© 2015. Published by The Company of Biologists Ltd | The Journal of Experimental Biology (2015) 218, 598-611 doi:10.1242/jeb.110692

REVIEW

Convergent evolution of neural systems in ctenophores

Leonid L. Moroz*

ABSTRACT

Neurons are defined as polarized secretory cells specializing in directional propagation of electrical signals leading to the release of extracellular messengers – features that enable them to transmit information, primarily chemical in nature, beyond their immediate neighbors without affecting all intervening cells en route. Multiple origins of neurons and synapses from different classes of ancestral secretory cells might have occurred more than once during ~600 million years of animal evolution with independent events of nervous system centralization from a common bilaterian/cnidarian ancestor without the bona fide central nervous system. Ctenophores, or comb jellies, represent an example of extensive parallel evolution in neural systems. First, recent genome analyses place ctenophores as a sister group to other animals. Second, ctenophores have a smaller complement of pan-animal genes controlling canonical neurogenic, synaptic, muscle and immune systems, and developmental pathways than most other metazoans. However, comb jellies are carnivorous marine animals with a complex neuromuscular organization and sophisticated patterns of behavior. To sustain these functions, they have evolved a number of unique molecular innovations supporting the hypothesis of massive homoplasies in the organization of integrative and locomotory systems. Third, many bilaterian/cnidarian neuron-specific genes and 'classical' neurotransmitter pathways are either absent or, if present, not expressed in ctenophore neurons (e.g. the bilaterian/cnidarian neurotransmitter, γ-amino butyric acid or GABA, is localized in muscles and presumed bilaterian neuron-specific RNA-binding protein *Elav* is found in non-neuronal cells). Finally, metabolomic and pharmacological data failed to detect either the presence or any physiological action of serotonin, dopamine, noradrenaline, adrenaline, octopamine, acetylcholine or histamine – consistent with the hypothesis that ctenophore neural systems evolved independently from those in other animals. Glutamate and a diverse range of secretory peptides are first candidates for ctenophore neurotransmitters. Nevertheless, it is expected that other classes of signal and neurogenic molecules would be discovered in ctenophores as the next step to decipher one of the most distinct types of neural organization in the animal kingdom.

KEY WORDS: Ctenophora, Neurons, Phylogeny, *Pleurobrachia*, *Mnemiopsis*, Genome, Neurotransmitters, Evolution

Introduction

Our understanding of the origins and early evolution of nervous systems is vague and highly controversial (Bullock and Horridge, 1965; Horridge, 1968; Jékely, 2011; Lentz, 1968; Mackie, 1970; Mackie, 1990; Miller, 2009; Moroz, 2009; Moroz, 2012; Moroz, 2014; Parker, 1919; Pennisi, 2013; Sakharov, 1974). The major obstacles in the field are the lack of molecular and physiological information about signaling systems from representatives of the basal animals. The phylum Ctenophora, or comb jellies, is of particular interest for two reasons. First, it is one of the earliest lineages of pre-bilaterian animals possessing 'true' nervous systems and mesoderm-derived muscles. Second, ctenophores are among the most challenging species when it comes to preparations for experimental analysis in neuroscience because of (1) their fragile nature that creates difficulties in working with them outside of their native habitats, (2) lack of reliable neuronal markers, and (3) lack of systematic behavioral or neuroplasticity studies. As a result, there is little information about nervous organization in Ctenophora, and not a single transmitter has been reliably identified in representatives of this important phylum. In this review, I will briefly summarize the history of neurobiological studies on ctenophores. Then, I will focus on the molecular basis of neuromuscular organization, starting with our recent analysis of ctenophore phylogeny and attempt to identify intercellular signal molecules in ctenophore neural circuits which could shed light on the origin of neurons and neurotransmitters in general (Moroz et al., 2014). As the hypotheses of single-origin of neurons have been widely discussed in the past (e.g. Lentz, 1968; Mackie, 1970; Mackie, 1990; Miller, 2009; Moroz, 2009; Parker, 1919; Sakharov, 1974) and recently (Marlow and Arendt, 2014; Moroz, 2014; Moroz et al., 2014), I will emphasize the alternative polygenesis scenario in this communication.

The comparative data suggest that at least some neuronal cell types and complex integrative structures (such as the aboral organ) evolved independently in the ctenophore lineage (Moroz, 2012; Moroz et al., 2014). The process could employ a subset of evolutionarily conserved gene modules that existed in the common ancestor of all animals to control directional propagation of electrical signals and polarized secretion, as well as novel neurogenic and signal molecules. Thus, ctenophores might have developed different transmitters (in addition to L-glutamate, the widespread eukaryotic intercellular messenger) and even neuronal 'master' genes or related transcriptional factors, as well as novel classes of non-coding regulatory RNAs.

Ctenophores as basal metazoans, sister to other animals

The reconstruction of the origins of neural systems requires a careful selection of reference species (Striedter et al., 2014). We can set the stage in this direction starting from the enigmatic Ctenophora. All 150+ described ctenophore species are carnivorous animals – ranging in habitat from tropical to polar seas (Hernandez-Nicaise, 1991; Hyman, 1940; Kozloff, 1990; Mayer, 1912). A long and controversial history of ctenophore biology started with the pioneering work on diversity and earliest developmental specification discovered by Chun (Chun, 1880). When Chun separated blastomers in two-cell embryos, he found that each half-embryo developed exactly half of adult structures in ctenophores suggesting the presence of highly deterministic mechanisms even after the first division during the cleavage. This pioneered work was challenged but later reproduced by Driesch and Morgan (Driesch

The Whitney Laboratory of Marine Biosciences and Department of Neuroscience and McKnight Brain Institute, University of Florida, FL 32080, USA. The Whitney Laboratory, University of Florida, 9505 Ocean Shore Boulevard, St. Augustine, FL 32080, USA.

*Author for correspondence (moroz@whitney.ufl.edu)

The Journal of Experimental Biology

and Morgan, 1895). The study of neural organization by Hertwig (Hertwig, 1880) was a logical expansion of the similar studies on cnidarians by Hertwig's brothers (Hertwig and Hertwig, 1878; Hertwig and Hertwig, 1879; Hertwig and Hertwig, 1880). This fundamental work led to the most well-known hypothesis of nervous system evolution (Parker, 1919).

For more than a century, ctenophores and cnidarians were superficially united as jelly-like diploblastic organisms with the simplest tissue organization derived from two embryonic layers (ectoderm and endoderm). But the phylogenetic relationships among five basal metazoan clades (Porifera, Placozoa, Ctenophora, Cnidaria and Bilateria) are still highly controversial. Virtually any key position of ctenophores at the animal tree of life was claimed: from a sister group to bilaterians to the most-basal metazoans (Dunn et al., 2008; Hejnol et al., 2009b; Nielsen, 2012; Nosenko et al., 2013; Philippe et al., 2009; Pick et al., 2010; Schierwater et al., 2009; Telford, 2009). One of the challenges from previous reconstructions was the lack of representative genomic data. Two most recent and independent genome-wide studies (Moroz et al., 2014; Moroz et al., 2012; Ryan et al., 2013b) came to the same conclusion – they suggested that Ctenophora is the earliest branching animal lineage, sister to all metazoans (Fig. 1). The verdict may not yet be considered final because of the limited amount of comparative data available and the complexity of statistical analysis in phylogenomic studies. For example, Ryan et al. (Ryan et al., 2013b) produced the summary tree using maximum-likelihood analysis of gene content. Here, Ctenophora, represented by *Mnemiopsis*, came as the most basal animal lineage. However, with the same 100% bootstrap support, molluscs (*Lottia*) and annelids (*Capitella*) were incorporated into chordates [see fig. 4 in Ryan (Ryan et al., 2013b)]. Additional comparative genomic data, especially from different lineages of sponges and ctenophores, would be indispensable to better resolve deep metazoan phylogeny.

In the past, ctenophores and cnidarians were considered as two sister lineages forming the clade Coelenterata. Although some phylogenomic studies still favor this classical classification (Nosenko et al., 2013; Philippe et al., 2009), independent analyses, including our recent large-scale phylogenomic studies on 10 ctenophore species (Moroz et al., 2014), reject the Coelenterata (Cnidaria and Ctenophrora) hypothesis (i.e. no support for sister relationships between Cnidaria and Ctenophora). Indeed, these two lineages are very dissimilar in many fundamental characteristics, including numerous differences at the ultrastructure and genomic levels; and their superficial jelly-like and sometimes transparent appearance is a result of convergent evolution as pelagic organisms. 'Whereas an ecologist might classify the ctenophores with Cnidaria, an electron microscopist would see the major differences in all tissues' (Horridge, 1974). However, extensive genomic analysis of cnidarians strongly supports their sister relationships with bilaterians (Chapman et al., 2010; Moroz et al., 2014; Putnam et al., 2007) including shared features in their neuronal and (neuro)transmitter systems.

Fossil records in the later Precambrian and lower Cambrian (Chen et al., 2007; Dzik, 2002; Shu et al., 2006; Tang et al., 2011) further support early ctenophore ancestry. For example, the ctenophore-type *Eoandromeda* is dated at 580-551 Mya (Tang et al., 2011), before the appearance of distinct sponge-type fossils around 548 Mya (Penny et al., 2014). The Cambrian explosion reflects a very rapid radiation of the majority of animal lineages (Erwin and Valentine, 2013) resulting in multiple events of parallel evolution of animal complexity and tissue organization. Thus, Ctenophores might possess derivatives of one of the earliest, independent designs for nervous systems and, possibly, muscular systems. Ctenophores evolved arrays of multiciliated cells supporting their highly efficient mode of locomotion.

Most notably, ctenophores have fully differentiated muscles and mesoderm. The majority of cnidarians do not possess 'true' muscle cells – they have so-called epitheliomuscular cells with mixed features of epithelial and contractive cells (Brusca and Brusca, 2003). Only a few cnidarian species have distinct striated muscles, suggesting their independent origins (Steinmetz et al., 2012). Muscles in ctenophores are primarily involved in prey catching rather than in locomotion. A few species secondarily, and independently from other animals, evolved muscular jet-like propulsion (*Ocyropsis crystalline*) and sinusoidal undulations of the whole body (*Cestum veneris*) during swimming escape responses. The muscle cells are supposedly derived from a type of mesenchyme cell in the mesoglea; they are segregated early in embryonic development and therefore can be considered as true mesodermal derivatives [separate from epidermis and gastrodermis (Burton, 2008; Derelle and Manuel, 2007)]. Some of them are giant and well characterized electrophysiologically (Anderson, 1984; Bilbaut et al., 1988; Dubas et al., 1988; Hernandez-Nicaise et al., 1980; Stein and Anderson, 1984). These muscles are used to control hydroskeleton tone, body shape and feeding, which might be original functions of muscle elements in animal ancestors.

The ciliated locomotion mode itself can be viewed as a primordial mode of movements mediated by specialized ctenes or the combs, organized in eight rows of comb plates. Each comb plate consists of hundreds of thousands of very long cilia which beat together as a unit (Tamm, 1982). Not surprisingly, the name of the phylum is derived from the Greek cteno-phora, or 'comb-bearers'. The cilia in multiciliated cells of the comb plates can reach 2 mm – the largest cilia in the animal kingdom.

In summary, the ctenophores are unique in virtually all aspects of their organization and development (Hernandez-Nicaise, 1991; Nielsen, 2012). The latest phylogenomics analyses confirm the hypothesis of the placement of ctenophores as the sister lineage to all other Metazoa (Borowiec et al., 2015; Whelan et al., 2015).

Neural systems in ctenophores

Using osmicated and partially dissociated whole mounts Hertwig published the first description of neural elements in ctenophores (Hertwig, 1880). These studies were extended using Methylene Blue vital staining and silver impregnation (Bethe, 1895; Heider, 1927a; Heider, 1927b; Korn, 1959) but with only moderate success. In fact, ctenophore neurons are very elusive cells to stain with convenient histological dyes. 'Apart from the fact that the net stains readily with Methylene Blue, the evidence that it is a conducting system is almost nonexistent' (Horridge, 1974). This statement still holds true today and we know nothing about any functional neural circuit in ctenophores.

In the 1960–1970s, with advances of the electron microscopy, Horridge and Hernandez-Nicaise teams performed extensive ultrastructural studies of ctenophore neurons and synapses (Hernandez-Nicaise, 1991; Hernandez-Nicaise, 1973a; Hernandez-Nicaise, 1973b; Hernandez-Nicaise, 1973c; Horridge, 1965b; Horridge, 1965c; Horridge et al., 1962; Horridge and Mackay, 1964). Fig. 2 summarizes these findings. Unfortunately, the neuronal architecture across ctenophores is not well described at the organ and microscopic levels. 'It is only an assumption that the axons, synapses and sensory cell bodies seen by electron microscopy are the same branched neurons that spread as a net over the whole surface. Only by analogy with higher animals are the synaptic vesicles thought to have this function and to be presynaptic' (Horridge, 1974).

