



Gap Junctions in Ctenophora

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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].

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

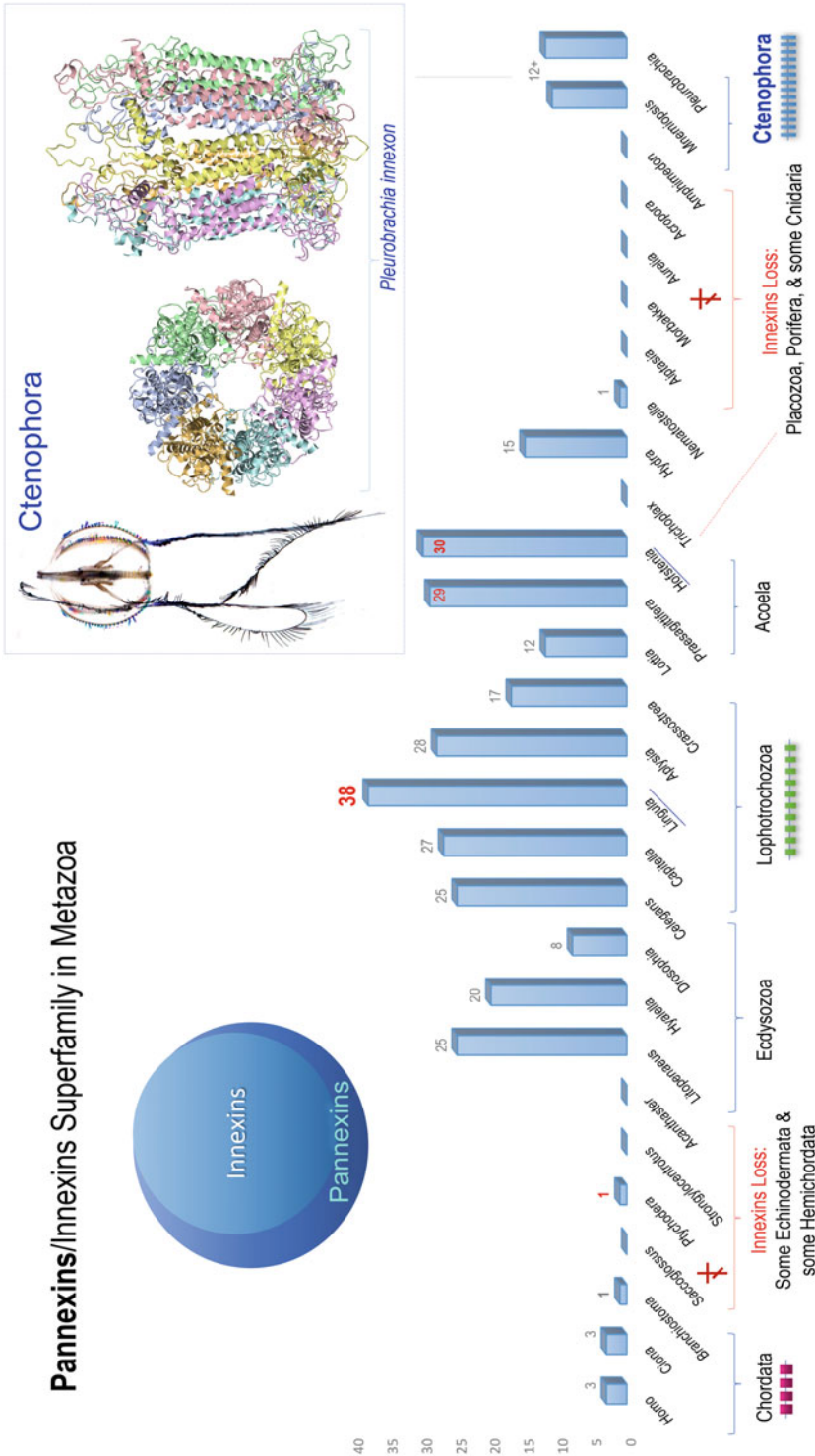


Fig. 1 The distribution of pannexin/innexin proteins across Metazoa with multiple examples of gene gains and losses. Y-axis indicates the presence and the number of genes encoding these gap junction proteins in selected reference species. Insert shows the three-dimensional structure of one innexin from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

