Chapter 16

Gap Junctions in Ctenophora

Andrea B. Kohn and Leonid L. Moroz

Abstract

Gap junction proteins form specialized intercellular communication channels, including electrical synapses, that regulate cellular metabolism and signaling. We present a molecular inventory of the gap junction proteins—innexins (INX-like) in ctenophores, focusing on two reference species, *Pleurobrachia bachei* and *Mnemiopsis leidyi*. Innexins were identified in more than 15 ctenophore species, including such genera as *Euplokamis*, *Pukia*, *Hormiphora*, *Bolinopsis*, *Cestum*, *Ocyropsis*, *Dryodora*, *Beroe*, benthic ctenophores, *Coeloplana* and *Vallicula*, and undescribed species of *Mertensiidae*. The observed diversity of innexins resulted from the independent expansion of this family from the common ancestor of ctenophores. Innexins show the conserved topology with four transmembrane domains connected by two extracellular loops, which bridge intracellular gaps. However, *INX*-like genes have highly diverse exon organization and low percentage identity for their amino acid sequences within the same species and between ctenophore species. Such a broad scope of molecular diversity differs from innexins in other phyla. We predicted posttranslational modifications in innexins: 249 and 188 for *M. leidyi* and *P. bachei,* respectively. Neither their number nor their locations were conserved within or between species. When the number of post-translational modifications is factored into the innexins' radiation, the potential for molecular and physiological diversity within gap junctions of ctenophores is almost unfathomable. RNA-seq and in situ hybridization data revealed that innexins are expressed across embryogenesis, including early cleavage stages and gastrulation. They are abundant in all adult tissues, with the highest expression level in the aboral organ (the major integrative center and the gravity sensor in ctenophores), followed by tentacles and comb plates. Nevertheless, each organ and tissue has a unique combination of innexins, suggesting their involvement in complex integrative functions and behaviors of ctenophores.

Key words Ctenophora, Innexin, Pannexin, Posttranslational modifications, *Pleurobrachia*, *Mnemiopsis*, Evolution, Phylogeny

1 Introduction

Gap junction proteins are critical components of electrical synapses, providing both fast electrical and metabolic coupling within cells across numerous animal tissues. Two distinct families of these channel proteins evolved convergently: pannexins (=innexins) and connexins [1–3]. Both families are metazoan innovations [1, 4, 5] with surprisingly similar membrane topologies [2, 4, 6].

Leonid L. Moroz (ed.), *Ctenophores: Methods and Protocols*, Methods in Molecular Biology, vol. 2757,
https://doi.org/10.1007/978-1-0716-3642-8_16,

Connexins are exclusively vertebrate- and tunicate-lineage-specific genes, whereas pannexins are present both in vertebrates and invertebrates. Before their discovery in humans, invertebrate pannexins were initially named innexins—i.e., invertebrate counterparts to gap junctions. Subsequently, Panchin and colleagues found innexin homologs in vertebrates and suggested a more general name for the entire protein superfamily as pannexins [1]. Nevertheless, in the literature, the term pannexins are usually employed in research on vertebrates, whereas a more traditional innexin terminology is widely preserved in references to invertebrates.

At this moment, neither innexins nor connexins were identified outside of animals. The sequenced genomes of choanoflagellates, other holozoans, and opisthokonts, including fungi, do not contain recognizable innexins. Thus, the origin of these two families in animal evolution is elusive, and studies of basal metazoan lineages are minimal.

The phylum Ctenophora represents the earliest branch on the animal tree of life—the ctenophore-sister hypothesis [7–11], which is strongly confirmed by the recent integrative genome analyses [12]. Thus, evolution of electrical synapses would be viewed within the phylogenetic framework of the ctenophore-first scenario. Gap junctions were experimentally discovered in ctenophores using ultrastructural [13–15] and functional [16] approaches in 1970–1980s [17, 18].

The initial characterization of the *Pleurobrachia bachei* genome revealed a dozen diverse genes encoding innexins [7] in this and ten other ctenophore species (by sequencing their transcriptomes). The sponge *Amphimedon* (representing the second branching metazoan lineage) and the placozoan *Trichoplax* (the third branch)—all lack recognized gap junction proteins. Thus, pannexins/innexins were present in the common ancestor of all animals and independently diversified in ctenophores [7], some cnidarians, and most bilaterians, as evidenced by the respected phylogenetic trees [4, 19–22]. In contrast, the lineages led to sponges and placozoans lost innexins [4, 7]. Notably, there are also multiple examples of gains and losses of innexins across Metazoa (*see* Fig. 1 and [23])—reasons why placozoans, some cnidarians, echinoderms, hemichordates, and lost innexins are unclear [24]. In basal chordates, functional innexins/pannexins were "substituted" by connexins [21], which can make equally efficient electrical synapses coupling various cell populations, including neurons.

Mechanistically, both gap junction families form hemichannels of contacting cells with similar molecular architecture [4, 25–27]: four transmembrane domains, one intracellular loop, and two extracellular loops (Figs. 2, 3, and 4). The connexin hemichannel comprises six subunits (connexon), whereas eight innexins (innexon) are required for such assembly [21]. Physically opposing hemichannels from coupling cells can be stabilized by disulfide

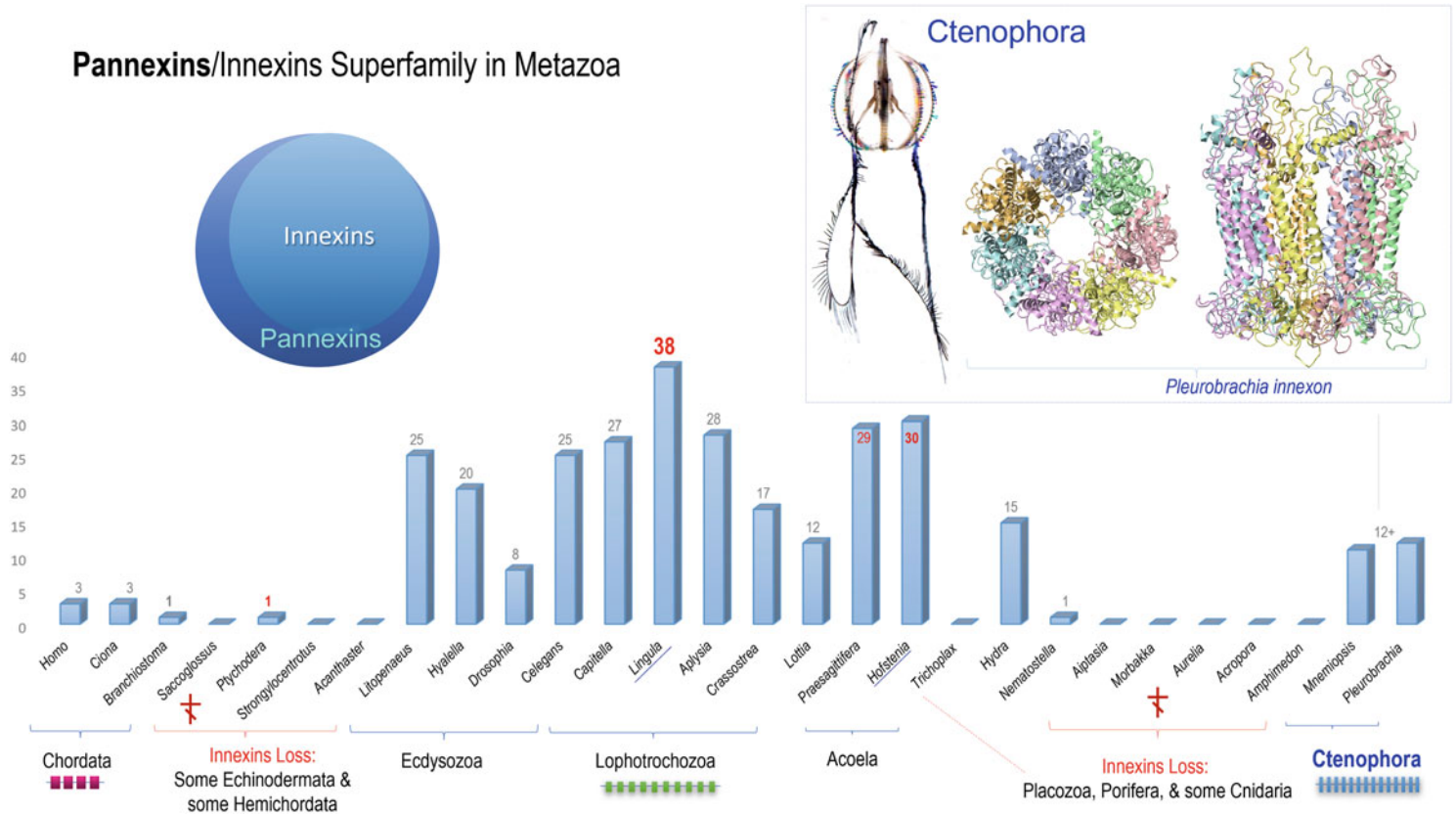

Fig. 1 The distribution of pannexin/innexin proteins across Metazoa with multiple examples of gene gains and losses. Y-axis indicates the presence and the number of genes encoding these gap junction proteins in selected reference species. Insert shows the three-dimensional structure of one innexin from the ctenophore *Pleurobrachia bachei* (photo). (Modified from Moroz et al. [23])