The Journal of Experimental Biology (2015) doi:10.1242/jeb.110692

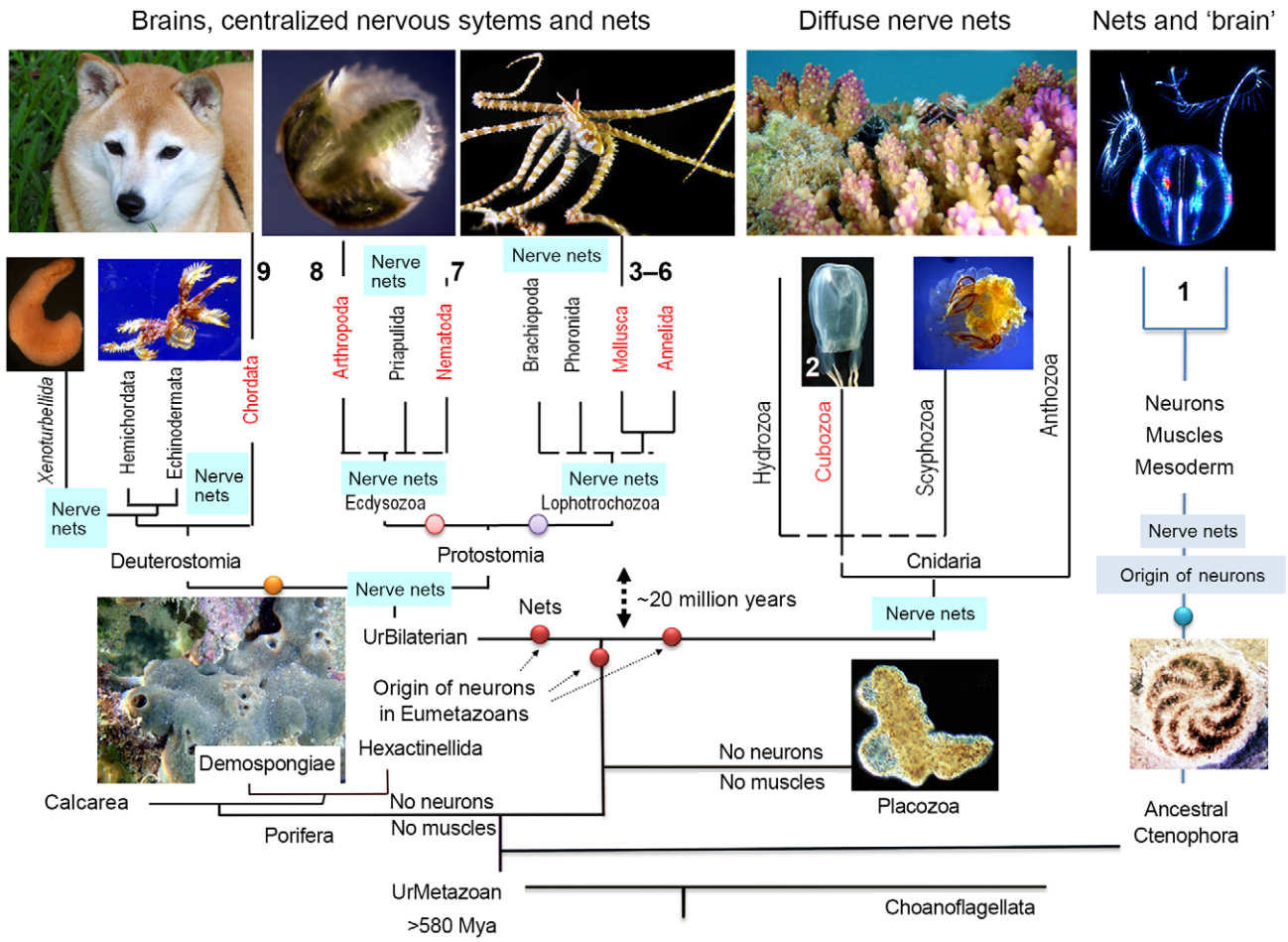

Fig. 1. Origins of neurons and parallel evolution of neural centralization. A simplified view of evolutionary relationships in the animal kingdom; the tree is combined with the presence or absence of a central nervous system (CNS) or brain in selected animal clades [modified and updated from Moroz (Moroz, 2009; Moroz, 2012; Moroz et al., 2014)]. Choanoflagellates are placed at the base of the tree as a sister group for Metazoa (King et al., 2008) followed by Ctenophora (represented by the photo of *Pleurobrachia bachei*) as the sister group to all other animals. The image representing the ancestral ctenophore is the fossil known as *Eoandromeda* (Tang et al., 2011). Porifera and Placozoa do not have recognized neurons. Cnidaria and Ctenophora have well defined neurons and muscles. Although neuronal organization in basal Metazoa can be superficially presented as a nerve net, many species have a prominent concentration of neuronal elements, and numerous and apparently autonomous networks governing surprisingly complex and well coordinated behaviors. Cubozoa have well-developed eyes and a ganglionic organization associated with rhopalia, which can be described in terms of a centralized nervous system in this and other cnidarians (Satterlie, 2011). Similarly, there is a well-defined concentration of neural elements associated with locomotory combs, the aboral organ in Ctenophora. Chordates, nematodes, molluscs and arthropods have well-defined CNSs, whereas in other bilaterians (e.g. phoronids, brachipods, Xenoturbellida, Nemertodermatida) the gross anatomical organization of their nervous systems can be similar or even simpler than those in selected cnidarians and ctenophores. Centralization of nervous systems occurred in parallel within several lineages representing all three major clades in bilaterians (Deuterostomes, Ecdysozoa and Lophotrochozoa). Red numbers indicate that at least nine independent events of neuronal centralization have occurred during evolution. Even in Mollusca, this centralization of the nervous system might occur 4–5 times in parallel (Kocot et al., 2011; Moroz, 2012). Only representative groups of the 34–36 recognized animal phyla are shown in the diagram (Nielsen, 2012). Colored circles indicate possible events of multiple origins of neurons: blue, the origin of neurons in Ctenophores; red, the origin of neurons in Bilateria/Cnidaria clade; it is also possible that some neural populations could originate in bilaterian lineages (yellow, orange and pink circles). This reconstruction of phylogenetic relationships among phyla is a combined view based upon recent large-scale molecular/phylogenomic analyses of several hundred proteins from representatives of more than 15 animal phyla (Hejnol et al., 2009a; Kocot et al., 2011; Moroz et al., 2014; Philippe et al., 2011; Ryan et al., 2013b). The origin of animals can be traced back to about 600 Mya (Erwin et al., 2011; Erwin and Valentine, 2013). However, the extant animal phyla might have more recent evolutionary history. It appears that the origin of major bilaterian groups occurred within a relatively short geological time (probably within 20 million years or even less). As a result the accurate evolutionary relationships among basal lineages and major bilaterian phyla might not be well resolved. Possible timing of the divergence in the diagram is indicated as Mya.

The ctenophore neural systems consist of at least four cell populations: (1) subepithelial nerve nets (neurons and neurites); (2) intramesogleal neural nets; (3) subgastrodermal elements and neural elements in tentacles; and (4) neural cells in the aboral organ. The activity of cilia is under control of the aboral organ composed of several cell types with gravity sensors and a statolith consisting of about 100 lithocytes (Tamm, 1973; Tamm, 1982). In addition, there is a diversity of mechano- and chemoreceptors (Aronova and Alekseeva, 2003; Kass-Simon and Hufnagel, 1992) as well as putative photosensors; but no morphologically defined eyes or photoreceptors were described (Anctil and Shimomura, 1984; Girsch and Hastings, 1978; Horridge, 1964b; Schnitzler et al., 2012; Vinnikov, 1990). The structural relationships and functional interactions among different neural elements are mostly unknown.

Superficially, the nervous system architecture in ctenophores looks like a simpler nerve net (Fig. 2E), but it is probably a subset of complex distributed networks controlling both stereotyped and learned behaviors (Hernandez-Nicaise, 1991; Horridge, 1974; Tamm, 1982).

The Journal of Experimental Biology (2015) doi:10.1242/jeb.110692

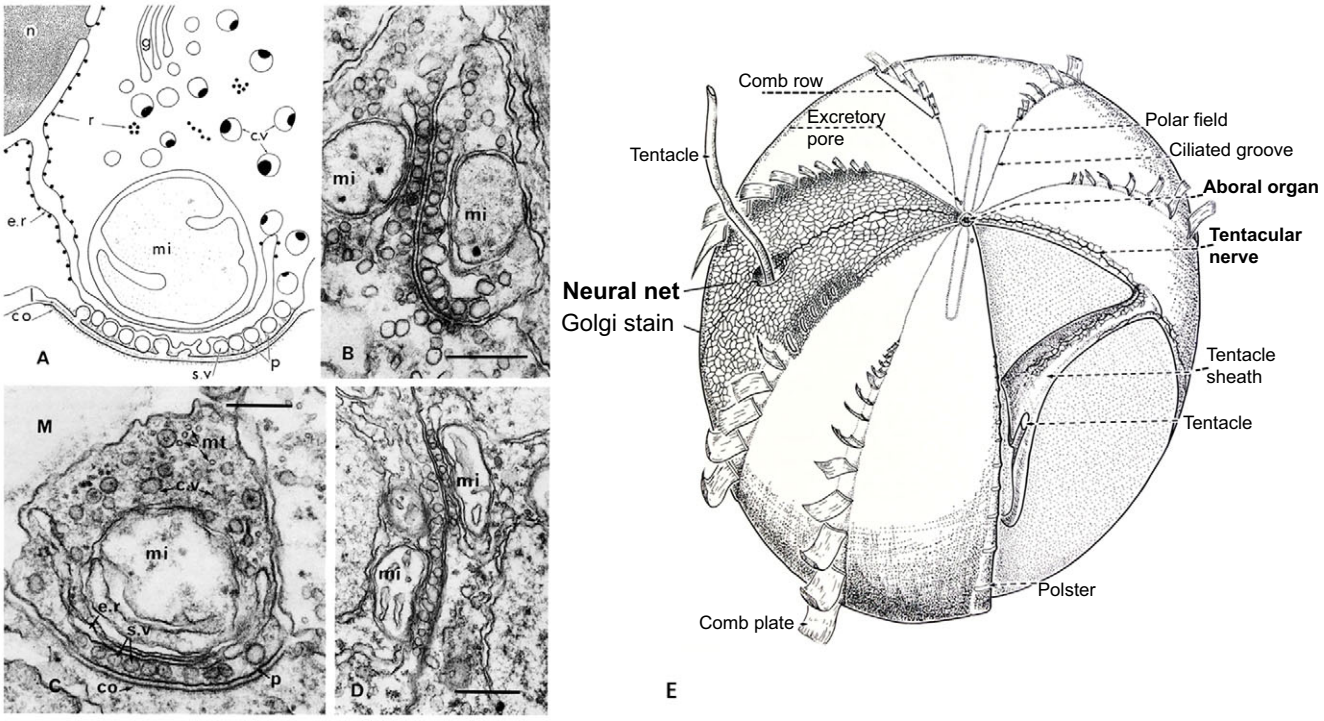

Fig. 2. Neural nets and synapses in ctenophores. (A–D) The basic features of synapses in ctenophores. (A) The generalized asymmetrical synapse. (B) Symmetrical neurite-to-neurite synapse in *Beroe*. Scale bar: 100 nm. (C) Asymmetrical synapse between a neurite and an epithelial cell (ep) in epidermis of *Pleurobrachia*. Scale bar: 200 nm. (D) Soma-to-soma reciprocal synapse in the epithelium of *Bolina hydatina*. Scale bar: 100 nm. c.v., cytoplasmic vesicles; co, dense coat on the postsynaptic membrane; e.r., endoplasmic reticulum; g, Golgi complex; l, intracleft dense line; M, mesoglea; mi, mitochondrion; mt, microtubules; n, nucleus; p, presynaptic dense projections; r, ribosomes; s.v., synaptic vesicle. (E) The schematic diagram of the subepithelial nerve system of a generalized cydippid (the aboral view). Images are reproduced and adapted from Hernandez-Nicaise (Hernandez-Nicaise, 1991) with permission from Wiley-Liss, Inc.

Some species have developed systems of relatively large neurons and axons (up to ~6–12 µm in diameter) to support fast escape (Mackie et al., 1992) and feeding (Tamm and Tamm, 1995) behaviors. The aboral organ (it is sometimes less correctly named as the apical organ because there is an analogous structure in bilaterian larvae) is the primary sensory ‘brain’-type structure located at the aboral pole of the animal. The anatomy of the aboral complex is well described (Aronova, 1974; Aronova, 1975; Aronova et al., 1979; Hernandez-Nicaise, 1991; Tamm, 1982; Tamm and Tamm, 2002). This complex controls locomotion, acting as gravity (statocyst) and possible light sensors (Aronova, 1979; Hernandez-Nicaise, 1991; Tamm, 1982). Axon-like processes of epithelial cells in the aboral organ resemble neurons; a fact that might be used to support earlier hypotheses about the possible evolution of nerves from epithelial conduction tissue (Tamm and Tamm, 2002). The impulses originating from primarily mechanosensory cells that bear the balancers reach the comb rows through the ciliary tracts. It appears that the transmission of this information depends upon mechanical forces and Ca^{2+} (Tamm, 1982), although all structures involved are densely innervated (Hernandez-Nicaise, 1991; Hernandez-Nicaise, 1968; Hernandez-Nicaise, 1973a; Hernandez-Nicaise, 1973b; Hernandez-Nicaise, 1973c; Hernandez-Nicaise, 1974; Tamm, 1982).

The beating of combs is highly coordinated. Different comb rows can beat synchronously or asynchronously, controlling various behaviors during prey capture or escape responses. Ciliated cells are under neuronal (primarily inhibitory) controls, with multiple different types of synapses described by electron microscopy (Hernandez-Nicaise, 1968; Hernandez-Nicaise, 1973a; Hernandez-Nicaise, 1973b; Hernandez-Nicaise, 1973c; Hernandez-Nicaise, 1974; Hernandez-Nicaise and Amsellem, 1980; Hernandez-Nicaise et al., 1982; Horridge, 1965c; Horridge et al., 1962; Horridge and Mackay, 1964). The frequency of beating can be accelerated, decreased, arrested or even completely reversed, presumably by neuronal-mediated stimuli; high Mg^{2+}, which suppresses synaptic transmission, eliminates these regulatory inputs (Horridge, 1974; Tamm, 1982). However, nothing is known about signal molecules or any neural circuit controlling complex ciliated locomotion, or any other behavior in ctenophores.

