Evolution of Synapses and Neurotransmitter Systems:
The Divide-and-Conquer Model for Early Neural Cell-Type Evolution

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Abstract

Nervous systems evolved around 560 million years ago to coordinate and empower animal bodies. Ctenophores – one of the earliest-branching lineages – are thought to share few neuronal genes with bilaterians and may have evolved neurons convergently. Here we review our current understanding of the evolution of neuronal molecules in non-bilaterians. We also reanalyse single-cell sequencing data in light of new cell-cluster identities from a ctenophore and uncover evidence supporting the homology of one ctenophore neuron-type with neurons in Bilateria. The specific coexpression of the presynaptic proteins Unc13 and RIM with voltage-gated channels, neuropeptides and homeobox genes pinpoint a spiking sensory-peptidergic cell in the ctenophore mouth. Similar Unc13-RIM neurons may have been present in the first eumetazoans to rise to dominance only in stem Bilateria. We hypothesize that the Unc13-RIM lineage ancestrally innervated the mouth and conquered other parts of the body with the rise of macrophagy and predation during the Cambrian explosion.

Keywords: choanoflagellates, sponges, ctenophores, placozoans, cnidarians, synapse, neuron, evolution, neuropeptides

Phylogenetic overview – set the stage for nervous system origins

Nervous systems probably first appeared on our planet shortly before 560 million years ago. The earliest indirect evidence for the presence of a nervous system are Precambrian trace fossils left by the earliest bilaterians ca. 558 Ma [1]. By this time, the major groups of animals, including the bilaterians, cnidarians, placozoans, ctenophores and sponges had likely already diverged.

With the exception of sponges and placozoans (and some highly reduced parasites), all animals have a nervous system. Animal nervous systems are morphologically, functionally and molecularly highly diverse and disparate. No universal neuronal markers have been found and the earliest transitions leading to nervous systems remain shrouded in mystery. It is clear that any attempt to reconstruct nervous system evolution requires a broad sampling across animal phylogeny. Non-bilaterian animals deserve special attention in such endeavours. These include the neuron-less sponges and placozoans (disk-shaped millimetre-sized ciliated marine animals) and the neuronal cnidarians (jellyfish, corals, sea anemones and relatives) and ctenophores or comb jellies (gelatinous marine invertebrates). In recent years, we have seen a revival of interest in these animals with an initial focus on genomics and phylogeny and more recently also on molecular function and neurophysiology.

Reconstructing ancestral states and transitions in nervous system evolution requires a robust phylogenetic framework. After a lot of flux, a reliable phylogeny for the major groups of non-bilaterian animals begins to emerge. Sponges seem to re-establish their position as the first animal group to branch from the rest of the animals. Their placement can only be explained by real phylogenetic

signal, while the other contestants for this key position, the ctenophores, were often pushed as first due to systematic error [2]. Ctenophores now seem to be the second branch off the main animal tree, from the perspective of the bilaterians, followed by cnidarians and their likely sisters, the placozoans [3]. More stable has been the position of the choanoflagellates, our closest protist relatives. These single-celled or colonial organisms, characterised by a cilium surrounded by a microvillar collar, also have special relevance to understanding the evolutionary assembly of neuronal pathways [4,5].

The timeline of animal origins has also been controversial with conflicting dates given by molecular clocks, fossil biomarkers and morphological fossils. A congruence of dates is now emerging, with molecular clock estimates becoming less credible [6,7] and with increasing ambiguity surrounding Ediacaran putative sponge fossils (e.g. [8]) and biomarkers [9–12]. At the same time, paleontologists increasingly succeed in placing fossils in a credible phylogenetic context providing minimum estimates for the appearance and ancestral morphology of the major animal lineages (e.g., [12,13]). Some Ediacaran fossils of previously enigmatic affinity (*Dickinsonia* and its relatives) have also been confirmed as animals based on steroid biomarker evidence, providing an important piece to the puzzle [14]. Our best understanding of the timeline and phylogeny of early animal diversification is summarised in Figure 1. This framework will form the basis of our discussion in this review.
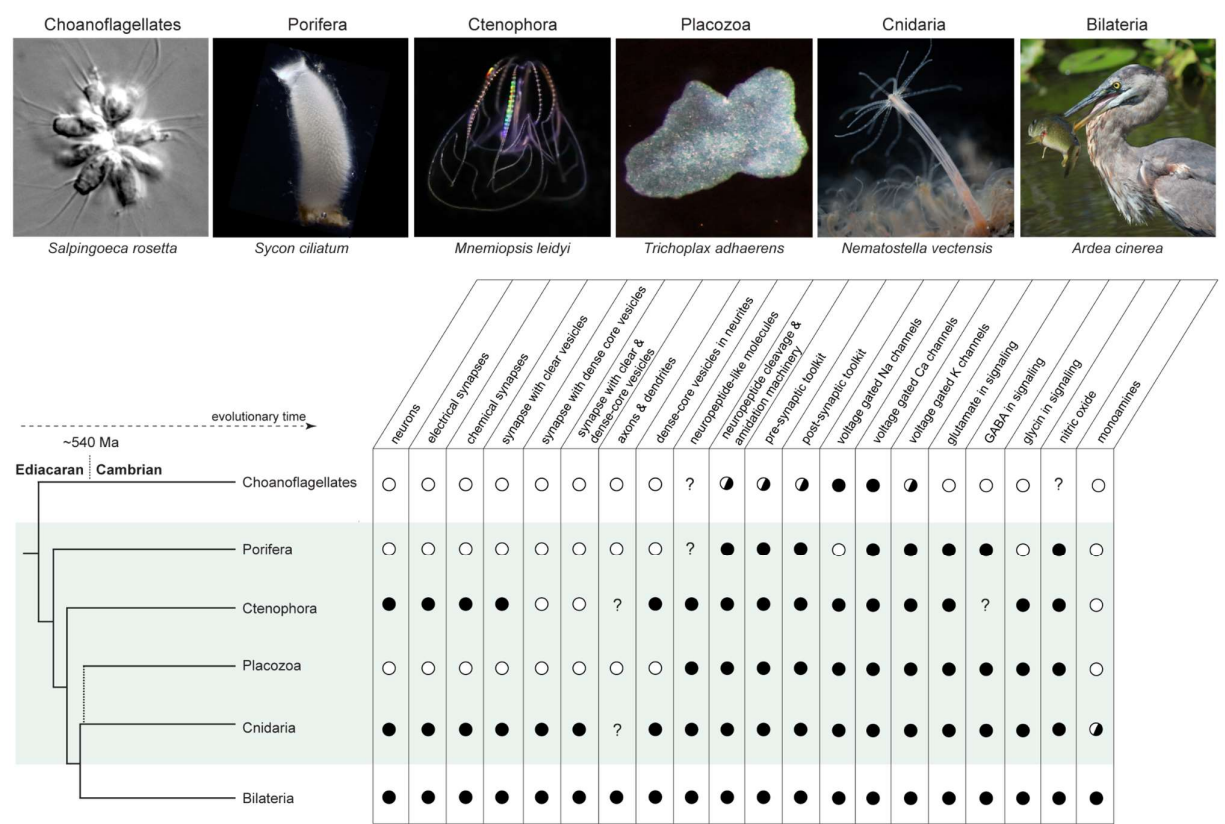
Figure 1: Animal phylogeny and the distribution of neuronal characters. (A) Representatives of the main animal lineages and their closest protist relatives. (B) Simplified animal phylogeny and character distribution across the main animal lineages and choanoflagellates. Phylogeny adapted from [2].