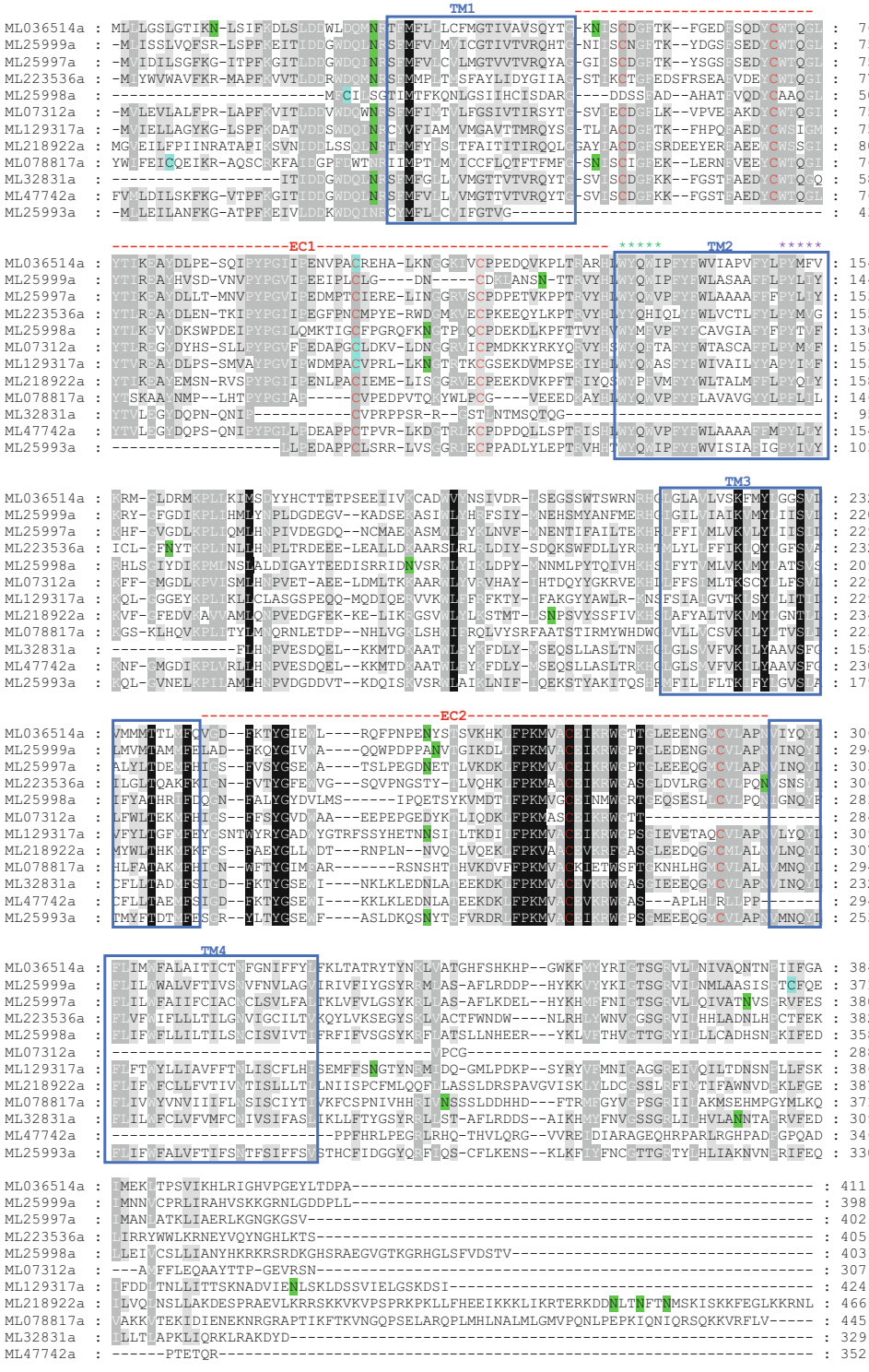

Fig. 2 Protein alignment of 12 innnexins in *Mnemiopsis leidyi*. Dark blue squares outline the four transmembrane domains (TM); the red dashes indicate the extracellular loops 1 and 2 EC1 and EC2. The conserved cysteine Cys residues are shown in red. The green highlights are N-glycosylation sites, and the turquoise highlights are S-nitrosylation sites. The green asterisks mark the "innexin sequence," and the blue asterisks correspond to the canonical "PXXXW" sequence within the second transmembrane domain

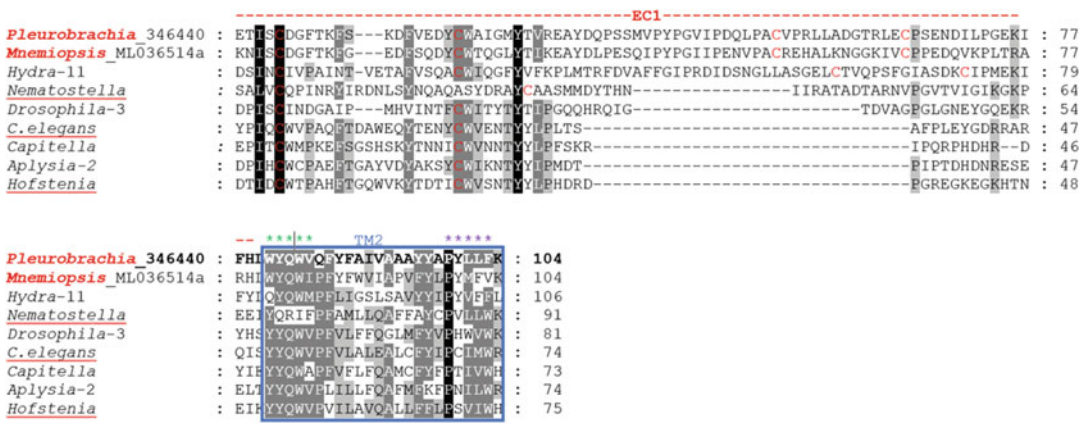

Fig. 3 Protein alignment of the extracellular loop, 1st and 2nd transmembrane domains innexins in representative species from different phyla. All sequences accession numbers are referenced in Table 2 for the phylogeny except for *C. elegans*, NP_001024407.1, *Hofstenia miamia*_g17543.t1, and *Capitella teleta*_ELT91918.1. The dark blue square outlines the TM2, and the red dashes indicate the extracellular loops 1. The conserved cysteine Cys residues are shown in red. The green asterisks indicate the "innexin sequence," and the blue asterisks indicate the "PXXXW" sequence motif within the TM2