Enigmatic ctenophore neurons

Earlier work on the neurobiology of ctenophores performed by Horridge (Bullock and Horridge, 1965; Horridge, 1965c; Horridge, 1974), Tamm (Moss and Tamm, 1986; Moss and Tamm, 1987; Tamm, 1982; Tamm, 1984; Tamm and Moss, 1985; Tamm and Tamm, 1981; Tamm and Tamm, 1987) and others (Haddock, 2007; Satterlie and Case, 1978) revealed a very complex behavioral repertoire in these marine predators. Nevertheless, we know little about ctenophore neurons because classical nerve stains are particularly unreliable in these animals. Although nerve cells in ctenophores were initially described more than 130 years ago (Hertwig, 1880), some follow up studies were unable to demonstrate even the existence of a nervous system in ctenophores (Samassa, 1892). For nearly a century, the only way to map neurons was vital staining with Methylene Blue and silver impregnation with Golgi stains (Bethe, 1895; Heider, 1927a; Heider, 1927b; Hernandez-Nicaise, 1973a). The majority of described neuronal cell bodies and their processes form a polygonal lattice on the body surface known as a subepidermal nerve net (Fig. 2E). Apparently, the same net has

The Journal of Experimental Biology (2015) doi:10.1242/jeb.110692

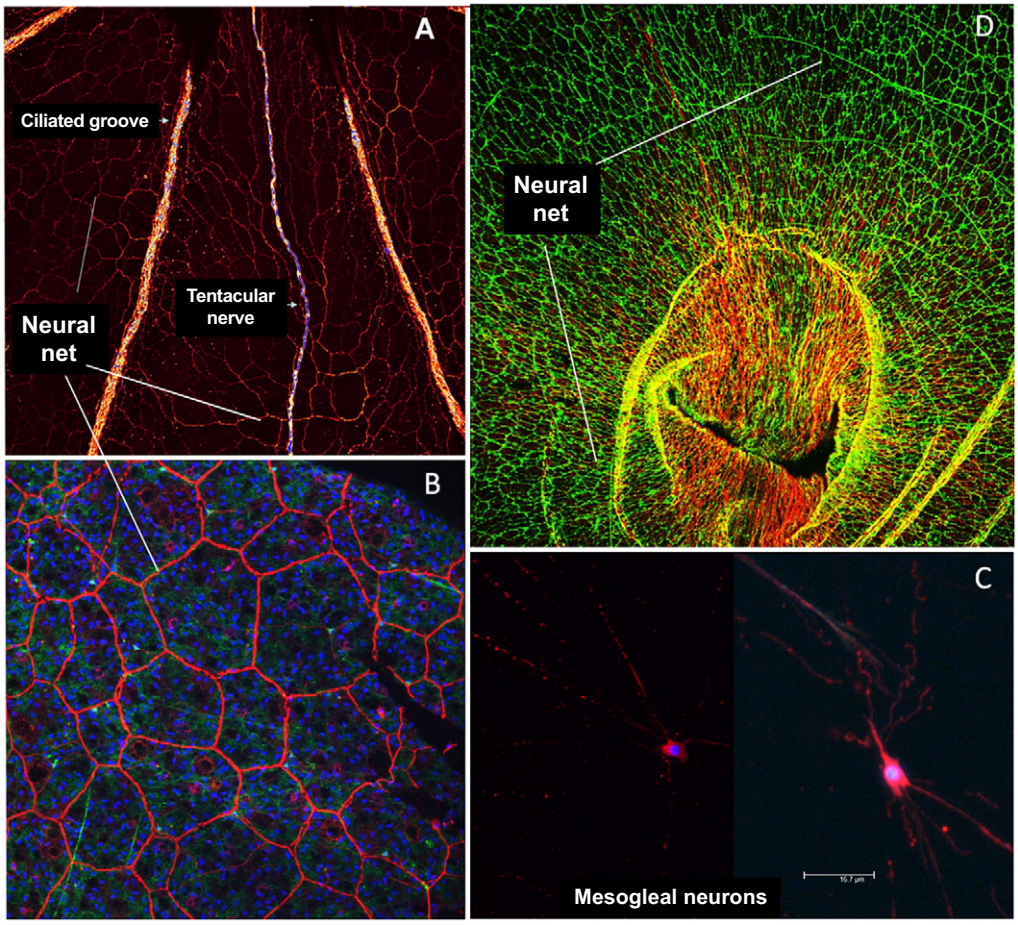

Fig. 3. Neural systems in the ctenophore *Pleurobrachia bachei*. (A) The subepithelial nerve net as revealed by acetylated β-tubulin immunostaining (L.L.M. and T. P. Norekian, unpublished results). Aboral side is located in the upper part of the photo. (B) The subepithelial net as revealed by tyrosinated α-tubulin immunolabeling (red); blue, nuclear (DAPI) staining; green, phalloidin (actin marker). Note neuronal somata within individual meshes. (C) Neural-type cells in mesoglea; red, tyrosinated α-tubulin immunolabeling; blue, nuclear (DAPI) staining. (D) The distributed neural networks around the mouth of *Pleurobrachia*. Modified from Moroz (Moroz et al., 2014); green, tyrosinated α-tubulin immunolabeling; red, phalloidin.

been recently labeled using antibodies against α- and β-tubulins in *Pleurobrachia* (Jager et al., 2011; Jager et al., 2013; Moroz et al., 2014) but with greater detail (Fig. 3A,B,D). Most likely, the revealed polygonal lattice results from a growth pattern where each polygon encircles a group of epithelial cells, even in very young larvae (Hernandez-Nicaise, 1991). In addition, there are intramesogleal nerve fibers and neurons (Hernandez-Nicaise, 1991; Hernandez-Nicaise, 1973a; Hernandez-Nicaise, 1973b; Hernandez-Nicaise, 1973c; Hernandez-Nicaise, 1974), also visualized using antibodies against tubulins (Fig. 3C) (Jager et al., 2011; Moroz et al., 2014). The developmental origin of these mesoglean neurons is unknown; they could be derivatives of the same precursor cells as mesoglean muscles. Experiments with fluorescent tracers could clarify the genealogy of different classes of mesoglean cells in development. Hypothetical glia-like cells were also described in *Beroe* (Aronova and Alekseeva, 2004), but the very concept of 'glia' in these neural systems should be reevaluated to avoid implementation of the terminology developed for vertebrates.

Electron microscopy of ctenophore neurons and synapses

Ctenophore neurons are better investigated at the ultrastructural level. They are quite diverse, with multiple recognized synapses between neurons and their potential effectors (Hernandez-Nicaise, 1991; Hernandez-Nicaise, 1973a; Hernandez-Nicaise, 1973b; Hernandez-Nicaise, 1973c; Hernandez-Nicaise, 1974) such as ciliated and gland cells, muscles, colloblasts, sensory structures and photocytes (a type of light-emitting cell located in the meridional canals and responsible for bioluminescence, with a few exceptions such as *Pleurobrachia*). A unique feature of ctenophore neurons is the absence of morphologically recognized polarity in these cells: any part of the neuronal membrane can form a synapse into other cells, including symmetrical (two-way) synapses (Horridge et al., 1962) where opposing presynaptic triads face each other (Fig. 2B) – such types of synapses do not occur in vertebrates (Hernandez-Nicaise, 1991). Interestingly, morphologically different symmetrical synapses were also discovered in cnidarians (Anderson, 1985; Anderson and Grünert, 1988) and they were considered as the morphological substrate of the diffuse conduction within apparently homogenous nerve nets (Anderson, 1985).

Ctenophores also possess soma–soma reciprocal (opposing triads staggered, Fig. 2D) and classical asymmetrical (highly polarized) synapses (Fig. 2A,B). Importantly, Hernandez-Nicaise (Hernandez-Nicaise, 1991) indicated that reciprocal and symmetrical synapses are less abundant than polarized synapses, except at the aboral organ and the core of tentacles. Thus, the directional chemical transmission might be the dominant way of communication within neural circuits of ctenophores.

Tripartite synapses in ctenophores

The ctenophore asymmetrical synapse is structurally quite organized, forming a so-called 'presynaptic triad' (Hernandez-Nicaise, 1991; Hernandez-Nicaise, 1973c; Hernandez-Nicaise, 1974). Each presynaptic element contains a tripartite complex of organelles: a single layer of synaptic vesicles lining the presynaptic membrane, a cistern of agranular endoplasmic reticulum just above the row of vesicles, followed by one or several mitochondria (Fig. 2A,B). The postsynaptic density and active zones, however, are less prominent in ctenophore synapses.

This tripartite synapse arrangement was used as a reliable electron microscopy marker for neurons (Hernandez-Nicaise, 1991). Synaptic vesicles are apparently very diverse at the ultrastructural level, suggesting the presence of multiple low molecular weight and neuropeptide-type transmitters of unknown identity.

FMRFamide-like and vasopressin-like immunereactivities were reported in some *Pleurobrachia* neurons (Grimmelikhuijzen, 1983; Jager et al., 2011), but none of these neuropeptide classes were found in the recent genomic studies (Moroz et al., 2014). Cholinergic and monoaminergic control of bioluminescent flashes were proposed for *Mnemiopsis* (Anctil, 1985) but neither effector neurons nor specific transmitter synthetic enzymes were identified. No genes encoding enzymes for synthesis of acetylcholine (i.e. choline acetyltransferase) and catecholamines were found in the sequenced ctenophore genomes. Furthermore, acetylcholine, classical catecholamines (dopamine, noradrenaline, adrenaline), serotonin and histamine were not detected in ctenophores using direct microchemical assays (Moroz et al., 2014).

For more than 50 years, two 'relaxed' morphological criteria to recognize ctenophore neurons and synapses were employed: the presence of 'neuro' tubules and 'synaptic' vesicles 30–50 nm in diameter at the presumed synaptic cleft. 'These structures occur quite consistently in all ctenophores examined, and the obvious conclusion from their structure and location is that they are synapses of the nervous system. Once this part of the circular argument is accepted the synapses becomes the best means of identifying nerve fibers. … The presynaptic component is usually interpretable as a nerve fiber, but realistic synapses have also been found with a muscle cell as the presynaptic element' (Horridge, 1974).

It is very difficult to classify conductive and secretory elements in ctenophores. Conductive elements can be either derivatives of muscles or neurons. Polarized secretory cells might not be genetically or developmentally related to neurons. Nevertheless, excitatory and inhibitory inputs on different effectors (cilia, tentacles, muscles, colloblasts, etc.) are eliminated by elevated levels of Mg^{2+}, suggesting the presence of directional neural circuits with functional chemical synapses (Horridge, 1974; Tamm, 1982).

It should be stressed that ctenophores have developed unique systems of conductive elements (Horridge, 1974; Tamm, 1982; Tamm, 1984). These 'neuroid' elements (possible muscle-derived with electrical synapses between cells) operate in parallel to synapse-based neural systems.

In summary, we know very little about ctenophore neural organization; and virtually nothing about the cellular or transmitter bases of their behaviors. This contrasts with very extensive studies on cnidarians, sponges and placozoans. The lack of neuronal molecular markers and genomic resources for ctenophores are major bottlenecks in the field.

Rise of ctenophore genomics – *Pleurobrachia bachei* as an emerging model

In collaboration with T. R. Gregory (Guelph University, Canada), we screened various ctenophore species using flow cytometry and densitometry, searching for small genome sizes following previously published protocols (DeSalle et al., 2005). The sea gooseberry *Pleurobrachia bachei* (Fig. 1) was found to have the smallest genome (~160 Mb) in the group surveyed and one of the smallest genomes within the animal kingdom (comparable to genomes of *C. elegans* and *Trichoplax*). A similarly small genome size was reported for *Mnemiopsis leidyi*. The pacific lobate *Bolinopsis infundibulum* has the second smallest genome (~220 Mb) whereas *Beroe abyssicola* has one of the largest genomes within the group: ~1 Gb. The largest ctenophore genome on record is a 3.1 Gb genome from the cydippid *Haeckelia rubra* (Gregory et al., 2007).

Unusually compact mitochondrial genomes (~10–11 kb) were sequenced from *Pleurobrachia* and *Mnemiopsis* (Kohn et al., 2012; Pett et al., 2011). Interestingly, the mitochondria in comb-plate cells are giant; they reach 10 µm in diameter (Horridge, 1964a). 'They are so crowded that they almost fill the comb plate, and presumably this is the fuel injection system of the giant cilia' (Horridge, 1974).

Pleurobrachia is a very abundant ctenophore species in the North Pacific Ocean; whereas the closely related *P. pileus* is found in the Atlantic Ocean. These species are perfectly amenable to various experimental manipulations, including reliable fixation (most other ctenophores simply disintegrate in the majority of common fixatives), development, neurobiological and behavioral tests (Tamm, 1982; Tamm, 1984; Tamm and Moss, 1985), as well as molecular manipulations (Alié et al., 2010; Jager et al., 2008; Moroz et al., 2014). Many ctenophores possess a characteristic cydippid larva that is similar to adult *Pleurobrachia* (Fig. 1), supporting the idea that basal characteristics have been retained in this lineage. Therefore, in 2007–2008, we selected *Pleurobrachia* as the major model for genomic sequencing. The results of *Pleurobrachia* whole-genome sequencing were formally reported at the SICB meeting in Charleston (SC) in January 2012 (Moroz et al., 2012), suggesting convergent evolution of ctenophore neural systems. The overall goals of the project were focused on questions about the origin of neural systems, functional and microchemical validation of the genome predictions (Moroz, 2013; Moroz et al., 2014; Pennisi, 2013). In parallel, a sequencing project was initiated and performed at the National Institutes of Health by Baxevanis and his team using *Mnemiopsis leidyi* as a model focusing on developmental and phylogenetic questions (Ryan et al., 2013a; Ryan et al., 2013b).

