NEUROEVOLUTION

Syncytial nerve net in a ctenophore adds insights on the evolution of nervous systems

Pawel Burkhardt[1]*, Jeffrey Colgren[1]†, Astrid Medhus[1]†, Leonid Digel[1], Benjamin Naumann[2], Joan J. Soto-Angel[1], Eva-Lena Nordmann[1], Maria Y. Sachkova[1], Maike Kittelmann[3]*

A fundamental breakthrough in neurobiology has been the formulation of the neuron doctrine by Santiago Ramón y Cajal, which stated that the nervous system is composed of discrete cells. Electron microscopy later confirmed the doctrine and allowed the identification of synaptic connections. In this work, we used volume electron microscopy and three-dimensional reconstructions to characterize the nerve net of a ctenophore, a marine invertebrate that belongs to one of the earliest-branching animal lineages. We found that neurons in the subepithelial nerve net have a continuous plasma membrane that forms a syncytium. Our findings suggest fundamental differences of nerve net architectures between ctenophores and cnidarians or bilaterians and offer an alternative perspective on neural network organization and neurotransmission.

For more than a century, the structure and evolutionary origin of the animal nervous system have been at the center of much debate among biologists. Fundamental progress in our structural understanding was put forward by Santiago Ramón y Cajal, who postulated that the nervous system is composed of discrete cells called neurons (*1*). This contrasts with Camillo Golgi's proposition that the nervous system is a syncytial continuum. The discovery of synaptic connections between individual neurons by electron microscopy later confirmed Cajal's theory. However, there is accumulating evidence that ctenophores, gelatinous marine invertebrates that move through the water column by ciliary comb rows, are among the earliest branching extant lineages of the animal kingdom (Fig. 1A) (*2*–*5*). Most ctenophore life cycles include a predatory cydippid stage during which, for some species, the ctenophore is able to reproduce only a few days after hatching (Fig. 1B) (*6*). Ancestral-state reconstruction suggests that the cydippid body plan is a plesiomorphic character of ctenophores (*7*).

The early split of ctenophores from other groups indicates that a nervous system, and maybe even neurons, could have evolved at least twice: once within the ctenophores and once within the lineage of the remaining animals (*8*). Initiated through genomic analyses (*2*, *3*), molecular and physiological features of the ctenophore nervous system were subsequently interpreted to support this scenario (*4*, *5*). In contrast to sponges and placozoans, ctenophores exhibit an elaborate nervous system consisting of a subepithelial nerve net (SNN), mesogleal neurons, a sensory aboral organ, tentacle nerves, and diverse sensory cells in all parts of their body (Fig. 1C and movie S1) (*9*–*14*). Deciphering the development, structure, and function of the ctenophore nervous system is a key element to understand the origin and evolution of animal nervous systems. We have recently shown that a large repertoire of lineage-specific neuropeptides has evolved in the ctenophore *Mnemiopsis leidyi* (*14*). Furthermore, we identified a distinctive feature of SNN neurons: the multiple neurites extending from one soma are interconnected through anastomoses and thus form an extensive continuous network within a single nerve-net neuron (*14*). This characteristic sets them apart from other animal neurons. Additionally, there was little evidence on how these nerve-net neurons connect to each other, to sensory neurons, and to cells within the mesoglea because of the lack of synaptic markers suitable for fluorescent labeling or large-scale electron microscopic data that spans multiple neurons. In this study, we used high-pressure freezing-fixation techniques in combination with serial block face scanning electron microscopy (SBFSEM) to establish the first ultrastructural three-dimensional (3D) network of SNN neurons and other cell types in a ctenophore.