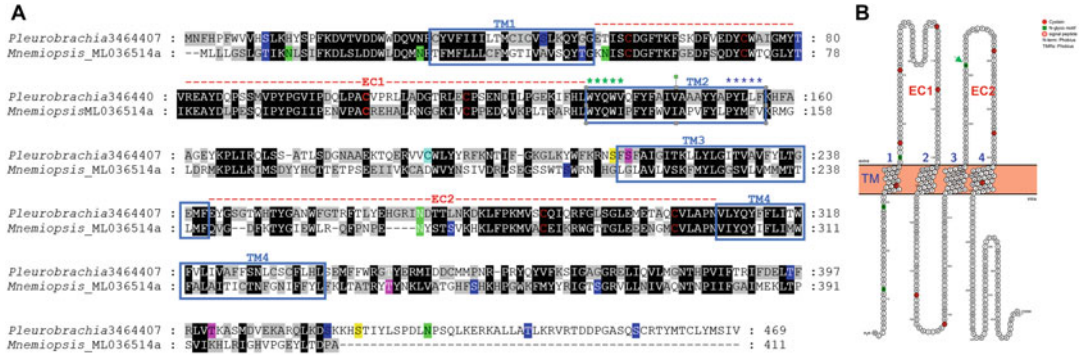

Fig. 4 (**a**) Protein alignment between representative innexins from *Pleurobrachia* (346440) and *Mnemiopsis* (ML036514a). Dark blue squares outline the four transmembrane domains (TM); the red dashes indicate the extracellular loops 1 and 2 EC1 and EC2. The conserved cysteine Cys residues are indicated in red. The green asterisks mark the "innexin sequence," and the blue asterisks correspond to the "PXXXW" sequence motif within the second transmembrane domain. The green highlights are N-glycosylation sites, the turquoise highlights are S-nitrosylation sites, the yellow highlights are S/T-cAMP- and cGMP-dependent protein kinase phosphorylation sites, and the bright blue highlights are S/T-protein kinase C phosphorylation sites and the pink highlights are S/T-protein kinase A phosphorylation sites. (**b**) Topographic representation of *Mnemiopsis* (ML036514a) with the Cys residues are indicated in red, and the PTMs are colored as described in (**a**) [47] (*See* Subheading 2)

bonds between cysteines in each extracellular loop [28]. The same cells might express multiple connexins and innexins, forming heteromeric hemichannels. Two opposing homo- and heteromeric connexons or innexons on different cells form a functional gap junction.

As a result of such combinatorial logic, N^6 and N^8 types of channels are possible for N subunits of genome-encoded connexins and innexins, respectively. It provides exceptional opportunities to generate enormous diversity of gap junction proteins and electrical synapses with different biophysical properties. For example, 14 and 12 innexin subunits, which we initially identified in two ctenophores (*Pleurobrachia* and *Mnemiopsis* [7, 19]), can theoretically form 1,475,789,056 and 429,981,696 different channels (14^8 or 12^8). It is astonishing numbers, but more likely, only a tiny fraction of possible combinations could exist in any given species yet provide a solid base for evolutionary selection.

Three pannexins identified in chordates do not form classical electrical synapses with coupling two hemichannels from contacting cells [21, 27, 29, 30]. Here, N-glycosylation prevents forming an actual conductance pore between two cells. Specifically, pannexins' first or second extracellular loop contains the Asn-X-Ser/Thr motif for asparagine glycosylation [21]. These sugars hinder intercellular gap junction formation by modifying the physical docking of the hemichannels. In other words, pannexins are membrane channels that mediate the release of low molecular weight compounds such as ATP [31, 32]. These single-channel properties might also justify the initial split in the channel terminology when the name of pannexins is preserved for chordates. At the same time, "innexins" refer to the homologous proteins in invertebrates.

More than 500 million years ago, pannexins had lost the ability to form gap junctions (due to N-glycosylation?) in ancestral chordates, which might have triggered an independent development of connexins (as an alternative or compensatory "route") and their subsequent expansion in vertebrates [21].

N-glycosylation of innexins with lots of gap junction functions is reported for some invertebrate species, such as insects (*Aedes aegypti)*, nematodes (*Caenorhabditis elegans*) [33], and predicted for mollusks (*Limax valentianus*), where large fractions of innexins are glycoproteins [21, 34]. N-glycosylation sites across innexins and species are not conserved (except vertebrates with a high level of evolutionary stabilization across taxa [21]). Whether N-glycosylation in innexins can hinder or modify actual functional electrical synapses between cells remains to be determined. At least seven innexins with N-glycosylation form gap junctions in annelids, nematodes, and insects [21]. The dynamic occurrence of sugar moieties in both pannexins and innexins subunits further expands the diversity and properties of this unique class of channels associated with rapid intercellular signaling. Yet, it is well-known that "the experimental identification of N-glycosylated proteins is technically demanding, time-consuming, and expensive" [21]. Thus, computational approaches are more favorable as the first steps for many comparative studies.

This chapter summarizes the presence and distribution of innexins in representatives of the most basally branching animal phylum—Ctenophora. This ancestral lineage is of utmost importance for deciphering the origin and evolution of animals and their tissue and organ innovations, including likely independent origins and evolution of neuromuscular, sensory, and digestive systems [7, 8, 16, 18, 35, 36]. Decoding complex behaviors and reconstructing the parallel evolution of many integrative systems in this clade [15–24] are linked with a deep understanding of gap junctions and their functional plasticity across multiple ctenophore species with distinct ecological adaptations. We focus on practical implementations in analyses of innexins from two reference species of ctenophores [7, 8]: the cydippid *Pleurobrachia bachei* and *Mnemiopsis leidyi*, with brief references to other ctenophores.

2 Methods

- All sequences were identified using BLAST searches on publicly available databases, including species-specific genome browsers with cutoff values of E-value $\leq 10^{-4}$.
- All *Pleurobrachia bachei* data was assembled, annotated, and available on the University of Florida Database: http://neurobase.rc.ufl.edu/.
- All *P. bachei* gene models used in the analysis were published and described here http://neurobase.rc.ufl.edu/ [7].
- *M. leidyi* gene models were obtained at http://research.nhgri.nih.gov/mnemiopsis/jbrowse/jbrowse.cgi [8].
- Alignments of all orthologs were performed with either ClustalX2 [37] or Muscle [38] and then, if appropriate, either trimmed manually or trimmed using GBlocks.
- Further comparative analysis of individual gene families used Pfam composition [39], Gene Ontology [40], and KEGG (Kyoto Encyclopedia of Genes and Genomes) [41] to validate orthologs.
- Once alignments were obtained, basic gene trees were constructed in MEGA 6 [42]. The molecular phylogenetic analysis used the maximum likelihood (ML) method based on the Whelan and Goldman (W.A.G.) matrix-based model.
- The bootstrap consensus tree was inferred from 100 replicates. All positions containing gaps and missing data were eliminated [42]. The tree is drawn to scale.
- We screened potential serine/threonine/tyrosine phosphorylation sites from 17 different kinases using the NetPhos 3.1Server,

https://services.healthtech.dtu.dk/service.php?NetPhos-3.1 [43].

- In addition, we used the PROSITE database, https://prosite.expasy.org [44], to validate and identify N-glycosylation sites.
- SMART (Simple Modular Architecture Research Tool) was used to identify protein domain architectures, http://smart.embl-heidelberg.de/ [45].
- N-glycosylation sites were also identified using GPS-SNO1.0 http://sno.biocuckoo.org/ [46].
- The Protter web application was used to visualize the sequence topology http://wlab.ethz.ch/protter/# [47].

3 Predictions, Analyses, and Discussion

3.1 Identification of Putative Innexin-Like Proteins and Their Topology

Using reported transcriptome databases [7, 11], we identify comparable diversity of innexins in more than 15 ctenophore species, including such genera as *Euplokamis*, *Pleurobrachia*, *Pukia*, *Hormiphora*, *Bolinopsis*, *Mnemiopsis*, *Cestum*, *Ocyropsis*, *Dryodora*, *Beroe*, benthic ctenophores, *Coeloplana* and *Vallicula*, and undescribed species of *Mertensiidae* (*see* Supplement).

Twelve putative innexin-like (INX) genes were initially reported in the *P. bachei* genome, and all were found in transcriptomes [7]. Upon further examination, at least 14 *INX*-like genes might occur in *Pleurobrachia*, and 12 genes exist in the *Mnemiopsis* genome [7, 19], as summarized in Table 1.