In addition, we performed deep transcriptome sequencing using other ctenophore species (*Euplokamis dunlapae*, *Coeloplana astericola*, *Vallicula multiformis*, *Pleurobrachia pileus*, *Dryodora glandiformis*, *Beroe abyssicola*, *Bolinopsis infundibulum*, undescribed mertensid, *Mnemiopsis leydi*), allowing comparative validation of initial predictions from the ctenophore genomes and to resolve internal ctenophore phylogeny (Moroz et al., 2014). The phylogeny revealed surprising relationships within ctenophores, with multiple examples of mosaic evolution, such as loss of cydippid larval stages consistent with earlier reconstructions using 18S rRNAs (Podar et al., 2001). The non-cydippid body plan of *Mnemiopsis* and *Bolinopsis* can be derived from a cydippid body plan of *Pleurobrachia*.

Quest for neurogenic genes and signal molecules in ctenophores

Extensive comparative genomic resources help address several fundamental questions: (1) are recognized genes associated with transmitter specification in other animals expressed in ctenophore neurons? (2) Are conventional pan-neuronal molecular markers found in other animals present in ctenophores and can they be used to label neurons? (3) How chemically heterogeneous are neuronal populations in ctenophores? (4) What are the neurotransmitters in ctenophores? (5) What are the systemic and behavioral functions of candidate neurotransmitters in ctenophores? (6) Can non-neuronal cells (e.g. ectodermal, glandular or muscle cells) synthesize and secrete transmitter-like substances? If so, what is the functional role of this non-neuronal transmitter secretion?

Even initial analysis of existing datasets from the *Pleurobrachia* genome and nine related species (Moroz, 2012; Moroz et al., 2014)

The Journal of Experimental Biology (2015) doi:10.1242/jeb.110692

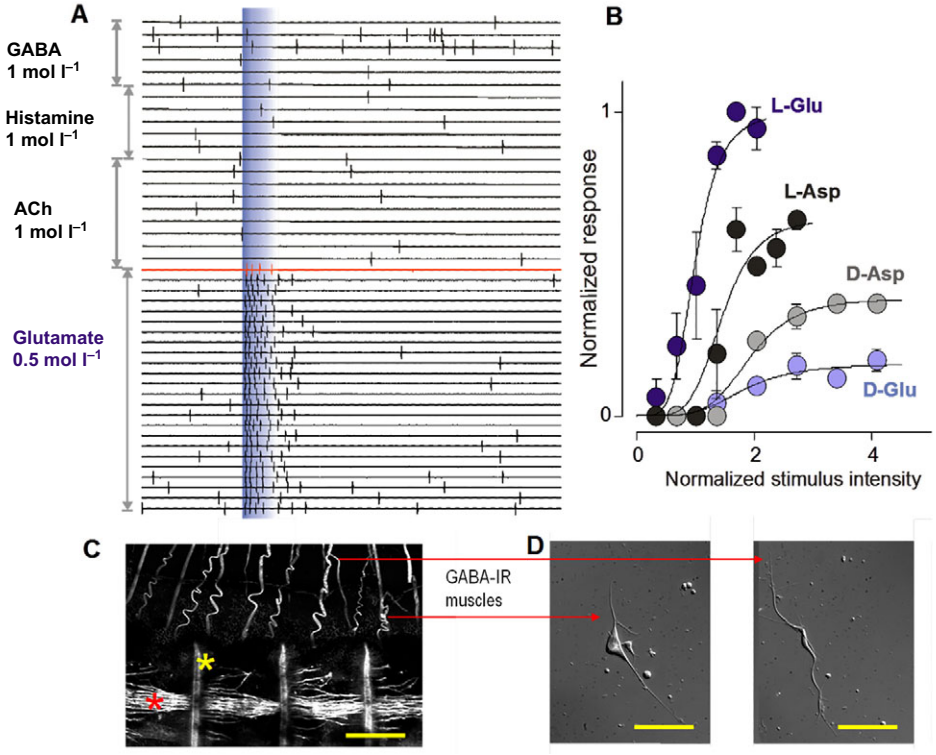

Fig. 4. Glutamate and aspartate as neuromuscular transmitter candidates in the ctenophore *Pleurobrachia bachei*. (A) L-glutamate (0.5–1 mmol l^{-1}) induced action potentials in mechanically isolated muscle cells whereas other transmitter candidates were ineffective even at concentrations up to 5 mmol l^{-1}. Typical responses of ctenophore muscle cells to local pulses of a transmitter application were externally recorded both as individual action potentials and video contractions from a single muscle cell (D). Image modified from Moroz (Moroz et al., 2014); see all details in this paper. (B) The graph shows normalized responses from the same muscle cell indicating L-glutamate is the most potential excitatory molecule compared with D-glutamate or L/D-aspartate. (C) GABA immunolabeling of muscle cells in *Pleurobrachia*. From Moroz (Moroz et al., 2014) and L.L.M. and T. P. Norekian, unpublished results. Red arrows indicate contractile muscle cells around comb plates; these cells were isolated for electrophysiological tests in A and B. Yellow asterisk marks the base of a single comb plate (polster, see Fig. 2E); red asterisk marks non-contractile muscle fibers possibly involved in cilia beat coordination across the entire comb row. Scale bars: 70 µm (C) and 20 µm (D). Duration of the recording in A is 50 s.

provided a number of surprises which can be summarized as the following:

(1) Classical Eumetazoans (i.e animals with nervous systems, Cnidaria, Ctenophora and Bilateria) are the polyphyletic clade (Moroz, 2012; Moroz et al., 2014). This hypothesis implies either massive loss of complex animal traits in sponges and placozoans (such as mesoderm, muscles and neurons) or massive homoplasies (molecular innovations) in ctenophores. In other words, the apparent similarity is a result of convergent and/or parallel evolution. If correct, the molecular make-up of ctenophore neurons, muscles and development can be different from those in cnidarians and bilaterians. Emerging genomic data supports this conclusion.

(2) Ctenophores have reduced complements of canonical neurogenic, synaptic, muscle, immune and developmental genes, as well as the apparent absence of *HOX* genes and microRNA machinery, suggesting their distinct molecular organization and the homoplasy for many characters in this lineage.

(3) Many bilaterian/cnidarian 'neuron-specific' genes and genes of 'classical' neurotransmitter pathways are either absent or, if present, are not expressed in neurons. For example, we found that GABA immunoreactivity is localized in muscles (Fig. 4), but it was not detected in neuronal populations revealed in Fig. 3. Using *in situ* hybridization, we also found that *Elav* genes are expressed in non-neuronal cells of ctenophores (Moroz et al., 2014) but not in neurons (unpublished observations for two other *Elav* genes).

(4) The majority of canonical low molecular weight transmitters are absent in ctenophores – consistent with the hypothesis that ctenophore neural systems evolved independently from those in other animals.

(5) Glutamate and a diversity of secretory peptides are candidates for ctenophore transmitters. In fact, L-glutamate is the only known transmitter candidate shared between ctenophores and other animals. It is not surprising because L-glutamate is an essential polar amino acid, and it can be recruited for signaling functions in all domains of life.

Combined, these data support the alternative 'polygenesis' scenario to the most widely accepted 'single-origin' hypotheses of nervous systems in animals (Fig. 1). Simply put, ancestral proneurons can be viewed as one of many populations of polarized secretory cells (Richards et al., 2008). Not surprisingly, the recruitment of different secretory cell lineages for more localized neural-like signaling might have occurred multiple times in evolution. The corollary for such hypotheses would be multiple origins of synapses as well. Below, I will expand and discuss some of these data and hypotheses.

Parallel evolution of neural organization in ctenophores

Ctenophores are not 'simpler' or 'primitive' animals or extant examples of 'early' or 'first' nervous systems. More than 550 million years of their evolutional history resulted in multiple unique innovations supported by at least 10,000 ctenophore-specific genes controlling highly deterministic development and other systemic functions (Moroz et al., 2014). For example, ctenophores have the most sophisticated system of ciliated locomotion in the animal kingdom. Their bioenergetics demands in comb-plate cells are supported by gigantic mitochondria, yet the smallest mitochondrial genomes (Kohn et al., 2012). Glue-based prey capture with unique tentacle/colloblast apparatus has no analogs across the animal kingdom. Ctenophores have both giant smooth and compact striated mesoderm-derived muscles with distinct molecular make-up. In addition to the aboral organ, which has a relatively small number of neural cells, there are two large and distinctive neural populations: the ectodermal hexagonal-type neural net and the more diffused mesoglea network of neural-type cells (Fig. 3). All described interneuronal and neuro-effector chemical synapses in all ctenophores have unique organization (Fig. 2), forming a 'presynaptic triad' (Hernandez-Nicaise, 1991) with highly heterogeneous populations of vesicles, suggesting the presence of multiple low molecular weight and peptide-type transmitters.

As most neurotransmitters and their synthesis pathways are highly conserved across bilaterians and cnidarians, we anticipated that ctenophores would share their neurotransmitter organization with other Eumetazoa, together with the presence of majority of cnidarian-bilaterian neurogenic and synapse-related genes. However, and in contrast to observations of all other animals with nervous systems, several genes controlling neuronal fate and patterning, such as neurogenins, NeuroD, Achaete–Scute, REST and HOX/otx, are absent in the ctenophores we sampled (Moroz et al., 2014). Orthologs of pre-and postsynaptic genes also have a reduced representation (Moroz et al., 2014) 'missing' components that are critical for synaptic function in other eumetazoans (i.e. organisms with nervous systems).

Our initial immunohistochemical data using serotonin-, dopamine- and histamine-specific antibodies failed to label specific cells in *Pleurobrachia*, *Beroe* and *Bolinopsis*. Likewise, NADPH-diaphorase reactivity [a robust marker for nitric oxide synthase across cnidarians and other bilaterians (Moroz, 2006; Moroz et al., 2004; Moroz et al., 2000)] did not reveal specific fixative-resistant activity in *Pleurobrachia*.

Next, we used direct ultrasensitive microchemical assays [capillary electrophoresis with different detection schemes from femtomolar (10^{-15}) to attomolar (10^{-18}) limits of detection (see Fuller et al., 1998; Moroz et al., 2005)] to look for canonical low molecular weight (neuro)transmitter candidates in four species – *Pleurobrachia bachei*, *Mnemiopsis leidy*, *Bolinopsis infundibulum* and *Beroe abyssicola*. Even if a candidate compound was not detected, we still performed extensive pharmacological screening for potential behavioral effects using semi-intact preparations with exposed ciliated combs (to be sure that the pharmacological agent gained access to the nervous system) and isolated muscle. In total, more than 20 compounds were tested in the broad range of concentrations up to 1 mmol l^{-1}. As a result, our combined microanalytical and pharmacological data suggest that ctenophores do not use acetylcholine, serotonin, dopamine, noradrenaline, adrenaline, octopamine, histamine or glycine as intercellular messengers (Moroz et al., 2014). Consistent with this conclusion, in the *Pleurobrachia* and *Mnemiopsis* genomes, as well as another 10 ctenophore transcriptomes (*Euplokamis*, *Coeloplana*, *Vallicula*, two *Pleurobrachia* species, *Dryodora*, *Beroe*, *Bolinopsis*, undescribed Mertensid, *Mnemiopsis*) we found neither genes encoding relevant ionotropic receptors nor genes encoding recognized synthetic enzymes for these molecules.

The majority of synthetic genes for neurotransmitter pathways are also not present in sequenced unicellular eukaryotes (such as *Monosiga* and *Capsaspora* recognized as sister groups for animals) suggesting they are cnidarian/bilaterian innovations. *Pleurobrachia* apparently also lack nitric oxide synthase (NOS) – a key synthetic enzyme involved in gaseous signaling mediated by nitric oxide (Moroz and Kohn, 2011), but NOS is present in the *Mnemiopsis* genome. Thus, ctenophores have the most dissimilar transmitter organization among all animals studied so far (see Kohn and Moroz, 2015b).

What are the ctenophore transmitters?

Using capillary electrophoresis, we detected L/D-glutamate and L/D-aspartate as well as γ-aminobutyric acid (GABA) in all four ctenophore species investigated (*Pleurobrachia*, *Beroe*, *Bolinopsis,* and *Mnemiopsis*) (Moroz et al., 2014). Physiological and pharmacological tests with application of all known low molecular weight neurotransmitters were performed using isolated muscles from *Pleurobrachia* and *Bolinopsis*: only glutamate and aspartate induced muscle contractions in ctenophores with rapid inward currents and the rise of intracellular Ca^{2+} in muscle cells (Moroz et al., 2014). The lowest threshold was determined for L-glutamate followed by L-aspartate, D-aspartate and D-glutamate (Fig. 4). Thus, potentially, each of these four molecules could be a neuromuscular transmitter in ctenophores; L-glutamate has a significantly higher affinity in these assays, and it is the most likely candidate.

The role of L-glutamate as an intercellular messenger is supported by an unprecedented diversity of ionotropic glutamate receptors, iGluR, in the *Pleurobrachia* genome and 10 other ctenophores we examined, including *Mnemiopsis* (Moroz et al., 2014). Combined, the diversity of iGluRs far exceeds the number of genes encoding iGluRs in other basal metazoans (Traynelis et al., 2010). Our initial phylogenetic reconstructions suggest that iGluRs might have undergone a substantial adaptive radiation within the ctenophore lineage (Moroz et al., 2014). Interestingly, the appearance of neurons in the *Pleurobrachia* development also correlates with co-expression of all iGlu receptors in cydippid larvae and the majority of iGluRs also show tissue-specific distribution in adults with predominant expression in tentacles, followed by combs and the apical organ (Moroz et al., 2014).