Defining a neuron - the hunt for elusive universal neuronal markers

A nervous system consists of excitable cells with long projections that connect to each other and other cells by synapses. Excitability is the ability of the cells to produce highly non-linear ionic responses that can propagate in the cells along the processes and spread to other cells at synapses. All of these characteristics of nervous systems are supported by dedicated cellular components and processes often specific to neuronal cells.

The neuronal components can include specialised sensory complexes in sensory neurons, neurotransmitter and neuromodulator synthesis and transport systems, transmitter receptors, various ion channels, or pre- and postsynaptic complexes. It is therefore not an unreasonable expectation that by using these molecules as markers, we should be able to identify neurons in any nervous system. This view has become less and less tenable over the last decade. Molecular studies in non-bilaterian animals instead suggested that the further away we move from the Bilateria, the more elusive the molecular signatures of neurons become [15]. This applies to neuronal specification molecules, neurotransmitters and their processing machinery, neuropeptides and their receptors, and synaptic proteins. Key molecular components of bilaterian nerves are often not expressed in neurons of non-bilaterians or not present in ctenophores [16]. The history of neuronally-expressed gene families is often difficult to reconstruct and in many cases these are not characterised by one-to-one but one-to-many or many-to-many orthology relationships. These patterns indicate extensive parallel evolution in neuronal systems and make specific functional assignments difficult. Single-cell sequencing studies also suggest a striking heterogeneity of gene expression in ctenophore neurons [17].

In other instances, the molecular players may be well conserved, but their expression diverges and while they can be robust neuron markers in bilaterians, they are not in non-bilaterians.

These patterns led to the suggestion that the nervous system of ctenophores and cnidarians+bilaterians evolved convergently [18]. Here we revisit this influential hypothesis in light of new evidence.

What were the first neurotransmitters?

The evolution of neurotransmitter systems has recently been extensively reviewed [19]. In this section, we give a brief overview of recent findings relevant to reconstruct the ancestry of neural systems.

The small neurotransmitters and monoamines familiar from bilaterians are only partially present in non-bilaterians. Only glycine and glutamate are generally involved in signalling in non-bilaterians and are the best candidates as the first neurotransmitters at the origin of nervous systems. In placozoans, glycine is the most abundant transmitter and exogenous glycine induces tissue contractility and changes ciliary activity [20]. In ctenophores, glutamate and glycine are the only small transmitters identified. Muscle cells in *Pleurobrachia bachei* generate action potentials upon the application of both D- and L-glutamate.

Ctenophore genomes encode over a dozen homologs of bilaterian ionotropic glutamate receptors (iGluRs). Biochemical and electrophysiological analysis of several of these from *Mnemiopsis leidyi* and *P. bachei* revealed that many are activated by glycine [21,22]. Other iGluRs are activated by glutamate with glycine acting as an antagonist. The presence of these receptors together with the physiological effects of glutamate and glycine makes glutamate and glycine strong candidates for neurotransmitters in ctenophores. The iGluR family has a more ancient origin and is also present in some sponges [23]. Broader sampling in choanoflagellates also revealed the sporadic presence of iGluR sequences in some species, indicating that the family predates animals (PB and Tarja Hoffmeyer, unpublished).