The deduced protein sequence alignment for all *Mnemiopsis* innexins shows four transmembrane domains (T.M.) connected by two extracellular (EC) loops that bridge the intracellular gap (Figs. 2 and 4b). The signature "innexin sequence" (green asterisk) Y.Y. (X)W(Z), where X is Q, R, M, E, or S and Z is V, M, A, I, S, or T, found at the beginning of the second transmembrane domain (TM2) is not conserved in the investigated ctenophore innexins and in the hydrozoan *Hydra magnipapillata* [48] (Figs. 2 and 3). The ctenophore uniqueness is true for the PXXXW sequence (blue asterisk) at the end of the second transmembrane domain. The tryptophan (W) amino acid is "missing" in all ctenophore and *Hydra* INX-like proteins examined. Still, the proline amino acid residue is present (Figs. 2 and 3). Studies suggest that this proline residue acts like a molecular hinge to change the conformational structure in response to voltage [2].

The cysteine (Cys) amino acid residues (red letter C) in the extracellular loops are highly conserved in all innexin sequences [2, 26]. Surprisingly, two conserved pairs of Cys are in the first EC1 of both *Pleurobrachia* and *Mnemiopsis* (Figs. 2, 3, and 4). Only one other studied non-bilaterian contains two pairs of Cys

Table 1
Summary of the number of the most common predicted posttranslational modifications in *Mnemiopsis* and *Pleurobrachia* deduced INX-like proteins

Menmiopsis	# Exons	Protein kinase C (PKC)	Protein kinase A (PKA)	Casein kinase 1 (CK1)	Casein kinase 2 (CK2)	cAMP PHOSPHO	N-glycosylation	S-nitrosylation	Total
ML036514a	8	7	1	2	3	0	4	1	**18**
ML25999a	17	7	0	1	5	0	3	1	**17**
ML25997a	18	7	1	1	7	0	3	0	**19**
ML223536a	4	6	2	0	6	1	4	0	**19**
ML25998a	8	7	2	0	6	1	2	1	**19**
ML07312a	9	4	1	1	5	0	1	1	**13**
ML129317a	1	6	2	0	7	1	4	1	**21**
ML218922a	8	12	5	1	15	3	6	0	**42**
ML078817a	2	12	2	0	2	1	2	1	**20**
ML32831a	6	7	4	0	8	2	2	1	**24**
ML47742a	8	12	2	2	8	3	1	0	**28**
ML25993a	12	4	2	0	2	0	1	0	**9**
Total		**91**	**24**	**8**	**74**	**12**	**33**	**7**	**249**
Pleurobrachia									
3464407	3	7	2	1	4	2	2	1	**19**
3463885	14	11	1	1	10	1	3	0	**27**
3468487	1	2	1	0	4	1	1	0	**9**
3469267	9	5	1	0	9	0	2	0	**17**
3471871	3	2	1	0	3	0	1	0	**7**
3473233	1	2	1	0	0	0	1	0	**4**
3479642	8	2	1	0	1	0	3	0	**7**
3464250	11	8	3	0	7	1	4	0	**23**
3465454	4	8	1	0	5	0	2	0	**16**
3465668	8	4	2	0	5	1	0	0	**12**
3465979	8	3	0	0	4	1	2	1	**11**
3466205	9	4	1	0	8	0	0	1	**14**
3466313	1	2	4	0	7	0	1	1	**15**
3468319	5	3	0	0	4	0	0	0	**7**
Total		**63**	**19**	**2**	**71**	**7**	**22**	**4**	**188**

Totals are in blue
Number of exons are also noted in the corresponding *INX*-like genes

residues in the EC1 loop of their predicted innexins—*Hydra* [48, 49] (Fig. 3).

The first pair of Cys residues located in the EC1 loop is highly conserved in cnidarians and bilaterians, including the acoel *Hofstenia miamia* (Fig. 3). Interestingly, the cnidarian *Nematostella vectensis* has only one pair of Cys residues in its EC1 loop (Fig. 3). The Cys residues in the EC1 and EC2 loops are critical to forming functional channels [2].

The EC1 loop in ctenophores and *Hydra* is larger (~75 amino acid residues, Fig. 3) than other innexins described (~60 amino acid residues) [2]. Such a larger EC1 loop may accommodate the extra pair of Cys residues in the EC1. Of note, in three *Mnemiopsis* innexins, one of the extra Cys can be a site for the predicted

posttranslational modification, S-nitrosylation (Fig. 4). It is consistent with the hypothesis that the gaseous messenger, nitric oxide (NO) might be a signal molecule in ctenophores [19, 50], where S-nitrosylation is one of the molecular targets for nitrergic transmission (potentially associated with NO-dependent modulation of electrical coupling across diverse cell populations in development and adult tissues).

The overall innexins' structure is similar between *P. bachei* and *M. leidyi*, including the three pairs of Cys residues (Fig. 4). But *posttranslational modifications* across innexins are not highly conserved (neither within the same species nor between these two ctenophore species).

3.2 Predicted Posttranslational Modifications (PTMs) in Innexin-Like Proteins

It has been shown that one possible PTM can alter the formation of functional gap junctions [51], so we investigated the most conserved predicted PTMs in innexins of *Pleurobrachia* and *Mnemiopsis*. To date, more than 400 different PTMs are possible [52]. Figures 2, 3, and 4 indicate potential N-glycosylation, S-nitrosylation sites, and phosphorylation (Fig. 4), and Table 1 lists the most conservative innexin modifications. Although no functional tests have been performed on any specific ctenophore innexin, PTMs suggest enormous ramifications to function [51].

The number of putative N-glycosylation sites varies considerably for *P. bachei* and *M. leidyi*, from six to zero (Table 1). The location within sequences also is inconsistent from the intracellular region N- and C-termini to the extracellular loops (Fig. 4). One N-glycosylation in the N-terminus, N28 in ML036514a, was relatively conserved across *Mnemiopsis* innexins (Fig. 2) but not in *Pleurobrachia* (Fig. 3a).

The predicted S-nitrosylation sites were far less abundant, with seven out of 12 innexins having a single S-nitrosylation site in *M. leidyi*. In contrast, only four out of 14 innexins had an S-nitrosylation site in *P. bachei*. More S-nitrosylation, potentially regulatory sites in *Mnemiopsis* might be associated with the presence of nitric oxide synthase and endogenous nitrergic transmission [50].

We also predicted the phosphorylation sites for multiple kinases (e.g., we chose a conservative set of kinases: protein kinase C (PKC), protein kinase A (PKA), casein kinase 1 (CK1), casein kinase 2 (CK2), cAMP- and cGMP-dependent protein kinase, etc.). The *Mnemiopsis* ML036514a could have 82 serine, threonine, or tyrosine phosphorylation sites from 17 different kinases (ATM, CKI, CKII, CaM-II, DNAPK, EGFR, GSK3, INSR, PKA, PKB, PKC, PKG, RSK, SRC, cdc2, cdk5, and p38MAPK), and 45 are above the threshold of probability [43]. Phosphorylation by these kinases regulates many protein functions in vertebrate connexins/pannexins, including intracellular trafficking, gap junction assembly, and stability [25, 51]. The number of phosphorylation

sites from this small cohort of enzymes ranged from 3 to 27 in a single innexin. The total number of predicted PTMs in innexins in is 249 and 188 for *M. leidyi* and *P. bachei,* respectively (Table 1).

The aligned *Pleurobrachia* and *Mnemiopsis* innexins had seven predicted PKC sites; however, only two were conserved (Fig. 4). Similarly, only one N-glycosylation site was conserved between these two species (Fig. 4).

In summary, the post-translation modifications in different innexins are not conserved within the same species or between *P. bachei* and *M. leidyi*. This situation contrasts with the molluscan innexins (e.g., from *Limax valentianus* species and across the entire molluscan phylum [34]). PTMs in molluscan innexins are likely conserved, because their amino acid sequences are highly conserved too. On the other hand, the % identity between innexins for *Pleurobrachia*_346440 and *Mnemiopsis*_ML036514a (Fig. 3) is only 38%. Besides low sequence identity, the genomic organization of innexin genes in ctenophores is highly variable, with the number of exons ranging from 14 to 1 for *P. bachei* and 17 to 1 for *M. leidyi* (Table 1).