The emerging genomic data on ctenophores reveals the unprecedented diversity of enzymes involved in glutamate synthesis (eight glutaminases) and glutamate transporters (eight sialins) – more than any other metazoan investigated (El Mestikawy et al., 2011; Omote et al., 2011). Most importantly, ctenophores have sialin class transporters, but not classical vesicular glutamate transporters, as in other eumetazoans. These data suggest both well-developed glutamate signaling and its remarkable parallel evolution in ctenophores.

Although we directly detected GABA by capillary electrophoresis assays in all four ctenophore species examined (*Pleurobrachia*, *Mnemiopsis*, *Bolinopsis* and *Beroe*), a lack of pharmacological effects of GABA on *Pleurobrachia* behaviors and major motor systems such as cilia, muscle and colloblasts suggest that GABA acts as a passive by-product of glutamate metabolism by glutamate acid decarboxylase. Our surprising immunohistochemical finding of GABA accumulation in muscle cells (Fig. 4C, but not in neurons as in other metazoans) implies that GABA is a metabolic intermediate that inactivates the action of glutamate at the neuromuscular synapse. Interestingly, a product of GABA metabolism itself can be a usable source of energy in ctenophore muscles. Indeed, the GABA transaminase gene, also found in the *Pleurobrachia* genome, encodes the enzyme that catalyses the conversion of GABA back into succinic semialdehyde and glutamate following formation of succinic acid that enters the citric acid cycle – the universal aerobic bioenergetics pathway.

The identity of other low molecular weight transmitters in ctenophores is unknown. The presence of P2X receptors encoded in ctenophore genomes (Moroz et al., 2014) suggest that purinergic (ATP/ADP-mediated) transmission might occur in ctenophores. Similarly, there is a possibility of proton-mediated transmission as recently reported in nematodes (Beg et al., 2008) and vertebrates (Highstein et al., 2014). However, I would not exclude the presence of other small signal molecules, including those with no analogs in other metazoans.

Small peptides as putative ctenophore signal molecules

The first nervous systems have been suggested to be primarily peptidergic in nature (Moroz, 2009). Although we did not find any previously identified neuropeptide homologs, the secretory peptide prohormone processing genes are present. We predicted several dozen novel peptide prohormones in *Pleurobrachia* and found more

than 50 of their homologs in other sequenced ctenophores (Moroz et al., 2014). These prohormone-derived peptides could have a variety of functions including cell-to-cell signaling, toxins or involvement in innate immunity, or a combination of all three. Interestingly, a number of predicted peptides are also differentially expressed in embryonic stages, implying their roles in early segregation of developmental potential. Several of these ctenophore-specific precursors are expressed in polarized cells around the mouth, in tentacles and polar fields (e.g. ctenophorin, tentillin, jasonin), suggesting a signaling role (Moroz et al., 2014).

There are at least two types of candidate for small peptide receptors in ctenophores (Moroz et al., 2014). The first class encodes more than 100 orphan G-protein coupled receptors (Palczewski and Orban, 2013). The second class of (neuro)peptide receptor candidates is amiloride-sensitive sodium channels (ASSCs or ENaCs), which are also known to be regulated by protons (Krishtal, 2003; Sherwood et al., 2012; Wemmie et al., 2002). The *Pleurobrachia* genome has 29 genes encoding ASSCs/ENaCs (including putative acid-sensing channels or ASICs), which is more than any organism sequenced so far (Moroz et al., 2014). Notably, the expression of most of ASSCs is correlated with the morphological appearance of neurons in development, and ASSC expression is most abundant in tentacles, combs and apical organs – structures that are highly enriched in neural elements and under complex synaptic control. Whether peptide- or proton-mediated (neuro)transmission exists in ctenophores is a subject for future studies.

In conclusion, a generalized chemical synapse in ctenophores has a mosaic combination of ancestral and derived features, yet with a reduced representation of orthologs of bilaterian/cnidarian pre- and postsynaptic genes. For example, *Pleurobrachia* and 10 other ctenophores lack neuroligin, but have a basal type of neurexins – a key component bringing together pre- and postsynaptic membranes in bilaterians (yet lost in *Nematostella*) (Bang and Owczarek, 2013). Surprisingly, predicted 'synaptic' proteins are consistently expressed during early development in the absence of recognized neurons, suggesting their additional functions as components of ubiquitous secretory and receptor machineries in eukaryotes. As a result, predicted 'synaptic' proteins alone cannot be used as pan-neuronal markers.

Electrical signaling in ctenophores is well developed, especially in non-neuronal conductive and locomotory systems (Bilbaut et al., 1988; Dubas et al., 1988; Horridge, 1965a). Ctenophores have more genes encoding ion channels than sponges and placozoans (Moroz et al., 2014). However, the overall diversity of voltage-gated ion channels is reduced compared with other eumetazoans (Liebeskind et al., 2011). For example, *Pleurobrachia* has voltage-gated sodium and many potassium channels that were apparently absent in sponges and a greater diversity of aquaporins (water channels) (Papadopoulos and Verkman, 2013) than all other basal metazoans combined. The apical organ, combs and tentacles have a large diversity of ion channels, possibly associated with the conduction mediated by non-neuronal elements (e.g. modified muscles and cilia), also highly abundant in these structures. Not surprisingly, ctenophores evolved a variety of electrical synapses (Hernandez-Nicaise, 1991; Satterlie and Case, 1978); these gap junctions are encoded by 12 pannexin/innexin genes in the *Pleurobrachia* genome (Moroz et al., 2014). We do not find any pannexins in choanoflagellates or other basal eukaryotic groups (see also Abascal and Zardoya, 2013; Panchin, 2005), suggesting that these are metazoan innovations with major expansion of this family in the ctenophore lineage. Interestingly, pannexins are apparently absent in sponges and placozoans, and this situation might represent secondary gene loss in these lineages.

Did neural systems evolve more than once?

The emerging new data from ctenophores allow us to revisit two scenarios of neuronal evolution: (1) polygenesis or independent origins of neural systems in ctenophores versus cnidarian/bilaterian clade neurons (Moroz, 2009; Moroz, 2012; Moroz, 2013; Moroz et al., 2014; Moroz et al., 2012; Pennisi, 2013) and (2) monophyly or a single origin of the neural system with massive loss of majority neurotransmitters and some neurogenic molecular components in ctenophores (Rokas, 2013). The corollary of this 'single-origin' scenario would be the catastrophic loss of the entire nervous system in both placozoans and sponges, but the preservation of the original molecular make-up in the bilaterian/cnidarian clade. We favor the polygenesis hypothesis because many components of the molecular machinery controlling (1) neurogenesis, (2) transmitter synthesis, (3) receptor pathways, (4) 'pre- and postsynaptic' genes (including neuroligins and neurexins) are also absent in unicellular eukaryotes recognized as sister groups of animals. (5) Given the current placement of ctenophores as one of the most basally branching animal clades, polygenesis of neurons seems the more plausible hypothesis for the origins of neuronal systems (Moroz et al., 2014). Below, I will further clarify three controversial points following the discussion on the origin and early evolution of neural systems.

First, ctenophores lack components of classical low molecular weight (neuro)transmitter systems – the feature well preserved in all eumetazoans, including species with compact genomes (e.g. nematodes and ascidians), as well as all in parasitic animals investigated so far. From a number of genes encoding transmitter synthesis and degradation, only orthologs of genes distantly related to phenylalanine hydroxylase (PH) are shared between the choanoflagellate *Monosiga*, the slime mold *Dictyostelium* and the ctenophore *Pleurobrachia*. Yet, as our capillary electrophoresis data suggest, these PH-related enzymes, if functional, do not produce any known catecholamines in ctenophores.

Moreover, neural transmitter systems are not only characterized by the presence of specific synthesis enzymes – the heterogeneity of secretory specificity of neurons is one of the most fundamental features of any nervous system. Transmitter phenotypes include very complex packing, uptake (transporters) and inactivation systems, as well as multipart receptor machinery, with several hundred of genes precisely co-expressed in a given neuronal cell type. Thus, the alternative possibility – the massive secondary loss in ctenophores of virtually all genes in the receptor and transmitter synthesis pathway – is a less-parsimonious scenario. All ctenophores are predators with complex behaviors and a nearly complete replacement of multiple signaling pathways, as in the single-origin hypothesis, seems to be a more complex reconstruction.

Of course, there is a possibility that ctenophores might have evolved complex behaviors later in evolution, suggesting their more recent radiation. According to 18S rRNA analysis by Podar and colleagues (Podar et al., 2001), this could have occurred at the K–T boundary 66 million years ago – yet the paleontological data suggest the presence of pre-Cambrian, Cambrian and Devonian ctenophore fossils with extensive comb organization (see Chen et al., 1991; Chen et al., 2007; Conway Morris and Collins, 1996; Dzik, 2002; Erwin and Valentine, 2013; Shu et al., 2006; Stanley and Stürmer, 1983; Tang et al., 2011). There is a possibility that the last common ancestor of extant ctenophores shared neuronal toolkits with other eumetazoans (Cnidaria and Bilateria) but this scenario, regardless of phylogenetic reconstructions (Moroz, 2014), still implies a situation

whereby modern ctenophores developed a very distinct molecular make-up and 'lost'(?) many of the eumetazoans signal molecules. Either way, the remarkable parallel evolution of neural organization in ctenophores is evident.

Second, we also found that orthologs of bilaterian and cnidarian 'pan-neuronal' markers are not expressed in ctenophore neurons, suggesting that they perform different functions. However, we identified two broad categories of other genes that are specifically expressed in ctenophore neurons (Moroz et al., 2014). One of the largest categories includes genes that are either ctenophore innovations (such as ctenophore-specific secretory peptides); or ctenophore lineage-specific isoforms (such as WntX). The second category is a group of genes that are specifically expressed in the *Pleurobrachia* neurons but are not specifically expressed in neurons in other eumetazoans (e.g. Argonaut or Dicers). Combined, these data suggest that the genes are independently recruited to the ctenophore neuronal machinery. An alternative explanation is that these genes or functions were also present in the common ancestor but were lost in all other metazoans; given conservation of neural components across eumetazoans, I consider that this scenario is less probable.

Third, we do find a number of genes encoding presumed pre- and post-synaptic proteins in the *Pleurobrachia* genome that are shared with Choanoflagellates and *Capsaspora*, suggesting a single-cell origin of the backbone of the canonical synaptic machinery revealed in proteomic studies on bilaterians (Ryan and Grant, 2009). However, our data indicate that these genes are not 'pure neuronal/synaptic' genes because, even in ctenophores, they are also expressed in non-neuronal tissues, including secretory cells. Most interestingly, the majority of canonical 'synapse-related' genes are highly expressed early in development (from 2- to 4-cell stages to gastrulation) when no neurons are present (Moroz et al., 2014). There is a possibility that zygotic transcription might start at or before first-cleavage and/or transcripts in high abundance would be maternally loaded rather than zygotically expressed. In any case, there is no evidence that neuronal cell lineages are involved in ctenophore gametogenesis. This feature indicates that these 'synaptic complex' genes are part of a ubiquitous secretory and signaling complex that couples Ca^{2+} signaling with cell cytoskeleton organization, as evidenced by their presence in unicellular eukaryotes. Thus, most of these 'synaptic complex' genes might not be considered alone as an evidence of early neuronal origin in the last common metazoan ancestor but rather as the evidence for deep ancestral roots for components of such secretory or receptive machinery in Opisthokonta and Holozoa (Mikhailov et al., 2009). In addition, the synaptic gene complement in ctenophores is significantly reduced compared with all other animals, suggesting that ctenophore synapses are distinct in their molecular make-up (Kohn and Moroz, 2015a), which is consistent with electron microscopic investigations (Hernandez-Nicaise, 1991). Combined, these data support a hypothesis of parallel evolution of synaptic organization in ctenophores, which also reflects the development of their unique neurotransmitter organization.

Similarly, ion channels cannot be considered as specific neuronal markers or the features indicating presence or absence of nervous systems in any evolutionary reconstruction because virtually the same genes are expressed in majority of non-neuronal tissues controlling cellular excitability in a broad spectrum of unicellular and multicellular eukaryotes. Not surprisingly, we found that ctenophores reveal examples of both gene gain and loss.

There are recent publications challenging the hypotheses of independent origins of neurons. For example, Ryan (Ryan, 2014) suggests 'a few lines of evidence uniting the nervous systems of ctenophores, cnidarians, and bilaterians'. Four of them are:

(1) 'The presence of selected genes known as neuronal fate and patterning genes (e.g. *Lhx*, *Hes*, *Bhlh*, *Sox*, *NKL* and *Tlx*)'. Yes, these genes are present in ctenophore genomes but they cannot be considered as pan-neuronal markers in ctenophores because they are expressed in many other cell types. Plus their neuronal colocalization at the cellular level has not been shown (Jager et al., 2006; Derelle and Manuel, 2007; Jager et al., 2008; Ryan et al., 2010; Simmons et al., 2012; Schnitzler et al., 2014). The fact that some of these genes are associated with the aboral organ or polar fields does not mean that in these structures these genes are expressed in neurons (e.g. in the aboral organ – the majority of cells are not neurons).

(2) 'The presence of many components critical for synaptic function in bilaterians (e.g. Cadherin, Ephrin, Pmca, mGluR, Magi, Pkc, Citron, Spar, Dlg, Syngap, Gkap, Nos, Lin-7 and Pick1)'. Yes, these genes are equally critical for many other non-neuronal functions; and, in bilaterians, they are also expressed in a diversity of non-neuronal tissues. Therefore, they can not be considered as unique synaptic markers, even in bilaterians.

