiling complete connectomes for organisms important for neuroscience research, including *Drosophila*, *Platynereis*, *Ciona*, zebrafish, and mouse[2,3,5–7].

Although most connectomics research focuses on synaptic connectivity, chemical synapses are only one of several paths through which neurons communicate in the brain. For example, many important interactions between neurons involve volume transmission, by which secreted molecules are released extrasynaptically and activate receptors on neurons that are not wired through synapses or gap junctions to the releasing neuron. Unlike synaptic and gap junction transmission, where signaling is restricted to neurons on either side of the synapse, volume transmission can mediate signaling across distances of microns[8–10]. Such extrasynaptic signaling has been described for classical neurotransmitters as well as monoamines, and is particularly prevalent for neuropeptides, which are released from dense core vesicles outside the synaptic active zone and act on a longer temporal and spatial scale[10,11]. Although these extrasynaptic interactions are largely independent from the synaptic wiring, they play key roles in neural circuits and are thus critical for understanding the neural basis of behavior[12–18].

Neuropeptides carry out particularly complex and important functions in the brains of all organisms[19–21]. Neuropeptides are thought to be among the most ancient neuronal signaling molecules, and their origin may in fact predate the actual evolution of neurons[22,23]. Biologically-active peptides

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71 are usually formed from larger genetically-encoded precursors, which are enzymatically processed

72 through proteolysis and/or chemical modification[24]. Although some neuropeptides have been shown

73 to activate ion channels or transmembrane kinases, they typically bind to G protein-coupled receptors

74 (GPCRs), which act through second messenger pathways to modulate diverse aspects of neuronal

75 physiology[25]. Neuropeptide-activated GPCRs fall into two main classes, the rhodopsin-like (Class A)

76 and secretin-like (Class B) families, and within these groups many receptors (e.g.

77 oxytocin/vasopressin, neuropeptide Y/F, neuromedin U, somatostatin) show significant conservation

78 across widely divergent animal phyla[26,27]. Neuropeptide systems also play broadly conserved roles in

79 the control of behavioral states, including those involved in feeding, sleep and arousal, reproductive

80 behavior, and learning[14,28–33]. In humans, the role of neuropeptides as neuromodulators has made this

81 signaling system a highly sought-after target for new neuropsychiatric treatments. Currently 50 drugs

82 acting via GPCRs have been approved by the FDA[34], including several neuropeptide-GPCRs such as:

83 orexin antagonists for treatment of insomnia[35], a substance P antagonist for treatment of

84 chemotherapy-induced nausea[36]. Besides new treatments are being developed like a GIP agonist as

85 treatment for Alzheimer and Parkinson's[37]. These promising therapies and the large number of

86 remaining neuropeptide-GPCRs in humans[18,38] indicate the therapeutic potential of this molecular

87 system.

88 The diversity and extent of neuropeptide signaling implies that the pathways for peptidergic

89 communication can also be considered as a network. The genomes of all animals encode many, in

90 some cases hundreds of neuropeptides, along with a similarly large number of GPCRs[18,39–41].

91 Moreover, in contrast to monoamines, which are typically expressed in only a small subset of neurons

92 in the brain, neuropeptides are expressed extremely broadly; indeed, recent single-cell transcriptomic

93 studies indicate that most if not all neurons in the mouse cerebral cortex express one or more

94 neuropeptides as well as multiple neuropeptide-binding GPCRs[39,42]. These data imply that peptidergic

95 signaling underpins a dense and pervasive interaction network involving most if not all neurons of an

96 animal's nervous system[43,44]. Since neuropeptide signaling is thought to be mostly extrasynaptic, the

97 topology and structure of this wireless neuropeptide connectome is expected to be fundamentally

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98 distinct from that of the wired synaptic connectome[45]. However, in most organisms there is

99 insufficient gene expression data mapped with single-cell resolution to identified neurons to map the

100 structure of these extrasynaptic networks.

101 The nematode *C. elegans* represents an attractive animal to investigate the structure and

102 organization of neuropeptide signaling networks due to its small and well-characterized nervous

103 system. *C. elegans* was the first and is currently still the only adult organism with a completely

104 mapped synaptic neuronal connectome, with each of its 302 neurons and approximately 2300 synaptic

105 connections identified through electron microscopy (EM) reconstructions[46–48]. Despite its small size,

106 the *C. elegans* nervous system has been found to share several structural features with that of larger

107 animals. For example, the *C. elegans* connectome, like those of larger nervous systems, exhibits a

108 small-world topology, with relatively high clustering paired with relatively short average path

109 lengths[49,50]. Likewise, the *C. elegans* nervous system is highly modular, with functionally segregated

110 local clusters of high within-group connectivity[51–54]. Finally, the worm connectome contains a small

111 number of highly connected hubs, which are interconnected in a core or rich club and facilitate

112 communication between modules[55]; similar rich club topology has been observed in bigger brains,

113 including the human cortex[56,57]. Shared structural features are also apparent at the microcircuit level;

114 for example, feed-forward motifs are significantly overrepresented in the nematode connectome, just

115 as in the mammalian cortex[58–60]. Thus, insights gained from analysis of neuropeptide signaling

116 networks in *C. elegans* may also reveal organizational principles that are conserved in larger brains.

117 Although the *C. elegans* nervous system is anatomically small, at the biochemical level its

118 neuropeptide signaling pathways show similar complexity and remarkable conservation with humans

119 and other larger-brained animals. Its genome contains 159 predicted neuropeptide precursor genes

120 (NPP) (including 40 insulin-like peptides) that produce over 300 different neuropeptides[24,61].

121 Likewise, approximately 150 *C. elegans* genes encode GPCRs known or predicted to be activated by

122 neuropeptides[25,62]. These numbers are of a similar order of magnitude to the numbers of neuropeptides

123 and peptide activated GPCRs in the human genome[18,63]. Each *C. elegans* neuron not only expresses at

124 least one neuropeptide and at least one neuropeptide receptor, as do vertebrate neurons,[18,39,40,42,43] but

125 each *C. elegans* neuron class expresses a unique combination of neuropeptide-encoding genes[40], a

126 notion that illustrates the tremendous potential complexity of neuropeptide signaling. Many *C.*

127 *elegans* neuropeptides and cognate receptors are phylogenetically conserved across animal phyla and

128 have clear human homologues, with some families (such as the RFamide peptides) having undergone

129 expansion in the nematode lineage[64]. Thus, neuropeptide signaling in nematodes shows surprising

130 conservation as well as similar diversity to neuropeptide signaling in the human brain, despite vast

131 differences in neuron number and anatomical complexity.

132   Here we present a draft connectome of neuropeptidergic signaling in *C. elegans,* built by

133 integrating gene expression, ligand-receptor interaction, and anatomical datasets. We identified

134 predicted neuron-to-neuron signaling interactions mediated by 91 neuropeptide-receptor pairs, and by

135 aggregating these individual neuropeptide-signaling networks generated a comprehensive network of

136 peptidergic signaling in the *C. elegans* nervous system. This resulting neuropeptidergic connectome

137 differs significantly in its structure from the previously-characterized wired connectome[47]; for example,

138 it is denser, contains distinct hubs, high-weight edges, and autocrine signaling foci, and has a

139 decentralized topology allowing direct communication among a large fraction of neurons. We expect

140 this nematode neuropeptide connectome will serve as a prototype for understanding the organization of

141 peptidergic signaling and how it interacts with the wired neural circuitry in other organisms, including

142 those with much larger brains.

143 **Results**

144

145 **Construction of a neuropeptidergic connectome**

146 To generate a draft neuropeptide connectome, we integrated information from three datasets to infer

147 potential pathways for neuropeptide signaling between individual *C. elegans* neurons (Figure 1A). To

148 identify biologically relevant molecular interactions between individual neuropeptides and receptors,

149 we used data from a large-scale reverse pharmacology screen which tested 55,386 potential

150   neuropeptide-receptor interactions *in vitro* and identified 461 peptide-GPCR couples encoded by 55

151   neuropeptide and 57 GPCR genes[40]. To determine which neurons express each neuropeptide and

152   receptor, we used publicly available single-neuron transcriptome data from the CeNGEN project, which

153   described single-cell RNA-sequencing transcription profiles of all predicted neuropeptide and peptide-

154   activated GPCR genes in *C. elegans*[40]. We validated these scRNA datasets with several CRISPR/Cas9-

155   engineered reporter alleles of ligands and receptors. Finally, we used previously published EM

156   anatomical reconstructions to assess physical constraints on potential diffusion of neuropeptides to their

157   target receptors[48,65,66]. In constructing the neuropeptide network, we consider that two neurons (nodes)

158   are connected by a directed edge if the first neuron expresses a particular neuropeptide ligand and the

159   second expresses a paired receptor, subject to spatial constraints on signaling (Figure 1B).

160   For each dataset, it was necessary to threshold for biologically relevant interactions.

161   For biochemical interactions, the raw dataset contained 461 neuropeptide-receptor pairs that showed

162   dose-dependent activation with a half-maximal effective concentration (EC_{50}) ranging between 0.1 pM

163   to 22 μM[64]. At the gene level, these pairs were encoded by 55 neuropeptide and 56 GPCR genes, with

164   148 unique gene-gene interactions. In assessing neuropeptide-receptor pairings we initially opted for a

165   conservative EC_{50} threshold of 500 nM, as many neuropeptide-receptor couples with EC_{50} values in this

166   range have been validated *in vivo*[14,25,28,32,62]. By this criterion, we defined 91 individual neuropeptide-

167   receptor gene couples, with a large number (51) of the predicted neuropeptide GPCRs having at least

168   one identified ligand. The ligand-receptor interactions were complex, with several peptides activating

169   more than one receptor (promiscuous receptors) and several receptors being activated by multiple

170   peptides encoded by distinct precursor genes (versatile peptides)[64].

171   We next sought to determine an appropriate threshold for the scRNA-based gene expression

172   dataset. The transcriptomic data from the CeNGEN project has been differentially thresholded based

173   on a ground-truth dataset of reliable gene expression from across the whole *C. elegans* nervous

174   system using fosmid or receptor tagged reporters[40]. Each threshold was given a false discovery rate

175   and a true positive rate depending on the number of correctly identified cells for a given gene by the

176   scRNAseq analysis[40]. To determine the most appropriate threshold for our analysis, we obtained

177   single-copy genomic knock-in reporters for 17 representative neuropeptide precursor genes and 9

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178   representative genes for neuropeptide receptors and characterized their expression patterns

179   comprehensively using the NeuroPAL marker strain (Figure 2A, B; Supplementary Figure 1). For

180   both we found that threshold 4, although on some occasions overly stringent, was a good

181   approximation of the expression pattern (Figure 2; Supplemental Table 1-3). Thus, although this

182   stringent threshold has lower discovery power and thus may undercount the number of neurons

183   expressing each gene, its stringency minimizes the likelihood that our resulting network will contain

184   edges that do not represent authentic paths for potential neuropeptide signaling.

185

186   **Evaluating spatial constraints on neuropeptide signaling**

187   These biochemical and gene expression datasets allowed us to infer which neurons express

188   neuropeptide and receptor genes that could mediate a neuromodulatory interaction; however, the

189   physics of diffusion and the neuroanatomy of the animal might potentially constrain some of these

190   interactions *in vivo*. We therefore considered several possible models of the neuropeptidergic network

191   based on different anatomical and spatial constraints (Figure 3B, C). We used EM reconstruction data

192   for each individual neuron in the *C. elegans* nervous system to identify its anatomical location (Figure

193   3A, Supplementary Table 4) and the neuropil bundles into which their axons and dendrites

194   project[48,65,66]. In the first and most permissive model, long-range signaling is permitted and

195   neuropeptidergic connections can take place between any neurons in the system. In the second ("mid-

196   range") model, neuropeptidergic connections can occur only between neuronal processes in the same

197   anatomical area, such as the head (including pharynx), tail and midbody (Figure 3A). In the third

198   ("short-range") model, we only considered potential peptidergic connections between neurons in the

199   same neuronal process bundle, that is, between neurons whose processes overlap in the nerve ring,

200   ventral or dorsal cord, or an auxiliary nerve. Finally, it has recently been shown that the nerve ring,

201   into which more than half the neurons (168) project, can be divided into four layers or strata based on

202   patterns of physical contact[67,68]. We therefore also considered a fourth model in which neuropeptide

203   signaling inside the nerve ring is constrained within individual strata and thus between neurons that

204   make physical contact.

205   To evaluate each model, we investigated whether the expression patterns of receptors and

206   ligands were consistent with neuropeptide signaling being restricted as described above (Figure 3B,

207   C). For example, if neuropeptide signaling occurred only between neurons in physical contact (as in

208   model 4) we would expect neurons expressing a given receptor to project to the same nerve ring strata

209   as neurons expressing its ligand. To simplify this analysis, we focused on the neuropeptide-receptor

210   couples in which the receptor has only one ligand and the neuropeptide only binds to that receptor,

211   assessing whether, under the constraints of each model, there would be receptor-expressing neurons

212   that were unable to receive a signal from any neurons expressing its ligand. Conversely, we also

213   analyzed couples where the ligand activated only one receptor, asking whether under a given model

214   there were ligand-expressing neurons that could not communicate with cells expressing its receptor.

215   This analysis appeared to argue against the most restrictive model, in which ligands could not

216   diffuse between nerve ring strata. Specifically, we observed many examples in which a neuropeptide

217   receptor was expressed only in strata that did not express its specific ligand (Figure 3B, C, orange

218   points). For instance, while the *capa-1* neuropeptide precursor gene is expressed only in the ASG

219   sensory neuron, which projects its axon into the fourth nerve ring stratum, the gene for its receptor

220   NMUR-1 is expressed in all four strata[32,69](Figure 3B, C). Thus, NMUR-1 receptors in strata 1-3 must

221   be activated by CAPA-1 peptides that diffuse from their release site in stratum 4. Overall, 37 of the 41

222   receptors analyzed were expressed in at least one neuron making no contact with a neuron expressing

223   its ligand. Likewise, 32 of the neuropeptide precursor genes were expressed in at least one neuron

224   making no contact with neurons expressing its receptor (Figure 3C, yellow points). Since the nerve

225   ring strata are not separated by glial or other barriers, and experimental evidence[14,32] indicates

226   neuropeptides can indeed travel between strata, it is reasonable to infer that neuropeptides are not

227   likely to be constrained by anatomical layers in the nerve ring or other neuronal bundles.

228   We likewise observed cases in which the expression of a neuropeptide or receptor gene

229   implies mid-range signaling between distinct nerve bundles in the same body region (Figure 3B, C).

230   For example, the *frpr-7* receptor gene is expressed in multiple pharyngeal neurons, while its ligand

231   FLP-1 is released exclusively from the AVK neuron whose processes lie in the nerve ring and ventral

232   cord[13]. Thus, FRPR-7 receptors in the pharynx must be activated by peptides released by AVK in the

9

233   nerve ring, consistent with published evidence that neuropeptides can signal between head neurons

234   and pharyngeal neurons[13,70]. Indeed, for a majority (14/22) of the receptors expressed in pharyngeal

235   neurons, their ligand was expressed only outside the pharynx (Figure 3B, red points). Likewise,

236   receptors such as NPR-3 and TRHR-1 are expressed in the CAN neurons, yet their ligands FLP-15

237   and NLP-54 respectively are not expressed in any neurons projecting into the canal-associated nerve[71]

238   (Figure 3B, C, CAN neurons labelled). Thus, we hypothesize that at least some neuropeptide

239   signaling occurs between different nerve bundles, in particular between the pharynx and the nerve

240   ring and between CAN and other nerves in the body. We therefore focused our subsequent analysis on

241   model 2, consisting only of stringently selected short-range interactions, and model 3, which includes

242   mid-range signaling between nerve bundles in the same body region.

243

244   **Neuropeptide networks exhibit diverse topologies**

245   Based on these criteria, we first constructed network graphs between neuropeptide-expressing

246   and receptor-expressing neurons for each of the 91 individual neuropeptide/receptor couples in our

247   dataset (Supplementary Figure 2-3). These networks were filtered by removing edges between

248   neurons that did not project an axon or dendrite into the same process bundle (for the short-range

249   network) or into the same body region (for the mid-range network). In their short-range version, 78 of

250   these ligand-receptor couples formed a single connected network, whereas 13 formed networks with

251   two or even three disconnected components (Figure 4B, Supplementary Figure 2). In their mid-range

252   versions, all 91 couples formed a single connected network (Supplementary Figure 3).

253   We observed a diverse range of topologies in the networks for individual peptide-receptor

254   couples. One topological measure in which the different networks varied was their assortativity, or the

255   extent to which nodes are preferentially connected to nodes of similar degree. Using the conservative

256   short-range model, we observed that most of the networks, including many of those highly conserved

257   in evolution, were local networks, in which both ligand and receptor were expressed in relatively

258   restricted sets of neurons (Figure 4A, Supplementary Figure 2). The neurons in these networks

259   showed relatively low average degree and often encompassed only a subregion of the total nervous

260   system. In addition, we found 8 highly disassortative *integrative* networks, with many low-degree

261   peptide-releasing neurons signaling to relatively few high-degree receptor-expressing neurons (Figure

262   4A). We also observed 23 disassortative *broadcasting* networks that are characterized by a small

263   number of high-degree peptide-releasing neurons that signal to many low-degree receiving neurons

264   with broadly expressed receptors (Figure 4A, Supplementary Figure 2). Interestingly, both integrative

265   and broadcasting networks involved promiscuous receptors with multiple ligands, specifically FRPR-

266   8, EGL-6, DMSR-7, and DMSR-1. DMSR-7 and DMSR-1 also figured prominently in a fourth

267   category of more assortative, pervasive networks, in which both the ligand and receptor show broad

268   expression and most neurons thus show relatively high degree (Figure 4A, B). Relaxing the spatial

269   restrictions on our model had relatively modest impact on network topology, with four networks that

270   are local in the short-range model becoming either integrative (one network) or broadcasting (3

271   networks) in the mid-range model (Supplementary Figure 3). Thus, the topologies of neuropeptide

272   networks appear relatively robust to our assumptions about the spatial scope of neuropeptidergic

273   signaling.

274   **The neuropeptide connectome is a decentralized, dense network**

275   By aggregating the networks from the individual neuropeptide-receptor couples, we next

276   compiled complete neuropeptide connectome networks based on short-range or mid-range signaling

277   (Figure 5). Even for the more conservative short-range network, in which signaling was restricted to

278   neurons with processes overlapping in the same bundle, the density of connections was remarkably

279   high, with more than a third of all possible connections or edges (0.3437) present in the network.

280   Allowing mid-range connections (0.4429) or long-range connections (0.5929) increased this density

281   further (Supplementary Figure 4A, B). By comparison, the *C. elegans* synaptic (0.0251) and

282   monoamine (0.0236) networks were far less dense, fourteen-fold lower than the short-range

283   neuropeptide network. We also computed edge weights for the connections in the neuropeptide

284   networks based to the number of neuropeptide-receptor pairs capable of signaling between two nodes.

285   We observed that a large number (35% in the short-range network) of neuron pairs were connected by

286   only a single neuropeptide-receptor interaction, but some (9%) were connected by 6 or more different

287   peptide-receptor couples (Figure 5). In the most extreme case, we observed that two neurons, the

288   AVD premotor interneurons and the PQR oxygen-sensing neurons, were linked by 18 different

289   neuropeptide-receptor pairs, suggesting extraordinarily complex patterns of signaling could exist

290   between these cells (Figure 5, highlighted). Interestingly many other high-weight, biochemically

291   complex connections occurred between neurons of the oxygen sensing circuit and were mediated by a

292   common set of promiscuous receptors (DMSR-1, DMSR-7 and FRPR-8) and versatile neuropeptides

293   (FLP-4, FLP-9, FLP-10 and FLP-13). These neurons are thus connected by multiple neuropeptidergic

294   channels of communication, potentially allowing complex regulation by context and experience.