3.3 Phylogenomic Analysis of PANX/INX-Like Predicted Proteins from Metazoans

As noted, gap junctions are metazoan innovations [1, 4, 24], essential for the origin and integration of multicellular organization. Genealogical relationships among pannexin/innexin illustrate multiple events of phylum-specific radiation of these channel proteins. Besides metazoans, *INX-like* genes have been found in a few viruses, possibly resulting from lateral gene transfer between a host and intracellular parasites [53]. For example, the endoparasitic wasps *Campoletis sonorensis* transmits a polydnavirus to their caterpillars during egg-laying [53]. The sequenced ichnovirus (IV) genome reveals a significant similarity to some parts of the wasp genome, including *INX* genes [53]. Predictably, these ichnovirus INX are closely related to innexins from arthropods.

Ctenophore innexins also exhibited a remarkable independent evolutionary diversification [19, 21], forming a distinct branch on the tree topology (e.g., Fig. 5 and Table 2). There are only four close sister-type relationships between *Pleurobrachia* and *Mnemiopsis*, red lines in Fig. 5. The low % identity found between the predicted amino acid sequences may result from early diversification of ctenophore innexins. There is a higher identity (44%) between the *Drosophila* INX-2 protein and the endoparasitic wasps *Campoletis sonorensis* INX-like d1 predicted sequences than between two *Pleurobrachia*_ 3464407 and *Pleurobrachi*a_ 3463885 INX-like predicted sequences.

Interestingly, there is no conservation in the exon–intron organization across *innexin/pannexin* genes [19]. The *pannexins* identified in mammals have four to five exons. However, the ctenophore *innexins* contain a far greater range of exons compared to their

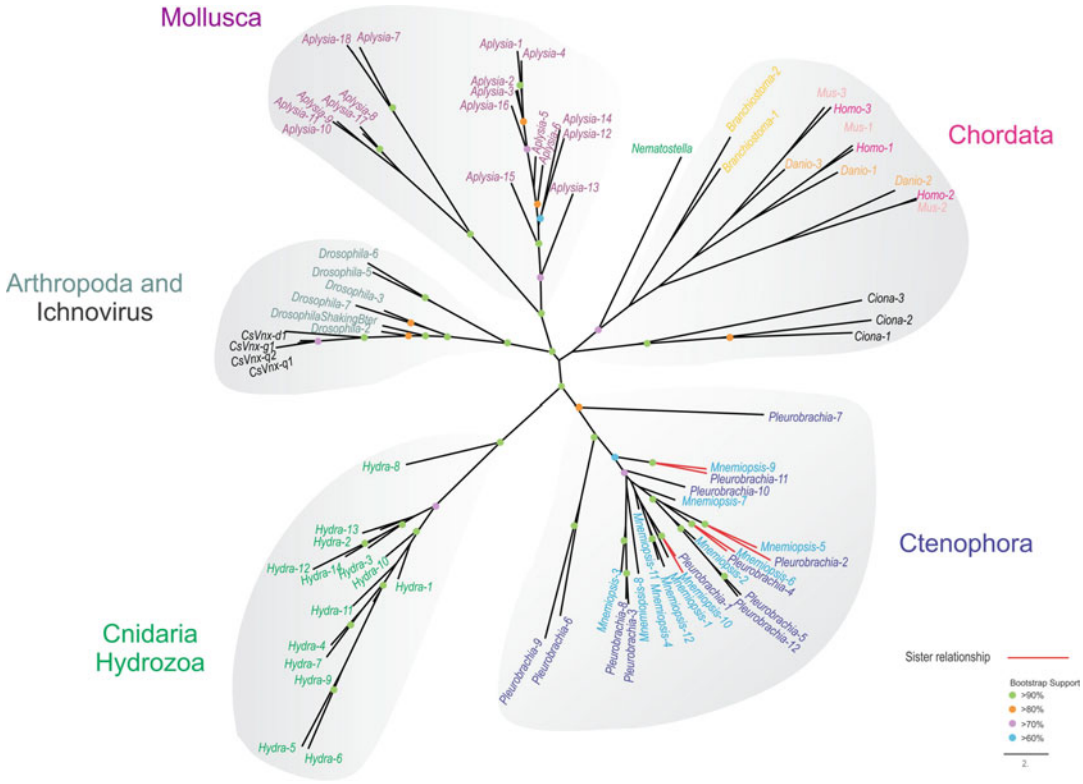

Fig. 5 Genealogical relationships among the putative protein family pannexin/innexin (PANX/INX) in metazoans (maximum likelihood method based on the Whelan and Goldman matrix-based model). The PANX/INX family shows lineage-specific diversification in major metazoan clades. Ctenophore PANX/INX proteins also share the highest identity, forming a distinct branch on the tree topology. Four close sister-type relationships exist between *Pleurobrachia* and *Mnemiopsis*, red lines. *See* Table 2 for names and accession numbers used in the analysis (*see* Subheading 2)

orthologs in Hydrozoa and bilaterians (the number of exons varies from 1 to 17, Table 1, *see* also Fig. 1) [19].

Cnidarians appear to have either dramatic losses or lineage-specific expansions of the *INX* genes (Fig. 1). The hydrozoan *Hydra* has at least 14 *INX*-like genes or even more [48]. In contrast, the anthozoan *Nematostella* has only one recognized *INX-like* gene (Fig. 5), possibly involved in the electrical coupling between blastomeres in embryos [54]. Moreover, the genomes of three other anthozoans, *Aiptasia* [55], *Acropora digitifera* [56], and *Stylophora pistillata* [57], had no *INX* genes detected. The scyphozoan *Cyanea capillata* also has no identified *INX* genes (based on our transcriptome profiling).

In summary, ctenophore innexins exhibited extraordinary phylum-specific diversification compared to other metazoans. The extreme range in the number of exons (1–17) and lack of conservation in protein identity, even between the same species, make the

Table 2
List of species accession numbers and names used on the tree in Fig. 5 to test genealogical relationships pannexin/innexin families in metazoans

Species	Database #/accession #	Name in tree
Mnemiopsis leidyi	ML036514a	*Mnemiopsis*-1
Mnemiopsis leidyi	ML07312a	*Mnemiopsis*-2
Mnemiopsis leidyi	ML078817a	*Mnemiopsis*-3
Mnemiopsis leidyi	ML129317a	*Mnemiopsis*-4
Mnemiopsis leidyi	ML218922a	*Mnemiopsis*-5
Mnemiopsis leidyi	ML223536a	*Mnemiopsis*-6
Mnemiopsis leidyi	ML25993a	*Mnemiopsis*-7
Mnemiopsis leidyi	ML25997a	*Mnemiopsis*-8
Mnemiopsis leidyi	ML25998a	*Mnemiopsis*-9
Mnemiopsis leidyi	ML25999a	*Mnemiopsis*-10
Mnemiopsis leidyi	ML32831a	*Mnemiopsis*-11
Mnemiopsis leidyi	ML47742a	*Mnemiopsis*-12
Pleurobrachia bachei	3463885	*Pleurobrachia*-1
Pleurobrachia bachei	3464250	*Pleurobrachia*-2
Pleurobrachia bachei	3464407	*Pleurobrachia*-3
Pleurobrachia bachei	3465454	*Pleurobrachia*-4
Pleurobrachia bachei	3465668	*Pleurobrachia*-5
Pleurobrachia bachei	3465979	*Pleurobrachia*-6
Pleurobrachia bachei	3466205	*Pleurobrachia*-7
Pleurobrachia bachei	3466313	*Pleurobrachia*-8
Pleurobrachia bachei	3468319	*Pleurobrachia*-9
Pleurobrachia bachei	3471871	*Pleurobrachia*-10
Pleurobrachia bachei	3468487	*Pleurobrachia*-11
Pleurobrachia bachei	3479642	*Pleurobrachia*-12
Homo sapiens	NP_056183.2	*Homo-1*
Homo sapiens	NP_443071.2	*Homo-2*
Homo sapiens	NP_443071.3	*Homo-3*
Mus musculus	NP_062355.2	*Mus-1*
Mus musculus	NP_443191.1	*Mus-2*
Mus musculus	NP_766042.2	*Mus-3*
Danio rerio	NP_957210.1	*Danio-1*