(3) 'Observed immunoreactivity to antisera targeted to bilaterian and cnidarian neurotransmitters (e.g. acetyl-cholinesterase, FMRFamide and vasopressin)' (Grimmelikhuijzen, 1983; Jager et al., 2011). Here, Ryan (Ryan, 2014) also used the example of a non-neuronal marker such acetylcholinesterase. Based on the existing sequencing data it is impossible to correctly predict enzymatic function of these gene orthologs in ctenophores. These gene orthologs belong to a broad cholinesterase family. Furthermore, the mentioned antisera to bilaterian or vertebrate peptides can not be used as any evidence because of the observed crossreactivity; and the fact that these peptide prohormones are not present in the ctenophore genomes sequenced (Moroz et al., 2014)..

Finally, (4) 'sensitivity of muscle (or any other effector) to L-glutamate'. Ryan's (Ryan, 2014) evidence uniting the nervous systems of ctenophores, cnidarians and bilaterians is not correct because it is an example of the broadest and non-specific category of intercellular messengers (also present in plants and bacteria) and an essential amino acid. This fact might only suggest that this type of L-glutamate-mediated signaling was in the common ancestor of all animals irrespective of the presence of neural systems. In summary, the presence of the above-mentioned genes in genomes (without further functional and co-localization studies) cannot be used as evidence to support or reject hypotheses of single versus multiple origins of neurons. Multiple examples of independent recruitment of similar gene batteries might occur in evolution as a reflection of modular or mosaic organization of both neural and non-neuronal tissues across the animal kingdom.

What is a neuron?

Here, I define neurons as polarized secretory cells specialized for directional propagation of electrical signals leading to the release of extracellular messengers – features that enable them to transmit information, primarily chemical in nature, beyond their immediate neighbors without affecting all intervening cells en route. Short- and long-term neuroplasticity, homeostatic plasticity as parts of learning and memory mechanisms, are inherent components of neural organization. Thus, the definition of a neuron is a functional, not a genetic category. As such, multiple origins of neurons from different classes of secretory cells might occur more than once during ~600 million years of animal evolution a part of transition from temporal to spatial differentiation (Mikhailov et al., 2009). Grundfest

(Grundfest, 1959) in the 1950–1960s and Sakharov (Sakharov, 1974) in the 1970s suggested that neurons arose from ancestral secretory cells, when the secretory activity became confined to the termination of elongated processes (Moroz, 2009; Moroz, 2014). Early neurons and synapses might have evolved as the next step in the development of compartmentalized transmitter secretion – the hallmark of neuronal organization – recruiting pre-existing molecular components for polarized transport and signaling from secretory and receptor machinery already well-developed in unicellular eukaryotes. This explains the recruitment in ctenophores of certain RNA binding proteins, which act as a cargo to transport selected localized RNA (e.g. secretory apparatus, receptors, ion channels, etc.) to distant neural processes.

The first neural circuits could reasonably have evolved from undifferentiated secretory-like cells (perhaps without recognized bona fide neurons as in *Trichoplax*) to control cilia and coordinate primary (ciliated) locomotion (Jékely, 2011) recruiting small peptides as early signal molecules. The first proto-neurons could mediate their action via volume transmission without structurally differentiated synapses. More localized, synaptic communication evolved later, with the increase of tissue complexity (and perhaps animal sizes); it could be an adaptation for faster transmission to distant and diverse effectors. The first muscles evolved to control the hydroskeleton and feeding/defensive movements, as in extant ctenophores with L-glutamate as one of the neuromuscular transmitters in addition to secretory peptides.

Even early synapses might use more than one secretory product. It is reasonable to assume that different ancestral cell lineages might eventually give a raise to different genetically unrelated classes of neurons and synapses. Thus, the corollary of the hypothesis of independent origins or parallel evolution of neurons might be independent origins or parallel evolution of synapses with different types of secretory specificity (Moroz, 2015), initially encoded in the ancestral cell lineages and later preserved in more complex neural systems. Selected gene regulatory circuits and secretory molecular toolkits might be used for homologization of neuronal classes in different animals.

What were the early transmitters?

L-glutamate may have been one of the first small molecules to be recruited as a neuromuscular transmitter in the ctenophores following profound diversification of iGluRs, components of glutamate synthesis and uptake. A diversity of secretory signal peptides and their receptors (including expansion of ASSCs/DEGs/ENaCs) may have also been recruited for this role in ctenophores independently from other metazoans, paralleled by the diversification of gap junction proteins most profoundly expressed in the apical organ of extant ctenophores. Polarized secretory (possible peptidergic) cells were probably involved in coordination of ciliated locomotion in many early animals and these types of cells can be considered as evolutionary precursors of different neuronal lineages with specific transmitter phenotypes. Our data also imply that classical low molecular weight transmitter systems such as cholinergic, GABAergic and three classes of monoaminergic systems (serotonergic, histaminergic, dopaminergic–adrenergic) were recruited for neuronal functions in cnidarian/bilaterians lineages. The presence of two existing neural nets in ctenophores with ectodermal and mesogleal neurons that are similar to mesogleal muscle-like precursors raises the possibility that some neuronal lineages evolved from muscle cells that lost contractility and gained a polarized secretory or synaptic apparatus. Finally, protons, ATP and related nucleotides as well as gaseous molecules such as NO, H_2S and CO might also be recruited as intercellular messengers early in evolution.

In conclusion, our genomic, expression and microchemical data (Moroz et al., 2014) indicate that the overall molecular make-up of the ctenophore nervous systems is remarkably different from all other nervous systems studied, suggesting extensive parallel evolution of neural organization in this lineage. Regardless of evolutionary interpretations, the sequenced *Pleurobrachia* genome, combined genomic, metabolomic and physiological data on 10 different species revealed extraordinary and unique molecular diversity of developmental and neural signaling pathways. Together with numerous ctenophore ‘innovations’, ctenophores may serve as a model to understand the origins or emergence of complex integrative functions and can be used in synthetic biology and regenerative medicine to design novel regulatory systems.

Acknowledgements

The author thanks FHL for facilities during animal collection and support Marine Genomics apprenticeships; Dr A. B. Kohn for fruitful discussions and analysis of multiple gene families during the *Pleurobrachia* genome project; Dr T. P. Norekian for tubulin and GABA immunohistochemistry; Dr Y. Bobkov for electrophysiology and imaging of isolated muscle cells. Yegor Moroz, Yelena Moroz Alpert and three anonymous reviewers for their editing and comments to the manuscripts. Many original results referred to in this review were parts of the large-scale ctenophore genome/transcriptome projects reported in *Nature* doi:10.1038/nature13400. This work was presented at the ‘Evolution of the First Nervous Systems II’ meeting, which was supported by the National Science Foundation (NSF) grant IOS 1339035.

Competing interests

The author declares no competing or financial interests.

Funding

The research was supported by National Science Foundation NSF-0744649 and NSF CNS-0821622; National Institute of Health 1R01GM097502, R01MH097062, R21RR025699 and 5R21DA030118; National Aeronautics and Space Administration (NASA) NNX13AJ31G; McKnight Brain Research and University of Florida Opportunity Funds to L.L.M. Deposited in PMC for release after 12 months.

References

Abascal, F. and Zardoya, R. (2013). Evolutionary analyses of gap junction protein families. *Biochim. Biophys. Acta* **1828**, 4-14.

Alié, A., Leclère, L., Jager, M., Dayraud, C., Chang, P., Le Guyader, H., Queinnec, E. and Manuel, M. (2011). Somatic stem cells express Piwi and Vasa genes in an adult ctenophore: ancient association of “germline genes” with stemness. *Dev. Biol.* **350**, 183-197.

Anctil, M. (1985). Cholinergic and monoaminergic mechanisms associated with control of bioluminescence in the ctenophore *Mnemiopsis leidyi*. *J. Exp. Biol.* **119**, 225-238.

Anctil, M. and Shimomura, O. (1984). Mechanism of photoinactivation and re-activation in the bioluminescence system of the ctenophore *Mnemiopsis*. *Biochem. J.* **221**, 269-272.

Anderson, P. A. V. (1984). The electrophysiology of single smooth muscle cells isolated from *Mnemiopsis*. *J. Comp. Physiol. B* **154**, 257-268.

Anderson, P. A. (1985). Physiology of a bidirectional, excitatory, chemical synapse. *J. Neurophysiol.* **53**, 821-835.

Anderson, P. A. and Grünert, U. (1988). Three-dimensional structure of bidirectional, excitatory chemical synapses in the jellyfish *Cyanea capillata*. *Synapse* **2**, 606-613.

Aronova, M. (1974). Electron microscopic observation of the aboral organ of Ctenophora. I. The gravity receptor. *Z. Mikrosk. Anat. Forsch.* **88**, 401-412.

Aronova, M. Z. (1975). [Electronmicroscopic study of the gravity receptors in ctenophores]. *Zh. Evol. Biokhim. Fiziol.* **11**, 503-508.

Aronova, M. Z. (1979). [Electron microscopic study of presumptive photoreceptor cells in the aboral organ of the ctenophore, *Beroë cucumis*]. *Zh. Evol. Biokhim. Fiziol.* **15**, 596-601.

Aronova, M. Z. and Alekseeva, T. M. (2003). [Development of chemoreceptor cells in oral epithelium of adult ctenophore *Beroe cucumis*]. *Zh. Evol. Biokhim. Fiziol.* **39**, 577-585.

Aronova, M. Z. and Alekseeva, T. M. (2004). [Ultrastructural identification of glial cells in the mouth of jellyfish *Beroe cucumis*]. *Zh. Evol. Biokhim. Fiziol.* **40**, 579-588.

Aronova, M. Z., Titova, L. K., Kharkeevitch, T. A. and Tsirulis, T. P. (1979). Ultrastructural peculiarities of the chemical transmission of the nerve impulse in the gravity receptors of some invertebrates. *Z. Mikrosk. Anat. Forsch.* **93**, 91-103.

Bang, M. L. and Owczarek, S. (2013). A matter of balance: role of neurexin and neuroligin at the synapse. *Neurochem. Res.* **38**, 1174-1189.

Beg, A. A., Ernstrom, G. G., Nix, P., Davis, M. W. and Jorgensen, E. M. (2008). Protons act as a transmitter for muscle contraction in *C. elegans*. *Cell* **132**, 149-160.