The cydippid SNN is organized in a syncytium

Recent 3D reconstruction of a nerve-net neuron in a cydippid-phase *M. leidyi* has revealed a wide network of anastomosed neurites extending from only one soma (*14*). However, to understand the nature of connections between multiple nerve-net neurons as well as other cell types, we collected a larger continuous SBFSEM dataset of an early cydippid that includes 5 nerve-net neurons, 6 mesogleal neurons, and 22 putative sensory cells. The neurites of all five SNN cells were connected through an anastomosed continuous network (Fig. 2A). Whereas gap junctions could readily be identified within comb plates (fig. S1), we detected neither electrical nor chemical synapses between the cells of the SNN. We confirmed this observation in smaller datasets of the nerve net beneath two comb rows and along the gut in two other cydippid individuals (fig. S2). Additionally, injection of the fluorescent lipophilic dye 1,1'-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI) into only one of the cells of two-cell staged embryos led to a fluorescent signal in only one-half of the cydippid body, which was seen in SNN cell bodies throughout the animal consistent with the syncytial nature of the SNN (fig. S3).

Morphologically, neurites within the SNN exhibited no obvious polarity (axon versus dendrite), showing similar diameter, dense-core vesicles throughout their length, and the lack of typical presynaptic triads (Fig. 2, A to C). Moreover, SNN neurites often showed a blebbed or "pearls-on-a-string" morphology (Fig. 2, D to G, and fig. S4). The narrow segments were often just wide enough for microtubules to pass (Fig. 2G and fig. S4), and bulged segments often contained larger clear or electron-dense vesicles and occasionally endoplasmic reticulum (Fig. 2D and fig. S4). A recently developed antibody against the neuropeptide ML02736a (*14*) confirmed the presence of neuropeptides within some of the vesicles of SNN neurons (Fig. 2E and fig. S5). Although SNN neurons seemed to lack synapses between each other, we identified chemical synapses from the SNN to polster cells (fig. S6), which suggest directional signal transmission from the SNN to effector cells.

Mesogleal neurons form direct contacts with the syncytial SNN

We identified and reconstructed six mesogleal neurons exhibiting a starlike morphology with extensive plasma membrane protrusions of variable lengths (Fig. 3A). Their somata were filled with a variety of vesicles and larger vacuoles (Fig. 3B), and the protrusions of these cells did not show the pearls-on-a-string morphology present in neurites of the SNN. Some of the protrusions formed plasma membrane juxtapositions to neurites of the SNN (Fig. 3, A, D, and E). However, we did not find ultrastructural evidence for electrical or chemical synapses (Fig. 3E). In contrast to SNN neurons, we did not observe any electron-dense vesicles in mesogleal neurons (Fig. 3B), but instead small electron-lucent vesicles of a similar size as synaptic vesicles (Fig. 3C), which suggests a different type of information transmission.

Sensory cells form simple circuits involving the syncytial SNN

We identified and reconstructed a total of 22 putative sensory cells from the present

[1]Michael Sars Centre, University of Bergen, 5008 Bergen, Norway. [2]Institut für Biowissenschaften, Allgemeine und Spezielle Zoologie, Universität Rostock, 18055 Rostock, Germany. [3]Department of Biological and Medical Sciences, Oxford Brookes University, Oxford OX3 0BP, UK.
*Corresponding author. Email: pawel.burkhardt@uib.no (P.B.); maike.kittelmann@brookes.ac.uk (M.K.)
†These authors contributed equally to this work.