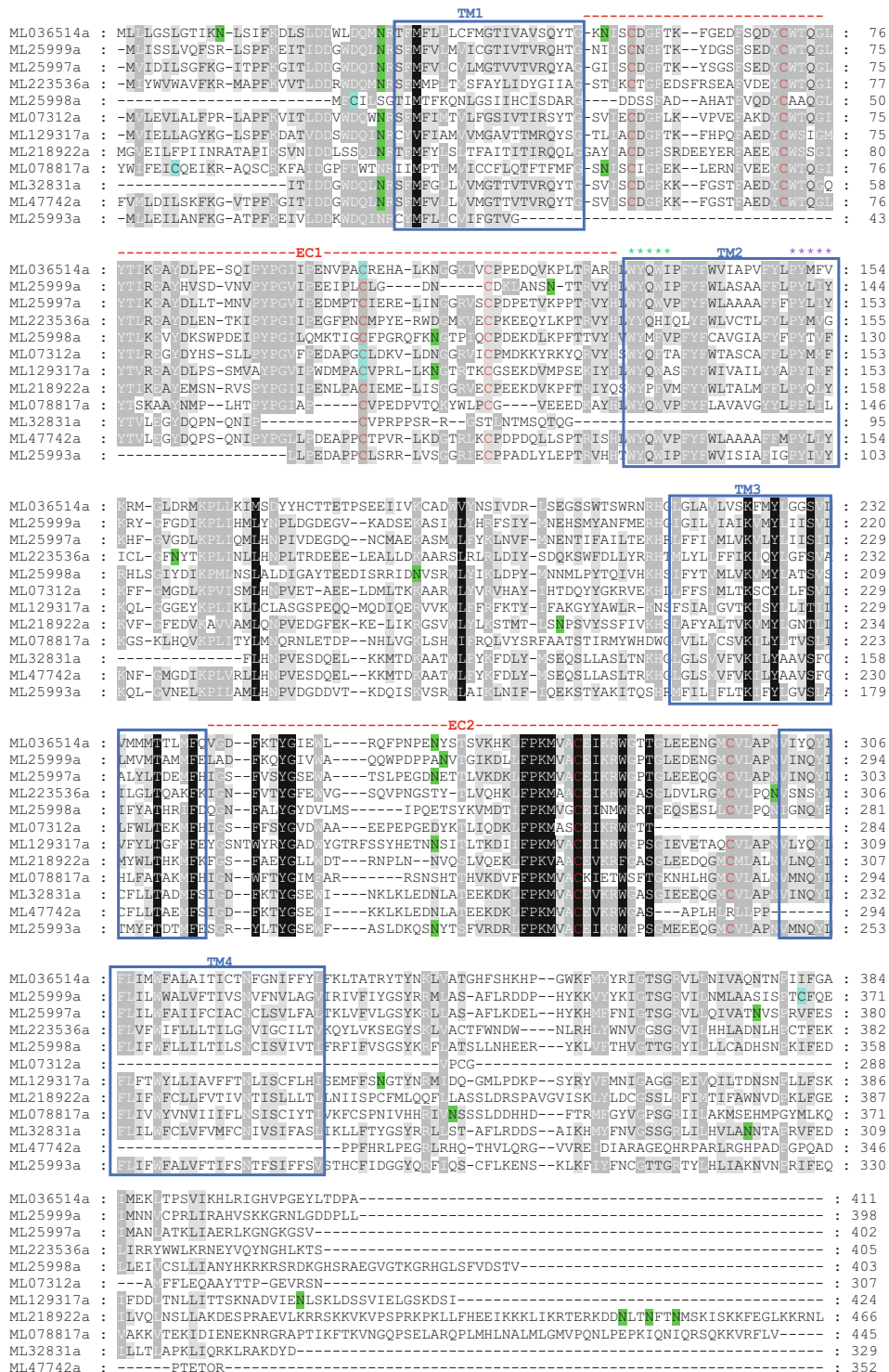


Fig. 2 Protein alignment of 12 innexins in *Mnemiopsis leidyi*. Dark blue squares outline the four transmembrane domains (TM); the red dashes indicate the extracellular loops 1 and 2 EC1 and EC2. The conserved cysteine Cys residues are shown in red. The green highlights are N-glycosylation sites, and 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