295   A striking feature of the midrange network in particular was its integration of neurons

296   disconnected from the synaptic connectome. For example, the 14 neuron classes of the pharyngeal

297   nervous system form a heavily synaptically interconnected, functionally autonomous network akin to

298   vertebrate enteric nervous systems, which is topologically isolated from the rest of the nervous

299   system[65,72,73]. We find that unlike the wired connectome, there are no neuropeptidergic networks that

300   define circuitry exclusively in the pharynx; to the contrary, pharyngeal neurons are fully integrated

301   into the somatic nervous system via strong reciprocal interconnectivity (Figure 5). All pharyngeal

302   neuron classes receive 90% or more of their incoming connections from outside the pharynx. Some

303   classes of pharyngeal neurons also broadcast extensively to the somatic nervous system, with several

304   (I1, I3, I4, I5, M5 and NSM) having more than 100 outgoing connections (more than 90% of their

305   total) to non-pharyngeal neurons. Likewise, it is notable that the CAN neurons, which completely lack

306   chemical synapses, show strong and reciprocal neuropeptidergic connectivity with the rest of the

307   nervous system, indicating that this unusual neuron class is well embedded in the neural network

308   (Figure 5, CAN highlighted).

309   We next investigated topological features of the aggregate neuropeptide network. In

310   particular, we analyzed the degree nodes of the network, defined as the number of incoming (in-

311   degree) and outgoing (out-degree) connections made by each neuronal node. Degree is often an

312   indicator of functional significance in networks, since high-degree nodes or hubs often play key

313   functional roles in the brain and other complex systems. As expected from the high density of

314   neuropeptide signaling, the average degree of both the short-range (Figure 6A) and mid-range

315   networks (Figure 6B) were significantly higher than for the previously characterized synaptic (Figure

316   6C) and monoamine (Figure 6D) networks. Moreover, when we analyzed the distribution of degree

317   across the neuropeptide networks, we observed relatively flat curves with many high-degree nodes;

318   more than half the neurons in the short-range network and nearly two thirds in the mid-range network

319   had a degree higher than 200, indicating that they have in and out connections with more than a third

320   of all other neurons (Figure 6A, B). In contrast, the synaptic network was much more centralized,

321   with only 10 neurons having degree greater than 50 (Figure 6C); similarly, the monoamine network

322   had only 18 high (>50) degree neurons (Figure 6D). The best-connected neurons in the neuropeptide

323   network have exceptionally high in-degree as well as out-degree, indicating that their status as hubs

324   depends as much on incoming connections as outgoing connections, and as much on their expression

325   of broadly-signaling neuropeptides as on integrating GPCRs (Figure 6A, B); this contrasts with the

326   monoamine network, where the hubs are exclusively monoamine-releasing neurons with a high out-

327   degree (Figure 6D).

328

329   **The neuropeptide connectome contains a highly connected core with unique peptidergic hubs**

330   A significant feature of many networks is the so-called rich club property. In such networks,

331   the most highly connected hubs form more connections between themselves than expected based on

332   their high degree alone; these hubs therefore comprise a rich club or core of the network that

333   facilitates communication between more peripheral network nodes. The *C. elegans* synaptic

334   connectome, for example, contains a rich club consisting of 11 premotor interneurons that appear to

335   play important roles in driving global brain states[55,74]. Remarkably, we found that the neuropeptide

336   connectome also shows the rich club property, but its rich club consists of 156 neurons (166 in mid-

337   range network), more than half of the nervous system (Figure 6F). Within the rich club the density of

338   connections is 0.6834 (p<0.00001), more than double the density of the overall network (0.3427).

339   Interestingly, compared to the wired synaptic connectome, the neuropeptide connectome as a whole

340   has significantly higher clustering and reciprocity (i.e. neurons are more likely to connect in both

341   directions) but lower modularity and disassortativity. This finding further supports the notion of a

342   decentralized neuropeptide connectome, with a broad-based densely-connected core allowing direct

343   communication between a large fraction of neuron pairs.

344   To further investigate the relationship between neuropeptide and synaptic signaling, we

345   looked at the correlation between synaptic and neuropeptidergic degree (Figure 6G). We observed

346   that neuropeptide degree and synaptic degree were positively correlated ($p < 0.0001$, $r = 0.53$). We

347   further observed that neurons with a high synaptic degree were among the most important

348   neuropeptidergic hubs; for example, all 11 members of the synaptic rich club (DVA, PVCL/R,

349   AVAL/R, AVBL/R, AVDL/R, AVEL/R) were among the 25 highest-degree nodes in the short-range

350   neuropeptide network (Figure 6G). However, there were also several neurons of very high degree in

351   the neuropeptide connectome but unexceptional synaptic degree (Figure 6G); these neurons can be

352   described as specialized for neuropeptide signaling. Six neurons (AVKL/R, PVQL/R, PVT and PVR)

353   had higher short-range neuropeptide degree than any of the synaptic rich club neurons (Figure 6A); if

354   mid-range signaling is considered these same six neurons remain the highest-degree hubs (Figure 6B).

355   Interestingly, the AVKL/R and PVT neurons are notable for expressing no classical neurotransmitters

356   or monoamines[75], while the PVQL/R neurons appear anatomically specialized for neuropeptide

357   signaling due to a preponderance of dense core vesicles[14,76]. AVKL/R, and PVT have been previously

358   linked to the control of global behavioral states related to sleep and arousal[14,73], but the functions of

359   the other neuropeptide hub neurons PVQ and PVR are not well-characterized.

360

361 The neuropeptide connectome core has a defined mesoscale structure

362   To further probe the structure of the neuropeptide connectome we investigated whether the

363   network contained modules or other forms of mesoscale substructure. We first applied standard

364   methods for modular decomposition, but the high density of the network precluded the identification

365   of any discrete modules, unless we aggressively filtered out lower-weight edges where neurons were

366   connected only by a small number of neuropeptide-receptor interactions. However, we hypothesized

367   that other types of meso-scale structure may be present[52,77]. We wondered for example whether we

368   could identify subgroups of neurons with similar patterns of incoming and outgoing neuropeptide

369   connections. We therefore applied dimensionality reduction methods (Principal Component Analysis

370   (PCA) and t-SNE) to the connectivity matrix of the aggregate neuropeptide networks to identify

371   groups of neurons that share a connectivity pattern. This analysis highlighted 3 clearly defined groups

372   of neurons that receive similar incoming connections, along with a more diffuse cloud of neurons with

373   more variable connectivity (Figure 7A, B). Interestingly the three clusters comprise most neurons of

374   the neuropeptidergic network (155 of 302), 76% of which are also members of the neuropeptidergic

375   rich club (112; Figure 7A, B). Interestingly, each group receives a characteristic range of incoming

376   connections, and this alone is enough to significantly sort them into these groups (p<0.0001) (Figure

377   7D): group 1 neurons have indegree of 134-156 (mean rank 162.2), group 2 neurons have an indegree

378   of 252-282 (mean rank 276.5), group 3 neurons have an indegree of 169-212 (mean rank 222.9), and

379   neurons in the unclustered cloud have an indegree below 169 (mean rank 77) (Figure 7D).

380   These groups also diverge in the neuron types that form them and to which they are connected

381   (Figure 7C, E). Group 1 is mostly motorneurons (motor core), including those involved in locomotion

382   (VA, DA, VB, and DB)[78] and the grouping of these neurons is driven by inputs from the posterior

383   touch mechanosensory neurons PVM and PLML/R[79] and the interneuron PVWL/R (Figure 7C).

384   Group 2 neurons are a mix of interneurons and motorneurons, including all top neuropeptidergic hubs

385   (hubs core), that receive connections from every other neuron type, with the neuropeptidergic hubs

386   (PQR, PVT and PVR) particularly important drivers for the grouping (Figure 7C). Finally, group 3

387   neurons are a mix of pharyngeal neurons, interneurons, motorneurons and sensory neurons (sensory

388   core) that receive connections from every other neuron type but motorneurons, with RIR and

389   pharyngeal neurons I5 and I4 driving the cluster (Figure 7C). Interestingly, although the characteristic

390   neural inputs of the groups differed substantially, the most important (highest indegree) neuropeptide-

391   receptor interactions for each group, involved versatile neuropeptides FLP-9, FLP-11 and FLP-16 and

392   promiscuous receptors DMSR-7 and DMSR-1 (Figure 7C), leading to interconnections between

393   groups (Figure 7E). For example, Group 1 forms connections with itself, Group 2 and with the

394   unclustered cloud but not with Group 3; Group 3 forms connections with Group 2 and unclustered

395   cloud neurons but not Group 1, and Group 2 forms connections with all other groups. Thus, Group 2

396   serves as a link with Groups 1 and 3, which have few direct connections with each other.

397

398   **Co-expression of neuropeptides with their target receptors generates autocrine pathways**

399 Signaling cascades, in which a neuropeptide receptor is specifically co-expressed with a non-

400 cognate peptide whose release it controls, are a classic hallmark of neuroendocrine pathways. To

401 investigate whether the *C. elegans* neuropeptide connectome contains such cascades, we evaluated co-

402 expression between neuropeptide and receptor genes. We performed a Fisher's test to look for

403 neuropeptide genes and GPCR genes that are co-expressed in the *C. elegans* nervous system more

404 often than expected from their individual expression frequencies. We identified 121 peptide-receptor

405 pairs meeting this criterion (Supplementary Figure 5), only 5% of which corresponded to cognate

406 neuropeptide-receptor pairs (autocrine connections). Using these peptide-receptor pairs as nodes and

407 the neuropeptide-receptor interaction data to form edges between them, we built a network of

408 overrepresented signaling cascades within the larger neuropeptide connectome (Supplementary Figure

409 5B). This network is fully connected and provides a simplified view of how neuropeptide signaling

410 pathways interact within the nervous system.

411 Co-expression of a neuropeptide receptor with its own ligand will generate a self-loop or

412 autocrine connection, in which release of the peptide can signal back onto the sending cell. If we

413 consider all cases where a peptide and its receptor are co-expressed in a given neuron, we find that 58%

414 of the *C. elegans* nervous system harbors putative autocrine peptide connections (Figure 8A and 8B).

415 Autocrine signals appear most prevalent in neurons categorized as either interneuron or motorneuron

416 (Figure 8B), although two of the three types with the largest number of autocrine connections (URX

417 and PQR) are sensory neurons. Promiscuous receptors participate more in autocrine peptide signaling

418 (Figure 8C, Suppl. Figure S6), the most prominent of which are DMSR-1 and DMSR-7 (Figure 8C).

419 We observed a strong positive correlation between the number of different autocrine peptide

420 connections that a neuron harbors to its degree within the neuropeptide network (Figure 8D, left panel),

421 with some but not all neuropeptide hubs showing a high diversity of autocrine signaling. In contrast,

422 weak to no correlations were observed between autocrine connection diversity and the synaptic, gap

423 junction, or monoamine connectomes.

424 Autocrine connections were especially prevalent in specific circuits in the *C. elegans* nervous

425 system. For example, both URX and PQR, which have among the highest diversity of autocrine

426 signaling, are key sensory neurons that mediate *C. elegans'* responses to aversive O_2 levels[80–82]; other

16

427   O_2 sensory neurons (AQR and BAG) also co-express ligand-receptor pairs, though to a lesser degree

428   (Figure 8E, bottom left). As O_2 sensing neurons tonically signal ambient O_2 concentrations[83], autocrine

429   signaling may play a stabilizing role in maintaining the homeostasis of neuronal activity. Autocrine

430   neuropeptide signals are also prevalent in the neuromuscular circuit of the ventral nerve cord (VNC –

431   Figure 8F, bottom right) which mediates locomotion[84]. These autocrine loops are primarily mediated

432   by eight RFamide-related neuropeptides that activate two promiscuous receptors, DMSR-1 and DMSR-

433   7, although other receptors such as NPR-5 are also involved (Supplementary Figure 6F). Interestingly,

434   autocrine signaling is restricted to the excitatory A- and B-class VNC motorneurons which drive

435   backward and forward locomotion, respectively[84]; in contrast, both D-type inhibitory motor neurons

436   and the excitatory AS motorneurons, which play modulatory roles in both forward and backward

437   crawling[85], do not co-express any neuropeptide-GPCR pairs. It should be noted that some autocrine

438   peptidergic pathways are shared between neighboring excitatory motorneurons in the VNC, raising the

439   possibility that they might coordinate activity and/or neurosecretion across excitatory motorneurons in

440   a paracrine manner[86–89]. Heterogeneity in autocrine neuropeptide pathways within the A- and B-class

441   motorneurons (Supplementary Figure 6F) hints at heterogeneity in the contribution of individual

442   motorneurons in this process.

443

444   **Discussion**

445   **Neuropeptide signaling forms a complex wireless network**

446   Neuropeptide signaling has long been recognized as critical to brain function, yet despite

447   recent advances in connectomics, the structures of neuropeptidergic signaling networks are largely

448   uncharacterized. We have generated a draft neuropeptide connectome by integrating information from

449   three datasets: a biochemical deorphanization screen matching neuropeptidergic GPCRs with their

450   ligands, a scRNAseq dataset characterizing the expression patterns of all peptide and receptor genes at

451   the single neuron level, and an anatomical dataset defining the morphologies and neuron-neuron

452   contacts of all *C. elegans* neurons. The resulting connectome is remarkably dense; even a short-range

453   network with the most conservative assumptions about the spatial diffusion of neuropeptides was

17

454   more than ten-fold denser than the previously mapped synaptic connectome of *C. elegans*. As we have

455   also used conservative assumptions regarding gene expression thresholds and ligand-receptor

456   affinities, and since approximately half the putative neuropeptide-activated GPCRs remain to be

457   deorphanized, the actual neuropeptide connectome is almost certainly even more dense than the draft

458   network described here. However, based on sensitivity analysis it seems clear that the overall

459   structure and topological features of the neuropeptide connectivity are likely to hold even when more

460   connections are added to the network (Supplementary Figure 4A-B).

461   A particularly salient feature of the neuropeptide connectome is its decentralized topology,

462   which contrasts sharply with the more centralized structure of wired neural connectomes. Synaptic

463   connectomes from worms to humans are characterized by a core of high-degree hubs, which are

464   interconnected to form a rich club. This rich club, which in *C. elegans* consists of 11 premotor

465   interneurons, occupies a central position in the connectome, connecting local modules and

466   coordinating their activity. Rich clubs have been previously linked to important functional properties;

467   for example, in *C. elegans* the synaptic rich club has been shown to be involved in global brain states

468   related to locomotion[55,74], and in *Drosophila melanogaster* the synaptic rich club constitutes the

469   sensorimotor integrative center of the organism[90]. The gap junction and monoamine connectomes of

470   *C. elegans* likewise contain relatively small rich clubs of less than 20 neurons of relatively high

471   degree. In contrast, the neuropeptide connectome contains a rich club of (in the short-range network)

472   156 neurons, more than half the neurons in the entire nervous system. The neurons in this

473   neuropeptidergic rich club are extremely well-connected to each other as well as to the rest of the

474   nervous system; all have a degree greater than 203 (in the mid-range network degree of 251), meaning

475   they both send and receive direct connections from at least 40% of all neurons. This remarkable

476   decentralization may be a feature of neuropeptide signaling networks in other organisms[43,44,91].

477   **Implications of neuropeptide network structure for neuronal computation**

478   The decentralized structure of the neuropeptidergic connectome implies that the strategies it

479   uses for computation and information flow may differ significantly from those employed by the more

480   centrally-organized synaptic network. Intriguingly, both nematode and mammalian neuronal types

481   appear to express nearly unique combinations of neuropeptides and receptors, serving as a molecular

482   bar code for neural identity[40,43]. Thus, the source of a signal may be encoded by the precise

483   combination of peptides released by the sending neuron. The dynamics of neuropeptide release are

484   also likely to be critical to the information conveyed by peptidergic signals. For example, acute

485   release of FLP-20 peptides by mechanosensory neurons has been shown to trigger short-term sensory

486   locomotor arousal in response to touch stimulation, while chronic release of FLP-20 peptides from the

487   same neurons mediates long-term cross-modal plasticity in olfactory circuits when the sense of touch

488   is lost[14]. Future studies of such mechanisms are likely to provide general insights into how

489   neuropeptide networks encode information in the brain.

490   Despite its highly dense connectivity, the core of the neuropeptide connectome exhibited a

491   clear substructure. When input patterns were analyzed using dimensionality reduction methods, the

492   neurons in the network core could be divided into three clear groups, one containing mainly

493   pharyngeal and sensory neurons, a second containing the highest degree interneurons, and a third

494   containing many motorneurons. These three groups are themselves connected in a defined pattern,

495   with the second group linking the first and third groups which show few direct links with each other.

496   This organization contrasts in interesting ways with the organization of many synaptic networks, in

497   which peripheral neurons form modules with high internal connectivity that connect to each other

498   through the hubs of the rich club core. In the neuropeptide connectome the core itself exhibits a clear

499   meso-scale organization, forming 3 distinct groups of neurons defined not by unusually high intra-

500   group connectivity, but by intra-group similarity of incoming and outgoing connections patterns. This

501   is in line with recent work showing the existence of different types of meso-scale organization[77] and is

502   also reminiscent of stochastic block modelling approaches previously applied to the *C. elegans*

503   synaptic connectome[52]. It will be interesting to see if neuromodulatory and synaptic networks from

504   other nervous systems show a similar diversity on meso-scale structure. We expect that looking

505   beyond classical modules is likely to become increasingly important as we begin to look at cellular-

506   scale connectomes of larger organisms, with classes of sensory neurons that are not interconnected but

507   have similar connectivity profiles and perform similar functions.

508   **Identification of neuropeptide signaling hubs**

19

509   Although the rich club of the neuropeptide connectome is extensive and encompasses a large

510   portion of the *C. elegans* nervous system, some neurons within this group are of particularly high

511   degree and therefore may play key roles in neuromodulatory signaling. Perhaps not surprisingly, the

512   premotor neurons of the synaptic rich club are also highly connected in the neuropeptide connectome;

513   all 11 of these neurons show a neuropeptide degree above 400 in the short-range network. Since these

514   neurons are known to play important roles in driving global brain states[14,74,76] it is logical that they

515   would also be important targets of neuromodulatory control. In addition, the neuropeptide

516   connectome contains 6 neurons whose neuropeptide degree is higher than any of the synaptic rich

517   club (degree > 490). Three of these neurons (PVT and the pair of AVK neurons) are specialized

518   peptidergic neurons that express no classical neurotransmitter or monoamine and have been linked to

519   arousal and sleep-like behaviors[14,92,93]. Specialized neuropeptidergic neurons have also been

520   described in other organisms, and in mice they have been linked to global behaviors such as fear[94].

521   The other peptidergic hubs (PVR and the pair of PVQ neurons) are tail neurons that extend long

522   processes to the nerve ring, and in the case of the PVQs (as well as PVT) these processes are rich in

523   dense-core vesicles[48,65,66]. Since AVK also has a long process (extended from its cell body in the head

524   through the nerve ring and the ventral cord to the tail) these neurons may be morphologically

525   specialized for local release of peptides throughout the nervous system. While AVK is known to play

526   roles in arousal and motor control, the functions of the remaining neuropeptide hubs (or of other

527   neurons of high neuropeptidergic degree such as PVN and PVP) are largely uncharacterized. Given

528   their importance in the neuropeptide signaling network, it will be interesting in the future to explore

529   the roles of these neurons in the control of behavioral states.