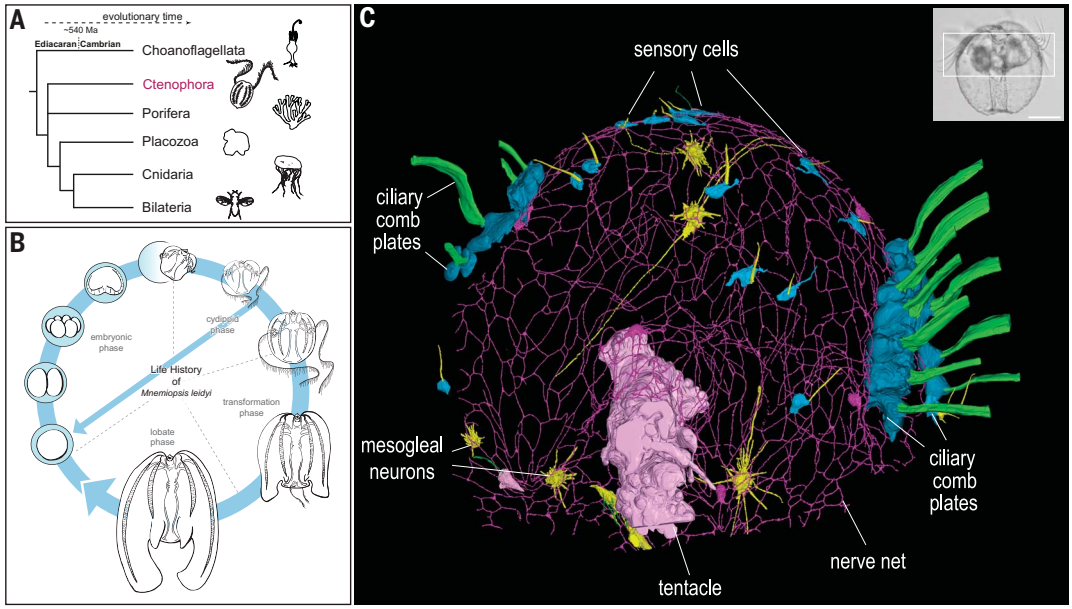

Fig. 1. Ctenophores and their nervous system. (**A**) Ctenophores as one of the earliest branching extant lineages of the animal kingdom. Silhouettes are from PhyloPic (www.phylopic.org). (**B**) The ctenophore *M. leidyi* exhibits complex life cycle stages, including a predatory cydippid phase that hatches from the egg and can reproduce after a few days. (**C**) 3D reconstruction of the nerve net, comb rows, sensory cells, mesogleal neurons, and a tentacle from SBFSEM data of a 1-day-old cydippid. (Inset) Phase contrast image of a 1-day-old cydippid. White box, area reconstructed in (C). Scale bar, 100 μm.

and an earlier dataset (*14*) that fit into five morphological groupings (Fig. 4, fig. S7, and table S1). Some of them resembled known ctenophore sensory cell types (types 1, 4, and 5) (*16*, *17*), whereas others exhibited a morphology that, to the best of our knowledge, has not been described previously (types 2 and 3) (Fig. 4, fig. S7, and table S1). We detected chemical synapses in several but not all putative sensory cells that contact neuronal or other effector cells (Fig. 4 and fig. S7). Type 1 sensory cells exhibited a single long cilium and onion-root basal body (Fig. 4 and fig. S7, A and B). Type 2 sensory cells exhibited a very short single cilium without an onion-root basal body. Long neurites extending from their somata formed chemical synapses to polster cells (Fig. 4B and fig. S7, A and C).

Type 3 sensory cells exhibited multiple cilia without onion-root basal bodies. Many large electron-dense vesicles are localized beneath the cilia (Fig. 4C and fig. S7, A and D). We found one of these cells near the tentacle with a synaptic connection to a mesogleal neuron (Fig. 4C). Type 4 sensory cells exhibited a single long filopodium. Some of them formed synapses to neurites of the SNN (Fig. 4, A and D), and some also received synaptic input from type 1 sensory cells (Fig. 4A). Type 5 sensory cells exhibited multiple long filopodia. They formed plasma membrane contact to polster cells, but we did not detect synaptic contacts from or to this cell type. Last, we used the 3D ultrastructural evidence to identify several discrete and simple neural circuits in early cydippid–phase *M. leidyi*. These circuits included synaptic signal transmission from sensory cells to other cell types including SNN neurons, mesogleal neurons, polster cells, or even other sensory cell types (Fig. 4, A to D).

