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Review

# Innexins: members of an evolutionarily conserved family of gap-junction proteins

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**Abstract**

Gap junctions are clusters of intercellular channels that provide cells, in all metazoan organisms, with a means of communicating directly with their neighbours. Surprisingly, two gene families have evolved to fulfil this fundamental, and highly conserved, function. In vertebrates, gap junctions are assembled from a large family of connexin proteins. Innexins were originally characterized as the structural components of gap junctions in *Drosophila*, an arthropod, and the nematode *Caenorhabditis elegans*. Since then, innexin homologues have been identified in representatives of the other major invertebrate phyla and in insect-associated viruses. Intriguingly, functional innexin homologues have also been found in vertebrate genomes. These studies have informed our understanding of the molecular evolution of gap junctions and have greatly expanded the numbers of model systems available for functional studies. Genetic manipulation of innexin function in relatively simple cellular systems should speed progress not only in defining the importance of gap junctions in a variety of biological processes but also in elucidating the mechanisms by which they act.


*Keywords:* Innexin; Connexin; Gap junction; Intercellular channel; *Drosophila*; *Caenorhabditis elegans*

**Contents**



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## 1. Introduction

From *Hydra* to human, neighbouring cells communicate directly with one another via gap junctions. These cellular junctions consist of tightly packed arrays of intercellular channels. Each channel is formed by the docking of a hexameric hemichannel in the membrane of one cell with a corresponding hemichannel in an adjacent cell membrane. A pore at the centre of the channel permits the cell–cell diffusion of ions and small signalling molecules.

Two gene families have evolved to form gap-junction channels. The well-known connexin (Cx) family appears to be confined to chordate lineages; no Cx homologues have been found in the fully sequenced genomes of *Drosophila*, an arthropod, and the nematode *Caenorhabditis* (*C.*) *elegans* [1,2]. Innexins (Inxs) were originally identified as the structural proteins of gap junctions in the fly and worm ([3], reviewed in Refs. [4,5]). More recently, *inx* genes have been cloned from several other invertebrates [6–9]. Genes with high homology to the arthropod innexins have been identified in the genomes of insect-associated viruses [10,11] and distantly related innexin homologues are present in mouse and human genomes [7,12,13]. Both Cxs and Inxs are members of large multigene families. There are over 20 Cxs in mammals [14]; the fly has 8, and the worm 25, *inx* genes [15,16] (reviewed in Ref. [5]). Connexin genes, with the exception of the divergent γ subgroup, do not have introns in their coding regions [17]. Many of the Inxs, on the other hand, are encoded on multiple exons and have the potential to produce more than one protein by differential splicing. The extent of such splicing remains to be determined; so far only one innexin gene, *Drosophila shak-B*, is known to produce multiple products [18–21].

Although there is no significant amino acid sequence similarity between Cxs and Inxs, in other respects the similarities are striking. Structurally, both are four-pass transmembrane (TM) proteins with intracellular N- and C-termini. The TM domains are connected by two extracellular (EC) loops, which bridge the intercellular gap in the docked hemichannel, and a single intracellular loop (reviewed in Refs. [4,5,22,23]). Evidence that Inxs are true functional analogues of the Cxs is compelling. Inx antibodies decorate gap junction profiles in immunogold-labelled electron microscope (EM) images [24]. Mutations in *Drosophila* and *C. elegans inx* genes lead to a loss of intercellular coupling [25–29] and a reduction in the numbers of gap junction profiles [30,31]. Expression of *inx* mRNAs in Cx-depleted paired *Xenopus* oocytes induces the formation of intercellular channels that display sensitivity to voltage and pH typical of Cx channels [9,12,32–34].

Gap junctions are found in essentially all tissues at some stage of development hinting at an enormous diversity of function beyond their traditional roles in coordinating electrical activity in excitable tissues (reviewed in Ref. [35]). Elucidating those functions has relied until relatively recently on analyses of connexins. Significant progress has been made through studies of Cx-knockout mice and human Cx-associated diseases (reviewed in Refs. [36–38]). Such studies are now being complemented by analyses of Inx function in *Drosophila* and *C. elegans*. Some of the work reviewed here illustrates how sophisticated genetic techniques in these simple model organisms are helping to unravel the functions of gap junctions at the single cell level.