530   It is interesting to note that even in the long-range network, where no spatial restrictions on

531   neuropeptide diffusion are imposed, the six short and mid-range hub neurons retain their central

532   importance in the network. This confirms that the high degree of the hubs in the short- and mid-range

533   networks is not merely an artifact of the spatial conditions we imposed but rather is a consequence of

534   expressing key combinations of neuropeptides and receptors. Notably, the long-range network

535   contains additional high-degree nodes that are not hubs in the short- and mid-range networks, in

536   particular the oxygen-sensing neurons URX, AQR, PQR and BDU and the tail motorneurons DA09

537 and VA12. One might speculate that these neurons could in fact engage in long-range neuroendocrine

538 signaling, and that the long-process morphology of the short- and mid-range hubs might allow them to

539 carry out neuropeptide modulation on a finer temporal or spatial scale. In the future, these questions

540 may be addressable using *in vivo* probes for neuropeptide receptor signaling[95,96].

Neuropeptide signaling links specific components of the nervous system

542 In addition to these high degree nodes, the neuropeptide connectome also contains edges of

543 unusually high weight, representing neuron pairs linked by multiple neuropeptide-signaling pathways.

544 While many neuron pairs in the network are connected by a single neuropeptide pathway, 17 pairs of

545 neurons are linked by 15 or more different neuropeptide-receptor couples. Most of these extremely

546 high-weight edges involve connections between the oxygen-sensing neurons PQR or URXL/R and the

547 motor circuit (either the DB motorneurons or the AVD premotor interneurons). Why might oxygen-

548 sensing neurons participate in so many complex neuromodulatory interactions? These neurons

549 strongly influence locomotor states and their tonic responses to ambient oxygen are influenced by

550 experience and other sensory cues. Complex neuropeptide signaling may allow feedback between the

551 oxygen sensors and motorneurons to fine-tune the activity of this circuit across time and space.

552 The oxygen-sensing neurons and the motor circuit were also found to be important sites for

553 autocrine signaling, in which a neuropeptide receptor and its ligand are co-expressed in the same

554 neuron. Autocrine peptide signaling is ubiquitous in other brains as well[97,98], suggesting it supports

555 important aspects of neural function. For example, autocrine neuropeptide pathways are known to

556 mediate cell-autonomous feedback[99–104], and are therefore often suggested to maintain neuronal

557 homeostasis; this may be particularly important to regulate the activity of tonically signaling neurons,

558 such as the oxygen-sensing neurons. In the motor circuit, ostensibly autocrine signaling may play a

559 further role by coordinating the activities of neighboring neurons with similar gene expression

560 patterns. Although proprioceptive and electrical coupling between adjacent motorneurons generates

561 waves of muscle contraction over adjacent body regions[88], autocrine pathways also have the capacity

562 to coordinate physiology across neighboring cells[105,106]. Indeed, mutants for several of the

563 neuropeptide ligands that act in motorneuron autocrine pathways have been shown to have

564 locomotion defects[107,108], suggesting an important role for these connections in patterning locomotor

565 behavior.

566 We also observe a critical role for neuropeptide signaling in linking disconnected components

567 of the nervous system to the broader network. In particular, the pharyngeal neurons, which form a

568 wired network analogous (if not homologous) to the vertebrate's enteric nervous system, and the CAN

569 neurons, whose processes lie in the excretory canal-associated nerve, are virtually completely

570 unconnected to the somatic synaptic and gap junction connectomes yet are extensively integrated into

571 the neuropeptide connectome. Both CAN and the pharyngeal neurons express multiple GPCRs whose

572 ligands are expressed exclusively outside the pharynx or canal-associated nerve, indicating that these

573 neurons are regulated by peptides released from physically-unconnected processes. Despite their

574 disconnection from the wired connectomes, the pharynx and CAN neurons carry out essential

575 physiological functions; indeed, CAN and the pharyngeal neuron M4 are the only neurons whose

576 ablation is lethal to the animal[109,110]. This provides a means for communication between the wider

577 nervous system and these isolated but biologically critical neurons.

578 **Prospects for future mapping of wireless brain connectomes**

579 We have described here the draft neuropeptide connectome of *C. elegans*, an animal with 302

580 neurons. In the future, we plan to refine this connectome, for example by expanding its scope through

581 deorphanization of neuropeptide receptors whose ligands are currently unknown. Differential

582 posttranscriptional processing may also lead to the synthesis of different peptides and receptors in

583 neurons that express the same gene, and alternative patterns of gene expression during development or

584 in response to environmental cues may likewise alter the structure and function of neuropeptide

585 signaling networks. Moreover, non-neuronal cells function as both senders and receivers of

586 neuropeptide signals; with the use of reporter lines for peptides and receptors it should be possible in

587 the future to incorporate these cells into the neuropeptide connectome and observe plasticity in the

588 network. We likewise plan to integrate functional information into the connectome map; for example,

589 in vitro experiments could identify the G-protein pathways downstream of individual receptors, and *in*

590 *vivo* sensors could provide empirical data on the spatial scope of neuropeptide signaling pathways.

591 Together these data will facilitate functional modeling of neuropeptidergic circuits revealed in the

592 connectome maps.

593   In principle, the approaches described here should also be applicable to mapping the

594 neuropeptide signaling networks of animals with much larger brains. Single-cell RNAseq data from

595 different vertebrate animals indicates that, as in *C. elegans*, most neurons express at least one

596 neuropeptide precursor and at least one neuropeptide receptor, facilitating dense and potentially

597 decentralized networks[18,39,43,44]. Although it is currently not possible to precisely link gene expression

598 clusters with individual neurons in brain circuits, the use of targeted reporters should eventually make

599 it possible to relate neuropeptide and receptor expression to increasingly detailed synaptic

600 connectome maps in flies and mice. Basic mechanisms of neuropeptide signaling are shared in all

601 animals, from nematodes to mammals: neuropeptides are released from dense core vesicles and

602 diffuse across space to neurons unconnected to the releasing cell by wired synapses. And although the

603 *C. elegans* nervous system is anatomically small, at the molecular level its neuropeptide systems are

604 highly complex and show significant homology to other animals. Thus, the neuropeptide connectome

605 of *C. elegans* may serve as a prototype to unravel general principles of neuromodulatory network

606 structure that also apply to much larger brains.

607 [34,35363718,38]

608

609

610 **Methods**

611 **Reporter Transgenic Strains**

612 *CRISPR based constructs*: Thirty-two transcriptional C-terminal GFP reporters of neuropeptides and

613 neuropeptide receptors were created where GFP was recombineered in the last coding exon of the

614 gene of interest. Of the 17 total neuropeptide precursor genes that we made reporters for, 8 had only a

615 T2A::3xNLS::GFP tag, 1 had a T2A::GFP::H2B tag, 6 had both T2A::3xNLS::GFP and

616 SL2::GFP::H2B tags, and 2 had only an SL2::GFP::H2B tag (Supplementary Table 1). For the GPCR

617   receptors, 8 gene expression reporters were made all with SL2::GFP::H2B tags (Supplementary Table

618   2). Most of these constructs were made by SUNY Biotech.

619

620   **Reporter Analysis**

621   Young adult animals were mounted on 5% agarose pads and immobilized with 100 mM sodium azide

622   and imaged on a Zeiss LSM880 using a 40X objective lens. GFP expression reporters were identified

623   at single neuron resolution as described[111]. GFP reporter expression of these constructs, as reported in

624   Table S1-2 were noted using three categories: moderate to high expression, low and variable

625   expression, and no detected expression. Additionally, we compared our GFP reporter expression data

626   to single-cell RNA-seq expression (scRNAseq) data from the CeNGEN project using their standard

627   thresholds (4 being the most stringent, 1 being the least stringent, and blank be unfiltered)[40].

628   In our analysis, for each gene and each CeNGEN threshold, we tallied 1) the number of neurons that

629   showed GFP expression but not scRNA expression, 2) the number of neurons showing both GFP

630   expression and scRNA expression, and 3) the number of neurons that showed scRNA expression but

631   no GFP expression (Table S3).

632   Based on the results of this analysis threshold 4, although in some occasions too conservative, had the

633   best correlation between GFP reporter and CeNGEN scRNAseq data expression per neuron for the

634   tested NPP and GPCR genes.

635

636   **Synaptic and gap junction networks**

637   The synaptic and gap junction networks used in this work were based on the full

638   hermaphrodite *C. elegans* connectome, containing all 302 neurons. This network was composed from

639   the somatic connectome[48], updated and released by the Chklovskii lab[47]; and the pharyngeal network

640   of Albertson and Thomson[65], made available by the Cybernetic *Caenorhabditis elegans* Program

641   (CCeP). The functional classifications referred to in the text (i.e., *sensory*

642   *neuron*, *interneuron*, *motorneuron*) are based on the classification scheme used in WormAtlas[112].

643   When there is double or triple classification in nerve ring neurons that can be sensory neurons,

644   interneurons, or motorneurons, the Zhen lab classification[66] was used to select one neuron type. URB

645   left and right neurons are the only ones in which the WormAtlas and the Zhen lab classification

646   diverge, leading us to classify them as sensory neuron following the later most recent classification.

647   DB neurons are identified as motorneurons although WormAtlas indicates that these could also be

648   interneurons[112]. The gap junction network was modelled as an undirected network with bidirectional

649   electrical synapses; note however that some gap junctions might be rectifying and thus exhibit

650   directionality. In the synaptic network reciprocal connections between nodes are considered as two

651   separate unidirectional connections.

652

653   **Monoamine network construction**

654   The monoamine network used in this work was made following the same procedure as in (Bentley et

655   al., 2016). The monoamine expression for the 302 neurons comes from the neurotransmitter atlas of

656   *C. elegans*[75] and receptor expression for the 302 neurons comes from the single-cell expression data

657   from the CeNGEN project (https://www.cengen.org)[40]. We used the expression data at CeNGEN

658   threshold 4. The interactions between ligand and receptors were previously described[45]. The

659   adjacency matrix was built using a binary version of the expression data for the 302 neurons. For a

660   given point $A^M(i,j)$ and for a given monoamine receptor pair M the connection between two neurons is

661   defined by $A^M(i,j) = Mon^M(i,j) \times Receptor^M(i,j)$. Each monoamine receptor interaction forms an

662   individual binary network. To get the overall monoamines network we add each individual

663   monoamine receptor network resulting in a weighted network where the weight indicates the number

664   of monoamine receptor pairs that connect two nodes. Reciprocal connections between nodes are

665   considered as two separate unidirectional connections.

666

667   **Neuropeptide network construction**

668   The neuropeptide network used in this work was made using a similar approach to that used for the

669   monoamines. The interactions between ligands and receptors were identified using a large-scale *in*

670   *vitro* reverse pharmacology pipeline in which over 87% of the predicted peptide GPCRs were

671   challenged with FMRFamide related peptides (FLP) and non-insulin non-FLP like peptides (NLP)[64].

672   Neuropeptide precursor and GPCR gene expression for the 302 neurons was extracted from the

673 single-cell transcriptome data of the CeNGEN project (https://www.cengen.org)[40]. We used the

674 expression data at CeNGEN threshold 4. The adjacency matrix was built using a binary version of the

675 expression data for the 302 neurons. For a given point $A^N(i,j)$ and for a given neuropeptide receptor

676 pair N the connection between two neurons is defined by $A^N(i,j) = NPP^N(i,j) \times GPCR^N(i,j)$. Each

677 neuropeptide receptor interaction forms an individual binary network. To get the overall

678 neuropeptides network we add each individual neuropeptide receptor network resulting in a weighted

679 network where the weight indicates the number of neuropeptide receptor pairs that connect two nodes.

680 Reciprocal connections between nodes are considered as two separate unidirectional connections.

681

Neuropeptide network spatial constraining

683 Neuropeptidergic networks were locally thresholded to filter out connections between neurons that

684 were anatomically far from each other. The anatomical EM data was obtained from The Mind of the

685 worm (https://www.wormatlas.org/MoW_built0.92/MoW.html) and other literature[48,65,66]. This data

686 was used to create a table of locations for each neuronal process, identifying 27 different neuronal

687 process bundles in the *C. elegans* nervous system as previously defined[48]. This classification was then

688 used to filter out neuropeptidergic connections based on putative signaling ranges. The stringent

689 short-range thresholding allows connections only in between neuronal processes that are in the same

690 process bundle and the pharynx is a separated system were all connections are allowed in between

691 pharyngeal neurons only. The mid-range stringency thresholding allows connections between neurons

692 with neuronal processes in the same anatomical area: head (including pharynx and the ventral cord

693 neurons that are in the ventral ganglion), midbody and tail. And in the unthresholded system all

694 neuropeptidergic connections are allowed.

695

Topological network measures

697 Edge counts, adjacency matrices and reducibility clusters were all computed using binary directed

698 versions of the networks. The same networks, excluding self-connections (i.e. setting all diagonal

699 elements to 0), were used to compute all other measures.

700 Network measures are compared to 100 null model networks generated using the degree-preserving

701 edge swap procedure from the Brain Connectivity Toolbox for MATLAB[113]. This is performed by

702 selecting a pair of edges ($A{\rightarrow}B$) ($C{\rightarrow}D$) and swapping them to give ($A{\rightarrow}D$)($C{\rightarrow}B$). If the resulting

703 edges already exist in the network, another pair of edges is selected instead. Each edge was swapped

704 10 times to ensure full randomization.

705

706 **Degree**

707 Degree is the number of edges connected to a given node. Indegree is the number of incoming

708 connections connected to a given node and outdegree is the number of outgoing connections.

709

710 **Density**

711 Density d is the fraction of present connections K to possible connections between the given nodes N:

712 $$d = \frac{K}{N^2 - N}$$

713 **Clustering coefficient**

714 Transitivity defines the ratio of triangles to triplets in the network (where a triple is a single node with

715 edges running to an unordered pair of others, and a triangle is a fully-connected triple). For a directed

716 network, this is equivalent to: $T = \frac{\sum_{i \in N} t_i}{\sum_{i \in N}[(k_i^{out} + k_i^{in})(k_i^{out} + k_i^{in} - 1) - 2\sum_{j \in N} A_{ij}A_{ji}]}$ where A is the

717 adjacency matrix, N is the number of nodes, k^{out} and k^{in} are the out-degree and in-degree, and t_i is the

718 number of triangles around a node: $t_i = \frac{1}{2}\sum_{j,h \in N}(A_{ij} + A_{ji})(A_{ih} + A_{hi})(A_{jh} + A_{hj})$

719

720 **Reciprocity**

721 Reciprocity is the fraction of reciprocal edges in the network: $r = \frac{|E^{\leftrightarrow}|}{M}$ where M is the number of edges,

722 and $|E^{\leftrightarrow}|$ is the number of reciprocal edges: $|E^{\leftrightarrow}| = \sum_{i \neq j} A_{ij}A_{ji}$

723 **Rich-club coefficient**

724   The rich-club coefficient measures the tendency for high-degree nodes in a network to form highly

725   interconnected communities[55]. These communities can be identified by creating subnetworks for each

726   degree level k and removing nodes with a degree $\leq k$. Then the rich-club coefficient $\Phi(k)$ for each

727   subnetwork is defined as the ratio of connections in the subnetwork M_k to the number of maximum

728   possible connections. For a directed network with no self-connections, where N_k is the number of

729   remaining nodes, this is given by: $$\Phi(k) = \frac{M_k}{N_k(N_k - 1)}$$

730   Thus, a fully connected subnetwork at a given degree k has a rich-club coefficient $\Phi(k) = 1$. We

731   normalize the rich-club coefficient by calculating: $$\Phi_{norm}(k) = \frac{\Phi(k)}{\langle \Phi_{random}(k) \rangle}$$ were $<\Phi_{random}(k)>$ is the

732   average value of the rich-club coefficient across random networks.

733   A rich-club exists when $\Phi_{norm}(k) \geq 1$, but in order to get a clear threshold range we use a probabilistic

734   approach. The threshold range of the rich-club is defined by $\Phi_{norm}(k) \geq 1 + 1\sigma$, where σ is the Standard

735   Deviation of $\Phi_{random}(k)$ for the 100 random networks.

736

737   **Dimensionality reduction analysis**

738   t-SNE is an algorithm for dimensionality reduction that facilitates visualizing high dimensionality

739   data. The analysis described here was performed using the MATLAB *t-sne* function on the adjacency

740   network of connections. The neuropeptide dimension was reduced, and clustering was performed

741   based on the pattern of connections due to receptor expression. Different distant measures were tested

742   to confirm the clustering: Euclidean distance, Chebychev distance, cosine distance and Mahalanobis

743   distance.

744

745   **Co-expression analysis**

746   The signaling networks used in this work represent connections between co-occurring genes. Nodes

747   are defined as pairs of neuropeptide precursor and GPCR genes that co-occur more than expected by

748   chance as measured by a Fisher's exact test[114] with FDR (false positive rate) correction[115]. The 2x2

749   contingency table for the Fisher's exact test contains the number of neurons for which both genes co-

750   occur and the number of neurons in which each NPP and GPCR gene is expressed without co-

751   occurring with the second gene. Thus, the Fisher's test is defined as:

752
$$p = \frac{(R_1!\, R_2!)(C_1!\, C_2!)}{N!\, \prod_{i,j} n_{i,j}!}$$

753
754   Where R_1 and R_2 are the row sums, C_1 and C_2 are the column sums, N is the total number of

755   observations in the contingency table, and n_{ij} is the value in the ith row and jth column of the table

756   Interactions between nodes are defined by the receptor-ligand interactions that the co-occurring genes

757   have with genes that co-occur in another node. The interactions between ligand and receptors were

758   identified using a large-scale *in vitro* reverse pharmacology pipeline[64].

759

760   **Software**

761   Network measures were computed in MATLAB (v9.8.0.1323502 (R2020a), The MathWorks Inc.

762   Natick, MA) using the Brain Connectivity Toolbox[113] (v2019-03-03) and the MATLAB/Octave

763   Networks Toolbox[116]. Clustering and visualization of multilayer plots was performed using

764   MuxViz[117]. And additional network visualizations were created using Cytoscape[118].

765

766   **Lead contact**

767   Requests for resources should be directed to the Lead Contact William Schafer (wschafer@mrc-

768   lmb.cam.ac.uk)

769

770   **Data and software availability**

771   The scRNAseq data are available at www.cengen.org. The biochemical deorphanization data are

772   available at https://worm.peptide-gpcr.org/project/neuropeptides/flps/ [64]. Analysis code is available at

773   https://github.com/LidiaRipollSanchez/Neuropeptide-Connectome.git

774   **Nematode Strains**

775   The strains used in this study are listed in Supplementary Tables 1 and 2.

776

Author contributions

L.R.S., W.R.S., P.E.V., J.W. and I.B. designed research. L.R.S. and J.W. performed analysis. H.S. and R.F. made strains and interpreted images. H.S., R.F., S.R.T., A.W., M.H., D.M.M., O.H. contributed with data and discussion. L.R.S., W.R.S., P.E.V., J.W. and I.B. contributed to writing the paper. All authors reviewed and approved the manuscript.

Declaration of Interests

The authors declare no competing interests

Figure titles and Legends

Figure 1. *In silico* reconstruction of the neuropeptidergic connectome of the *C. elegans* nervous system by combining *in vitro* neuropeptide-GPCR interaction, gene expression and anatomical datasets.

(A) Datasets used to build the network *in silico*. Left: biochemical neuropeptide-GPCR interaction dataset defining 91 neuropeptide - G protein-coupled receptor (GPCR) pairs below

30

805   500 nM EC_{50} affinity, coupling 51 GPCRs to 48 neuropeptides[64]. Neuropeptides from the

806   same precursor gene (NPP) are considered the same. Middle: anatomical and morphological

807   description of the *C. elegans* hermaphrodite nervous system[48]. Right: single-cell RNAseq

808   expression of the 302 *C. elegans* neurons, adapted from[40].

809   (B) Representation of the *C. elegans* nervous system connectome network in which all types of

810   neurons (pharyngeal, interneuron, CAN neurons, motorneurons and sensory neurons) are

811   connected through neuropeptidergic connections.