Discussion

In the debate at the end of the 19th century about the organization of the animal nervous system, Joseph von Gerlach (1871) (*18*) and Camillo Golgi (1885) (*19*) put forward the reticular theory (also known as the syncytial theory). Both proposed the cellular continuity of neurons. This view was challenged by Cajal (1888) (*1*), who proposed an organization from discrete cellular units connected through synapses. Both contestant theories were founded on Golgi's newly invented black staining that enabled scientists to study the detailed morphology of neurons and their neurites (*20*). Golgi and Cajal were honored with the Nobel Prize in Physiology or Medicine in 1906 for their efforts in elucidating the architecture of the nervous system (*20*). However, with the advent of electron microscopy in the 1950s and the discovery of the synaptic cleft, the reticular theory was put to rest in favor of Cajal's hypothesis (*21*, *22*). In our present study, volume electron microscopy revealed the 3D ultrastructural architecture of the SNN in an early cydippid–phase ctenophore, providing evidence for its reticular—or syncytial—organization. Previous work suggested anastomosed nerve cords in adult ctenophores on the basis of chemical staining (*9*) and multiple parallel strands of neurites stained with anti–tyrosylated-α-tubulin (*10*). In this work, we showed that a syncytial nerve net already exists in cydippid-phase *M. leidyi*. This syncytium may be reinforced in adult animals through the anastomosis of additionally formed neurites; however, confirmation of such connectivity will require further detailed, high-resolution analysis of the nerve net throughout development.

Using high-pressure freezing and freeze substitution techniques to preserve fine ultrastructural details with minimal fixation artifacts, we showed that the SNN forms a continuous structure. This is further supported by the unrestricted spread of DiI throughout the nerve net.

Whereas gap junctions could be identified within the comb plates, as previously reported (*15*) in our SBFSEM data as well as in TEM micrographs, we found no evidence of similar structures between neurites of nerve-net neurons that would suggest the presence of electrical synapses. Additionally, a recent characterization of the complete set of *M. leidyi* innexins—responsible for the formation of gap junctions in invertebrates—did not show any mRNA expression in in situ hybridization experiments in nerve-net cell bodies (*23*). However, we did observe synaptic triads and plasma membrane contacts of unknown molecular structure that connect the SNN externally to polster and mesogleal neurons.

Previous characterizations of ctenophore nerve nets have been predominantly based on traditional histochemical staining techniques (*9*, *24*) and more recently on fluorescence microscopy of antibody staining against α-tubulin (*10*, *12*, *13*, *25*). Although both techniques provide valuable insight into the general organization and location of ctenophore neurons, they do not allow the investigation of the ultrastructure and nature of neuronal connections. Data from transmission electron microscopic serial sections (*26*, *27*) may also have overlooked this distinct