The evidence is more fragmentary for GABA. GABA is present in placozoans [20], cnidarians (in the jellyfish *Aequorea*, GABA is a dominant analyte) and sponges. In sponges, GABA can induce

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contractions or block the action of glutamate, depending on the species [24,25]. However, the application of GABA did not seem to have an effect on muscle cells in *Pleurobrachia bachei* [16] and ctenophores seem to have lost most genes of the GABA pathway [26]. No specific GABA receptors have been cloned and characterised from any non-bilaterian, but candidate receptors exist in sponges [26]. Acetylcholine as a transmitter is likely a bilaterian innovation as non-bilaterians lack its synthesis, transport and degradation pathways [19].

The monoamine transmitters known from bilaterian neurons (dopamine, serotonin, octopamine, tyramine, noradrenaline) also likely appeared only in stem Bilateria. No monoamine receptors, full synthesis pathway or vesicular transporters have been found in non-bilaterian genomes [19,26]. In placozoans and ctenophores, capillary electrophoresis failed to detect serotonin, dopamine, or octopamine [20]. The G-protein coupled receptor (GPCR) families for octopamine, tyramine and noradrenaline diversified in stem bilaterians with no evidence for direct non-bilaterian orthologs [27]. Although chemical analyses identified both serotonin and dopamine for example in the hydrozoan *Hydra magnipapillata* [28] or the sea anemone *Aiptasia pallida* [29], there is no evidence that these molecules function as neurotransmitters or are produced by neurons. Only rarely, such as in the anthozoan *Renilla koellikeri,* was serotonin localised to neurons by immunohistochemistry [30].

Melatonin may be the only monoamine hormone shared between cnidarians and bilaterians. It was detected in the sea anemones *Actinia equina* [31] and *Nematostella vectensis* [32] where its levels showed diel oscillations, consistent with a role in circadian physiology. Intriguingly, the jellyfish *Cassiopea* has a sleep-like state but the involvement of melatonin in this state is unclear [33]. Cnidarians and also placozoans have melatonin receptor orthologs [34], but these have not been characterised experimentally.

There is also the intriguing possibility that other, non-canonical monoamines may be deployed in neuronal signalling outside the Bilateria. This is suggested by the large number of orphan monoamine-GPCR-like sequences in Cnidaria (GJ and Luis A Yañez-Guerra, unpublished).

Other non-conventional transmitters also occur in some non-bilaterians. In the jellyfish *Cyanea capillata*, taurine is a candidate neurotransmitter as it is present in the motor nerve net [35] and – together with beta-alanine – can depolarise neurons [36]. Furthermore, the D and L enantiomers of both glutamate and aspartate in non-bilaterians deserve more attention, with D-glutamate having potentially unique transmitter roles in ctenophores [37].

Paracrine signalling molecules

Paracrine signalling through the non-synaptic release of small molecules is ubiquitous in animals. This mode of signalling may represent the first form of intercellular communication that allowed multicellular animals to coordinate tissue activity (e.g. contractions, secretion, ciliary movement) [37]. A widespread and ancient paracrine signalling molecule is nitric oxide (NO), produced by the enzyme nitric oxide synthase (NOS) [19]. NO can influence smooth muscle contraction, neuronal and developmental signalling. Placozoans have three NOS genes expressed in different subpopulations of cells close to the edge regions and capillary electrophoresis supports the presence of active NOS enzymes [38]. In ctenophores, only highly derived NOS enzymes have been found, and it is unclear whether these animals have functional NO signalling [38]. In the demosponge *Spongilla lacustris*, NOS is expressed in pinacocytes – cells that line the canal system – and exogenous NO induces contractions in cells of the incurrent canals [39].

A functionally and structurally more diverse class of paracrine signalling molecules are secreted neuropeptide-like molecules. Such peptides are produced from prepropeptides that undergo proteolytic cleavage and modifications in the secretory pathway. The mature peptides are released

from dense core vesicles and can influence behaviour and development across eumetazoans [40–43].

Signalling peptides were already present in the eumetazoan common ancestor [37] and potentially before. They are abundant in ctenophores [16,44], placozoans [16,42,45–47], cnidarians [48,49] and bilaterians [50–52] (Figure 2A). In sponges, no neuropeptide-like molecule has yet been identified but some may exist. This is suggested by the presence of prohormone convertases in sponges, enzymes that cleave prepropeptides, and of peptidylglycine α-amidating monooxygenase (PAM), an enzyme required for peptide amidation (Figure 2B). PAM is an ancient enzyme also present across ciliated protists [53] and involved in the processing of amidated signalling peptides in the green alga *Chlamydomonas* [54]. In sponges, the substrates of PAM are unknown but sponge tissues contain dense core vesicles [55] [56] similar to peptidergic vesicles in eumetazoans nerves, suggesting the regulated release of peptides.