812

813   **Figure 2**. Single-copy genomic knock-in reporters corroborate the expression profiles of neuropeptide

814   and GPCR genes obtained from scRNAseq. GFP-positive neurons were identified using the

815   NeuroPAL multicolor transgene [111]Figure pictures all segments (head, tail, vulva and midbody)

816   showing neuronal expression, within which individual neurons are circled and labeled. Scale bars

817   represent 10 μm. Tables of expression data and comparisons between cells identified in reporters and

818   scRNAseq detection thresholds are in Supplementary Tables S1-3.

819   (A) Fluorescent GFP reporters for the expression of two representative neuropeptide genes, *flp-20*

820   and *nlp-45,* showing high correlation between reporter expression and CeNGEN scRNAseq

821   expression. Data for additional neuropeptide gene reporters are shown in Supplementary

822   Figure S1A.

823   (B) Fluorescent GFP reporters for the two representative neuropeptide-activated GPCRs,

824   expression of *tkr-1* and *dmsr-6*, showing high correlation between reporter expression and

825   CeNGEN scRNAseq expression.[111] Data for additional GPCR gene reporters are shown in

826   Supplementary Figure S1B.

827

828   **Figure 3**. Analysis of neuropeptide and receptor expression allows assessment of the spatial scale of

829   neuropeptide signaling.

830   (A) Full anatomical description of the *C. elegans* hermaphrodite nervous system[112]. Neuronal

831   bundles represented in red, and the pharynx in green. Short-range connections are defined as

832   occurring within the same neuronal bundle (axons and dendrites considered equally). Mid-

833   range connections occur between neurons whose processes are present in one of following

834   distinct anatomical areas: head (ranging from nose to deirid commissures including the

835   ventral and retrovesicular ganglion), midbody (between but not including deirid commissures

836   and the preanal ganglion), or tail (from and including the preanal ganglion to the tail's tip).

837   Classifications of individual neurons are in Supplementary Table S4.

838   (B) Expression matrix for 23 GPCRs activated *in vitro* by a single neuropeptide ligand. Neurons

839   are sorted by neuron type on the x axis and the expression of each GPCR on the y axis. Colors

840   indicate the range of diffusion distance required for communication with at least one ligand-

841   expressing neuron: blue indicates that the receptor expressing neuron makes contact with a

842   neuron expressing its ligand, either within the same stratum of the nerve ring (dark blue) or in

843   a thinner neuronal bundle (light blue); orange indicates short-range connections between

844   strata in the nerve ring (i.e. between neurons in same bundle but not in physical contact); red

845   indicates connections between neurons in the same anatomical area but not in the same

846   bundle. Nerve ring stratum as defined[67,6867,6867,68]

847   (C) Expression matrix for 23 neuropeptide precursor genes with a single target receptor in vitro.

848   Neurons are sorted by neuron type on the x axis and the expression of each neuropeptide gene

849   on the y axis. Colors indicate the range of diffusion distance required for communication with

850   a receptor-expressing neuron, as described above.

851 **Figure 4**. Assortativity analysis of 91 individual NPP-GPCR networks shows different topologies

852 depending on the NPP-GPCR interaction.

853   (A) Representation of network topologies depending on assortativity, reflecting the preference of

854   neuropeptides to signal to GPCRs that are expressed in the same number of neurons.

855   Networks are classified either as local, pervasive, broadcaster or integrative. Bottom left:

856   scatter plot showing the number of neurons expressing a particular GPCR versus the number

857   of neurons expressing the corresponding NPP gene for each of the 91 individual networks.

858   Local networks show restricted NPP and GPCR expression in 50 neurons or less. Pervasive

859   networks have broad NPP and GPCR expression in more than 50 neurons. Broadcaster

860   networks show broad GPCR (>50 neurons) but restricted NPP expression (≤50 neurons),

861   while integrative networks display broad NPP (>50 neurons) and restricted GPCR expression

862   (≤50 neurons). Filled circles indicate receptor presence in that neuron and empty circles

863   indicate neuropeptide release from that neuron. Left top: example of local network for CAPA-

864   1/NMUR-1 that has been linked to learning[32]. Left middle top: example of pervasive network

865   FLP-18/NPR-5 of which the receptor is implicated within locomotory behavior[119]. Right

866   middle top: example of broadcaster network FLP-1/FRPR-7 that modulates the interaction

867   between food sensation and locomotion[13]. Right top: example of integrative network NLP-

868   47/GNRR-1, the receptor of which is linked to egg-laying behavior [120] and is only activated by

869   NLP-47 *in vitro* [64,120]. Diagrams of all 91 networks are in Supplementary Figures S2 and S3.

870   (B) The topology of the network is defined by the individual NPP-GPCR pairing. Networks of the

871   same receptor can show different topologies depending on its neuropeptide ligand. In each

872   example, the same receptor can form both pervasive and broadcasting networks (DMSR-2,

873   DMSR-7) or both local and integrative networks (FRPR-8 and EGL-6) depending on the

874   activating ligand.

875

876   **Figure 5**. The aggregate neuropeptide connectome in *C. elegans* connects all 302 neurons in a dense

877   network. Adjacency matrix representation of the aggregate 91 NPP-GPCR pair network considering

878   short-range (color) and mid-range (gray) diffusion models. Axes represent the number of NPP and

879   GPCR genes per neuron. Virtually all neurons express at least 1 NPP and 1 GPCR gene, the only

880   exception of which is the IL1 neuron class that does not show GPCR expression. Neurons can

881   communicate with each other using up to 18 different NPP-GPCR pathways, a feature conserved in

882   both short- and mid-range diffusion models. Dense and diverse connections are mainly seen from

883   oxygen sensing neurons to inter- and motorneurons. 5% of all connections are putative autocrine

884   connections in which a single neuron co-expresses both NPP and GPCR genes of a cognate pair.

885   Supplementary Figure 4 shows adjacency matrices for long-range (A) and mid-range (B) models.

886

887   **Figure 6**. Topological analysis of the *C. elegans* nervous system highlights neuropeptide signaling

888   hubs and a large rich club.

889   (A) Degree distribution of the short-range model aggregate neuropeptide network. Degree (sum of

890   incoming and outgoing connections) is shown in orange, the indegree (sum of incoming

891   connections) is shown in green and the outdegree (sum of outgoing connections) is shown in

892   purple. . The 10 hubs with the highest degree are indicated, including hubs of the synaptic

893   connectome (AVAL/R, PVCL/R and DVA) as well as 6 neurons with specialised roles in the

894   neuropeptide signaling network (AVKL/R, PVT, PVQL/R, PVR).

895   (B) Degree distribution of the mid-range model aggregate neuropeptide network. Similar to the

896   short-range model, degree distribution is equally influenced by both in- and outdegree: the 10

897   hub neurons of highest degree are indicated.

898   (C) Degree distribution of the synaptic network of *C. elegans*. Compared to the neuropeptide

899   connectome, the synaptic connectome contains a much smaller number of high-degree hub

900   nodes.

901   (D) Degree distribution of the monoamine network of *C. elegans*. The overall degree distribution

902   shows two distinct phases, correlating with the network having a star-like topology centered

903   around a small core of high degree hubs. This topological feature is further reflected in the

904   low outdegree of most neurons.

905   (E) Conceptual network representation highlighting: node (neuron), edge (connection between

906   neurons), degree (k, sum of incoming and outgoing connections), hub (highly connected

907   neurons), and rich club (group of hubs that connect more to one another than to the rest of the

908   network).

909   (F) Rich club of the *C. elegans* neuropeptidergic connectome. The rich club coefficient $\Phi(k)$ for

910   the *C. elegans* neuropeptidergic network is shown in black and the randomized rich club

911   curve $\Phi_{random}(k)$ generated by averaging the rich club coefficients of 100 randomized versions

912   of the *C. elegans* neuropeptidergic network that preserve degree distribution) is depicted in

913   gray. The red curve is the normalized coefficient. $\Phi(k) \geq \Phi_{random}(k) + 10\sigma$ over the range 203

914         $\leq k \leq 298$, indicating the rich club. The large number of connections in the range k > 298

915   make the random networks more unreliable but since $\Phi(k)$ is very close and mostly higher

916   than $\Phi_{random}(k) + 10\sigma$ we can infer that all neurons where $203 \leq k$ are part of the rich club.

917   This means the short-range aggregate neuropeptide network has a rich club of 156 neurons

918   (166 in the mid-range, Suppl. Figure 6)

919   (G) Correlation between synaptic and neuropeptidergic degrees. A positive correlation was

920   observed over all neurons ($r = 0.54$, $p = 3.1\ e^{-14}$). Dark red dots indicate neurons in the *C.*

921   *elegans* neuropeptidergic rich club. Synaptic hubs, which also exhibit a high neuropeptide

922   degree ($k>400$) (ranking among the top 30 neuropeptide hubs) are highlighted. Also

923   highlighet are 6 neuropeptidergic hubs, with low synaptic degree ($k < 30$, below the

924   correlation line), as well as low monoamine ($22 > k \geq 10$) and gap junction ($14 > k \geq 3$)

925   degrees (Suppl. Figure 6), appear to be specialized for neuropeptide signaling.

926

927   **Figure 7**. Mesoscale structure of the neuropeptide connectome

928   (A) t-SNE dimensionality reduction of the adjacency matrix of the mid-range aggregate network

929   (Euclidean distance, perplexity 30). This plot we identifies 3 clear clusters (1, 2, 3) which

930   encompass 112 of out the 166 mid-range neuropeptide rich club neurons), in addition to a

931   periphery of loosely clustered neurons. Datapoint markers represent their neuronal

932   classification.

933   (B) PCA dimensionality reduction of the adjacency matrix of the mid-range aggregate network

934   (Euclidean distance, perplexity 30). Neurons in this plot are colored based on that of the

935   groups defined in (A). A clustering pattern similar to that following t-SNE appears,

936   highlighting the robustness of these defined groups across dimensionality reduction

937   techniques. Datapoint markers represent their neuronal classification.

938   (C) Mid-range aggregate network sorted in both dimensions (sending and receiving neurons)

939   based on neuronal clusters defined in (A) and (B). Neuronal clusters divisions are highlighted

940   with discontinuous lines. Group 3 does not receive nor send connections to group 1. All three

941   groups, specially 2 and 3, send a large number of connections to peripheral neurons while not

942   receiving significant input from the periphery. Group 2 is the only group that sends and

35

943   receives connections to and from all 4 neuronal clusters. The neurons with higher weight of

944   connections (number of NPP-GPCR pairs used to make the connection) are mainly found in

945   groups 2 and 3. The neurons defining the patterns that drive the clustering of each group are

946   shown at the bottom: group 1 is mainly driven by the connections of DA, VC and VA

947   terminal motorneurons; group 2 is mainly driven by the connections of neuropeptidergic hubs

948   PVQL/R and PVR, synaptic hubs AVAL/R and PVNL/R neurons; group 3 is mainly driven

949   by the connections of pharyngeal neurons I5 and I4 and RIR, RICL/R interneurons.

950   (D) Violin plots showing indegree values for the 3 clusters and the periphery. Indegree cleanly

951   defines the 3 groups and the periphery (median group 1: 139, median group 2: 267, median

952   group 3: 198, median periphery: 54), and therefore is the determinant factor in the definition

953   of these groups. Significances determined by Kruskal-Wallis test followed by Tukey-Kramer

954   test for multiple comparisons with rank sums. The mean ranks for the 4 groups were shown to

955   be significantly different (mean rank group 1: 162, mean rank group 2: 276, mean rank group

956   3: 223, mean rank periphery: 77). n.s. not significant; ***$p \leq 0.001$.

957   (E) Diagram showing the organization of connections between the 3 defined clusters. Group 1

958   contains the main motorneurons core. These connect between themselves, with the periphery

959   and with group 2. Group 2 contains the main interneurons core including the top 10 hubs.

960   These neurons connect to almost every other neuron in the nervous system. Group 3 contains

961   the main sensory neurons and pharyngeal neurons core. These neurons connect to group 2

962   neurons and the periphery but do not connect to group 1 neurons. This structure indicates an

963   intrinsic organization of the *C. elegans* neuropeptidergic connectome rich club and how it

964   coordinates the function of the overall neuropeptidergic connectome.

965

966 **Figure 8**. Co-expression between GPCRs and their corresponding neuropeptide ligand(s) facilitates

967 potential autocrine and paracrine signaling. Additional data on putative autocrine signaling is in

968 Supplementary Figure S6.

36

(A) Neuronal expression matrix for both NPP and GPCR genes of the 91 NPP-GPCR pairs. Grey dots represent neuronal expression of only the NPP (upper panel) or GPCR (lower panel) gene, which black dots indicating co-expression of both.

(B) Percentage of each neuron type showing peptide autocrine connections (upper panel). The number of different NPP-GPCR pairs being co-expressed in each neuron type is figured in the bottom panel. Neurons showing the highest diversity of different NPP-GPCR pairs being co-expressed are highlighted, including the sensory neurons PQR and URX and the interneuron PVR.

(C) Scatter plot showing the number of neurons with co-expression for each of the 91 NPP-GPCRs. Pairs with promiscuous GPCRs, specially DMSR-1 and DMSR-7 are co-expressed in many different neurons, highlighting their central role in autocrine neuropeptidergic signaling.

(D) Correlation between number of autocrine connections and neuropeptide (left) or synaptic (right) degree for each of the 302 neurons. Point shapes indicate degree (round), indegree (incoming arrow), outdegree (outgoing arrow).

(E) Representation of the autocrine connections in each neuron of the *C. elegans* worm (anterior part of the worm in the left side, posterior in the right, dorsal upper part, and ventral lower part). The size of each cell body indicates the number of autocrine NPP-GPCR pairs expressed in that neuron. Circuits in which autocrine connections are prominent are the locomotion and oxygen sensing circuit. The colors indicate the neurons in the oxygen sensing and locomotion circuits that have the largest number of autocrine connections. Left bottom: oxygen sensing circuits with autocrine connections, URX and PQR are the neurons with the largest number of connections (arrow size indicates number of NPP-GPCR pairs). Right bottom: description of the locomotion system with autocrine connections, VA/VB and DA/DB are excitatory motorneurons that actuate worm crawling and which show many autocrine connections while inhibitory DD/VD and minor excitatory AS motorneurons do not have autocrine connections.

997 **Supplementary Figure S1.** Expression patterns of neuropeptide and GPCR reporters. Cells were

998 identified as described in Figure 2. A) Fluorescent GFP reporters for the expression of 17

999 representative neuropeptide precursor genes. B) Fluorescent GFP reporters for the expression of 8

1000 representative GPCR precursor genes. Images of the *trhr-1* expression strain show some bleed-

1001 through signal that does not represent neuronal expression.

1002

1003 **Supplementary Figure S2.** Graphical representations of the 91 NPP-GPCR networks with short-

1004 range connections. Colors given by assortativity like Figure 4; yellow indicates local, green pervasive,

1005 blue integrative and red promiscuous networks.

1006

1007 **Supplementary Figure S3.** Graphical representations of the 91 NPP-GPCR networks with mid-range

1008 connections. Networks are sorted in the same order as in Supplementary Figure 2 for comparison.

1009 Colors given by assortativity as defined in Figure 4, yellow indicates local, green pervasive, blue

1010 integrative and red promiscuous networks. Networks that change assortativity between short and mid-

1011 range spatial models of neuropeptide transmission are highlighted.

1012

1013 **Supplementary Figure 4.** Adjacency matrix representation of the long and mid-range neuropeptide

1014 networks. A) Model 1, aggregate matrix of the long-range neuropeptide connections, the histogram

1015 lining the matrix indicate the number of GPCR or NPP that the neuron expresses. B) Model 2,

1016 aggregate matrix of the mid-range neuropeptide connections.

1017

1018 **Supplementary Figure 5.** Co-expression analysis of NPP and GPCR genes. A) Hierarchical

1019 clustering matrix showing NPP – GPCR pairs with significant co-expression. The y axis indicates

1020 GPCR and the x axis indicates NPP expression. The significance of their co-expression is indicated by

1021 the legend. B) Adjacency matrix representation of the co-expression network. The nodes represent

1022 NPP – GPCR pairs with significant neuronal coexpression, and edges are formed when the NPP of

1023 one node is the ligand of the GPCR of another node. C) Hierarchical clustering matrix of the NPP vs

1024 NPP co-expression. D) Hierarchical clustering matrix of the GPCR vs. GPCR co-expression. E)

38

1025 Visual representation of one of the possible signaling cascades based on the co-expression network.

1026 DMSR-3 co-expresses with FLP-34 and NLP-54 releasing neurons, which means that secretion could

1027 be activated by FLP-14 binding to DMSR-3.

1028

1029 **Supplementary Figure 6.** Autocrine connections sensitivity analysis. A) comparison of co-

1030 expression of cognate NPP-GPCR pairs in the 4 different combinations of CeNGEN and

1031 deorphanization datasets with different stringencies (CeNGEN threshold 2 or 4; EC_{50} threshold 1 μM

1032 or 500 nM), each depicted in a different color. B) Number of NPP-GPCR co-expressed per fraction of

1033 neurons in these 4 different combinations of datasets. C) Number of neurons with co-expression for

1034 the FLP or NLP neuropeptides show that FLP are more prevalent. D) Comparison of number of

1035 autocrine connections between specific and promiscuous NPP-GPCR pairs, showing that promiscuous

1036 pairs tend to have more autocrine connections. E) Correlation of autocrine connections with gap

1037 junction and monoamine networks. F) Number of autocrine connections in each motorneuron for each

1038 of the main promiscuous NPP-GPCR pairs present in those neurons.

1039

1040

1041 **References**

1042 1.   Eichler, K., Li, F., Litwin-Kumar, A., Park, Y., Andrade, I., Schneider-Mizell, C.M.,

1043   Saumweber, T., Huser, A., Eschbach, C., Gerber, B., et al. (2017). The complete connectome

1044   of a learning and memory centre in an insect brain. Nature *548*, 175–182.

1045   10.1038/nature23455.

1046 2.   Scheffer, L.K., Xu, C.S., Januszewski, M., Lu, Z., Takemura, S., Hayworth, K.J., Huang, G.B.,

1047   Shinomiya, K., Maitlin-Shepard, J., Berg, S., et al. (2020). A connectome and analysis of the

1048   adult Drosophila central brain. Elife *9*, e57443. 10.7554/eLife.57443.

1049 3.   Verasztó, C., Jasek, S., Gühmann, M., Shahidi, R., Ueda, N., Beard, J.D., Mendes, S., Heinz,

1050   K., Bezares-Calderón, L.A., Williams, E., et al. (2020). Whole-animal connectome and cell-

1051   type complement of the three-segmented *Platynereis dumerilii* larv.

1052   4.   Hulse, B.K., Haberkern, H., Franconville, R., Turner-Evans, D., Takemura, S., Wolff, T.,

1053   Noorman, M., Dreher, M., Dan, C., Parekh, R., et al. (2021). A connectome of the Drosophila

1054   central complex reveals network motifs suitable for flexible navigation and context-dependent

1055   action selection. Elife *10*, e66039. 10.7554/eLife.66039.

1056   5.   Ryan, K., Lu, Z., and Meinertzhagen, I.A. (2016). The CNS connectome of a tadpole larva of

1057   Ciona intestinalis (L.) highlights sidedness in the brain of a chordate sibling. Elife *5*, e16962.

1058   10.7554/eLife.16962.

1059   6.   Kunst, M., Laurell, E., Mokayes, N., Kramer, A., Kubo, F., Fernandes, A.M., Förster, D., Dal

1060   Maschio, M., and Baier, H. (2019). A Cellular-Resolution Atlas of the Larval Zebrafish Brain.

1061   Neuron *103*, 21-38.e5. 10.1016/j.neuron.2019.04.034.

1062   7.   Oh, S.W., Harris, J.A., Ng, L., Winslow, B., Cain, N., Mihalas, S., Wang, Q., Lau, C., Kuan,

1063   L., Henry, A.M., et al. (2014). A mesoscale connectome of the mouse brain. Nature *508*, 207–

1064   214. 10.1038/nature13186.