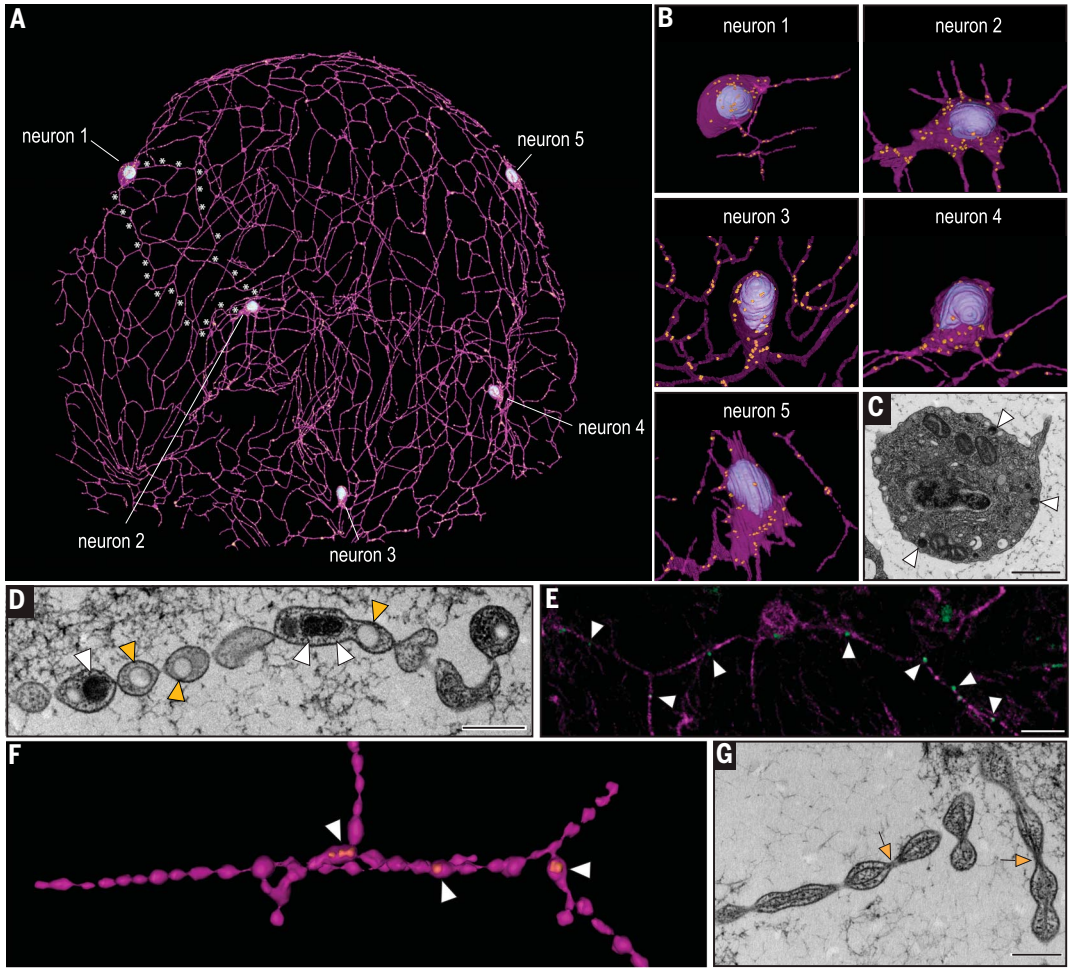

Fig. 2. Connectivity and ultrastructure of the ctenophore SNN. (**A**) 3D reconstruction of five SNN neurons. White asterisks indicate examples of the continuous membrane between the cell bodies of neurons 1 and 2. (**B**) 3D reconstruction of the SNN neuron cell bodies showing the nucleus (blue) and dense-core vesicles (orange). (**C**) TEM cross section of an SNN neuron cell body that shows ultrastructural details, including large, dense-core vesicles (white arrowhead). Scale bar, 1 μm. (**D**) TEM cross section of a SNN neurite with dense-core and clear-core vesicles localized in blebbed areas (white and orange arrowheads, respectively). Scale bar, 500 nm. (**E**) Antibody staining against neuropeptide ML02736a (green) in SNN neurites (magenta) stained for tubulin. (**F**) TEM 3D reconstruction of SNN neurite (violet) and dense-core vesicles (orange), highlighting the blebbed morphology. (**G**) TEM cross section of SNN neurites showing continuous microtubules (orange arrows) passing through narrow segments. Scale bar, 500 nm.

syncytial architecture because of the difficulty to produce continuous section series over such a large volume. Aside from reports on single self-anastomosing neurites in other animals (*28*–*30*), the presence of a complete syncytial nerve net has only been reported for the cnidarian, medusae-like colonial polyp, *Velella* (*31*, *32*). However, to the best of our knowledge, the syncytial organization of this nerve net has not yet been verified on an ultrastructural level. At this time, we have found this feature only in the ctenophore *M. leidyi* nerve net, but further analysis across nerve net–bearing animals may provide exciting insights into early nervous system evolution and modes of neuronal connectivity.

Although neurite fusion and pruning seem to be a common principle during the early neural development in many animals (*33*, *34*), we do not consider the syncytial cydippid SNN to be completely remodeled by such a process later in development. It was suggested that the early cydippid phase is not a larval phase but rather an autonomous life history phase of *M. leidyi* and other ctenophores (*6*). Indeed, cydippid-phase *M. leidyi* are free-swimming pelagic predators, able to reproduce and exhibit complex behaviors as described for their second, reproductive, lobate phase (*35*–*37*).