## 2. Phylogenetic analysis of innexins

One of the questions raised by the original discovery of innexins in the fly and worm was whether these proteins would be unique to Arthropods and Nematodes, protostomes of the clade *Ecdysozoa*, or more highly conserved across phylogenies. Studies over the last few years have provided unambiguous evidence for the latter and have highlighted, perhaps, an unexpected degree of conservation. In addition to the full complement of innexins identified in the genomes of *Drosophila melanogaster* and *C. elegans* [3] (reviewed in Refs. [4,5]), one or more complete sequences have been isolated from several other invertebrate organisms: the grasshopper *Schistocerca americana* (two orthologues of the fly genes) [6], the fall armyworm *Spodoptera frugiperda* (one gene), the poly-

chaete worm *Chaetopterus variopedatus* (one gene) [8], the leech *Hirudo medicinalis* (two genes) [9], the flatworm *Girardia tigrina* (one gene) and the sea butterfly *Clione limacina* (one gene) [7]. Partial sequences have been isolated from the moth, *Bombyx mori* and the black tiger shrimp, *Penaeus monodon* (Table 1). Thus, innexins are found both in Ecdysozoans and Lophotrochozoans, the two major protostome clades [39]. Interestingly, innexins are also present in the genomes of polydnaviruses (PDVs), symbiotic proviruses of parasitic Hymenoptera (Table 1). To date, these viral innexins have been identified in *Campoletis sonorensis* (four genes), *Hyposoter didymator* (four genes) and *Hyposoter fugitivus* (one gene) ichnoviruses (a subgroup of PDVs), that are carried by Ichneumonidae wasps of the same names [10,11].

The finding that Inxs span protostome lineages is unsurprising. Remarkable, given the well-established role of Cxs in vertebrate cell–cell communication, is the presence of innexin homologues, referred to as pannexins (panx), in vertebrate genomes [7,12,13]. Three human genes, *panx1*, *panx2* and *panx3*, and orthologues in mouse, rat and zebrafish have been identified (Table 1). It seems very unlikely that these genes are simply nonfunctional relics of ancestral innexins. Recent studies have demonstrated, first, that pannexins are transcribed in several human and mouse tissues [12,13] and, second, that Panx proteins form intercellular channels in paired *Xenopus* oocytes [12]. These data argue strongly that Panxs, although fewer in number and less widely expressed than Cxs, complement the latter in playing tissue specific roles in cell–cell communication in deuterostome lineages.

Table 1
Innexin sequences in the databases, by phylum

| Phylum | Organism | Sequence |
|---|---|---|
| Arthropoda | *Drosophila melanogaster* (fly) | *Dm*-Shak-B; *Dm*-Ogre; *Dm*-Inx2 to Inx7 |
| | *Schistocerca americana* (grasshopper) | *Sa*-Inx1; *Sa*-Inx2 |
| | *Bombyx mori* (silk moth) | *Bm*-Inx2 |
| | *Spodoptera frugiperda* (fall armyworm) | *Sf*-Inx2 |
| | *Penaeus monodon* (black tiger shrimp) | *Pm*-Inx1; *Pm*-Inx2 |
| Nematoda | *Caenorhabditis elegans* (nematode worm) | *Ce*-UNC-7; *Ce*-UNC-9; *Ce*-EAT-5; *Ce*-INX-1 to INX-22 |
| Annelida | *Hirudo medicinalis* (medicinal leech) | *Hm*-Inx1; *Hm*-Inx2 |
| | *Chaetopterus variopedatus* (polychaete worm) | *Cv*-Inx |
| Platyhelminthes | *Girardia tigrina* (flatworm) | *Gt*-Panx1 |
| Mollusca | *Clione limacina* (sea butterfly) | *Cl*-Panx1 |
| Vertebrata | *Danio rerio* (zebrafish) | *Dr*-Panx1 |
| | *Mus musculus* (mouse) | *Mm*-Panx1; *Mm*-Panx2; *Mm*-Panx3 |
| | *Rattus norvegicus* (rat) | *Rn*-Panx1; *Rn*-Panx2; *Rn*-Panx3 |
| | *Homo sapiens* (human) | *Hs*-Panx1; *Hs*-Panx2; *Hs*-Panx3 |
| Ichnovirus | *Campoletis sonorensis* | *Cs*-Vnxd1; *Cs*-Vnxg1; *Cs*-Vnxq1; *Cs*-Vnxq2 |
| | *Hyposoter fugitivus* | *Hf*-Vnxd1 |
| | *Hyposoter didymator* | *Hd*-Vnx1; *Hd*-Vnx2; *Hd*-Vnx3; *Hd*-Vnx4 |

Sequences listed are those in the NCBI database (http://www.ncbi.nlm.nih.gov) as of April 2004. The *Bombyx mori* and *Penaeus monodon* sequences are partial sequences.