1065   8.   Nässel, D.R. (2009). Neuropeptide signaling near and far: how localized and timed is the

1066   action of neuropeptides in brain circuits? Invertebrate Neuroscience *9*, 57–75.

1067   10.1007/s10158-009-0090-1.

1068   9.   van den Pol, A.N. (2012). Neuropeptide Transmission in Brain Circuits. Neuron *76*, 98–115.

1069   10.1016/j.neuron.2012.09.014.

1070   10. Atkinson, L.E., Liu, Y., McKay, F., Vandewyer, E., Viau, C., Irvine, A., Rosa, B.A., Li, Z.,

1071   Liang, Q., Marks, N.J., et al. (2021). *Ascaris suum* Informs Extrasynaptic Volume

1072   Transmission in Nematodes. ACS Chem Neurosci *12*, 3176–3188.

1073   10.1021/acschemneuro.1c00281.

1074   11. Nurrish, S. (2014). Dense Core Vesicle Release: Controlling the Where as Well as the When.

1075   Genetics *196*, 601–604. 10.1534/genetics.113.159905.

1076   12. Marder, E. (2012). Neuromodulation of Neuronal Circuits: Back to the Future. Neuron *76*, 1–

1077   11. 10.1016/j.neuron.2012.09.010.

1078   13. Oranth, A., Schultheis, C., Tolstenkov, O., Erbguth, K., Nagpal, J., Hain, D., Brauner, M.,

1079   Wabnig, S., Costa, W.S., McWhirter, R.D., et al. (2018). Food Sensation Modulates

1080   Locomotion by Dopamine and Neuropeptide Signaling in a Distributed Neuronal Network.

1081   Neuron *100*, 1414-1428.e10. 10.1016/j.neuron.2018.10.024.

1082   14.   Chew, Y.L., Tanizawa, Y., Cho, Y., Zhao, B., Yu, A.J., Ardiel, E.L., Rabinowitch, I., Bai, J.,

1083   Rankin, C.H., Lu, H., et al. (2018). An Afferent Neuropeptide System Transmits

1084   Mechanosensory Signals Triggering Sensitization and Arousal in C. elegans. Neuron *99*, 1233-

1085   1246.e6. 10.1016/j.neuron.2018.08.003.

1086   15.   Lin, S., Senapati, B., and Tsao, C.-H. (2019). Neural basis of hunger-driven behaviour in

1087   Drosophila. Open Biol *9*, 180259. 10.1098/rsob.180259.

1088   16.   Ramachandran, S., Banerjee, N., Bhattacharya, R., Lemons, M.L., Florman, J., Lambert, C.M.,

1089   Touroutine, D., Alexander, K., Schoofs, L., Alkema, M.J., et al. (2021). A conserved

1090   neuropeptide system links head and body motor circuits to enable adaptive behavior. Elife *10*,

1091   e71747. 10.7554/eLife.71747.

1092   17.   Melzer, S., Newmark, E.R., Mizuno, G.O., Hyun, M., Philson, A.C., Quiroli, E., Righetti, B.,

1093   Gregory, M.R., Huang, K.W., Levasseur, J., et al. (2021). Bombesin-like peptide recruits

1094   disinhibitory cortical circuits and enhances fear memories. Cell *184*, 5622-5634.e25.

1095   10.1016/j.cell.2021.09.013.

1096   18.   Zhong, W., Barde, S., Mitsios, N., Adori, C., Oksvold, P., Feilitzen, K. von, O'Leary, L.,

1097   Csiba, L., Hortobágyi, T., Szocsics, P., et al. (2022). The neuropeptide landscape of human

1098   prefrontal cortex. Proceedings of the National Academy of Sciences *119*, e2123146119.

1099   10.1073/pnas.2123146119.

1100   19.   Bargmann, C.I. (2012). Beyond the connectome: How neuromodulators shape neural circuits.

1101   BioEssays *34*, 458–465. 10.1002/bies.201100185.

1102   20.   Schoofs, L., de Loof, A., and van Hiel, M.B. (2017). Neuropeptides as Regulators of Behavior

1103   in Insects. Annu Rev Entomol *62*, 35–52. 10.1146/annurev-ento-031616-035500.

1104   21.   Bhat, U.S., Shahi, N., Surendran, S., and Babu, K. (2021). Neuropeptides and Behaviors: How

1105   Small Peptides Regulate Nervous System Function and Behavioral Outputs. Front Mol

1106   Neurosci *14*.

1107   22.   Jékely, G. (2021). The chemical brain hypothesis for the origin of nervous systems.

1108   Philosophical Transactions of the Royal Society B: Biological Sciences *376*, 20190761.

1109   10.1098/rstb.2019.0761.

1110   23.   Yañez-Guerra, L.A., Thiel, D., and Jékely, G. (2022). Premetazoan Origin of Neuropeptide

1111   Signaling. Mol Biol Evol *39*, msac051. 10.1093/molbev/msac051.

1112   24.   van Bael, S., Watteyne, J., Boonen, K., de Haes, W., Menschaert, G., Ringstad, N., Horvitz,

1113   H.R., Schoofs, L., Husson, S.J., and Temmerman, L. (2018). Mass spectrometric evidence for

1114   neuropeptide-amidating enzymes in. Journal of Biological Chemistry *293*, 6052–6063.

1115   10.1074/jbc.RA117.000731.

1116   25.   Frooninckx, L., van Rompay, L., Temmerman, L., van Sinay, E., Beets, I., Janssen, T.,

1117   Husson, S., and Schoofs, L. (2012). Neuropeptide GPCRs in C. elegans. Front Endocrinol

1118   (Lausanne) *3*.

1119   26.   Jékely, G. (2013). Global view of the evolution and diversity of metazoan neuropeptide

1120   signaling. Proceedings of the National Academy of Sciences *110*, 8702–8707.

1121   10.1073/pnas.1221833110.

1122   27.   Rizzo, M.J., and Johnson, E.C. (2020). Homodimerization of Drosophila Class A neuropeptide

1123   GPCRs: Evidence for conservation of GPCR dimerization throughout metazoan evolution.

1124   Biochem Biophys Res Commun *523*, 322–327. 10.1016/j.bbrc.2019.12.019.

1125   28.   Rogers, C., Reale, V., Kim, K., Chatwin, H., Li, C., Evans, P., and de Bono, M. (2003).

1126   Inhibition of Caenorhabditis elegans social feeding by FMRFamide-related peptide activation

1127   of NPR-1. Nat Neurosci *6*, 1178–1185. 10.1038/nn1140.

1128   29.   Beets, I., Janssen, T., Meelkop, E., Temmerman, L., Suetens, N., Rademakers, S., Jansen, G.,

1129   and Schoofs, L. (2012). Vasopressin/Oxytocin-Related Signaling Regulates Gustatory

1130   Associative Learning in *C. elegans*. Science (1979) *338*, 543–545. 10.1126/science.1226860.

1131   30.   Iannacone, M.J., Beets, I., Lopes, L.E., Churgin, M.A., Fang-Yen, C., Nelson, M.D., Schoofs,

1132   L., and Raizen, D.M. (2017). The RFamide receptor DMSR-1 regulates stress-induced sleep in

1133   C. elegans. Elife *6*, e19837. 10.7554/eLife.19837.

1134   31.   Nässel, D.R., and Zandawala, M. (2019). Recent advances in neuropeptide signaling in

1135   Drosophila, from genes to physiology and behavior. Prog Neurobiol *179*, 101607.

1136   10.1016/j.pneurobio.2019.02.003.

1137   32.   Watteyne, J., Peymen, K., van der Auwera, P., Borghgraef, C., Vandewyer, E., van Damme,

1138   S., Rutten, I., Lammertyn, J., Jelier, R., Schoofs, L., et al. (2020). Neuromedin U signaling

1139   regulates retrieval of learned salt avoidance in a C. elegans gustatory circuit. Nat Commun *11*,

1140   2076. 10.1038/s41467-020-15964-9.

1141   33.   Topilko, T., Diaz, S.L., Pacheco, C.M., Verny, F., Rousseau, C. v, Kirst, C., Deleuze, C.,

1142   Gaspar, P., and Renier, N. (2022). Edinger-Westphal peptidergic neurons enable maternal

1143   preparatory nesting. Neuron *110*, 1385-1399.e8. 10.1016/j.neuron.2022.01.012.

1144   34.   Davenport, A.P., Scully, C.C.G., de Graaf, C., Brown, A.J.H., and Maguire, J.J. (2020).

1145   Advances in therapeutic peptides targeting G protein-coupled receptors. Nat Rev Drug Discov

1146   *19*, 389–413. 10.1038/s41573-020-0062-z.

1147   35.   Coleman, P.J., Gotter, A.L., Herring, W.J., Winrow, C.J., and Renger, J.J. (2017). The

1148   Discovery of Suvorexant, the First Orexin Receptor Drug for Insomnia. Annu Rev Pharmacol

1149   Toxicol *57*, 509–533. 10.1146/annurev-pharmtox-010716-104837.

1150   36.   Hargreaves, R., Ferreira, J.C.A., Hughes, D., Brands, J., Hale, J., Mattson, B., and Mills, S.

1151   (2011). Development of aprepitant, the first neurokinin-1 receptor antagonist for the

1152   prevention of chemotherapy-induced nausea and vomiting. Ann N Y Acad Sci *1222*, 40–48.

1153   10.1111/j.1749-6632.2011.05961.x.

1154   37.   Zhang, Z.Q., and Hölscher, C. (2020). GIP has neuroprotective effects in Alzheimer and

1155   Parkinson's disease models. Peptides (N.Y.) *125*, 170184. 10.1016/j.peptides.2019.170184.

1156   38.   Hoyer, D., and Bartfai, T. (2012). Neuropeptides and Neuropeptide Receptors: Drug Targets,

1157   and Peptide and Non-Peptide Ligands: a Tribute to Prof. Dieter Seebach. Chem Biodivers *9*,

1158   2367–2387. 10.1002/cbdv.201200288.

1159   39.   Yao, Z., van Velthoven, C.T.J., Nguyen, T.N., Goldy, J., Sedeno-Cortes, A.E., Baftizadeh, F.,

1160   Bertagnolli, D., Casper, T., Chiang, M., Crichton, K., et al. (2021). A taxonomy of

1161   transcriptomic cell types across the isocortex and hippocampal formation. Cell *184*, 3222-

1162   3241.e26. 10.1016/j.cell.2021.04.021.

1163   40.   Taylor, S.R., Santpere, G., Weinreb, A., Barrett, A., Reilly, M.B., Xu, C., Varol, E.,

1164   Oikonomou, P., Glenwinkel, L., McWhirter, R., et al. (2021). Molecular topography of an

1165   entire nervous system. Cell *184*, 4329-4347.e23. 10.1016/j.cell.2021.06.023.

1166   41.   Li, H., Janssens, J., de Waegeneer, M., Kolluru, S.S., Davie, K., Gardeux, V., Saelens, W.,

1167   David, F.P.A., Brbić, M., Spanier, K., et al. (2022). Fly Cell Atlas: A single-nucleus

1168   transcriptomic atlas of the adult fruit fly. Science (1979) *375*, eabk2432.

1169   10.1126/science.abk2432.

1170   42.   Tasic, B., Yao, Z., Graybuck, L.T., Smith, K.A., Nguyen, T.N., Bertagnolli, D., Goldy, J.,

1171   Garren, E., Economo, M.N., Viswanathan, S., et al. (2018). Shared and distinct transcriptomic

1172   cell types across neocortical areas. Nature *563*, 72–78. 10.1038/s41586-018-0654-5.

1173   43.   Smith, S.J., Sümbül, U., Graybuck, L.T., Collman, F., Seshamani, S., Gala, R., Gliko, O.,

1174   Elabbady, L., Miller, J.A., Bakken, T.E., et al. (2019). Single-cell transcriptomic evidence for

1175   dense intracortical neuropeptide networks. Elife *8*, e47889. 10.7554/eLife.47889.

1176   44.   Smith, S.J. (2021). Transcriptomic evidence for dense peptidergic networks within forebrains

1177   of four widely divergent tetrapods. Curr Opin Neurobiol *71*, 100–109.

1178   10.1016/j.conb.2021.09.011.

1179   45.   Bentley, B., Branicky, R., Barnes, C.L., Chew, Y.L., Yemini, E., Bullmore, E.T., Vértes, P.E.,

1180   and Schafer, W.R. (2016). The Multilayer Connectome of Caenorhabditis elegans. PLoS

1181   Comput Biol *12*, e1005283. 10.1371/journal.pcbi.1005283.

1182   46.   Cook, S.J., Jarrell, T.A., Brittin, C.A., Wang, Y., Bloniarz, A.E., Yakovlev, M.A., Nguyen,

1183   K.C.Q., Tang, L.T.-H., Bayer, E.A., Duerr, J.S., et al. (2019). Whole-animal connectomes of

1184   both Caenorhabditis elegans sexes. Nature *571*, 63–71. 10.1038/s41586-019-1352-7.

1185   47.   Varshney, L.R., Chen, B.L., Paniagua, E., Hall, D.H., and Chklovskii, D.B. (2011). Structural

1186   Properties of the Caenorhabditis elegans Neuronal Network. PLoS Comput Biol *7*, e1001066.

1187   10.1371/journal.pcbi.1001066.

1188 48. White, J.G., Southgate, E., Thomson, J.N., and Brenner, S. (1986). The structure of the

1189   nervous system of the nematode *Caenorhabditis elegans*. Philosophical Transactions of the

1190   Royal Society of London. B, Biological Sciences *314*, 1–340. 10.1098/rstb.1986.0056.

1191 49. Watts, D.J., and Strogatz, S.H. (1998). Collective dynamics of 'small-world' networks. *393*, 3.

1192 50. Bassett, D.S., and Bullmore, E.T. (2017). Small-World Brain Networks Revisited. The

1193   Neuroscientist *23*, 499–516. 10.1177/1073858416667720.

1194 51. Reigl, M., Alon, U., and Chklovskii, D.B. (2004). Search for computational modules in the C.

1195   elegans brain. BMC Biol *2*, 25. 10.1186/1741-7007-2-25.

1196 52. Pavlovic, D.M., Vértes, P.E., Bullmore, E.T., Schafer, W.R., and Nichols, T.E. (2014).

1197   Stochastic Blockmodeling of the Modules and Core of the Caenorhabditis elegans

1198   Connectome. PLoS One *9*, e97584. 10.1371/journal.pone.0097584.

1199 53. Pathak, A., Chatterjee, N., and Sinha, S. (2020). Developmental trajectory of Caenorhabditis

1200   elegans nervous system governs its structural organization. PLoS Comput Biol *16*, e1007602.

1201   10.1371/journal.pcbi.1007602.

1202 54. Helm, A., Blevins, A.S., and Bassett, D.S. (2021). The growing topology of the C. elegans

1203   connectome (bioRxiv).

1204 55. Towlson, E.K., Vertes, P.E., Ahnert, S.E., Schafer, W.R., and Bullmore, E.T. (2013). The Rich

1205   Club of the C. elegans Neuronal Connectome. Journal of Neuroscience *33*, 6380–6387.

1206   10.1523/JNEUROSCI.3784-12.2013.

1207 56. van den Heuvel, M.P., and Sporns, O. (2011). Rich-Club Organization of the Human

1208   Connectome. Journal of Neuroscience *31*, 15775–15786. 10.1523/JNEUROSCI.3539-11.2011.

1209 57. Schroeter, M.S., Charlesworth, P., Kitzbichler, M.G., Paulsen, O., and Bullmore, E.T. (2015).

1210   Emergence of Rich-Club Topology and Coordinated Dynamics in Development of

1211   Hippocampal Functional Networks In Vitro. Journal of Neuroscience *35*, 5459–5470.

1212   10.1523/JNEUROSCI.4259-14.2015.

1213 58. Milo, R., Shen-Orr, S., Itzkovitz, S., Kashtan, N., Chklovskii, D., and Alon, U. (2002).

1214   Network Motifs: Simple Building Blocks of Complex Networks. Science (1979) *298*, 824–

1215   827. 10.1126/science.298.5594.824.

1216   59.   Alon, U. (2007). Network motifs: theory and experimental approaches. Nat Rev Genet *8*, 450–

1217   461. 10.1038/nrg2102.

1218   60.   Matelsky, J.K., Norman-Tenazas, R., Davenport, F., Reilly, E.P., and Gray-Roncal, W. (2021).

1219   Circuit motifs and graph properties of connectome development in C. elegans (bioRxiv).

1220   61.   McKay, F.M., McCoy, C.J., Crooks, B., Marks, N.J., Maule, A.G., Atkinson, L.E., and

1221   Mousley, A. (2022). In silico analyses of neuropeptide-like protein (NLP) profiles in parasitic

1222   nematodes. Int J Parasitol *52*, 77–85. 10.1016/j.ijpara.2021.07.002.

1223   62.   Hobert, O. (2013). The neuronal genome of Caenorhabditis elegans. WormBook, 1–106.

1224   10.1895/wormbook.1.161.1.

1225   63.   Foster, S.R., Hauser, A.S., Vedel, L., Strachan, R.T., Huang, X.-P., Gavin, A.C., Shah, S.D.,

1226   Nayak, A.P., Haugaard-Kedström, L.M., Penn, R.B., et al. (2019). Discovery of Human

1227   Signaling Systems: Pairing Peptides to G Protein-Coupled Receptors. Cell *179*, 895-908.e21.

1228   10.1016/j.cell.2019.10.010.

1229   64.   Beets, I., Zels, S., Vandewyer, E., Demeulemeester, J., Caers, J., Baytemur, E., Schafer, W.R.,

1230   Vértes, P.E., Mirabeau, O., and Schoofs, L. (2022). System-wide discovery of neuropeptide-

1231   GPCR pairs in C. elegans. Preprint in bioRxiv. https://doi.org/10.1101/2022.10.30.514428.

1232   65.   Albertson, D.G., and Thomson, J.N. The pharynx of Caenorhabditis elegans. Philos Trans R

1233   Soc Lond B Biol Sci *275*, 299–325. https://doi.org/10.1098/rstb.1976.0085.

1234   66.   Witvliet, D., Mulcahy, B., Mitchell, J.K., Meirovitch, Y., Berger, D.R., Wu, Y., Liu, Y., Koh,

1235   W.X., Parvathala, R., Holmyard, D., et al. (2021). Connectomes across development reveal

1236   principles of brain maturation. Nature *596*, 257–261. 10.1038/s41586-021-03778-8.

1237   67.   Moyle, M.W., Barnes, K.M., Kuchroo, M., Gonopolskiy, A., Duncan, L.H., Sengupta, T.,

1238   Shao, L., Guo, M., Santella, A., Christensen, R., et al. (2021). Structural and developmental

1239   principles of neuropil assembly in C. elegans. Nature *591*, 99–104. 10.1038/s41586-020-

1240   03169-5.

1241   68.   Brittin, C.A., Cook, S.J., Hall, D.H., Emmons, S.W., and Cohen, N. (2021). A multi-scale

1242   brain map derived from whole-brain volumetric reconstructions. Nature *591*, 105–110.

1243   10.1038/s41586-021-03284-x.

1244   69.   Maier, W., Adilov, B., Regenass, M., and Alcedo, J. (2010). A Neuromedin U Receptor Acts

1245   with the Sensory System to Modulate Food Type-Dependent Effects on C. elegans Lifespan.

1246   PLoS Biol *8*, e1000376. 10.1371/journal.pbio.1000376.

1247   70.   Pocock, R., and Hobert, O. (2010). Hypoxia activates a latent circuit for processing gustatory

1248   information in C. elegans. Nat Neurosci *13*, 610–614. 10.1038/nn.2537.

1249   71.   van Sinay, E., Mirabeau, O., Depuydt, G., van Hiel, M.B., Peymen, K., Watteyne, J., Zels, S.,

1250   Schoofs, L., and Beets, I. (2017). Evolutionarily conserved TRH neuropeptide pathway

1251   regulates growth in *Caenorhabditis elegans*. Proceedings of the National Academy of Sciences

1252   *114*. 10.1073/pnas.1617392114.