Bethe, A. (1895). Der subepitheliale nervenplexus der Ctenophoren. *Biol. Zbl* **15**, 140-145.
Bilbaut, A., Hernandez-Nicaise, M. L., Leech, C. A. and Meech, R. W. (1988). Membrane currents that govern smooth muscle contraction in a ctenophore. *Nature* **331**, 533-535.
Borowiec, M. L., Lee, E. K., Chiu, J. C. and Plachetzki, D. C. (2015). Dissecting phylogenetic signal and accounting for bias in whole-genome data sets: a case study of the Metazoa. *Mol. Biol. Evol.* (in press).
Brusca, R. C. and Brusca, G. J. (2003). *Invertebrates*. Sunderland, MA: Sinauer Associates, Inc.
Bullock, T. H. and Horridge, G. A. (1965). *Structure and Function in the Nervous Systems of Invertebrates*. San Francisco, CA: Freeman.
Burton, P. M. (2008). Insights from diploblasts; the evolution of mesoderm and muscle. *J. Exp. Zool. B* **310**, 5-14.
Chapman, J. A., Kirkness, E. F., Simakov, O., Hampson, S. E., Mitros, T., Weinmaier, T., Rattei, T., Balasubramanian, P. G., Borman, J., Busam, D. et al. (2010). The dynamic genome of *Hydra*. *Nature* **464**, 592-596.
Chen, J. Y., Bergstrom, J., Lindstrom, M. and Hou, X. G. (1991). Fossilized soft-bodied fauna. *Natl. Geogr. Res.* **7**, 8-19.
Chen, J. Y., Schopf, J. W., Bottjer, D. J., Zhang, C. Y., Kudryavtsev, A. B., Tripathi, A. B., Wang, X. Q., Yang, Y. H., Gao, X. and Yang, Y. (2007). Raman spectra of a Lower Cambrian ctenophore embryo from southwestern Shaanxi, China. *Proc. Natl. Acad. Sci. USA* **104**, 6289-6292.
Chun, C. (1880). Die Ctenophoren des Golfes von Neapel. *Fauna Flora Neapel Monogr*. 1-313.
Conway Morris, S. and Collins, D. H. (1996). Middle Cambrian Ctenophores from the Stephen Formation, British Columbia, Canada. *Philos. Trans. R. Soc. B* **351**, 279-308.
Derelle, R. and Manuel, M. (2007). Ancient connection between NKL genes and the mesoderm? Insights from Tlx expression in a ctenophore. *Dev. Genes Evol.* **217**, 253-261.
DeSalle, R., Gregory, T. R. and Johnston, J. S. (2005). Preparation of samples for comparative studies of arthropod chromosomes: visualization, in situ hybridization, and genome size estimation. *Methods Enzymol.* **395**, 460-488.
Driesch, H. and Morgan, T. H. (1895). Zur Analysis der ersten Entwickelungsstadien des Ctenophoreneieees. *Archiv für Entwicklungsmechanik der Organismen* **2**, 204-215.
Dubas, F., Stein, P. G. and Anderson, P. A. (1988). Ionic currents of smooth muscle cells isolated from the ctenophore *Mnemiopsis*. *Proc. R. Soc. B* **233**, 99-121.
Dunn, C. W., Hejnol, A., Matus, D. Q., Pang, K., Browne, W. E., Smith, S. A., Seaver, E., Rouse, G. W., Obst, M., Edgecombe, G. D. et al. (2008). Broad phylogenomic sampling improves resolution of the animal tree of life. *Nature* **452**, 745-749.
Dzik, J. (2002). Possible ctenophoran affinities of the Precambrian "sea-pen" *Rangea*. *J. Morphol.* **252**, 315-334.
El Mestikawy, S., Wallén-Mackenzie, A., Fortin, G. M., Descarries, L. and Trudeau, L. E. (2011). From glutamate co-release to vesicular synergy: vesicular glutamate transporters. *Nat. Rev. Neurosci.* **12**, 204-216.
Erwin, D. H. and Valentine, J. W. (2013). *The Cambrian Explosion. The Construction of Animal Biodiversity*, pp. 406. Greenwood Village, CO: Roberts and Company.
Erwin, D. H., Laflamme, M., Tweedt, S. M., Sperling, E. A., Pisani, D. and Peterson, K. J. (2011). The Cambrian conundrum: early divergence and later ecological success in the early history of animals. *Science* **334**, 1091-1097.
Fuller, R. R., Moroz, L. L., Gillette, R. and Sweedler, J. V. (1998). Single neuron analysis by capillary electrophoresis with fluorescence spectroscopy. *Neuron* **20**, 173-181.
Girsch, S. J. and Hastings, J. W. (1978). The properties of mnemiopsin, a bioluminescent and light sensitive protein purified by hollow fiber techniques. *Mol. Cell. Biochem.* **19**, 113-124.
Gregory, T. R., Nicol, J. A., Tamm, H., Kullman, B., Kullman, K., Leitch, I. J., Murray, B. G., Kapraun, D. F., Greilhuber, J. and Bennett, M. D. (2007). Eukaryotic genome size databases. *Nucleic Acids Res.* **35**, D332-D338.
Grimmelikhuijzen, C. J. (1983). FMRFamide immunoreactivity is generally occurring in the nervous systems of coelenterates. *Histochemistry* **78**, 361-381.
Grundfest, H. (1959). Evolution of conduction in the nervous system. In *Evolution of Nervous Control from Primitive Organisms to Man* (ed. A. D. Bass), pp. 43-86. Washington, DC: American Association for Advancement of Science.
Haddock, S. H. (2007). Comparative feeding behavior of planktonic ctenophores. *Integr. Comp. Biol.* **47**, 847-853.
Heider, K. (1927a). Ueber das Nervensystem von *Beroe ovata*. *Nachr. Ges. Wiss. Göttingen*, 145-157.
Heider, K. (1927b). Vom nervensystem der ctenophoren. *Z. Morphol. Oekol. Tiere* **9**, 638-678.
Hejnol, A., Obst, M., Stamatakis, A., Ott, M., Rouse, G. W., Edgecombe, G. D., Martinez, P., Baguñà, J., Bailly, X., Jondelius, U. et al. (2009a). Assessing the root of bilaterian animals with scalable phylogenomic methods. *Proc. Biol. Sci.* **276**, 4261-4270.
Hejnol, A., Obst, M., Stamatakis, A., Ott, M., Rouse, G. W., Edgecombe, G. D., Martinez, P., Baguñà, J., Bailly, X., Jondelius, U. et al. (2009b). Assessing the root of bilaterian animals with scalable phylogenomic methods. *Proc. Biol. Sci.* **276**, 4261-4270.
Hernandez-Nicaise, M. L. (1968). Specialized connexions between nerve cells and mesenchymal cells in ctenophores. *Nature* **217**, 1075-1076.
Hernandez-Nicaise, M. L. (1973a). [The nervous system of ctenophores. I. Structure and ultrastructure of the epithelial nerve-nets]. *Z. Zellforsch. Mikrosk. Anat.* **137**, 223-250.
Hernandez-Nicaise, M. L. (1973b). [The nervous system of ctenophores. II. The nervous elements of the mesoglea of beroids and cydippids (author's transl)]. *Z. Zellforsch. Mikrosk. Anat.* **143**, 117-133.
Hernandez-Nicaise, M. L. (1973c). The nervous system of ctenophores. III. Ultrastructure of synapses. *J. Neurocytol.* **2**, 249-263.
Hernandez-Nicaise, M. L. (1974). Ultrastructural evidence for a sensory-motor neuron in Ctenophora. *Tissue Cell* **6**, 43-47.
Hernandez-Nicaise, M.-L. (1991). Ctenophora. In *Microscopic Anatomy of Invertebrates: Placozoa, Porifera, Cnidaria, and Ctenophora*, Vol. 2 (ed. F. W. F.W. Harrison and J. A. Westfall), pp. 359-418. New York, NY: Wiley.
Hernandez-Nicaise, M. L. and Amsellem, J. (1980). Ultrastructure of the giant smooth muscle fiber of the ctenophore *Beroe ovata*. *J. Ultrastruct. Res.* **72**, 151-168.
Hernandez-Nicaise, M. L., Mackie, G. O. and Meech, R. W. (1980). Giant smooth muscle cells of *Beroë*. Ultrastructure, innervation, and electrical properties. *J. Gen. Physiol.* **75**, 79-105.
Hernandez-Nicaise, M. L., Bilbaut, A., Malaval, L. and Nicaise, G. (1982). Isolation of functional giant smooth muscle cells from an invertebrate: structural features of relaxed and contracted fibers. *Proc. Natl. Acad. Sci. USA* **79**, 1884-1888.
Hertwig, R. (1880). *Ueber Den Bau der Ctenophoren*. Jena: G. Fischer.
Hertwig, O. and Hertwig, R. (1878). *Das Nervensystem und die Sinnesorgane der Medusen (The Nervous System and the Sensory Organs of the Medusa)*. Leipzig: Vogel.
Hertwig, O. and Hertwig, R. (1879). Die actinien anatomisch und histologisch mit besonderer Berucksichtigung des Nervenmuskelsystems untersucht. *Jenaische Z. Naturwiss.* **13**, 457-460.
Hertwig, O. and Hertwig, R. (1880). Die actinien anatomisch und histologisch mit besonderer berücksichtigung des nervenmuskelsystems untersucht. *Jenaische Z. Naturwiss.* **14**, 39-80.
Highstein, S. M., Holstein, G. R., Mann, M. A. and Rabbitt, R. D. (2014). Evidence that protons act as neurotransmitters at vestibular hair cell-calyx afferent synapses. *Proc. Natl. Acad. Sci. USA* **111**, 5421-5426.
Horridge, G. A. (1964a). The giant mitochondria of ctenophore comb plates. *Q. J. Microsc. Sci.* **105**, 301-310.
Horridge, G. A. (1964b). Presumed photoreceptive cilia in a ctenophore. *Q. J. Microsc. Sci.* **105**, 311-317.
Horridge, G. A. (1965a). Intracellular action potentials associated with the beating of the cilia in ctenophore comb plate cells. *Nature* **205**, 602.
Horridge, G. A. (1965b). Non-motile sensory cilia and neuromuscular junctions in a ctenophore independent effector organ. *Proc. R. Soc. B* **162**, 333-350.
Horridge, G. A. (1965c). Relations between nerves and cilia in Ctenophores. *Am. Zool.* **5**, 357-375.
Horridge, G. A. (1968). The origin of the nervous system. In *Structure and Function of Nervous Tissue* (ed. G. H. Bourne), pp. 1-33. New York, NY: Academic Press.
Horridge, G. A. (1974). Recent studies on the Ctenophora. In *Coelenterate Biology* (ed. L. Muscatine and H. M. Lenhoff), pp. 439-468. New York, NY: Academic Press.
Horridge, G. A. and Mackay, B. (1964). Neurociliary synapses in *Pleurobrachia* (Ctenophora). *Q. J. Microsc. Sci.* **105**, 163-174.
Horridge, G. A., Chapman, D. M. and Mackay, B. (1962). Naked axons and symmetrical synapses in an elementary nervous system. *Nature* **193**, 899-900.
Hyman, L. H. (1940). *Invertebrates: Protozoa through Ctenophora*. New York, NY: London: McGraw-Hill.
Jager, M., Quéinnec, E., Houliston, E. and Manuel, M. (2006). Expansion of the sox gene family predated the emergence of the Bilateria. *Mol. Phyl. Evol.* **39**, 468–477.
Jager, M., Quéinnec, E., Chiori, R., Le Guyader, H. and Manuel, M. (2008). Insights into the early evolution of SOX genes from expression analyses in a ctenophore. *J. Exp. Zool. B* **310**, 650-667.
Jager, M., Chiori, R., Alié, A., Dayraud, C., Quéinnec, E. and Manuel, M. (2011). New insights on ctenophore neural anatomy: immunofluorescence study in *Pleurobrachia pileus* (Müller, 1776). *J. Exp. Zool. B* **316B**, 171-187.
Jager, M., Dayraud, C., Mialot, A., Quéinnec, E., le Guyader, H. and Manuel, M. (2013). Evidence for involvement of Wnt signalling in body polarities, cell proliferation, and the neuro-sensory system in an adult ctenophore. *PLoS ONE* **8**, e84363.
Jékely, G. (2011). Origin and early evolution of neural circuits for the control of ciliary locomotion. *Proc. Biol. Sci.* **278**, 914-922.
Kass-Simon, G. and Hufnagel, L. A. (1992). Suspected chemoreceptors in coelenterates and ctenophores. *Microsc. Res. Tech.* **22**, 265-284.
King, N., Westbrook, M. J., Young, S. L., Kuo, A., Abedin, M., Chapman, J., Fairclough, S., Hellsten, U., Isogai, Y., Letunic, I. et al. (2008). The genome of the choanoflagellate *Monosiga brevicollis* and the origin of metazoans. *Nature* **451**, 783-788.
Kocot, K. M., Cannon, J. T., Todt, C., Citarella, M. R., Kohn, A. B., Meyer, A., Santos, S. R., Schander, C., Moroz, L. L., Lieb, B. et al. (2011). Phylogenomics reveals deep molluscan relationships. *Nature* **477**, 452-456.
Kohn, A. B. and Moroz, L. L. (2015a). Genomic portrait of synapses and their evolution. *Integr. Comp. Neurol.* **Abstracts**, S5.3.
Kohn, A. B. and Moroz, L. L. (2015b). Unbiased views of synaptic evolution. *Integr. Comp. Biol.* (in press).
Kohn, A. B., Citarella, M. R., Kocot, K. M., Bobkova, Y. V., Halanych, K. M. and Moroz, L. L. (2012). Rapid evolution of the compact and unusual mitochondrial genome in the ctenophore, *Pleurobrachia bachei*. *Mol. Phylogenet. Evol.* **63**, 203-207.
Korn, H. (1959). Zum nervensystem der ctenophore *Pleurobrachia pileus* O. Muller. *Zool. Anz.* **163**, 351-359.
Kozloff, E. N. (1990). *Invertebrates*. Philadelphia, PA: Saunders College Publishing.