Clarification of the identity of gap-junction genes in organisms such as coelenterates, which predated the protostome–deuterostome bifurcation, is eagerly awaited. Based on present knowledge, the odds are in favour of innexins as the gap-junction genes in these more primitive organisms. Connexins appeared to have evolved in chordate lineages. A search of the genome of the urochordate, *Ciona intestinalis* (sea squirt) revealed 17 candidate Cxs; innexin sequences are also present but in fewer numbers mirroring the situation in vertebrates [40]. An interesting question, for which there is at present no conclusive answer, is whether Cxs diverged from an ancestral innexin, or other common ancestor, or arose de novo by convergent evolution [41,42]. While the lack of significant sequence similarity between the two groups of proteins may be suggestive of structural convergence, one cannot rule out the possibility that the sequences have diverged dramatically so that only a few key residues have been preserved.

To compare the sequence relatedness of innexin proteins in different organisms a global alignment of all available sequences (61 in total, as of April 2004, excluding one of two virtually identical Shak-B proteins and some partial sequences, Table 1) was generated, and used to construct a phylogenetic tree, using Clustal software [43,44]. In keeping with previous reports, the unrooted tree (Fig. 1) shows that insect, *C. elegans*, and vertebrate innexins form distinct groups [5,42,46]. The few identified annelid and mollusc sequences form a separate cluster but clearly are more closely related to the nematode, than to the arthropod, sequences. The single plathyhelminthes sequence (*Gt-Panx*) branches from the centre of the tree very close to the annelid/mollusc group [8,9,46]. While this may imply the existence of two groups of Lophotrochozoan Inxs, it is not possible to infer this at present because the numbers of sequences available for analysis was limited. The Ichnovirus proteins are closely related to the insect Inxs with a mean similarity of approximately 50%. They form a tight cluster that originates from the insect branch of the tree (Fig. 1).

Orthologues (homologues that arose by speciation) can be identified within individual Inx clusters by the short branch lengths. For example, the vertebrate cluster contains human, rat and mouse orthologues of the three Panxs; zebrafish *panx1*, although likely to be orthologous, is more divergent than its mammalian counterparts. In the insect cluster, *Dm-ogre* and *Sa-inx1* are orthologous as are *Dm-inx2*, *Bm-inx2*, *Sa-inx2* and *Sf-inx2*. The similarity of viral and insect genes suggests that the viral innexins are