1253   72.   Cook, S.J., Crouse, C.M., Yemini, E., Hall, D.H., Emmons, S.W., and Hobert, O. (2020). The

1254   connectome of the Caenorhabditis elegans pharynx. J Comp Neurol *528*, 2767–2784.

1255   10.1002/cne.24932.

1256   73.   Vidal, B., Gulez, B., Cao, W.X., Leyva-Díaz, E., Reilly, M.B., Tekieli, T., and Hobert, O.

1257   (2022). The enteric nervous system of the C. elegans pharynx is specified by the Sine oculis-

1258   like homeobox gene ceh-34. Elife *11*, e76003. 10.7554/eLife.76003.

1259   74.   Kaplan, H.S., Nichols, A.L.A., and Zimmer, M. (2018). Sensorimotor integration in

1260   Caenorhabditis elegans: A reappraisal towards dynamic and distributed computations.

1261   Philosophical Transactions of the Royal Society B: Biological Sciences *373*.

1262   10.1098/rstb.2017.0371.

1263   75.   Pereira, L., Kratsios, P., Serrano-Saiz, E., Sheftel, H., Mayo, A.E., Hall, D.H., White, J.G.,

1264   LeBoeuf, B., Garcia, L.R., Alon, U., et al. (2015). A cellular and regulatory map of the

1265   cholinergic nervous system of C. elegans. Elife *4*, e12432. 10.7554/eLife.12432.

1266   76.   Niu, L., Li, Y., Zong, P., Liu, P., Shui, Y., Chen, B., and Wang, Z.-W. (2020). Melatonin

1267   promotes sleep by activating the BK channel in C. elegans. Proc Natl Acad Sci U S A *117*,

1268   25128–25137. 10.1073/pnas.2010928117.

1269   77.   Betzel, R.F., Medaglia, J.D., and Bassett, D.S. (2018). Diversity of meso-scale architecture in

1270   human and non-human connectomes. Nat Commun *9*, 346. 10.1038/s41467-017-02681-z.

1271   78.   Wen, Q., Po, M.D., Hulme, E., Chen, S., Liu, X., Kwok, S.W., Gershow, M., Leifer, A.M.,

1272   Butler, V., Fang-Yen, C., et al. (2012). Proprioceptive Coupling within Motor Neurons Drives

1273   C. elegans Forward Locomotion. Neuron *76*, 750–761. 10.1016/j.neuron.2012.08.039.

1274   79.   Schafer, W.R. (2015). Mechanosensory molecules and circuits in C. elegans. Pflugers Arch

1275   *467*, 39–48. 10.1007/s00424-014-1574-3.

1276   80.   Fenk, L.A., and de Bono, M. (2017). Memory of recent oxygen experience switches

1277   pheromone valence in *Caenorhabditis elegans*. Proceedings of the National Academy of

1278   Sciences *114*, 4195–4200. 10.1073/pnas.1618934114.

1279   81.   Guillermin, M.L., Carrillo, M.A., and Hallem, E.A. (2017). A Single Set of Interneurons

1280   Drives Opposite Behaviors in C. elegans. Current Biology *27*, 2630-2639.e6.

1281   10.1016/J.CUB.2017.07.023.

1282   82.   Laurent, P., Soltesz, Z., Nelson, G.M., Chen, C., Arellano-Carbajal, F., Levy, E., and de Bono,

1283   M. (2015). Decoding a neural circuit controlling global animal state in C. Elegans. Elife *2015*.

1284   83.   Busch, K.E., Laurent, P., Soltesz, Z., Murphy, R.J., Faivre, O., Hedwig, B., Thomas, M.,

1285   Smith, H.L., and de Bono, M. (2012). Tonic signaling from O2 sensors sets neural circuit

1286   activity and behavioral state. Nat Neurosci *15*, 581–591. 10.1038/nn.3061.

1287   84.   Haspel, G., Deng, L., Harreguy, M.B., and Tanvir, Z. (2020). Elegantly. The Neural Control of

1288   Movement, 3–29. 10.1016/B978-0-12-816477-8.00001-6.

1289   85.   Tolstenkov, O., Van der Auwera, P., Costa, W.S., Bazhanova, O., Gemeinhardt, T.M., Bergs,

1290   A.C.F., and Gottschalk, A. (2018). Functionally asymmetric motor neurons contribute to

1291   coordinating locomotion of Caenorhabditis elegans. Elife *7*. 10.7554/ELIFE.34997.

1292   86.   Butler, V.J., Branicky, R., Yemini, E., Liewald, J.F., Gottschalk, A., Kerr, R.A., Chklovskii,

1293   D.B., and Schafer, W.R. (2015). A consistent muscle activation strategy underlies crawling

1294   and swimming in Caenorhabditis elegans. J R Soc Interface *12*. 10.1098/RSIF.2014.0963.

1295   87.   Faumont, S., Rondeau, G., Thiele, T.R., Lawton, K.J., McCormick, K.E., Sottile, M.,

1296   Griesbeck, O., Heckscher, E.S., Roberts, W.M., Doe, C.Q., et al. (2011). An Image-Free Opto-

1297   Mechanical System for Creating Virtual Environments and Imaging Neuronal Activity in

1298   Freely Moving Caenorhabditis elegans. PLoS One *6*, e24666. 10.1371/journal.pone.0024666.

1299   88.   Wen, Q., Po, M., Hulme, E., Chen, S., Liu, X., Kwok, S., Gershow, M., Leifer, A., Butler, V.,

1300   Fang-Yen, C., et al. (2012). Proprioceptive coupling within motor neurons drives C. elegans

1301   forward locomotion. Neuron *76*, 750–761. 10.1016/J.NEURON.2012.08.039.

1302   89.   Gao, S., Guan, S.A., Fouad, A.D., Meng, J., Kawano, T., Huang, Y.-C., Li, Y., Alcaire, S.,

1303   Hung, W., Lu, Y., et al. (2018). Excitatory motor neurons are local oscillators for backward

1304   locomotion. Elife *7*. 10.7554/eLife.29915.

1305   90.   Worrell, J.C., Rumschlag, J., Betzel, R.F., Sporns, O., and Mišić, B. (2017). Optimized

1306   connectome architecture for sensory-motor integration. Netw Neurosci *1*, 415–430.

1307   10.1162/NETN_a_00022.

1308   91.   Williams, E.A., Verasztó, C., Jasek, S., Conzelmann, M., Shahidi, R., Bauknecht, P.,

1309   Mirabeau, O., and Jékely, G. (2017). Synaptic and peptidergic connectome of a neurosecretory

1310   center in the annelid brain. Elife *6*, e26349. 10.7554/eLife.26349.

1311   92.   Turek, M., Besseling, J., Spies, J.-P., König, S., and Bringmann, H. (2016). Sleep-active

1312   neuron specification and sleep induction require FLP-11 neuropeptides to systemically induce

1313   sleep. Elife *5*, e12499. 10.7554/eLife.12499.

1314   93.   Xu, Y., Zhang, L., Liu, Y., Topalidou, I., Hassinan, C., Ailion, M., Zhao, Z., Wang, T., Chen,

1315   Z., and Bai, J. (2021). Dopamine receptor DOP-1 engages a sleep pathway to modulate

1316   swimming in C. elegans. iScience *24*, 102247. 10.1016/j.isci.2021.102247.

1317   94.   Priest, M.F., Freda, S.N., Badong, D., Dumrongprechachan, V., and Kozorovitskiy, Y. (2021).

1318   Peptidergic modulation of fear responses by the Edinger-Westphal nucleus (bioRxiv).

1319   95.   Kim, M.W., Wang, W., Sanchez, M.I., Coukos, R., von Zastrow, M., and Ting, A.Y. (2017).

1320   Time-gated detection of protein-protein interactions with transcriptional readout. Elife *6*,

1321   e30233. 10.7554/eLife.30233.

1322   96.   Kim, C.K., Cho, K.F., Kim, M.W., and Ting, A.Y. (2019). Luciferase-LOV BRET enables

1323   versatile and specific transcriptional readout of cellular protein-protein interactions. Elife *8*,

1324   e43826. 10.7554/eLife.43826.

97. Smith, S.J., Smbül, U., Graybuck, L.T., Collman, F., Seshamani, S., Gala, R., Gliko, O., Elabbady, L., Miller, J.A., Bakken, T.E., et al. (2019). Single-cell transcriptomic evidence for dense intracortical neuropeptide networks. Elife *8*, 1–35. 10.7554/eLife.47889.

98. Chen, R., Wu, X., Jiang, L., and Zhang, Y. (2017). Single-Cell RNA-Seq Reveals Hypothalamic Cell Diversity. Cell Rep *18*, 3227–3241. 10.1016/J.CELREP.2017.03.004.

99. Terauchi, A., Johnson-Venkatesh, B., Bullock, B., Lehtinen, M., and Umemori, H. (2016). Retrograde fibroblast growth factor 22 (FGF22) signaling regulates insulin-like growth factor 2 (IGF2) expression for activity-dependent synapse stabilization in the mammalian brain. Elife *5*. 10.7554/ELIFE.12151.

100. Ghosh, D.D., Sanders, T., Hong, S., McCurdy, L.Y., Chase, D.L., Cohen, N., Koelle, M.R., and Nitabach, M.N. (2016). Neural Architecture of Hunger-Dependent Multisensory Decision Making in C. elegans. Neuron *92*, 1049–1062. 10.1016/j.neuron.2016.10.030.

101. Root, C.M., Ko, K.I., Jafari, A., and Wang, J.W. (2011). Presynaptic facilitation by neuropeptide signaling mediates odor-driven food search. Cell *145*, 133–144. 10.1016/j.cell.2011.02.008.

102. Horowitz, L., Brandt, J., and Ringstad, N. (2019). Repression of an activity-dependent autocrine insulin signal is required for sensory neuron development in C. elegans. Development *146*. 10.1242/DEV.182873.

103. Mardinly, A., Spiegel, I., Patrizi, A., Centofante, E., Bazinet, J., Tzeng, C., Mandel-Brehm, C., Harmin, D., Adesnik, H., Fagiolini, M., et al. (2016). Sensory experience regulates cortical inhibition by inducing IGF1 in VIP neurons. Nature *531*, 371–375. 10.1038/NATURE17187.

104. Choi, C., Cao, G., Tanenhaus, A., McCarthy, E., Jung, M., Schleyer, M., Shang, Y., Rosbash, M., Yin, J., and Nitabach, M. (2012). Autoreceptor control of peptide/neurotransmitter corelease from PDF neurons determines allocation of circadian activity in drosophila. Cell Rep *2*, 332–344. 10.1016/J.CELREP.2012.06.021.

105. Yoshioka, J., Prince, R., Huang, H., Perkins, S., Cruz, F., MacGillivray, C., Lauffenburger, D., and Lee, R. (2005). Cardiomyocyte hypertrophy and degradation of connexin43 through

1352   spatially restricted autocrine/paracrine heparin-binding EGF. Proc Natl Acad Sci U S A *102*,

1353   10622–10627. 10.1073/PNAS.0501198102.

1354   106.   Tse, L., and Wong, Y. (2019). GPCRs in Autocrine and Paracrine Regulations. Front

1355   Endocrinol (Lausanne) *10*. 10.3389/FENDO.2019.00428.

1356   107.   Yemini, E., Jucikas, T., Grundy, L.J., Brown, A.E.X., and Schafer, W.R. (2013). A database of

1357   Caenorhabditis elegans behavioral phenotypes. Nat Methods *10*, 877–879.

1358   10.1038/nmeth.2560.

1359   108.   Brown, A.E.X., Yemini, E.I., Grundy, L.J., Jucikas, T., and Schafer, W.R. (2013). A

1360   dictionary of behavioral motifs reveals clusters of genes affecting Caenorhabditis elegans

1361   locomotion. Proceedings of the National Academy of Sciences *110*, 791–796.

1362   10.1073/pnas.1211447110.

1363   109.   Avery, L., and Horvitz, H.R. (1989). Pharyngeal pumping continues after laser killing of the

1364   pharyngeal nervous system of C. elegans. Neuron *3*, 473–485. 10.1016/0896-6273(89)90206-

1365   7.

1366   110.   Forrester, W.C., and Garriga, G. (1997). Genes necessary for C. elegans cell and growth cone

1367   migrations. Development *124*, 1831–1843. 10.1242/dev.124.9.1831.

1368   111.   Yemini, E., Lin, A., Nejatbakhsh, A., Varol, E., Sun, R., Mena, G.E., Samuel, A.D.T.,

1369   Paninski, L., Venkatachalam, V., and Hobert, O. (2021). NeuroPAL: A Multicolor Atlas for

1370   Whole-Brain Neuronal Identification in C. elegans. Cell *184*, 272-288.e11.

1371   10.1016/j.cell.2020.12.012.

1372   112.   Hall, D.H., and Altun, Z.F. (2011). Nervous system, general description. WormAtlas: The

1373   Handbook of C.elegans Anatomy. doi:10.3908/wormatlas.1.18.

1374   113.   Rubinov, M., and Sporns, O. (2010). Complex network measures of brain connectivity: Uses

1375   and interpretations. Neuroimage *52*, 1059–1069. 10.1016/j.neuroimage.2009.10.003.

1376   114.   Fisher, R.A. (1934). Statistical methods for research workers, 5th ed. (Edinburgh).

1377   115.   Storey, J.D. (2002). A direct approach to false discovery rates. J R Stat Soc Series B Stat

1378   Methodol *64*, 479–498. 10.1111/1467-9868.00346.

1379   116.   Bounova, G., and de Weck, O. (2012). Overview of metrics and their correlation patterns for

1380   multiple-metric topology analysis on heterogeneous graph ensembles. Phys Rev E *85*, 016117.

1381   10.1103/PhysRevE.85.016117.

1382   117.   de Domenico, M., Porter, M.A., and Arenas, A. (2015). MuxViz: a tool for multilayer analysis

1383   and visualization of networks. J Complex Netw *3*, 159–176. 10.1093/comnet/cnu038.

1384   118.   Shannon, P., Markiel, A., Ozier, O., Baliga, N.S., Wang, J.T., Ramage, D., Amin, N.,

1385   Schwikowski, B., and Ideker, T. (2003). Cytoscape: A Software Environment for Integrated

1386   Models of Biomolecular Interaction Networks. Genome Res *13*, 2498–2504.

1387   10.1101/gr.1239303.

1388   119.   Bhardwaj, A., Thapliyal, S., Dahiya, Y., and Babu, K. (2018). FLP-18 Functions through the

1389   G-Protein-Coupled Receptors NPR-1 and NPR-4 to Modulate Reversal Length in

1390   Caenorhabditis elegans. The Journal of Neuroscience *38*, 4641–4654.

1391   10.1523/JNEUROSCI.1955-17.2018.

1392   120.   Lindemans, M., Liu, F., Janssen, T., Husson, S.J., Mertens, I., Gäde, G., and Schoofs, L.

1393   (2009). Adipokinetic hormone signaling through the gonadotropin-releasing hormone receptor

1394   modulates egg-laying in Caenorhabditis elegans. Proceedings of the National Academy of

1395   Sciences *106*, 1642–1647. 10.1073/pnas.0809881106.

1396

A

HEAD

Nerve ring

Amphid commissures
Anterior ventral nerve cord
Deirid commissures

MIDBODY

Dorsal sublateral left and right
Dorsal Nerve Cord

Ventral sublateral left and right
Ventral Nerve cord
Canal associated left and right

TAIL

Preanal ganglion
Canal associated
Tail tip bundle

B

CAN neurons

GPCR expression

Pharynx | Sensory neurons | Interneurons | Motorneurons

C

CAN neurons

Cognate NPP expression

Pharynx | Sensory neurons | Interneurons | Motorneurons

Type of connection required to explain expression

Mid range | Between strata | Within bundle (outside NR) | Within stratum
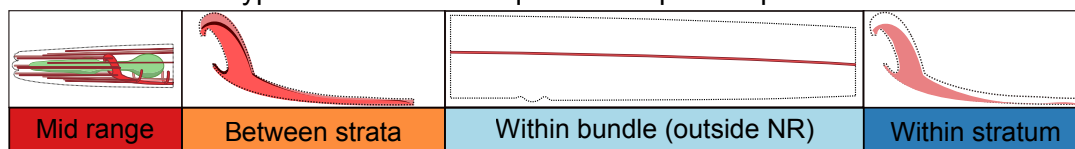
A

Local

Pervasive

Broadcasting

Integrative

○ CAPA-1 ● NMUR-1
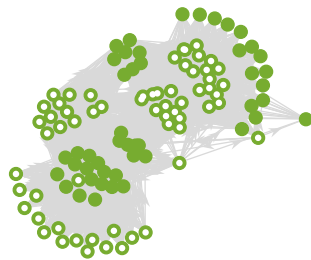
○ FLP-18 ● NPR-5
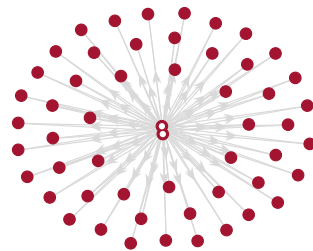
○ FLP-1 ● FRPR-7
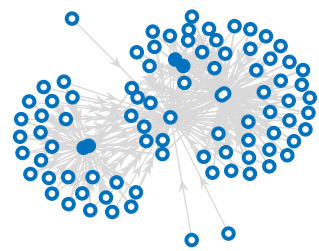
○ NLP-13 ● B0334.6