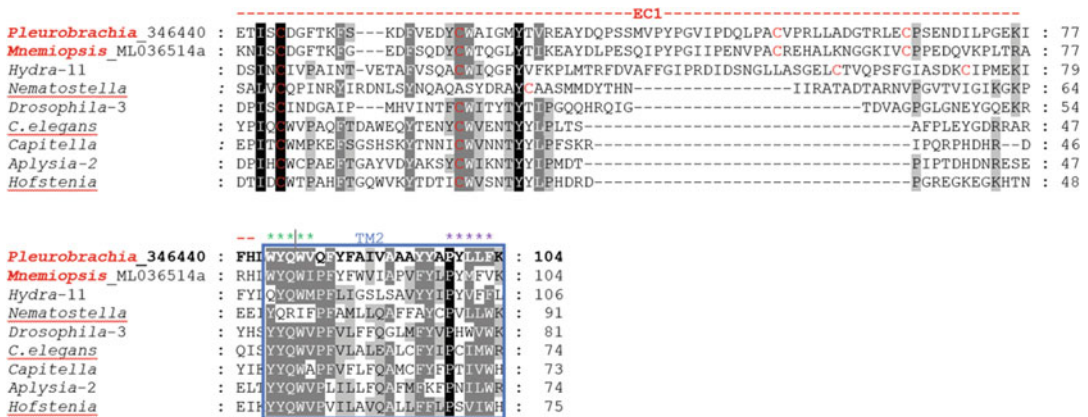


Fig. 3 Protein alignment of the extracellular loop, 1st and 2nd transmembrane domains in innexins in representative species from different phyla. All sequences accession numbers are referenced in Table 2 for the phylogeny except for *C. elegans*, NP_001024407.1, *Hofstenia miamia*_g17543.t1, and *Capitella tele-ta*_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

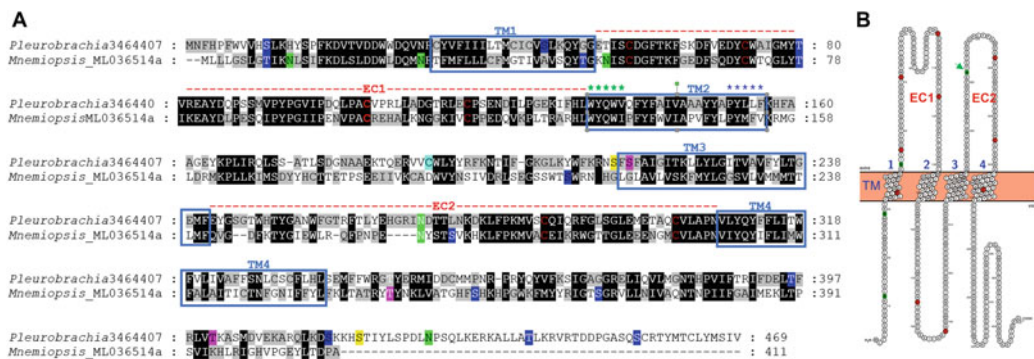


Fig. 4 (a) Protein alignment between representative innexins from *Pleurobrachia* (346440) and *Mnemiopsis* (ML036514a). Dark blue squares outline the four transmembrane domains (TM); the red dashes indicate the extracellular loops 1 and 2 EC1 and EC2. The conserved cysteine Cys residues are indicated 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 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 3.1 Server,

<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

<i>Mnemiopsis</i>	# 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
<i>Pleurobrachia</i>									
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 (CKI), 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 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 polydnvirus 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 *Pleurobrachia*_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