We know a lot less about peptide receptors. Although all non-bilaterian genomes contain many GPCRs – the most common peptide receptors – so far only one has been matched to a ligand, the receptor of the maturation inducing peptide (MIH) in the jellyfish *Clytia hemisphaerica* [40]. In *Hydra*, RFamide-like peptides signal by ligand-gated ion channels [57]. These DEG/ENAC family channels are present in placozoans and also (greatly expanded) in ctenophores [16]. How widespread their involvement is in ionotropic peptidergic signalling is unknown.
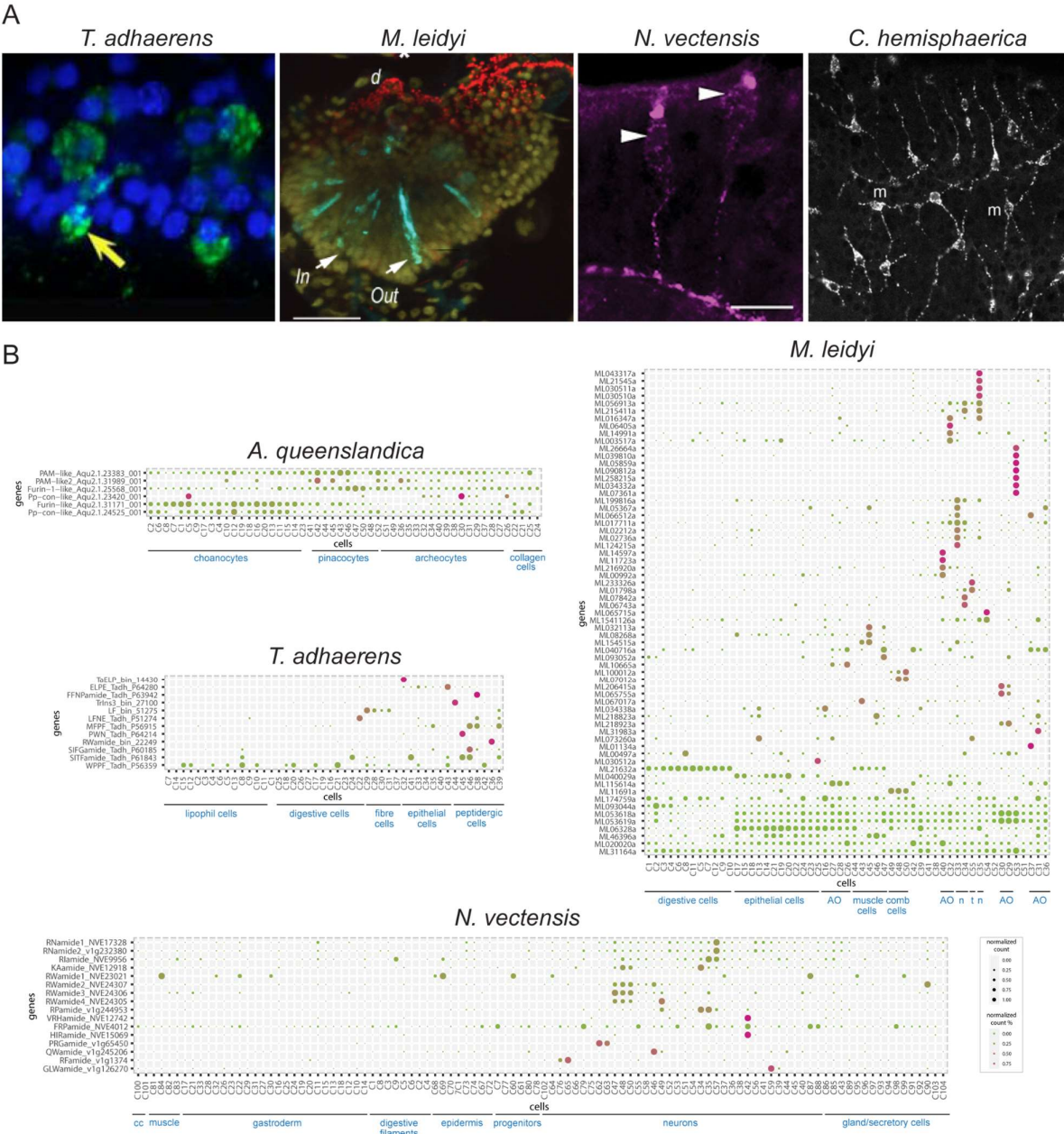
Figure 2: Neuropeptides in non-bilaterian animals. (A) Neuropeptide localization in the placozoan *T. adhaerens*, the ctenophore *M. leidyi* and the two cnidarians *N. vectensis* and *C. hemisphaerica* (modified from [43,44,58,59]) **(B)** Single-cell RNA sequencing data of prepropeptide processing enzyme genes across the annotated metacells in the sponge *A. queenslandica*, and prepropeptide genes in the placozoan *T. adhaerens*, the ctenophore *M. leidyi* and the cnidarian *N. vectensis*, based on [44,60,61]. Abbreviations: AO, aboral organ; n, neurons; pp-con, proprotein-convertase; t, tentacles, cc, cnidocytes

Tissue stainings and single-cell RNA sequencing approaches revealed the highly specific expression of most prepropeptides in all animals studied. To illustrate this, we plotted the expression of prepropeptide genes across cell clusters from the cnidarian *Nematostella vectensis*, the placozoan *Trichoplax adhaerens*, and the ctenophore *Mnemiopsis leidyi* based on the published datasets [60,61]

(Figure 2B). In all three species, the prepropeptides show highly cell-type-specific expression, with the exception of some uncharacterised peptides in the ctenophore. The specific expression of these genes has recently allowed the identification of some of the cell clusters as neuronal-peptidergic cells in the ctenophore *M. leidyi* [44]. In the sponge *Amphimedon queenslandica*, PAM and secretory prohormone-convertase homologs – potentially involved in prepropeptide cleavage – are broadly expressed, suggesting the potentially broad deployment of signalling by amidated peptides.

These data suggest that secreted peptides were already produced and secreted by the first neurons and due to their highly specific expression and signalling through specific receptors contributed to these neurons' identity and signalling specificity. These signalling functions of peptides may have evolved before synapses.

Synaptic proteins and synapse structure

Synapses are one of the defining features of neurons and are contact points between neurons or neurons and effector cells, like muscle cells and secretory cells. Synapses evolved early in animals. Two different types can be distinguished, chemical and electrical. Both types of synapses have been identified in ctenophores and cnidarians at an ultrastructural level [62] [63] [64]. Here we focus on the presynaptic release machinery of chemical synapses and dense core vesicles.