B
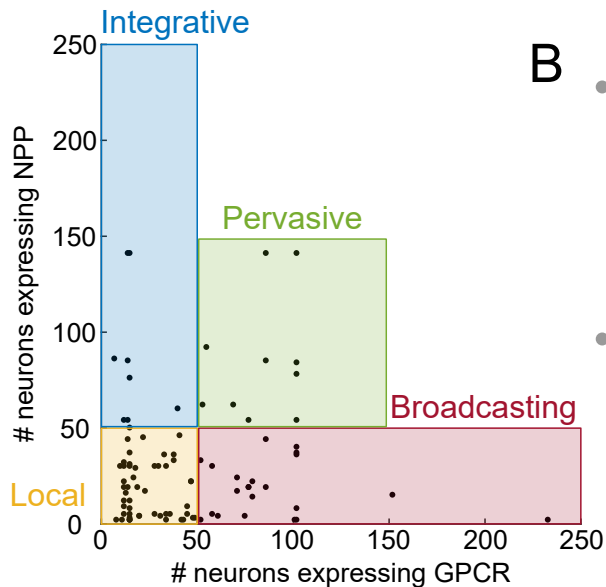
● DMSR-1

○ FLP-11   ○ FLP-10

● DMSR-7
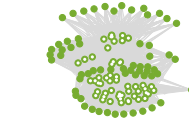
○ FLP-11   ○ FLP-1

● FRPR-8

○ FLP-13   ○ FLP-11
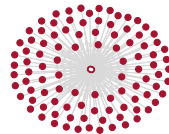
● EGL-6
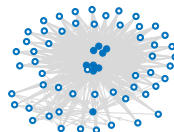
○ FLP-10   ○ FLP-5
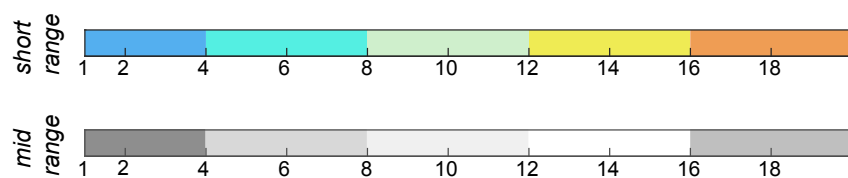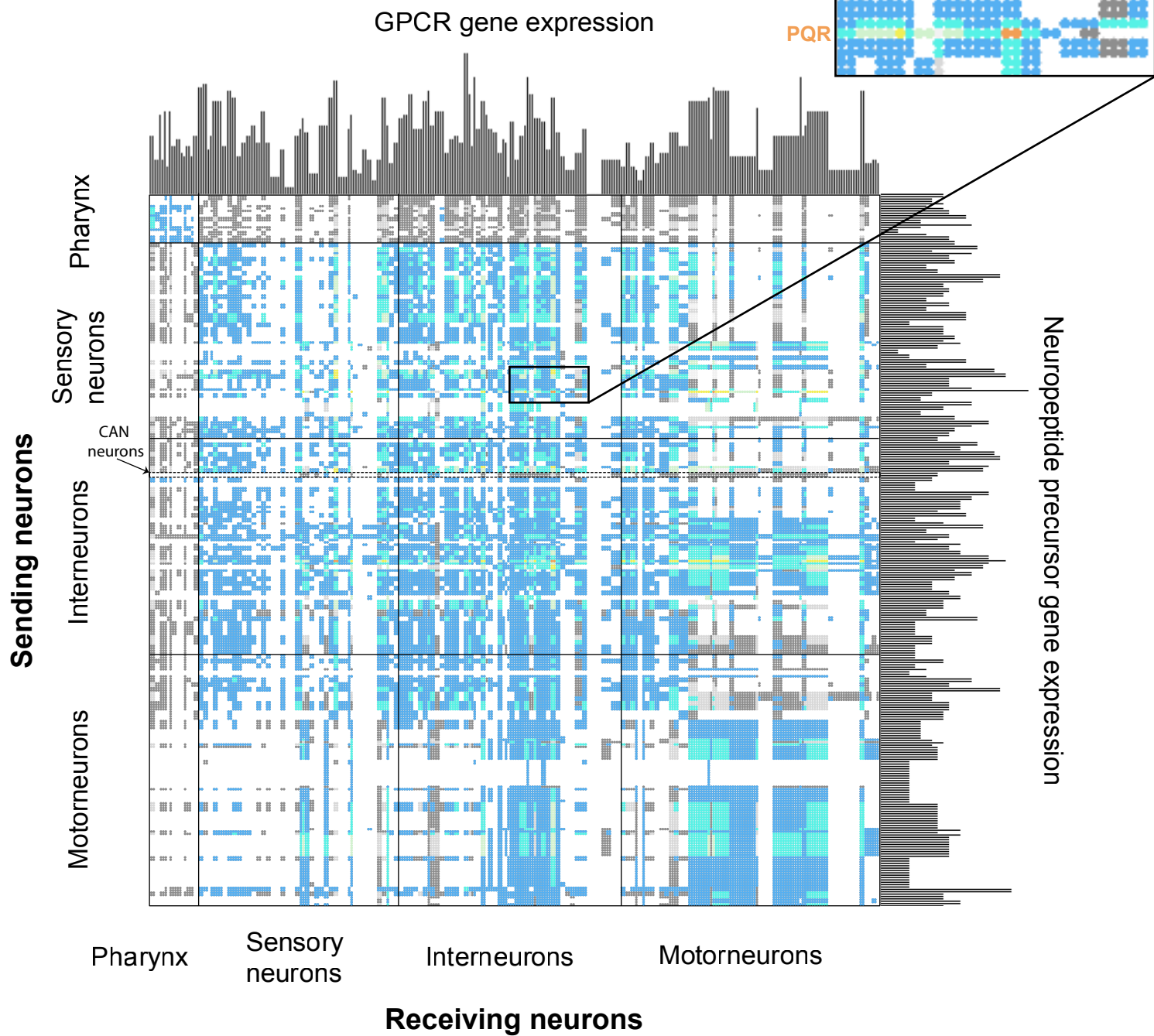
Edge weight (number of linking NPP-GPCR pairs)
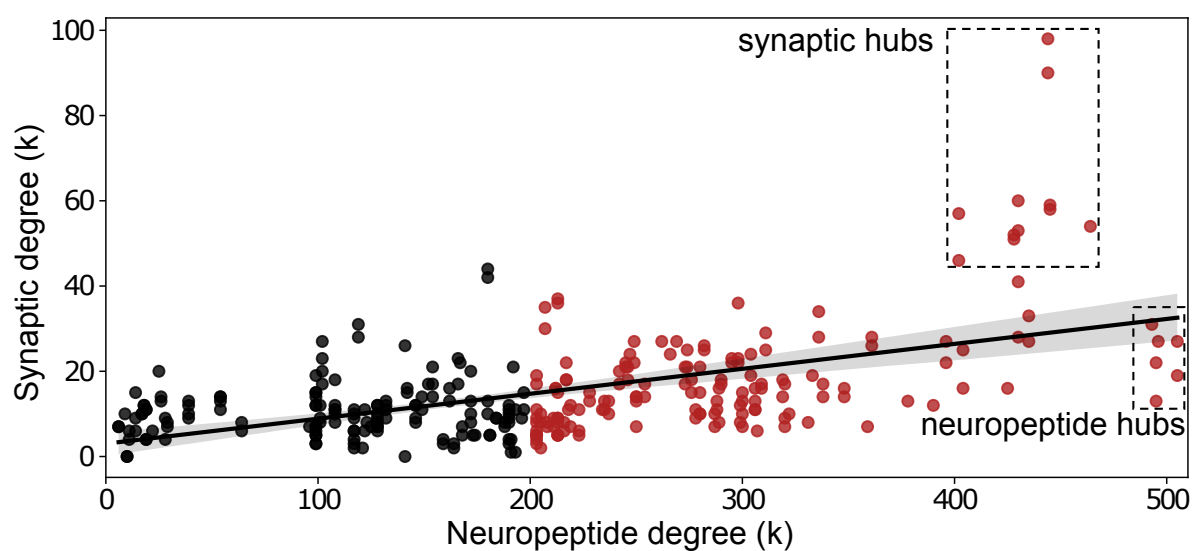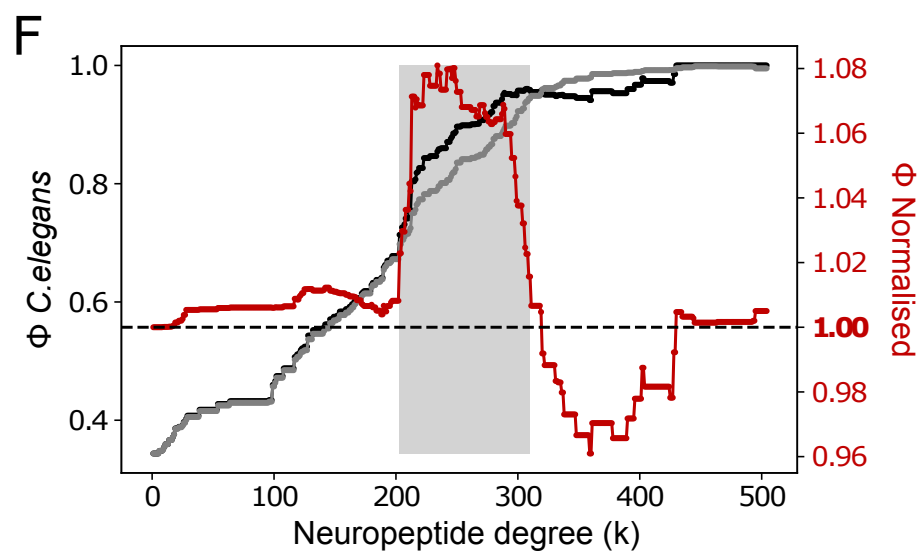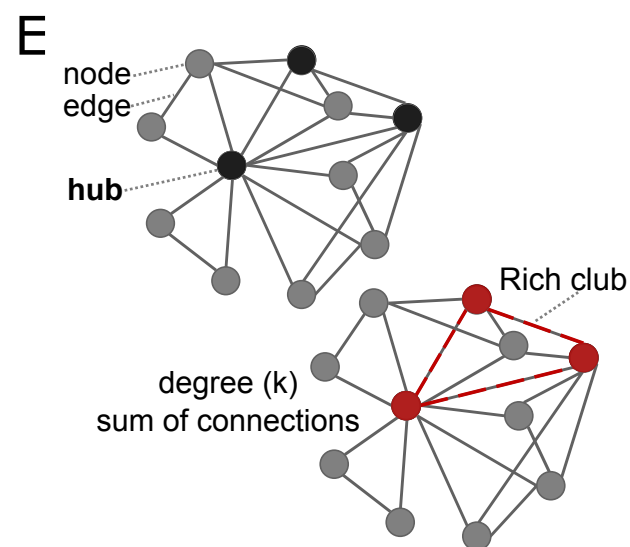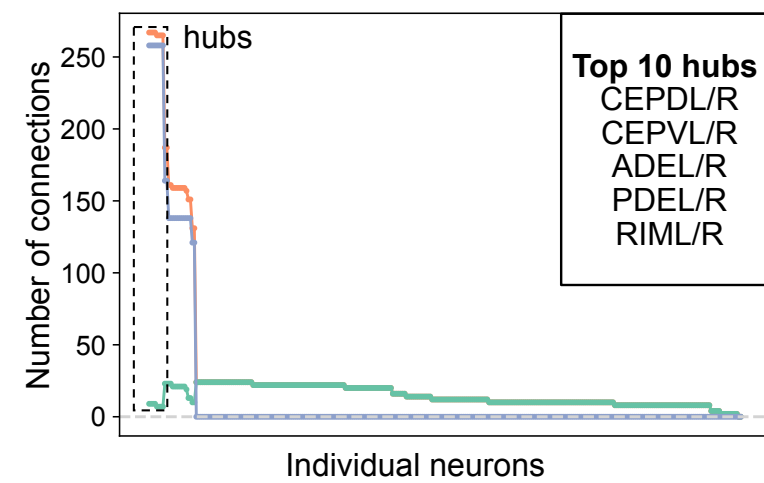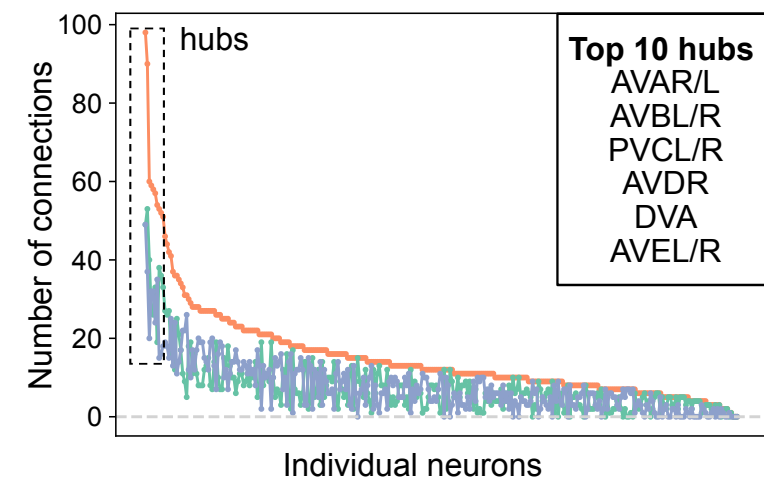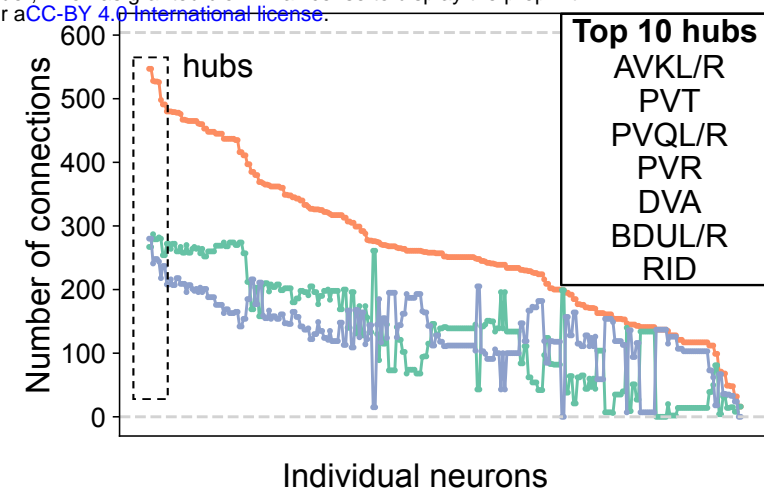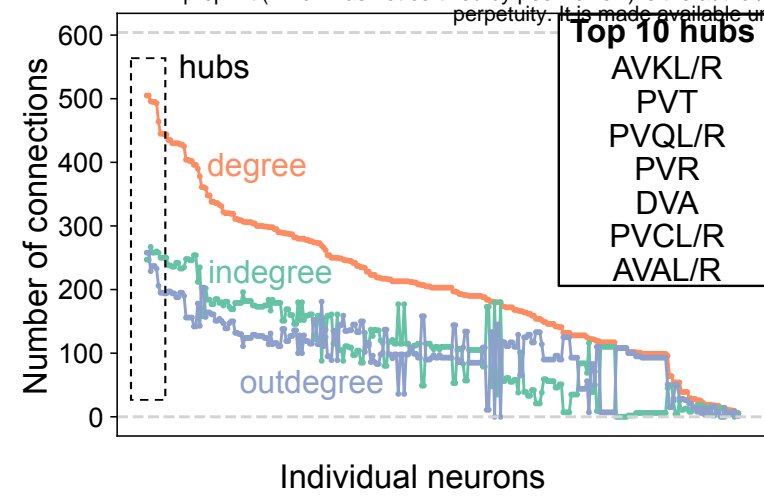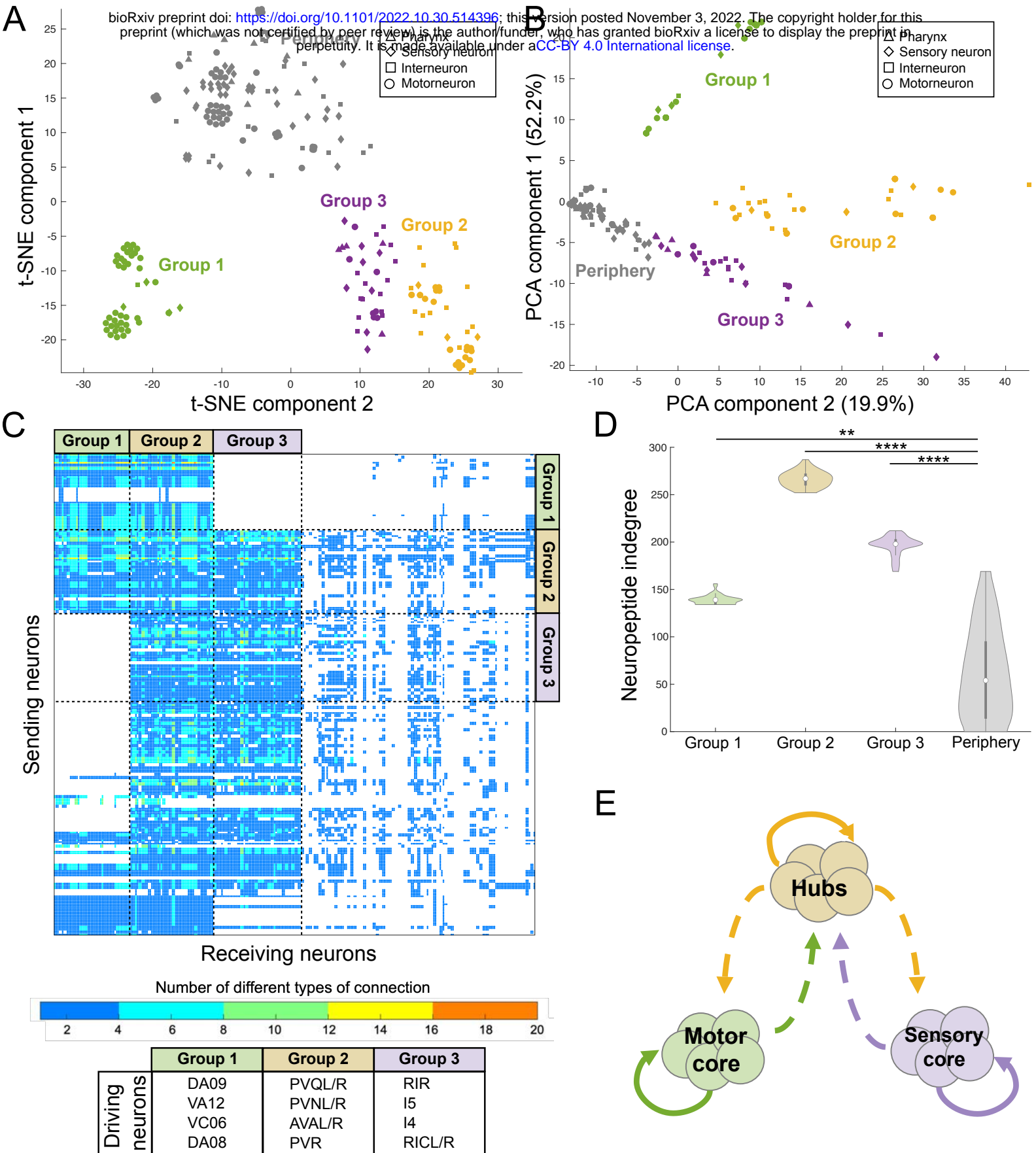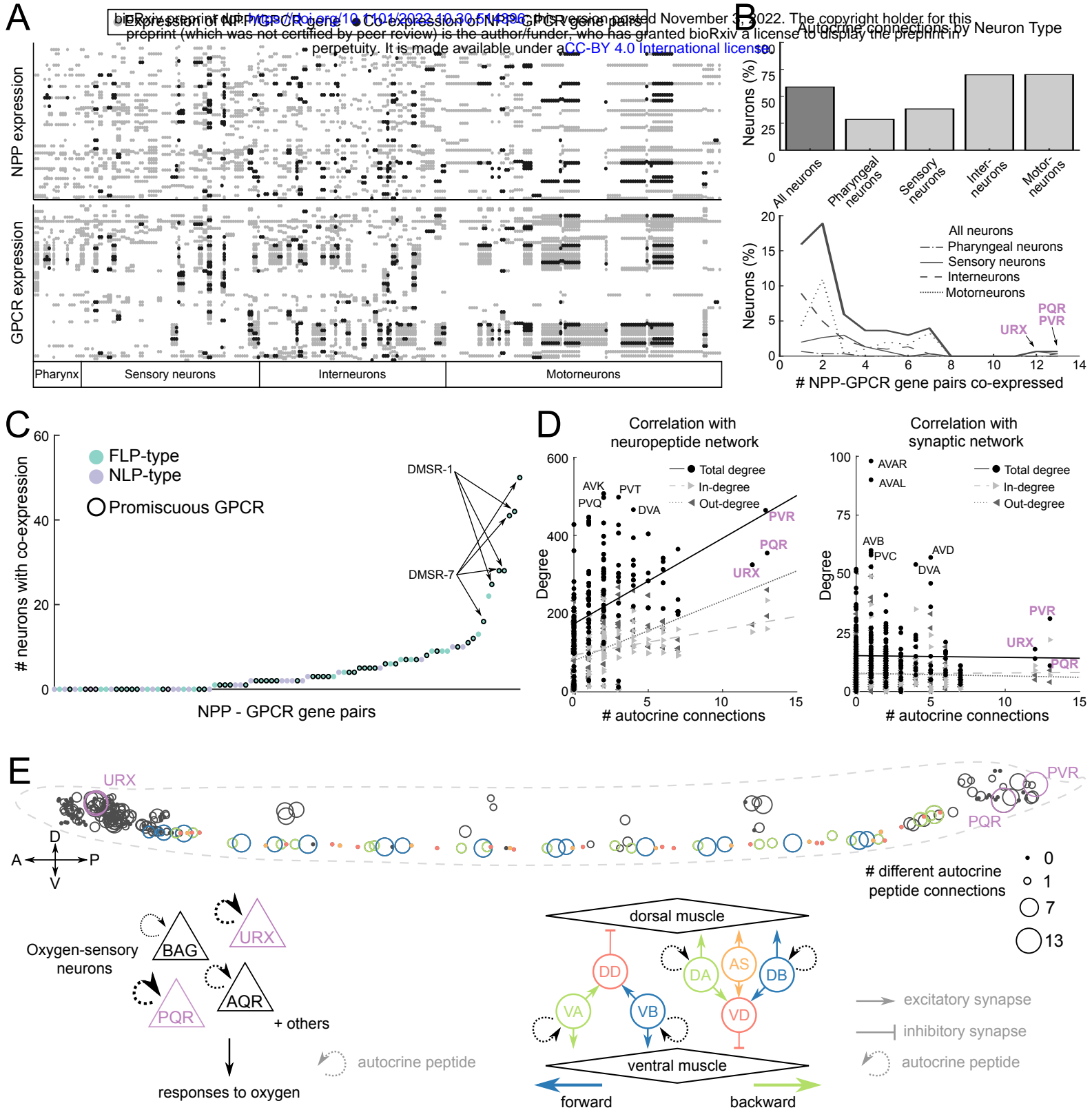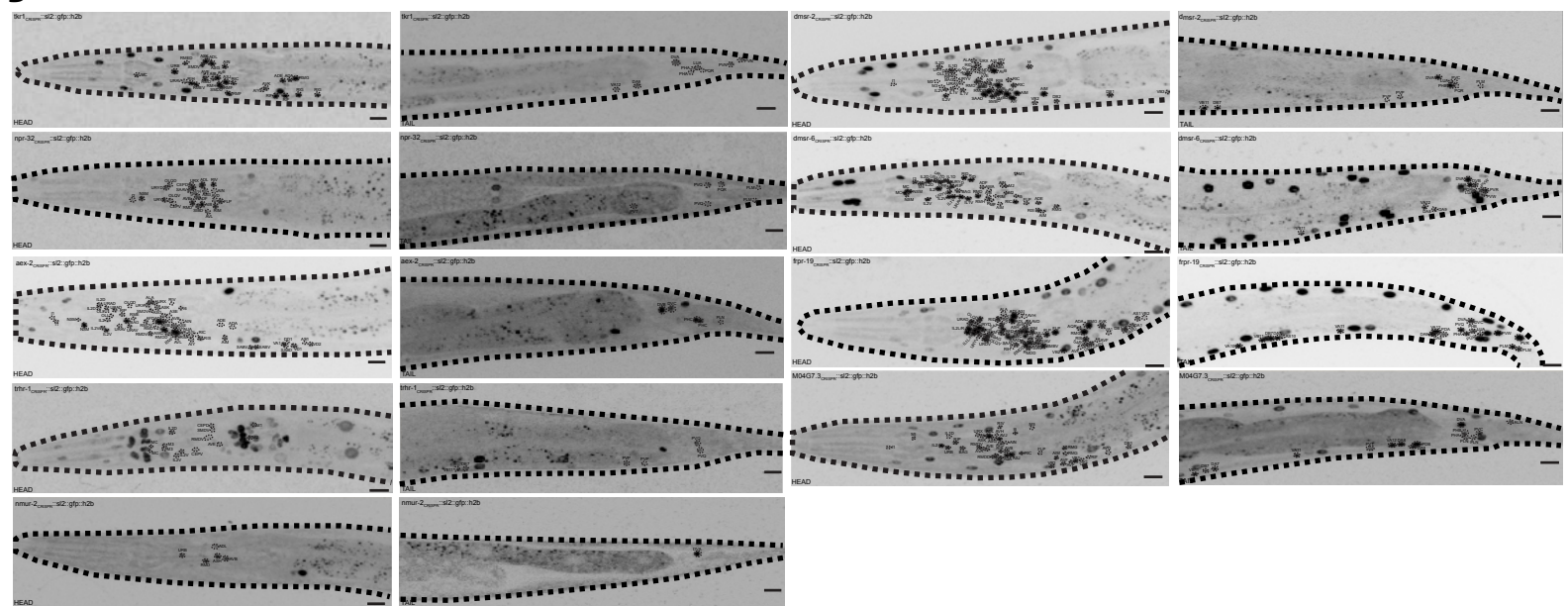
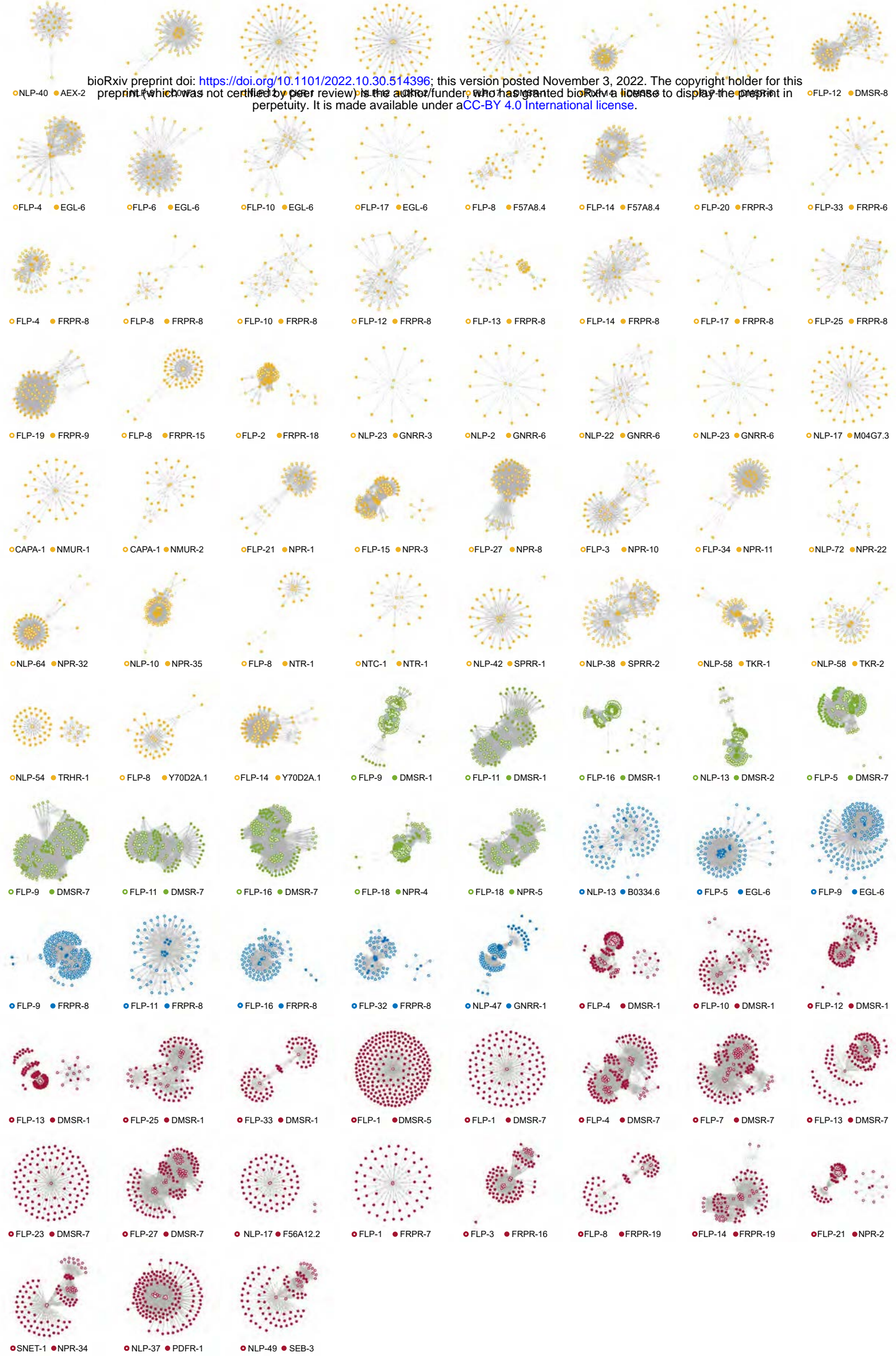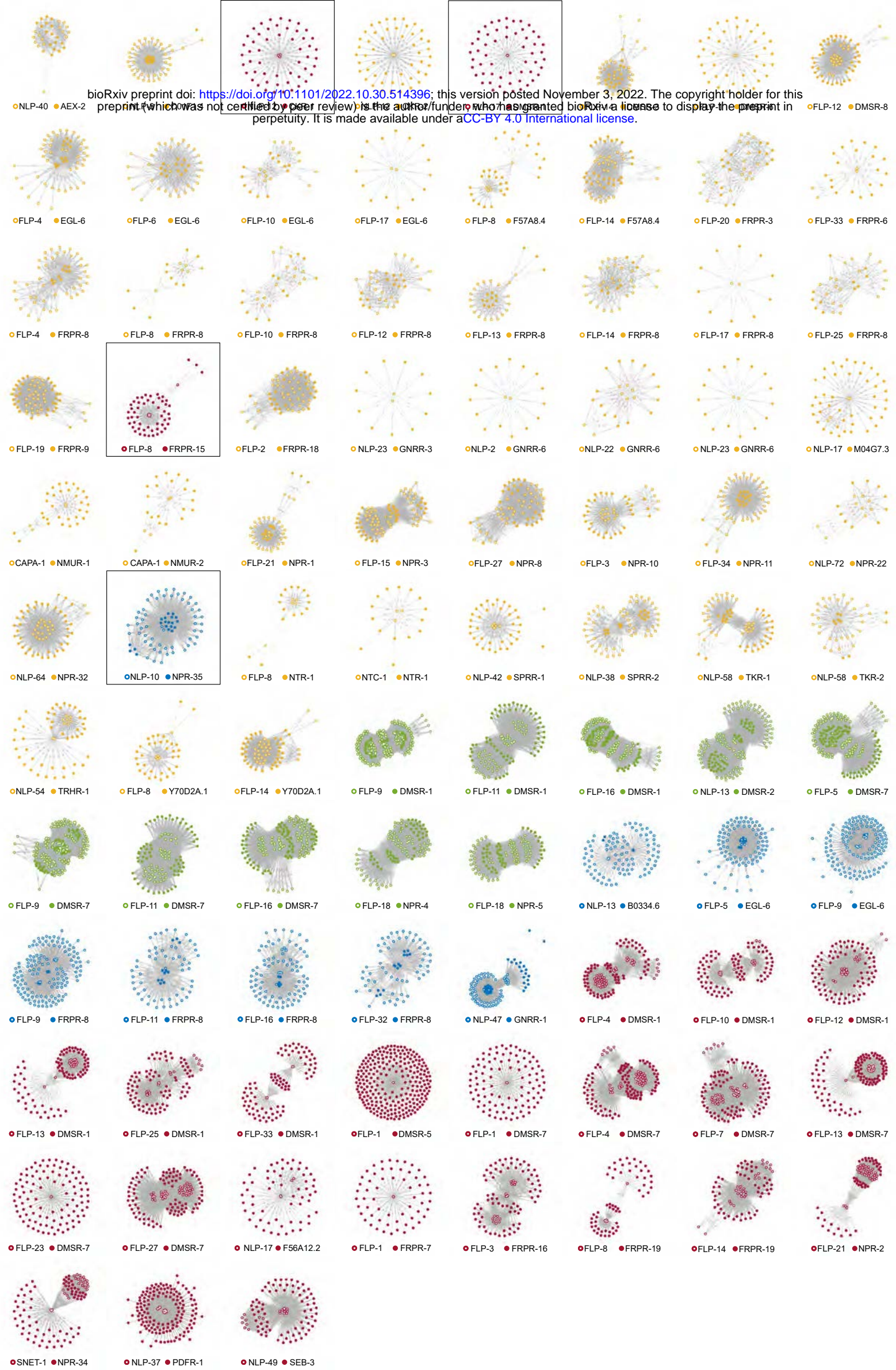**A** Expression of NPP/GPCR gene ● Co-expression of NPP-GPCR gene pairs