Our identification of the nonsynaptic architecture of the cydippid-phase SNN raises the question of the mechanism of signal propagation. Genome and single-cell transcriptome analyses revealed that *M. leidyi* SNN neurons express 1 voltage-gated calcium (Ca_v), 35 potassium (K_v), and 2 nonspecific sodium (Na_v) channels (*14*, *38*, *39*). These numbers are similar to those in neurons of other animals, and ctenophore SNN neurons may therefore be able to produce membrane potential or even action potentials (*40*). Moreover, the presence of numerous peptidergic vesicles in the SNN suggests that signal transmission also occurs through neuropeptide release, and the Ca_v channel expressed in these cells might be involved in exocytosis (*14*, *41*). Therefore, we can speculate that the SNN could function as a neuroendocrine system that is able to release transmitters into the mesoglea through vesicle fusion with the plasma membrane at different neurite sites. Such a system would require only a minimum number of chemical synapses and, if acting at short distances, may reach enough effector cells. Studies on the conduction velocity in ctenophores have shown a slower speed of signal propagation compared with that of nerve nets and conducting epithelia of other animals (*42*), indicating that signal propagation could be nonsynaptic.

Additionally, our ultrastructural identification of simple circuits now provides a basis that allows for better understanding of how mechanoreception, swimming, and prey-capture behavior in young cydippid-phase ctenophores could be facilitated. Numerous sensory neurons are connected through chemical synapses to the nerve net, which in turn forms chemical synapses onto effector cells such as the comb rows or ciliated groove cells (*14*). Type 1 ciliated sensory cells and type 4 filopodiated sensory cells, previously described as Tastborsten and Taststifte (*9*), have been postulated to be sensitive to water vibrations and touch (*17*, *43*, *44*). Their abundance throughout the epidermis and direct cell-to-cell contact to the nerve net (many through chemical synapses) underscore the importance of direct transmission of localized vibration and touch information to the SNN. Morphological analysis allows us to speculate that a type 2 sensory