Most, if not all of the molecular components of synapses (synaptic modules) evolved prior to the appearance of the first neurons and many proteins prior to the origin of the first animals [65][5]. For example, key proteins involved in the tightly regulated process of neurosecretion (e.g., SNAREs, Unc18, Unc13 and Complexin), active zone proteins (e.g., Rimbp) and core neurosecretory vesicle proteins (e.g., Synaptophysin and Synaptogyrin) are present in unicellular organisms closely related to animals. In addition, postsynaptic scaffolding proteins (e.g., PSD-95, Homer, Shank) and ionotropic glutamate receptors existed before the first animals. These findings highlight the difficulty to pinpoint specific "neuronal" proteins as molecular markers for synapses, as the ancestral functions of most of these proteins were likely non-neuronal. Below we highlight recent work characterizing the function of "synaptic" genes or proteins in non-bilaterian animals without and with synapses and neurons.

In an elegant study using whole body scRNAseq in the sponge *Spongilla lacustris* the authors found that sponge "presynaptic" genes are specifically expressed in the sensory contractile pinacocyte epithelium, and in a new cell type the authors refer to as neuroid cells [39]. The neuroid cell contains many different dense-core vesicle secretion genes and wraps around choanocytes, the feeding cells of sponges. Complementing this, "postsynaptic" genes are predominantly enriched in choanocytes. These experiments indicate a close interaction of two cell types expressing pre-and postsynaptic genes and thus may identify these two cell types as proto-neurons. The placozoan *Trichoplax adhaerens* has no recognizable neurons but possesses neuropeptide-like molecules that are expressed in elongated, vesicle-rich epithelial cells. Immunostaining experiments have revealed that these cells co-express synaptobrevin, complexin, and synaptophysin, proteins enriched in vertebrate synapses [45,66]. When compared to other animals with neurons, ctenophores possess a synaptic protein repertoire comparable to sponges, placozoans and cnidarians, although a few key molecules like Synaptotagmin1 and Neuroligins have not been identified in the genomes of *M. leidyi* and *P. pileus* [15]. However, localisation studies and functional characterization of putative synaptic proteins in ctenophores have yet to be performed. The recent identification of the neuropeptide repertoire in a ctenophore allowed for the annotation of many previously unknown ctenophore cell types and led to the identification of the molecular identity of the nerve net [44]. This work showed that the ctenophore nerve net expresses Ferlin and Segretagogin (putative synaptic vesicle markers) and multiple ion channels important for the generation of action potentials. It is so far unclear how activity is spreading through the whole ctenophore nerve net as neurons within the nerve net display a unique structure

with neurites fused to each other (anastomoses) and no directionality (axons vs dendrites) can be distinguished [44].

Ultrastructural analysis of ctenophore neurons and many sensory cells revealed multiple different synapse arrangements (Figure 3A-C). Synapses with clear or dense-cored vesicles on either one side or both sides of the synaptic cleft have been identified (Figure 3A) [64], but the exact function of these uni- and bidirectional synapses remains to be investigated. All ctenophore synapses studied so far are characterized by a presynaptic triad [62] [63] [44] (Figure 3D-F) and this structure can be considered a morphological marker for neuronal cell types. Strikingly, synapses with an unusual localization can be found in a variety of different sensory cells and it appears that any part of a sensory cell can form a synapse onto another cell (Figure 3C).

In cnidarians, synapses are rather sparse and usually contain fewer and slightly larger vesicles [64]. As described above for ctenophores, dense-cored and clear vesicles on either one side or both sides of the synaptic cleft have been identified in interneurons (Figure 3A). Two-way synapses (bi-directional synapses) were the first to be described in cnidarians [66] and later studies confirmed that these synapses were physiologically nonpolarized and thus conducted equally well in either direction [67]. In addition, dense-core vesicles at two-way interneuronal synapses and at neuromuscular synapses have been labelled with antibodies against the neuropeptides RFamide and RWamide indicating the involvement of neuropeptides in synaptic signalling. Consistent with bidirectional signalling, at least one neuron type in *Nematostella* lacks segregated axo-dendritic compartments as suggested by the live imaging of vesicle transport [68].