(continued)

Table 2
(continued)

Species	Database #/accession #	Name in tree
Danio rerio	NP_001243570.1	*Danio-2*
Danio rerio	XP_001919861.1	*Danio-3*
Ciona intestinalis	XP_002124490.2	*Ciona-1*
Ciona intestinalis	XP_002119287.2	*Ciona-2*
Ciona intestinalis	XP_009858431.1	*Ciona-3*
Nematostella vectensis	XP_001623899.1	*Nematostella*
Branchiostoma floridae	XP_002604233.1	*Branchiostoma-1*
Branchiostoma floridae	XP_002585873.1|	*Branchiostoma-2*
Drosophila melanogaster	NP_001162684.1	*Drosophila-2*
Drosophila melanogaster	NP_524730.1	*Drosophila-3*
Drosophila melanogaster	NP_573353.2	*Drosophila-5*
Drosophila melanogaster	NP_572374.1	*Drosophila-6*
Drosophila melanogaster	NP_788872.1	*Drosophila-7*
Drosophila melanogaster	NP_728361.1	*Drosophila-8*
Campoletis sonorensis	AAO45828.1	*CsVnx-d1*
Campoletis sonorensis	AAO45831.1	*CsVnx-q2*
Campoletis sonorensis	AAO45830.1	*CsVnx-q1*
Campoletis sonorensis	AAO45829.1	*CsVnx-g1*
Aplysia californica	NP_001191577.1	*Aplysia-1*
Aplysia californica	NP_001191579.1	*Aplysia-2*
Aplysia californica	NP_001191578.1	*Aplysia-3*
Aplysia californica	NP_001191576.1	*Aplysia-4*
Aplysia californica	NP_001191595.1	*Aplysia-5*
Aplysia californica	NP_001191594.1	*Aplysia-6*
Aplysia californica	NP_001191616.1	*Aplysia-7*
Aplysia californica	NP_001191596.1	*Aplysia-8*
Aplysia californica	NP_001191461.1	*Aplysia-9*
Aplysia californica	NP_001191462.1	*Aplysia-10*
Aplysia californica	XP_005110953.1	*Aplysia-11*
Aplysia californica	XP_005100630.1	*Aplysia-12*
Aplysia californica	XP_005109439.1	*Aplysia-13*

(continued)

Table 2
(continued)

Species	Database #/accession #	Name in tree
Aplysia californica	XP_005110669.1	*Aplysia-14*
Aplysia californica	XP_005103400.1	*Aplysia-15*
Aplysia californica	XP_005104811.1	*Aplysia-16*
Aplysia californica	XP_005110954.1	*Aplysia-17*
Aplysia californica	XP_005101166.1	*Aplysia-18*
Hydra vulgaris	NP_001274699.1	*Hydra-1*
Hydra vulgaris	XP_002160488.1	*Hydra-2*
Hydra vulgaris	XP_002166931.2	*Hydra-3*
Hydra vulgaris	XP_004213297.2	*Hydra-4*
Hydra vulgaris	XP_004212712.1	*Hydra-5*
Hydra vulgaris	XP_004212713.1	*Hydra-6*
Hydra vulgaris	XP_012561363.1	*Hydra-7*
Hydra vulgaris	XP_004208200.2	*Hydra-8*
Hydra vulgaris	XP_002170241.2	*Hydra-9*
Hydra vulgaris	XP_002164718.2	*Hydra-10*
Hydra vulgaris	XP_012566780.1	*Hydra-11*
Hydra vulgaris	XP_002165350.1	*Hydra-12*
Hydra vulgaris	XP_012554059.1	*Hydra-13*
Hydra vulgaris	XP_002170247.1	*Hydra-14*

ctenophore lineage unique. Although functional analysis of the electrical synapses in ctenophores is in its infancy, it will help to shed light on the contribution of this unusual complement of innexins associated with rapid intracellular signaling [16, 19, 58] and volume transmission (e.g., when N-nitrosylation or other post-translational modifications prevent to form electrical synapses—*see* also [23]).

3.4 Expression of Predicted INX-Like Proteins in Ctenophores

Ideally, innexins' temporal and spatial distribution is performed using both in situ hybridization and RNA-seq analyses. These two approaches are complementary; we applied them as illustrated in Figs. 6 and 7. Of note, because of more tough organization and texture, species of *Pleurobrachia* are ideal models for in situ hybridization studies, especially in adult stages. In contrast, adult *Mnemiopsis* and related lobate species are so fragile that even initial

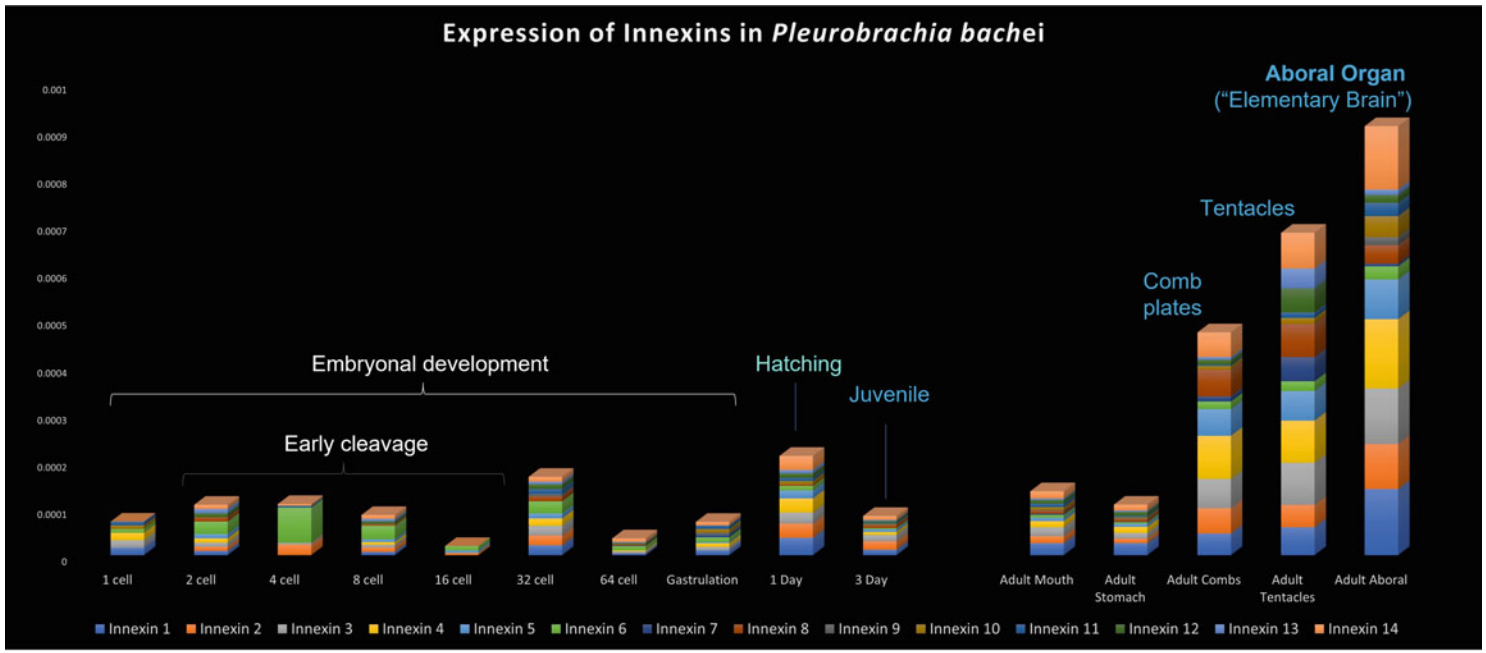

Fig. 6 RNA-seq analysis of innexin expression during development and in adult organs of *Pleurobrachia bachei*. The aboral organ has the greatest diversity and highest combined expression levels of 14 innexins, suggesting elaborated and diverse signaling pathways in this complex integrative organ—an analog of an elementary brain in ctenophores. Different innexins are co-expressed in every adult structure and developmental stage tested (it is shown as a summation of normalized frequencies of respective sequencing reads in each RNA-seq dataset; *y* axis is "expression frequency"). (Modified from Moroz et al. [7])