Krishtal, O. (2003). The ASICs: signaling molecules? Modulators? *Trends Neurosci.* **26**, 477-483.
Lentz, T. L. (1968). *Primitive Nervous Systems*. New Haven, CT; London: Yale University Press.
Liebeskind, B. J., Hillis, D. M. and Zakon, H. H. (2011). Evolution of sodium channels predates the origin of nervous systems in animals. *Proc. Natl. Acad. Sci. USA* **108**, 9154-9159.
Mackie, G. O. (1970). Neuroid conduction and the evolution of conducting tissues. *Q. Rev. Biol.* **45**, 319-332.
Mackie, G. O. (1990). The elementary nervous sytems revisited. *Am. Zool.* **30**, 907-920.
Mackie, G. O., Mills, C. E. and Singla, C. L. (1992). Giant axons and escape swimming in *Euplokamis dunlapae* (Ctenophora, Cydippida). *Biol. Bull.* **182**, 248-256.
Marlow, H. and Arendt, D. (2014). Evolution: ctenophore genomes and the origin of neurons. *Curr. Biol.* **24**, R757-R761.
Mayer, A. G. (1912). Ctenophores of the Atlantic coast of North America. *Carnegie Institution of Washington Publication* **162**, 1-58.
Mikhailov, K. V., Konstantinova, A. V., Nikitin, M. A., Troshin, P. V., Rusin, L. Y., Lyubetsky, V. A., Panchin, Y. V., Mylnikov, A. P., Moroz, L. L., Kumar, S. et al. (2009). The origin of Metazoa: a transition from temporal to spatial cell differentiation. *BioEssays* **31**, 758-768.
Miller, G. (2009). Origins. On the origin of the nervous system. *Science* **325**, 24-26.
Moroz, L. L. (2006). Localization of putative nitrergic neurons in peripheral chemosensory areas and the central nervous system of *Aplysia californica*. *J. Comp. Neurol.* **495**, 10-20.
Moroz, L. L. (2009). On the independent origins of complex brains and neurons. *Brain Behav. Evol.* **74**, 177-190.
Moroz, L. L. (2012). Phylogenomics meets neuroscience: how many times might complex brains have evolved? *Acta Biol. Hung.* **63 Suppl. 2**, 3-19.
Moroz, L. L. (2013). Genomic bases for independent origins of neurons and complex brains: new insights from RNA-seq and genomic sequencing of basal metazoans, basal deuterostomes and molluscs. *Integr. Comp. Biol. Abstracts*. Available at: http://www.sicb.org/meetings/2013/schedule/abstractdetails.php?id=235
Moroz, L. L. (2014). Genealogy of genealogy of neurons. *Commun. Integr. Biol.* **7**, 1-6.
Moroz, L. L. (2015). Convergent evolution of neurons and synapses from distinct cell lineages. NeuroSystematics: from ctenophores to vertebrates. *Integr. Comp. Biol. Abstracts*. Available at: http://www.sicb.org/meetings/2015/schedule/abstractdetails.php?id=466.
Moroz, L. L. and Kohn, A. B. (2011). Parallel evolution of nitric oxide signaling: diversity of synthesis and memory pathways. *Front. Biosci. (Landmark Ed.)* **16**, 2008-2051.
Moroz, L. L., Norekian, T. P., Pirtle, T. J., Robertson, K. J. and Satterlie, R. A. (2000). Distribution of NADPH-diaphorase reactivity and effects of nitric oxide on feeding and locomotory circuitry in the pteropod mollusc, Clione limacina. *J. Comp. Neurol.* **427**, 274-284.
Moroz, L. L., Meech, R. W., Sweedler, J. V. and Mackie, G. O. (2004). Nitric oxide regulates swimming in the jellyfish *Aglantha digitale*. *J. Comp. Neurol.* **471**, 26-36.
Moroz, L. L., Dahlgren, R. L., Boudko, D., Sweedler, J. V. and Lovell, P. (2005). Direct single cell determination of nitric oxide synthase related metabolites in identified nitrergic neurons. *J. Inorg. Biochem.* **99**, 929-939.
Moroz, L. L., Kohn, A., Citarella, M., Grigorenko, A., Kokot, K., Halanych, K. and Rogaev, E. (2012). The genome of the ctenophore *Pleurobrachia* bachei: molecular insights into independent origins of nervous systems. *Integr. Comp. Biol. Abstracts*. Available at: http://sicb.org/meetings/2012/schedule/abstractdetails.php?id=527
Moroz, L. L., Kocot, K. M., Citarella, M. R., Dosung, S., Norekian, T. P., Povolotskaya, I. S., Grigorenko, A. P., Dailey, C., Berezikov, E., Buckley, K. M. et al. (2014). The ctenophore genome and the evolutionary origins of neural systems. *Nature* **510**, 109-114.
Moss, A. G. and Tamm, S. L. (1986). Electrophysiological control of ciliary motor responses in the ctenophore *Pleurobrachia*. *J. Comp. Physiol. A* **158**, 311-330.
Moss, A. G. and Tamm, S. L. (1987). A calcium regenerative potential controlling ciliary reversal is propagated along the length of ctenophore comb plates. *Proc. Natl. Acad. Sci. USA* **84**, 6476-6480.
Nielsen, C. (2012). *Animal Evolution: Interrelationships of the Living Phyla*. Oxford: Oxford University Press.
Nosenko, T., Schreiber, F., Adamska, M., Adamski, M., Eitel, M., Hammel, J., Maldonado, M., Müller, W. E., Nickel, M., Schierwater, B. et al. (2013). Deep metazoan phylogeny: when different genes tell different stories. *Mol. Phylogenet. Evol.* **67**, 223-233.
Omote, H., Miyaji, T., Juge, N. and Moriyama, Y. (2011). Vesicular neurotransmitter transporter: bioenergetics and regulation of glutamate transport. *Biochemistry* **50**, 5558-5565.
Palczewski, K. and Orban, T. (2013). From atomic structures to neuronal functions of G protein-coupled receptors. *Annu. Rev. Neurosci.* **36**, 139-164.
Panchin, Y. V. (2005). Evolution of gap junction proteins – the pannexin alternative. *J. Exp. Biol.* **208**, 1415-1419.
Papadopoulos, M. C. and Verkman, A. S. (2013). Aquaporin water channels in the nervous system. *Nat. Rev. Neurosci.* **14**, 265-277.
Parker, G. H. (1919). *The Elementary Nervous Systems*. Philadelphia, PA: Lippincott.
Pennisi, E.; Society for Integrative and Comparative Biology Annual Meeting (2013). 2013 Society for Integrative and Comparative Biology Annual Meeting. Nervous system may have evolved twice. *Science* **339**, 391.
Penny, A. M., Wood, R., Curtis, A., Bowyer, F., Tostevin, R. and Hoffman, K. H. (2014). Early animals. Ediacaran metazoan reefs from the Nama Group, Namibia. *Science* **344**, 1504-1506.
Pett, W., Ryan, J. F., Pang, K., Mullikin, J. C., Martindale, M. Q., Baxevanis, A. D. and Lavrov, D. V. (2011). Extreme mitochondrial evolution in the ctenophore *Mnemiopsis leidyi*: Insight from mtDNA and the nuclear genome. *Mitochondrial DNA* **22**, 130-142.
Philippe, H., Derelle, R., Lopez, P., Pick, K., Borchiellini, C., Boury-Esnault, N., Vacelet, J., Renard, E., Houliston, E., Quéinnec, E. et al. (2009). Phylogenomics revives traditional views on deep animal relationships. *Curr. Biol.* **19**, 706-712.
Philippe, H., Brinkmann, H., Copley, R. R., Moroz, L. L., Nakano, H., Poustka, A. J., Wallberg, A., Peterson, K. J. and Telford, M. J. (2011). Acoelomorph flatworms are deuterostomes related to *Xenoturbella*. *Nature* **470**, 255-258.
Pick, K. S., Philippe, H., Schreiber, F., Erpenbeck, D., Jackson, D. J., Wrede, P., Wiens, M., Alié, A., Morgenstern, B., Manuel, M. et al. (2010). Improved phylogenomic taxon sampling noticeably affects nonbilaterian relationships. *Mol. Biol. Evol.* **27**, 1983-1987.
Podar, M., Haddock, S. H., Sogin, M. L. and Harbison, G. R. (2001). A molecular phylogenetic framework for the phylum Ctenophora using 18S rRNA genes. *Mol. Phylogenet. Evol.* **21**, 218-230.
Putnam, N. H., Srivastava, M., Hellsten, U., Dirks, B., Chapman, J., Salamov, A., Terry, A., Shapiro, H., Lindquist, E., Kapitonov, V. V. et al. (2007). Sea anemone genome reveals ancestral eumetazoan gene repertoire and genomic organization. *Science* **317**, 86-94.
Richards, G. S., Simionato, E., Perron, M., Adamska, M., Vervoort, M. and Degnan, B. M. (2008). Sponge genes provide new insight into the evolutionary origin of the neurogenic circuit. *Curr. Biol.* **18**, 1156-1161.
Rokas, A. (2013). Genetics. My oldest sister is a sea walnut? *Science* **342**, 1327-1329.
Ryan, J. F. (2014). Did the ctenophore nervous system evolve independently? *Zoology* **117**, 225-226.
Ryan, T. J. and Grant, S. G. (2009). The origin and evolution of synapses. *Nat. Rev. Neurosci.* **10**, 701-712.
Ryan, J. F., Pang, K., Mullikin, J. C., Martindale, M. Q. and Baxevanis, A. D. (2010). The homeodomain complement of the ctenophore *Mnemiopsis leidyi* suggests that Ctenophora and Porifera diverged prior to the Parahoxozoa. *EvoDevo* **1**, 9.
Ryan, J. F., Pang, K., Schnitzler, C. E., Koch, B. J., Nguyen, A.-D., Moreland, R. T., Mullikin, J. C., Wolfsberg, T. G., Martindale, M. Q. and Baxevanis, A. D. (2013a). The genome of the Ctenophore, *Mnemiopsis leidyi*: insights into the genetics of innovation and the evolution of multicellularity. *Integr. Comp. Biol. Abstracts*. Available at: http://www.sicb.org/meetings/2013/schedule/abstractdetails.php?id=1280
Ryan, J. F., Pang, K., Schnitzler, C. E., Nguyen, A. D., Moreland, R. T., Simmons, D. K., Koch, B. J., Francis, W. R., Havlak, P., Smith, S. A. et al.; NISC Comparative Sequencing Program (2013b). The genome of the ctenophore *Mnemiopsis leidyi* and its implications for cell type evolution. *Science* **342**, 1242592.
Sakharov, D. A. (1974). *Genealogy of Neurons*. Moscow: Nauka.
Samassa, I. (1892). Zur histologie der ctenophoren. *Archiv für Mikroskopische Anatomie* **40**, 157-243.
Satterlie, R. A. (2011). Do jellyfish have central nervous systems? *J. Exp. Biol.* **214**, 1215-1223.
Satterlie, R. A. and Case, J. F. (1978). Gap junctions suggest epithelial conduction within the comb plates of the ctenophore *Pleurobrachia bachei*. *Cell Tissue Res.* **193**, 87-91.
Schierwater, B., Eitel, M., Jakob, W., Osigus, H. J., Hadrys, H., Dellaporta, S. L., Kolokotronis, S. O. and Desalle, R. (2009). Concatenated analysis sheds light on early metazoan evolution and fuels a modern "urmetazoon" hypothesis. *PLoS Biol.* **7**, e20.
Schnitzler, C. E., Pang, K., Powers, M. L., Reitzel, A. M., Ryan, J. F., Simmons, D., Tada, T., Park, M., Gupta, J., Brooks, S. Y. et al. (2012). Genomic organization, evolution, and expression of photoprotein and opsin genes in Mnemiopsis leidyi: a new view of ctenophore photocytes. *BMC Biol.* **10**, 107.
Schnitzler, C. E., Simmons, D. K., Pang, K., Martindale, M. Q. and Baxevanis, A. D. (2014). Expression of multiple sox genes through embryonic development in thectenophore *Mnemiopsis leidyi* is spatially restricted to zones of cell proliferation. *EvoDevo* **5**, 15.
Sherwood, T. W., Frey, E. N. and Askwith, C. C. (2012). Structure and activity of the acid-sensing ion channels. *Am. J. Physiol.* **303**, C699-C710.
Shu, D. G., Morris, S. C., Han, J., Li, Y., Zhang, X. L., Hua, H., Zhang, Z. F., Liu, J. N., Guo, J. F., Yao, Y. et al. (2006). Lower Cambrian vendobionts from China and early diploblast evolution. *Science* **312**, 731-734.
Simmons, D. K., Pang, K. and Martindale, M. Q. (2012). Lim homeobox genes in the ctenophore *Mnemiopsis leidyi*: the evolution of neural cell type specification. *EvoDevo* **3**, 2.
Stanley, G. D. and Stürmer, W. (1983). The first fossil ctenophore from the lower Devonian of West Germany. *Nature* **303**, 518-520.
Stein, P. G. and Anderson, P. A. (1984). Maintenance of isolated smooth muscle cells of the ctenophore *Mnemiopsis*. *J. Exp. Biol.* **110**, 329-334.
Steinmetz, P. R., Kraus, J. E., Larroux, C., Hammel, J. U., Amon-Hassenzahl, A., Houliston, E., Wörheide, G., Nickel, M., Degnan, B. M. and Technau, U. (2012). Independent evolution of striated muscles in cnidarians and bilaterians. *Nature* **487**, 231-234.
Striedter, G. F., Belgard, T. G., Chen, C. C., Davis, F. P., Finlay, B. L., Güntürkün, O., Hale, M. E., Harris, J. A., Hecht, E. E., Hof, P. R. et al. (2014). NSF workshop report: discovering general principles of nervous system organization by comparing brain maps across species. *J. Comp. Neurol.* **522**, 1445-1453.
Tamm, S. L. (1973). Mechanisms of ciliary co-ordinations in ctenophores. *J. Exp. Biol.* **59**, 231-245.
Tamm, S. L. (1982). Ctenophora. In *Electrical Conduction and Behavior in "Simple" Invertebrates* (ed. G. A. B. Shelton), pp. 266-358. Oxford: Clarendon Press.

Tamm, S. L. (1984). Mechanical synchronization of ciliary beating within comb plates of ctenophores. *J. Exp. Biol.* **113**, 401-408.
Tamm, S. L. and Moss, A. G. (1985). Unilateral ciliary reversal and motor responses during prey capture by the ctenophore *Pleurobrachia*. *J. Exp. Biol.* **114**, 443-461.
Tamm, S. L. and Tamm, S. (1981). Ciliary reversal without rotation of axonemal structures in ctenophore comb plates. *J. Cell Biol.* **89**, 495-509.
Tamm, S. L. and Tamm, S. (1987). Massive actin bundle couples macrocilia to muscles in the ctenophore *Beroë*. *Cell Motil. Cytoskeleton* **7**, 116-128.
Tamm, S. and Tamm, S. L. (1995). A giant nerve net with multi-effector synapses underlying epithelial adhesive strips in the mouth of *Beroë* (Ctenophora). *J. Neurocytol.* **24**, 711-723.
Tamm, S. L. and Tamm, S. (2002). Novel bridge of axon-like processes of epithelial cells in the aboral sense organ of ctenophores. *J. Morphol.* **254**, 99-120.
Tang, F., Bengtson, S., Wang, Y., Wang, X. L. and Yin, C. Y. (2011). Eoandromeda and the origin of Ctenophora. *Evol. Dev.* **13**, 408-414.
Telford, M. J. (2009). Animal evolution: once upon a time. *Curr. Biol.* **19**, R339-R341.
Traynelis, S. F., Wollmuth, L. P., McBain, C. J., Menniti, F. S., Vance, K. M., Ogden, K. K., Hansen, K. B., Yuan, H., Myers, S. J. and Dingledine, R. (2010). Glutamate receptor ion channels: structure, regulation, and function. *Pharmacol. Rev.* **62**, 405-496.
Vinnikov, I. A. (1990). [Evolution of the locomotor-sensory systems]. *Arkh. Anat. Gistol. Embriol.* **99**, 5-25.
Wemmie, J. A., Chen, J., Askwith, C. C., Hruska-Hageman, A. M., Price, M. P., Nolan, B. C., Yoder, P. G., Lamani, E., Hoshi, T., Freeman, J. H., Jr et al. (2002). The acid-activated ion channel ASIC contributes to synaptic plasticity, learning, and memory. *Neuron* **34**, 463-477.
Whelan, N. V., Kocot, K. and Halanych, K. M. (2015). Resolving the metazoan tree of life with advanced bioinformatic pipelines and phylogenetic methods. *Integ. Comp. Biol. Abstracts*. Available at: www.sicb.org/meetings/2015/schedule/abstractdetails.php?id=851.