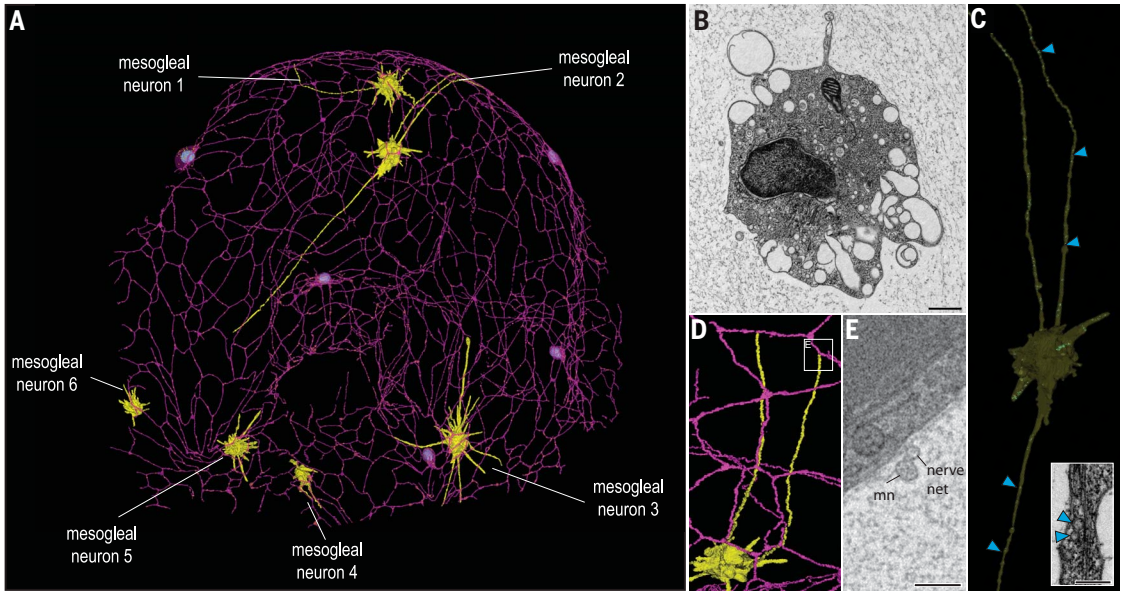

Fig. 3. Close association of mesogleal neurons and the SNN. (**A**) 3D reconstruction of SNN (violet) and mesogleal neurons (yellow) from SBFSEM data. (**B**) TEM cross section of a mesogleal neuron cell body. Different types of clear vesicles and vacuoles but no dense-core vesicles are present. Scale bar, 1 μm. (**C**) 3D reconstructed mesogleal neuron with three long neurites that contain small clear vesicles (blue arrowheads). (Inset) TEM cross section of mesogleal neurites with small clear vesicles. Scale bar, inset, 200 nm. (**D**) 3D reconstruction of mesogleal neuron with contact site (white box) to SNN. (**E**) Corresponding SBFSEM image of contact site between mesogleal neuron and SNN neuron. No chemical or electric synapse structures could be observed. Mn, mesogleal neuron. Scale bar, 500 nm.

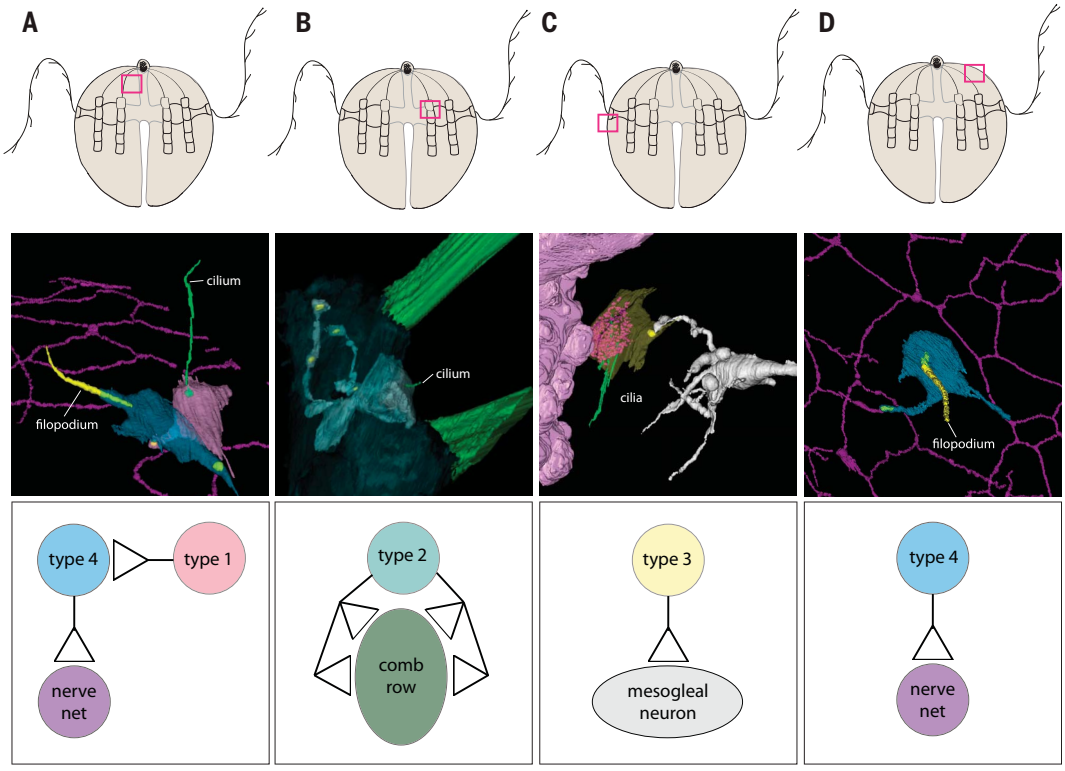

Fig. 4. 3D reconstruction of sensory cells allows for the identification of simple circuits. (Top) Localization of each circuit (pink square). (Middle) 3D reconstructions of sensory and effector cells. Mitochondria are shown in yellow to represent synaptic tripartite complexes in all circuits. (Bottom) Proposed wiring diagram. (**A**) Circuit between type 1 and type 4 sensory cells and SNN. (**B**) Multiple synaptic connections between a type 2 sensory cell with short cilium and comb cells. (**C**) Synaptic connection between a type 3 sensory cell near a tentacle and a mesogleal neuron. (**D**) A type 4 sensory cell with single filopodium synapses onto nerve net.