B Autocrine connections by Neuron Type

C

D Correlation with neuropeptide network

Correlation with synaptic network

E

A Model 1: long-range
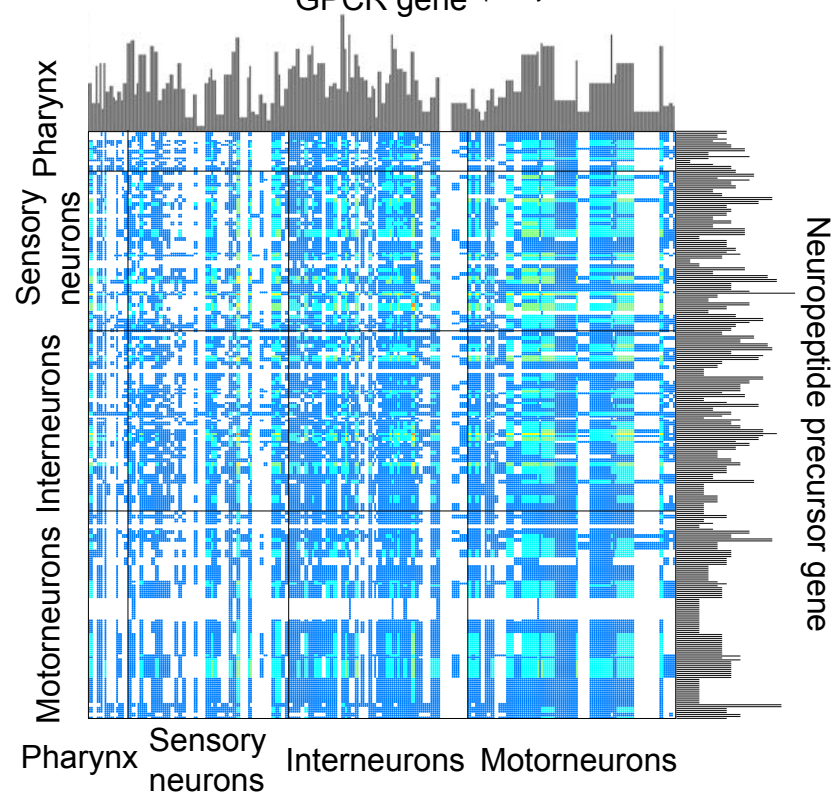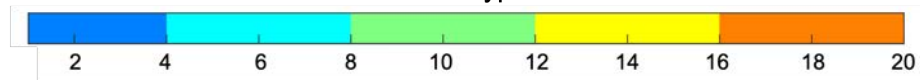
B Model 2: mid-range
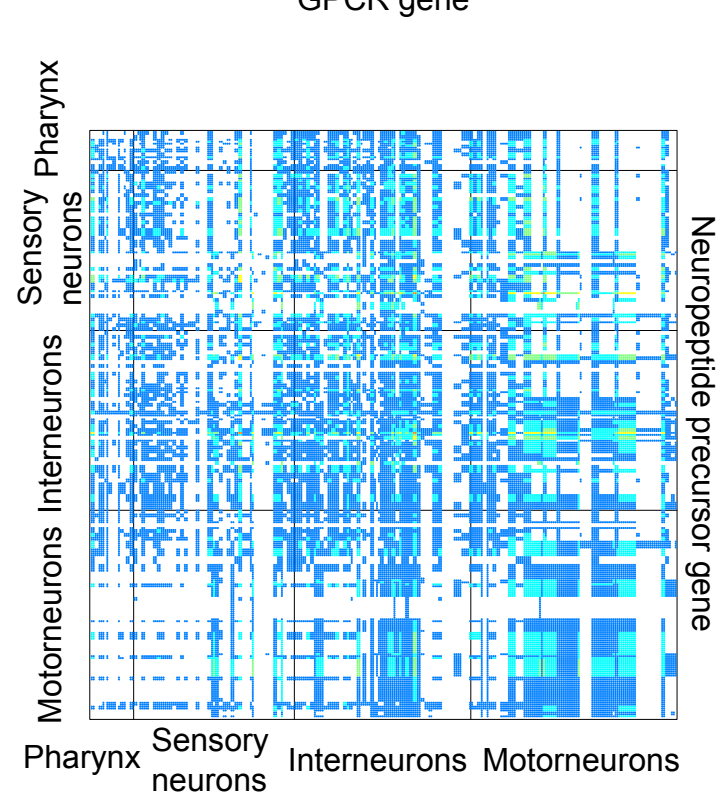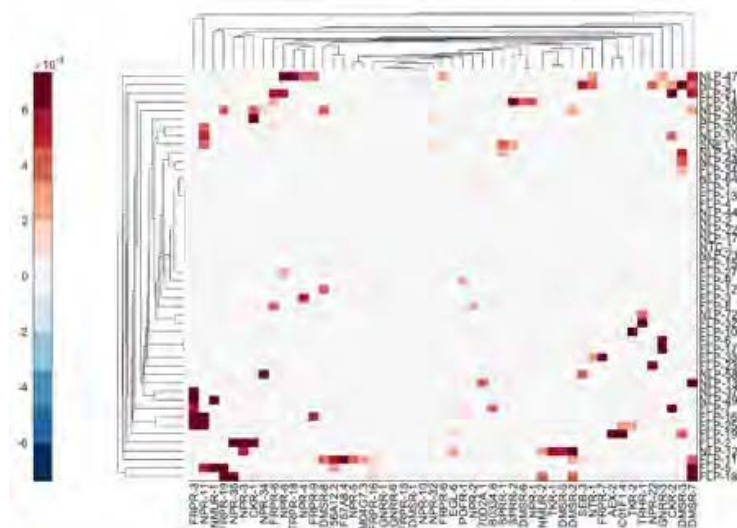
Number of different types of connection

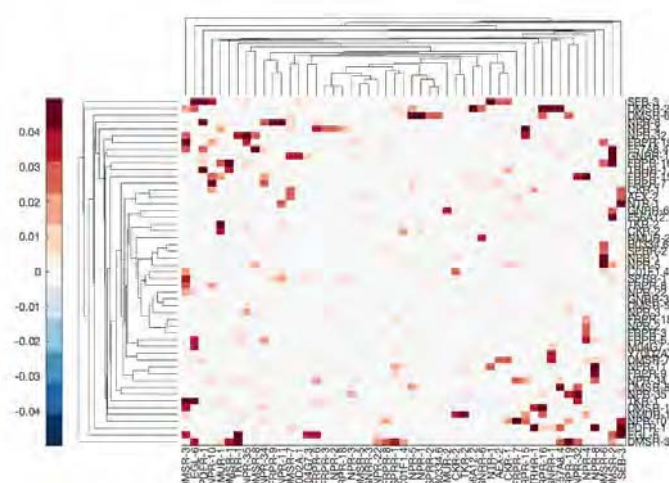**A** Co-expression of NPP-GPCR gene pairs

Pharynx | Sensory neurons | Interneurons | Motorneurons

B

Fraction of neurons

CeNGEN threshold 2 - EC$_{50}$ 1 µM
CeNGEN threshold 2 - EC$_{50}$ 500 nM
CeNGEN threshold 4 - EC$_{50}$ 1 µM
CeNGEN threshold 4 - EC$_{50}$ 500 nM

Number of NPP-GPCR gene pairs co-expressed

All neurons
Pharyngeal neurons
Sensory neurons
Interneurons
Motorneurons

C

neurons with co-expression

FLP-type   NLP-type

**

D

neurons with co-expression

Specific   Promiscuous

GPCR showing autocrine connections (%)

Specific   Promiscuous

E

Correlation with gap junction network

Degree

Total degree

AVAL
AVAR
AVBR
AVBL

PVR
URX   PQR

autocrine connections

Correlation with monoaminergic network

Degree

Total degree
In-degree
Out-degree

CEP
ADE   PDE

URX   PVR
PQR

autocrine connections

F

DA

VA

DB

VB

DMSR-1
DMSR-7
NPR-5