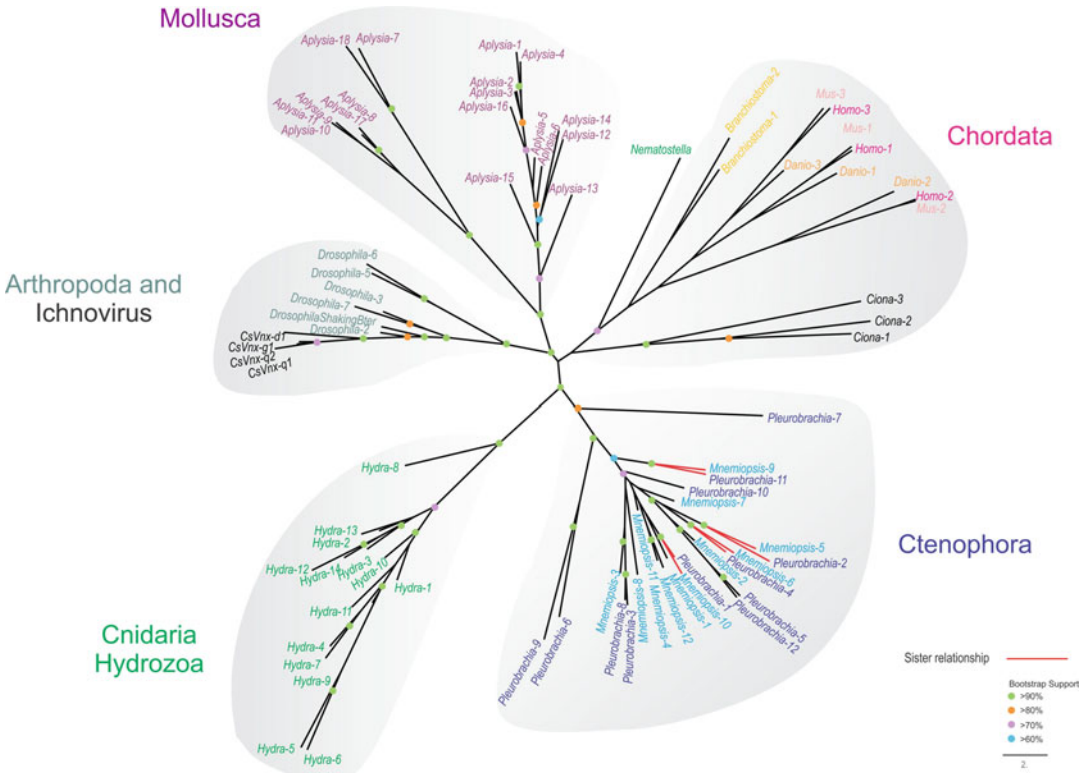


Fig. 5 Genealogical relationships among the putative protein family pannexin/innexin (PANX/INX) in metazoans (maximum likelihood method based on the Whelan and Goldman matrix-based model). The PANX/INX family shows lineage-specific diversification in major metazoan clades. Ctenophore PANX/INX proteins 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
<i>Mnemiopsis leidyi</i>	ML036514a	<i>Mnemiopsis</i> -1
<i>Mnemiopsis leidyi</i>	ML07312a	<i>Mnemiopsis</i> -2
<i>Mnemiopsis leidyi</i>	ML078817a	<i>Mnemiopsis</i> -3
<i>Mnemiopsis leidyi</i>	ML129317a	<i>Mnemiopsis</i> -4
<i>Mnemiopsis leidyi</i>	ML218922a	<i>Mnemiopsis</i> -5
<i>Mnemiopsis leidyi</i>	ML223536a	<i>Mnemiopsis</i> -6
<i>Mnemiopsis leidyi</i>	ML25993a	<i>Mnemiopsis</i> -7
<i>Mnemiopsis leidyi</i>	ML25997a	<i>Mnemiopsis</i> -8
<i>Mnemiopsis leidyi</i>	ML25998a	<i>Mnemiopsis</i> -9
<i>Mnemiopsis leidyi</i>	ML25999a	<i>Mnemiopsis</i> -10
<i>Mnemiopsis leidyi</i>	ML32831a	<i>Mnemiopsis</i> -11
<i>Mnemiopsis leidyi</i>	ML47742a	<i>Mnemiopsis</i> -12
<i>Pleurobrachia bachei</i>	3463885	<i>Pleurobrachia</i> -1
<i>Pleurobrachia bachei</i>	3464250	<i>Pleurobrachia</i> -2
<i>Pleurobrachia bachei</i>	3464407	<i>Pleurobrachia</i> -3
<i>Pleurobrachia bachei</i>	3465454	<i>Pleurobrachia</i> -4
<i>Pleurobrachia bachei</i>	3465668	<i>Pleurobrachia</i> -5
<i>Pleurobrachia bachei</i>	3465979	<i>Pleurobrachia</i> -6
<i>Pleurobrachia bachei</i>	3466205	<i>Pleurobrachia</i> -7
<i>Pleurobrachia bachei</i>	3466313	<i>Pleurobrachia</i> -8
<i>Pleurobrachia bachei</i>	3468319	<i>Pleurobrachia</i> -9
<i>Pleurobrachia bachei</i>	3471871	<i>Pleurobrachia</i> -10
<i>Pleurobrachia bachei</i>	3468487	<i>Pleurobrachia</i> -11
<i>Pleurobrachia bachei</i>	3479642	<i>Pleurobrachia</i> -12
<i>Homo sapiens</i>	NP_056183.2	<i>Homo</i> -1
<i>Homo sapiens</i>	NP_443071.2	<i>Homo</i> -2
<i>Homo sapiens</i>	NP_443071.3	<i>Homo</i> -3
<i>Mus musculus</i>	NP_062355.2	<i>Mus</i> -1
<i>Mus musculus</i>	NP_443191.1	<i>Mus</i> -2
<i>Mus musculus</i>	NP_766042.2	<i>Mus</i> -3
<i>Danio rerio</i>	NP_957210.1	<i>Danio</i> -1