Both cnidarian and ctenophore inter-neuronal and neuromuscular synapses lack recognizable postsynaptic specializations in electron micrographs (although in the cnidarian *Chrysaora* postsynaptic cisternae in the muscle have been described [64](Figure 3B). However, fluorescently tagged Homer and synaptobrevin (putative markers for post and presynapses) showed contact sites between neurites in *Nematostella [68]*. Consistent with the role of Homer in postsynaptic sites, another study found Homer puncta in transgenic *Nematostella* at tentacle-retractor and mesentery-retractor muscles and Homer co-expressed with Shank and iGluRs in muscle-cell transcriptomes [69]. In ctenophores, Homer, PSD95 and Shank are expressed broadly and do not reveal any specific postsynaptic cells. However, several iGluR types show highly cell-type-specific expression, potentially marking cells receiving glutamatergic or glycinergic presynaptic input (Figure 3H).
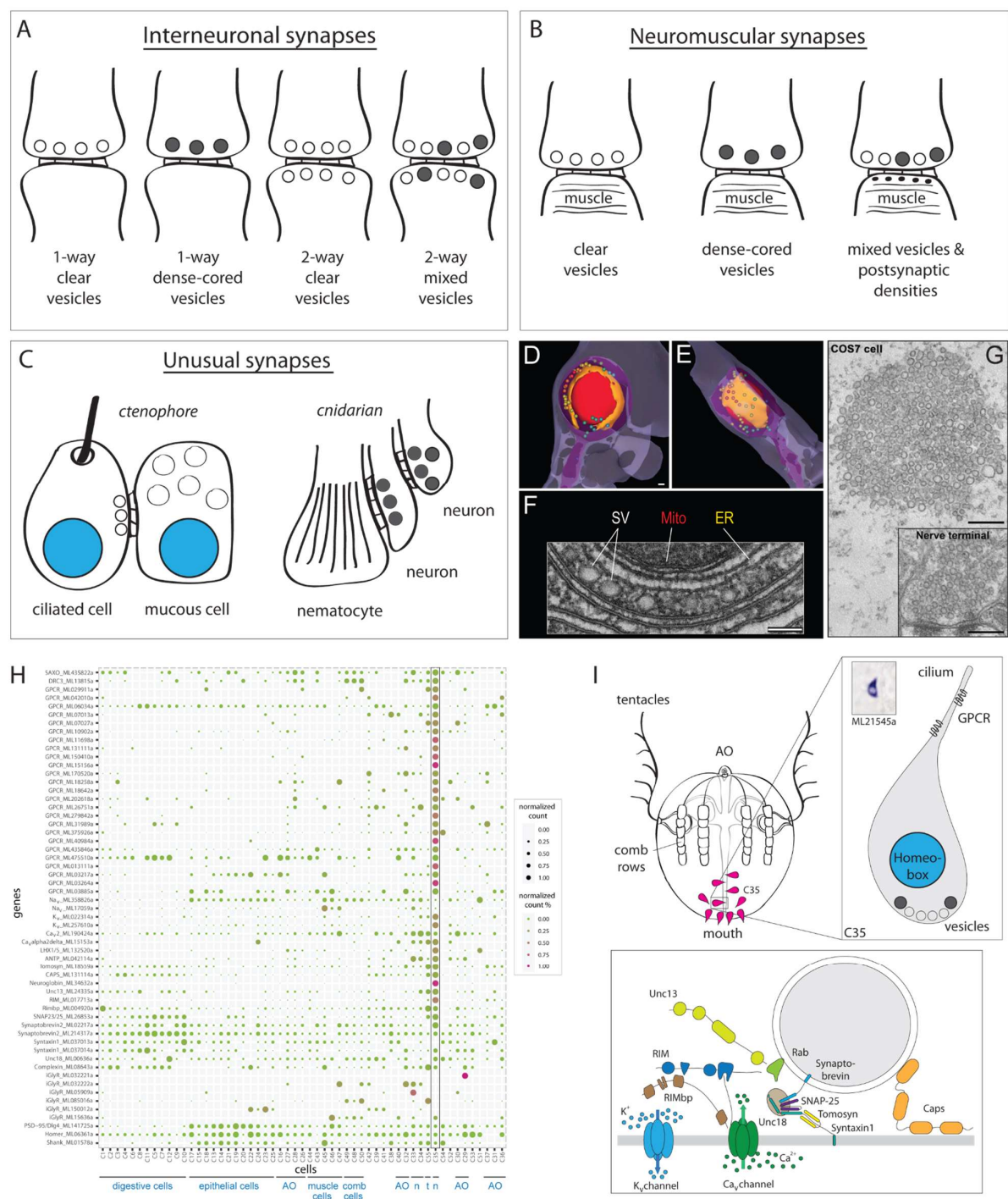
Figure 3: Diversity of synapses in cnidarians and ctenophores, and the ctenophore Unc13-RIM neuron, a potential homolog of bilaterian neurons. (A-B) Schematics of the different synapse arrangements that can be found in both cnidarians and ctenophores (modified from [64]). (C) Unusual synapses in ctenophores and cnidarians. (D-F) 3D reconstruction and electron micrograph of a ctenophore synapse with the characteristic pre-synaptic triad (modified from [44]). (G) The presynaptic proteins synaptophysin and synapsin, when expressed in non-neuronal COS7 cells, induce active zone-like structures (modified from [70]). (H) Single-cell RNA sequencing data of synaptic components, ciliary genes, GPCRs and transcription factors from the ctenophore *M. leidyi* reveal a ciliated-peptidergic spiking neuron (C35). (I) Drawing of a ctenophore (modified from [71])

with the position of the C35 cells highlighted, neuropeptide ML21545a expression pattern and a schematic of a C35 cell. The lower panel shows a putative presynaptic site in the C35 cell. Some components may be involved in the release of both clear and dense core vesicles. Note that *M. leidyi* RIM lacks a zinc finger domain due to loss (it is present in the sponge *Oscarella*) [72].

Voltage-gated ion channels and action potentials

The ability to generate action potentials evolved early in eukaryotes and is thus not restricted to neurons or animals. Action potentials have been detected in green algae [73], diatoms [74] and in a variety of other protists like Stentor, ciliates and heliozoans [75]. In diatoms, monomeric voltage-gated sodium channels [76] with rapid kinetics resembling animal tetrameric Ca_V/Na_V channels mediate gliding motility. In the green algae *Chlamydomonas*, action potentials are mediated by voltage dependent Ca_V channels that control the fast switch of flagellar beating [75,77]. Thus, Na_V/Ca_V channels evolved early in evolution (likely were present in the last eukaryotic common ancestor) and a picture is emerging that action potentials may have first evolved on flagellar membranes, which is in line with the absence of Na_V/Ca_V channels in eukaryotes without flagella [75]. Action potentials may then have spread from the cilium across the whole cell thus allowing rapid propagation of signals across the cell body [75].