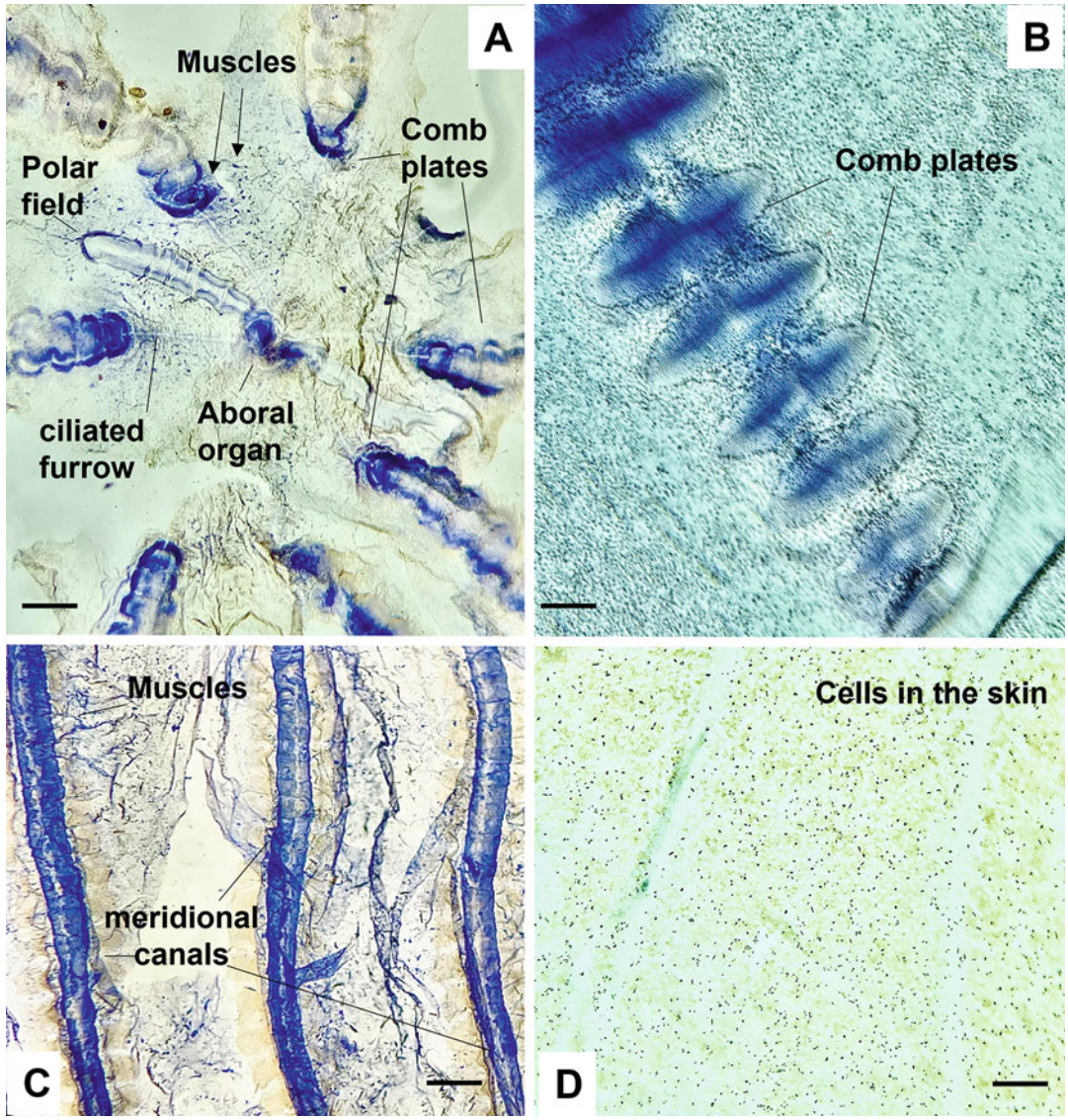

Fig. 7 Illustrative examples of spatial expression of four innexins in adult *Pleurobrachia bachei* (in situ hybridization). (**a**) The aboral view shows a specific expression of innexin 1 in the aboral organ, combs, ciliated furrows, and polar fields. Arrows indicate differential expression in selected muscle cells in subepithelial regions. (**b**) Particular expression of innexin 2 in comb plates. (**c**) Innexin 5 is differentiated and expressed in meridional canals underlying the comb rows (unstained). (**d**) Expression of innexin 3 in skin cells. Scale: 400 μm (**a**, **c**); 80 μm (**b**, **d**)

fixation of adult tissues is highly challenging, with rapid dissociation of virtually every structure restricting expression analysis to the earlier developmental stages only.

Multiple innexins are highly co-expressed in the adult aboral organ of *P. bachei* (Figs. 6 and 7a), also known as the major integrative center and the gravity sensor in ctenophores [18, 35,

58]. Most innexins are co-localized in combs, conductive tracts, mouth, pharynx, stomach (Fig. 6), and some neuronal-like subepithelial cells (Fig. 7d).

In ctenophores, gap junctions were previously identified by electron microscopy in the ciliated grooves, which run from the apical organ to the first comb plate of each comb row, and in comb plates themselves, as well as through the endoderm of the meridional canals [16], suggesting that the primary form of electrical coupling. Using in situ hybridization, we also detected expression of innexins in the ciliated furrows as well as in the polar fields (putative chemosensory structures) as well as selected subsets of subepithelial muscles and meridional canals (Fig. 7), consistent with earlier electron microscopy studies [13–15, 35].

Gap junctions also allow the direct exchange of small molecules among neighboring cells and sometimes form channel aggregates in the plasma membrane that might explain the expression of selected types of innexins during *Pleurobrachia* development. RNA-seq data revealed that distinct sets of innexins are expressed across embryogenesis, including early cleavage stages and gastrulation, with all identified innexins expressed at hatching. (Fig. 6).

4 Conclusion

Studies of the distribution and functions of innexins across different ctenophores are still in infancy, with more questions than answers. This family of gap junction proteins underwent extensive parallel evolution, and many preserved features might be ancestral to metazoans. Yet, the biodiversity of ctenophores in the context of gap junctions and other functional modules remains unexplored, and we anticipate an array of discoveries in this frontier of comparative biology.

Ctenophore innexins might be the most divergent gap junction protein subfamily compared to other metazoan innexins subfamilies with elaborated functional diversification and plasticity, which are predictably comparable, if not exceed, those observed in *C. elegans* [59]. When the number of PTMs is considered, the functional potential for enormous diversity within innexins of ctenophores is almost unfathomable.

Although innexins are broadly expressed across most structures, each organ and tissue has a unique combination of innexins, suggesting their involvement in complex integrative functions and behaviors of ctenophores.

Acknowledgments

This work was supported in part by the Human Frontiers Science Program (RGP0060/2017) and National Science Foundation (IOS-1557923) grants to LLM. Research reported in this publication was also supported in part by the National Institute of Neurological Disorders and Stroke of the National Institutes of Health under Award Number R01NS114491 (to LLM). The content is solely the authors' responsibility and does not necessarily represent the official views of the National Institutes of Health.