(continued)

Table 2
(continued)

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

(continued)

Table 2
(continued)

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

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

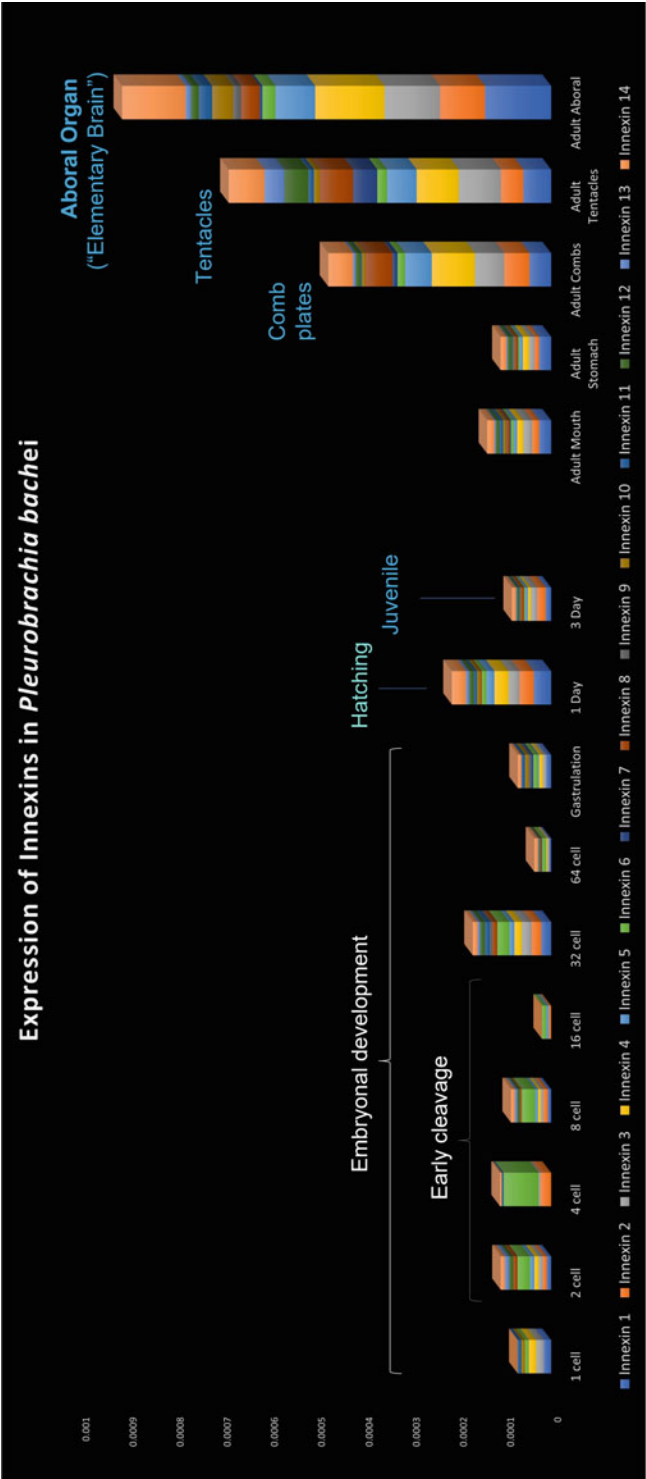


Fig. 6 RNA-seq analysis of innexin expression during development and in adult organs of *Pleurobrachia bachei*. The aboral organ has the greatest diversity and highest combined expression levels of 14 innexins, suggesting elaborated and diverse signaling pathways in this complex integrative organ—an 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])