In animals, excitable cells express K_V channels, which are responsible for terminating action potentials, alongside Na_V/Ca_V channels. The evolutionary history of K_V channels is potentially even older than Na_V/Ca_V channels, as they can be found across protists including choanoflagellates and even in some prokaryotes (archaea *Aeropyrum* and bacteria *Listeria*) [78] [79]. Putative K_V channels are present in all animals, except sponges. A staggering 44 K_V channels are present in *Nematostella*, a surprising finding given the rather simple anatomy of its nervous system [80].

The evolutionary origin of action potentials in animals is still unresolved. Previous analyses revealed that Na_V/Ca_V channels exist in choanoflagellates [81], but it remains unclear what their function is in these organisms. Most sponges lack Na_V channels, however Ca_V channels and hydrogen channels [26]) are present and action potentials, although slow, have been recorded from glass sponges [82]. Recent electrophysiological experiments in placozoans revealed the presence of fast Na_V dependent action potentials [83] and comparative analyses uncovered a surprising diversity of sodium voltage-gated channels in placozoans [83], hinting at the independent expansion of Na_V channels in placozoans. In ctenophores, various voltage-gated ion channels exist. Ca_V channels mediate increases in intraciliary Ca^{2+} concentration and thus control cilia beating of the comb plate [84]. Two different Na_V channels have been identified in ctenophores, but their function and potential contribution to action potentials has not yet been determined. In cnidarians, sodium-based action potentials have been reported in *Nematostella* [85], *Cyanea [86]* and *Polyorchis [87]*. Calcium action potentials are also very common in non-bilaterians [88] and have likely mediated excitability in the first neurons. Sodium action potentials evolved later independently in placozoans, cnidarians and bilaterians, with unique sodium selectivity filters in each of the three groups [81].

An enigmatic ciliated-peptidergic ctenophore spiking neuron and the homology of (some) synapses

Genomic comparisons alone have so far not been conclusive about the single or multiple origins of synapses and neurons. The advent of single-cell sequencing has dramatically changed the level of analysis and the insights that can be gained.

The first single-cell study of the ctenophore *M. leidyi* identified over 50 cell clusters, however, due to the lack of pan-neuronal markers [15], it has initially not been possible to identify neural cells in these data [61]. The recent mapping of prepropeptides to this dataset, combined with tissue stainings, has allowed the identification of several ctenophore neuronal clusters [44]. Building on this development, we have reanalysed the distribution of synaptic and other key molecules in the *M. leidyi* single cell data [61].

To our great surprise, a single cell cluster, C35, stood out. C35 expresses a unique and very interesting combination of genes which allow us to infer its cellular phenotype. It is a ciliated cell likely to have a motile cilium based on the presence of the ciliary markers SAXO [89] and DRC3 [90] (Figure 3H) and several axonemal dyneins (not shown). The cell is able to receive specific chemical signals as suggested by the expression of a large number of orphan GPCRs (many of them highly specific to C35). Based on the specific expression of Ca_V channel subunits and K_V channels, the cell is likely able to generate calcium-mediated action potentials. The cell seems to be specified by a unique homeobox transcription factor code comprising the LIM homeobox transcription factor Lhx1/5 [91] and an ANTP class homeobox transcription factor [92]. C35 also very specifically co-expresses the synaptic active zone factors Unc13 and RIM – previously postulated as key markers of synapse homology [93] – and other presynaptic proteins including Rimbp, SNAP23/25 and a synaptobrevin, suggesting the presence of bilaterian-like small molecule synapses. C35 also specifically expresses at least seven neuropeptides (Figure 3H) and the calcium-regulated dense-core-vesicle secretion factor CAPS, indicating that it releases peptidergic vesicles by a canonical pathway. A neuroglobin, related to the bilaterian pan-neuronal neuroglobins [94], is also very specifically expressed in C35. Based on the highly specific neuropeptide markers this cell has been localised to the mouth and pharynx region of *Mnemiopsis* [44].

Clearly, C35 is not the only neuron type in *Mnemiopsis*. Many other cells have neuronal morphology, express neuropeptides or have morphologically identified synapses. There are also several synaptic proteins that are expressed more broadly, including Unc18, synaptobrevin, complexin and syntaxin genes, suggesting the presence of molecularly distinct types of neurons and synapses in ctenophores [17]. This agrees with the high morphological diversity of synapses and neuronal cell types.

We interpret the similarities of the C35 cell to bilaterian neural cells as homology and suggest that the eumetazoan common ancestor already had spiking neurons with Unc13-RIM glutamatergic synapses and CAPS-controlled dense core vesicles. The postsynaptic cells likely expressed iGluR and neuropeptide receptors. The presynaptic cell may have also been ciliated and sensory. Based on the position of C35 in *Mnemiopsis* we speculate that the first neuron may have localised to the mouth region of the animal receiving food-derived signals and signalling to postsynaptic cells that coordinated mouth and pharynx movements during feeding.

If this homology assignment is correct, it does not necessarily mean that all neuronal cells are homologous. Ctenophores underwent extensive parallel evolution in their neural systems, and for example greatly expanded iGluRs, ASICs and innexins [16]. They may have also invented other types of synapses independent of other animals (Fig.3C). It is interesting to note in this context that in vertebrates the presynaptic proteins synaptophysin and synapsin, when expressed in non-neuronal cells, induce active zone-like structures in non-neuronal cells (Figure 3G) [70]. These findings indicate that it is rather easy to induce synapse-like structures and thus, synapses could have "easily" evolved several times separately in different animal lineages.