cell, which wraps around polster cells, may be able to detect water flow and thus alter comb-beat frequency, whereas a type 3 sensory cell, the multiple cilia of which are in close contact with the tentacle, may be triggered by food capture. Functional experiments are needed to fully understand the activity of these circuits and to unravel the different modes of signal transmission used by the different ctenophore neuronal cell types. This study is limited to the analysis of an early developmental stage in which fixation of whole animals with high-pressure freezing is still possible. Comparison with other ctenophore species and investigation of later life history stages of *M. leidyi* are needed to clarify whether a syncytial SNN is a feature restricted to an early ontogenetic phase in only a few species or is a common feature of all ctenophores. This approach will also provide valuable insights into the development of the syncytial SNN on whether neurons divide but remain connected in the cydippid SNN, or whether neurites from different cell bodies reach out and fuse.

Whether neurons of animals have a single origin or possibly originated more than once during evolution is a debated topic. The existing data on the ctenophore nervous system show a specific mosaic of cellular and syncytial components with distinct evolutionary histories. It will be a major future challenge to clearly identify the parts of the mosaic that may have evolved independently and the preexisting parts that were strongly modified. Our study underscores that the resemblance between the nerve net of ctenophores and the nerve nets of cnidarians and bilaterians might only be superficial because it appears that their connectivity is fundamentally different. Our ultrastructural analysis of the ctenophore SNN not only puts ctenophores at the center of nervous system evolution but also provides an opportunity to explore the boundaries of nervous system organization and function.

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ACKNOWLEDGMENTS

We thank C. Le Goff and A. Jan for the phase contrast image of a 1-day-old ctenophore and R. Gohde for help with the Western blot experiments. High-pressure freezing, SBFSEM, and TEM imaging was done in the Oxford Brookes Centre for Bioimaging. **Funding:** This work was supported by the Michael Sars Centre core budget and the European Research Council Consolidator Grant (101044989, "ORIGINEURO") awarded to P.B. **Author contributions:** Conceptualization: P.B. and M.K.; Methodology: P.B., J.C., M.Y.S., and M.K.; Investigation: P.B., J.C., A.M., J.J.S.-A., E.-L.N., M.Y.S., and M.K.; Funding acquisition: P.B.; Supervision: P.B.; Visualization: P.B., J.C., A.M., L.D., M.Y.S., and M.K.; Writing – original draft: P.B., B.N., and M.K.; Writing – review and editing: P.B., J.C., B.N., M.Y.S., and M.K. **Competing interests:** The authors declare no competing interests. **Data and materials availability:** All data are available in the manuscript or the supplementary materials.

SUPPLEMENTARY MATERIALS

science.org/doi/10.1126/science.ade5645
Materials and Methods
Figs. S1 to S7
Table S1
References (*45*–*50*)
Movie S1
MDAR Reproducibility Checklist

View/request a protocol for this paper from *Bio-protocol*.

Submitted 24 August 2022; accepted 24 February 2023
10.1126/science.ade5645

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Pawel Burkhardt, Jeffrey Colgren, Astrid Medhus, Leonid Digel, Benjamin Naumann, Joan J. Soto-Angel, Eva-Lena Nordmann, Maria Y. Sachkova, and Maike Kittelmann

Science, **380** (6642), .
DOI: 10.1126/science.ade5645

View of an ancient brain

The evolutionary origin of nervous systems remains a fundamental question in biology. A hallmark of nervous systems is that they are composed of discrete cells (neurons) that communicate through synapses. Ctenophores, a sister group to all animals with nervous systems, play a key role in comparative studies into the evolutionary origin(s) of neurons and their connections. To establish neuronal circuits that facilitate ctenophore behavior, Burkhardt *et al*. used high-resolution three-dimensional electron microscopy, revealing that nerve-net neurons are not separate entities, but rather are interconnected through continuous neurite plasma membranes without evidence of synapses (see the Perspective by Dunn). The findings offer a new perspective on the evolution of neuronal networks and neurotransmission. —MMa

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