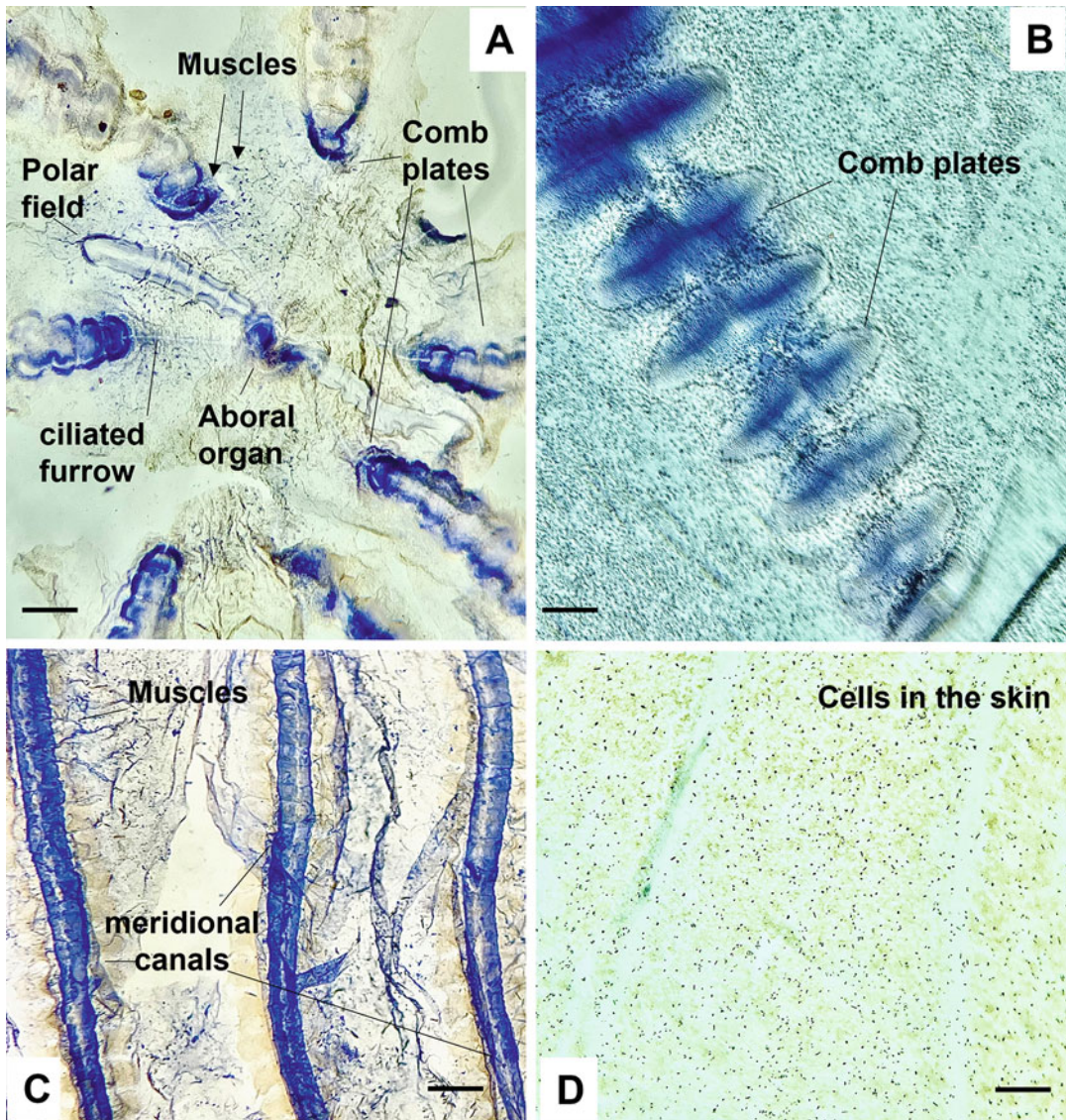


Fig. 7 Illustrative examples of spatial expression of four innexins in adult *Pleurobrachia bachei* (in situ hybridization). (a) The aboral view shows a specific expression of innexin 1 in the aboral organ, combs, ciliated furrows, and polar fields. Arrows indicate differential expression in selected muscle cells in sub-epithelial 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 μ m (a, c); 80 μ m (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.

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