The Divide-and-Conquer model for early neuronal cell type evolution

The patterns of origin and distribution of neuronal components are consistent with what we call the Divide-and-Conquer model for neuronal evolution. This model suggests that in early animals

intercellular signalling was more democratic with many more cell types involved in intercellular signalling on many different channels. This included the release and reception of peptides, glutamate, glycine, NO and ATP in paracrine cell networks ('chemical brains') and not yet synaptic networks [19,37]. As recently formulated by Leonid Moroz and co-workers "ancestral neural systems evolved from genetically heterogeneous populations of polarized secretory cells without canonical synapses and capable of volume (non-synaptic) transmission" [19]. Synapses evolved in stem Eumetazoa in some cell types as clusters of secretory vesicles through the scaffolding of pre-existing vesicle-secretion proteins. Several synapse types may have evolved early on and also later lineage-specifically as suggested by synapse diversity in ctenophores and cnidarians. Nostalgic placozoans lost synapses and reverted to the chemical-brain state.

One spiking cell lineage, which we call the Unc13-RIM lineage, was also part of the ancient eumetazoan cell-type mix. The tethering of a Ca_V channel to the vesicle release machinery by RIM allowed this cell to couple action potentials and calcium influx to rapid vesicle release. Based on the *Mnemiopsis* data, we speculate that this cell may have occurred around the mouth and may potentially have specialised in chemosensing and the regulation of feeding. After the separation of ctenophores from Cnidaria+Bilateria the democratic unity of cell types had been divided when the Unc13-RIM faction started to become the dominant spiking neuronal lineage. Due to the rapid coupling of firing and vesicle release the Unc13-RIM neuron-type and its synapses gradually conquered the body and took over the control of effectors. This streamlining may have coincided with the advent of predation, where specialised neurons able to do rapid firing and to trigger rapid responses became a life-and-death question [95]. Going from chemical brains to spiking brains may have started in the region of the mouth, suggesting a savoury connection between macrophagy, predation and brain evolution.

Acknowledgements

Conflict of Interest

The authors declare that they have no conflicts of interest.

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Annotated subset of particularly important papers:

**Stone, Michelle C., Gregory O. Kothe, Melissa M. Rolls, and Timothy Jegla. 2020. "Cytoskeletal and Synaptic Polarity of LWamide-Like+ Ganglion Neurons in the Sea Anemone." The Journal of Experimental Biology 223 (Pt 21). https://doi.org/10.1242/jeb.233197.

This paper uses transgenesis and live imaging in the sea anemone *Nematostella vectensis*. The authors demonstrate that in one neuron type all neurites have axon-like microtubule polarity and they could observe the trafficking of both presynaptic and postsynaptic markers.

**Sebé-Pedrós, Arnau, Elad Chomsky, Kevin Pang, David Lara-Astiaso, Federico Gaiti, Zohar Mukamel, Ido Amit, Andreas Hejnol, Bernard M. Degnan, and Amos Tanay. 2018. "Early Metazoan Cell Type Diversity and the Evolution of Multicellular Gene Regulation." Nature Ecology & Evolution 2 (7): 1176–88.

This paper reports single-cell sequencing data from the sponge *Amphimedon queenslandica*, the ctenophore *M. leidyi* and the placozoan *T. adhaerens*. The authors report the cell-type diversity and transcription-factor regulatory programmes for these non-bilaterians.

*Romanova DY, Smirnov IV, Nikitin MA, Kohn AB, Borman AI, Malyshev AY, Balaban PM, Moroz LL: Sodium action potentials in placozoa: Insights into behavioral integration and evolution of nerveless animals. Biochem Biophys Res Commun 2020, 532:120–126.

Here the authors demonstrate the presence of sodium-dependent action potentials in placozoans. They also analyse the evolution of Na$_V$ channels and uncover an independent evolutionary radiation of voltage gated sodium channels in these animals.

*Park, Daehun, Yumei Wu, Sang-Eun Lee, Goeun Kim, Seonyoung Jeong, Dragomir Milovanovic, Pietro De Camilli, and Sunghoe Chang. 2021. "Cooperative Function of Synaptophysin and Synapsin in the Generation of Synaptic Vesicle-like Clusters in Non-Neuronal Cells." Nature Communications 12 (1): 263.

This paper reports that synapsin, when expressed together with the synaptic-vesicle membrane protein synaptophysin in fibroblasts, can organise vesicle clusters similar to synaptic-vesicle clusters. This minimal system also has implications for understanding the evolution of synapses.

**Luxmi, Raj, Dhivya Kumar, Richard E. Mains, Stephen M. King, and Betty A. Eipper. 2019. "Cilia-Based Peptidergic Signaling." PLoS Biology 17 (12): e3000566.

Here the authors report the presence of PAM and amidated peptides in ciliary ectosomes from the green alga *Chlamydomonas*. They show that one amidated peptide acts as a chemoattractant. This beautiful work uncovers a deep ancestry for metazoan neuropeptide signalling.

**Kapli, Paschalia, and Maximilian J. Telford. 2020. "Topology-Dependent Asymmetry in Systematic Errors Affects Phylogenetic Placement of Ctenophora and Xenacoelomorpha." Science Advances 6 (50). https://doi.org/10.1126/sciadv.abc5162.

In this paper the authors use simulations and empirical data to show that the Ctenophora-first but not the Porifera-first topology can be explained by systematic errors. Porifera-first can only be

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explained by real phylogenetic signal. This large asymmetry in systematic bias strongly supports Porifera-first.