References

1. Panchin YV (2005) Evolution of gap junction proteins--the pannexin alternative. J Exp Biol 208(Pt 8):1415–1419
2. Phelan P (2005) Innexins: members of an evolutionarily conserved family of gap-junction proteins. Biochim Biophys Acta 1711(2): 225–245
3. Guiza J et al (2018) Innexins: expression, regulation, and functions. Front Physiol 9:1414
4. Abascal F, Zardoya R (2013) Evolutionary analyses of gap junction protein families. Biochim Biophys Acta 1828(1):4–14
5. Connors BW, Long MA (2004) Electrical synapses in the mammalian brain. Annu Rev Neurosci 27:393–418
6. Skerrett IM, Williams JB (2017) A structural and functional comparison of gap junction channels composed of connexins and innexins. Dev Neurobiol 77(5):522–547
7. Moroz LL et al (2014) The ctenophore genome and the evolutionary origins of neural systems. Nature 510(7503):109–114
8. Ryan JF et al (2013) The genome of the ctenophore *Mnemiopsis leidyi* and its implications for cell type evolution. Science 342(6164): 1242592
9. Li Y et al (2021) Rooting the animal tree of life. Mol Biol Evol 38(10):4322–4333
10. Whelan NV et al (2015) Error, signal, and the placement of Ctenophora sister to all other animals. Proc Natl Acad Sci U S A 112(18): 5773–5778
11. Whelan NV et al (2017) Ctenophore relationships and their placement as the sister group to all other animals. Nat Ecol Evol 1(11): 1737–1746
12. Schultz DT et al (2023) Ancient gene linkages support ctenophores as sister to other animals. Nature 618(7963):110–117
13. Hernandez-Nicaise ML, Amsellem J (1980) Ultrastructure of the giant smooth muscle fiber of the ctenophore *Beroe ovata*. J Ultrastruct Res 72(2):151–168
14. Hernandez-Nicaise ML, Nicaise G, Malaval L (1984) Giant smooth muscle fibers of the ctenophore *Mnemiopsis leidyi*: ultrastructural study of in situ and isolated cells. Biol Bull 167:210–228
15. Hernandez-Nicaise ML, Nicaise G, Reese TS (1989) Intercellular junctions in ctenophore integument. In: Anderson PAV (ed) Evolution of the first nervous systems. Plenum Press, New York, pp 21–32
16. Satterlie RA, Case JF (1978) Gap junctions suggest epithelial conduction within the comb plates of the ctenophore *Pleurobrachia bachei*. Cell Tissue Res 193(1):87–91
17. Horridge GA (1974) Recent studies on the Ctenophora. In: Muscatine L, Lenhoff HM (eds) Coelenterate biology. Academic Press, New York, pp 439–468
18. Tamm SL (1982) Ctenophora. In: Electrical conduction and behavior in "simple" invertebrates. Clarendon Press, Oxford, pp 266–358
19. Moroz LL, Kohn AB (2016) Independent origins of neurons and synapses: insights from ctenophores. Philos Trans R Soc Lond Ser B Biol Sci 371(1685):20150041
20. Moroz LL, Romanova DY (2022) Alternative neural systems: what is a neuron? (ctenophores, sponges and placozoans). Front Cell Dev Biol 10:1071961
21. Welzel G, Schuster S (2022) Connexins evolved after early chordates lost innexin diversity. elife 11:e74422
22. Yen MR, Saier MH Jr (2007) Gap junctional proteins of animals: the innexin/pannexin superfamily. Prog Biophys Mol Biol 94(1–2): 5–14
23. Moroz LL, Romanova DY, Kohn AB (2021) Neural versus alternative integrative systems: molecular insights into origins of

neurotransmitters. Philos Trans R Soc Lond Ser B Biol Sci 376(1821):20190762

24. Slivko-Koltchik GA, Kuznetsov VP, Panchin YV (2019) Are there gap junctions without connexins or pannexins? BMC Evol Biol 19(1):46
25. Pogoda K et al (2016) Regulation of gap junction channels and hemichannels by phosphorylation and redox changes: a revision. BMC Cell Biol 17(Suppl 1):11
26. Oshima A (2017) Structure of an innexin gap junction channel and cryo-EM sample preparation. Microscopy (Oxf) 66(6):371–379
27. Mikalsen SO, Kongsstovu SÍ, Tausen M (2021) Connexins during 500 million years-from cyclostomes to mammals. Int J Mol Sci 22(4):1584
28. Dahl G et al (1991) Cell/cell channel formation involves disulfide exchange. Eur J Biochem 197(1):141–144
29. Dahl G, Muller KJ (2014) Innexin and pannexin channels and their signaling. FEBS Lett 588(8):1396–1402
30. Wang J et al (2014) The membrane protein Pannexin1 forms two open-channel conformations depending on the mode of activation. Sci Signal 7(335):ra69
31. Chiu YH, Ravichandran KS, Bayliss DA (2014) Intrinsic properties and regulation of Pannexin 1 channel. Channels (Austin) 8(2):103–109
32. Dahl G (2015) ATP release through pannexon channels. Philos Trans R Soc Lond Ser B Biol Sci 370(1672):20140191
33. Sangaletti R, Dahl G, Bianchi L (2014) Mechanosensitive unpaired innexin channels in *C. elegans* touch neurons. Am J Physiol Cell Physiol 307(10):C966–C977
34. Sadamoto H et al (2021) Identification and classification of innexin gene transcripts in the central nervous system of the terrestrial slug *Limax valentianus*. PLoS One 16(4): e0244902
35. Hernandez-Nicaise M-L (1991) Ctenophora. In: Harrison FWFW, Westfall JA (eds) Microscopic anatomy of invertebrates: Placozoa, Porifera, Cnidaria, and Ctenophora. Wiley, New York, pp 359–418
36. Moroz LL (2018) NeuroSystematics and periodic system of neurons: model vs reference species at single-cell resolution. ACS Chem Neurosci 9(8):1884–1903
37. Larkin MA et al (2007) Clustal W and Clustal X version 2.0. Bioinformatics 23(21): 2947–2948
38. Edgar RC (2004) MUSCLE: a multiple sequence alignment method with reduced time and space complexity. BMC Bioinform 5: 113
39. Mistry J et al (2021) Pfam: the protein families database in 2021. Nucleic Acids Res 49(D1): D412–D419
40. Gene Ontology C (2021) The Gene Ontology resource: enriching a GOld mine. Nucleic Acids Res 49(D1):D325–D334
41. Kanehisa M et al (2012) KEGG for integration and interpretation of large-scale molecular data sets. Nucleic Acids Res 40(Database issue): D109–D114
42. Tamura K et al (2013) MEGA6: molecular evolutionary genetics analysis version 6.0. Mol Biol Evol 30(12):2725–2729
43. Blom N et al (2004) Prediction of post-translational glycosylation and phosphorylation of proteins from the amino acid sequence. Proteomics 4(6):1633–1649
44. Sigrist CJ et al (2013) New and continuing developments at PROSITE. Nucleic Acids Res 41(Database issue):D344–D347
45. Letunic I, Khedkar S, Bork P (2021) SMART: recent updates, new developments and status in 2020. Nucleic Acids Res 49(D1):D458–D460
46. Xue Y et al (2010) GPS-SNO: computational prediction of protein S-nitrosylation sites with a modified GPS algorithm. PLoS One 5(6): e11290
47. Omasits U et al (2014) Protter: interactive protein feature visualization and integration with experimental proteomic data. Bioinformatics 30(6):884–886
48. Takaku Y et al (2014) Innexin gap junctions in nerve cells coordinate spontaneous contractile behavior in *Hydra* polyps. Sci Rep 4:3573
49. Chapman JA et al (2010) The dynamic genome of *Hydra*. Nature 464(7288):592–596
50. Moroz LL, Mukherjee K, Romanova DY (2023) Nitric oxide signaling in ctenophores. Front Neurosci 17:1125433
51. D'Hondt C et al (2013) Regulation of connexin- and pannexin-based channels by post-translational modifications. Biol Cell 105(9): 373–398
52. Ramazi S, Zahiri J (2021) Post-translational modifications in proteins: resources, tools and prediction methods. Database (Oxford) 2021: baab012
53. Tanaka K et al (2007) Shared and species-specific features among ichnovirus genomes. Virology 363(1):26–35
54. Popova LB et al (2012) Gap Junctions in *Nematostella vectensis* Sea Anemone embryos. Biol Bull Rev 2(5):368–389

55. Baumgarten S et al (2015) The genome of *Aiptasia*, a sea anemone model for coral symbiosis. Proc Natl Acad Sci U S A 112:11893
56. Shinzato C et al (2011) Using the *Acropora digitifera* genome to understand coral responses to environmental change. Nature 476(7360):320–323
57. Voolstra CR et al (2017) Comparative analysis of the genomes of *Stylophora pistillata* and *Acropora digitifera* provides evidence for extensive differences between species of corals. Sci Rep 7(1):17583
58. Tamm SL (2014) Cilia and the life of ctenophores. Invertebr Biol 133(1):1–46
59. Bhattacharya A et al (2019) Plasticity of the electrical connectome of *C. elegans*. Cell 176(5):1174–1189 e16