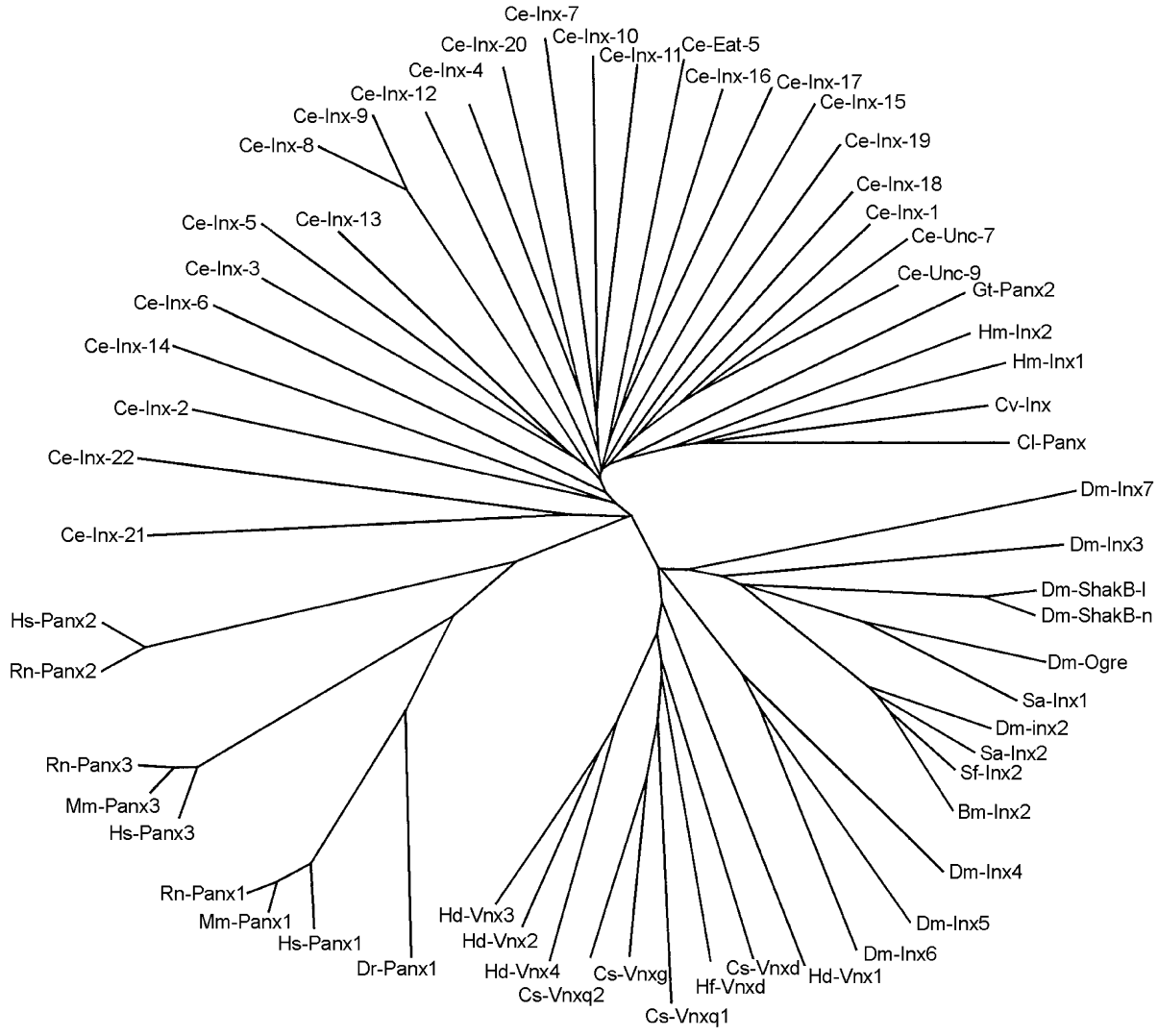


Fig. 1. Dendrogram of a multiple amino acid sequence alignment of innexins. The unrooted tree was generated in ClustalX [44], using a multiple sequence alignment (Supplementary material, Fig. S1) imported from ClustalW [43], and plotted using TreeView 1.6.6 [45]. Inx, innexin; Panx, pannexin; Vnx, viral innexin; shak-B-n, shak-B(neural); shak-B-l, shak-B(lethal); Ce, *Caenorhabditis elegans*; Dm, *Drosophila melanogaster*; Sa, *Schistocerca americana*; Bm, *Bombyx mori*; Sf, *Spodoptera frugiperda*; Cv, *Chaetopterus variopedatus*; Hm, *Hirudo medicinalis*; Cl, *Clione limacina*; Gt, *Girardia tigrina*; Cs, *Campoletis sonorensis*; Hd, *Hyposoter didymator*; Hf, *Hyposoter fugitivus*; Dr, *Danio rerio*; Mm, *Mus musculus*; Rn, *Rattus norvegicus*, Hs, *Homo sapiens*. Note: The *Clione* and *Girardia* sequences, although named Panx, are invertebrate innexins. All other pannexins are vertebrate homologues of innexins.

orthologues of host insect innexins. There is no evidence for orthologues between different phyla. Within a given phylum, gene numbers have been assigned arbitrarily; thus, for example, *Ce-inx-1* and *Ce-inx-2* are not orthologous to the insect *inx1* and *inx2* genes, respectively.

## 3. Comparative sequence analysis of innexins

Over the past few years, several authors have attempted to define amino acid sequence motifs or single residues characteristic of Inx family members [4,5,42,47]. It is clear that as sequence alignments have grown over time, to incorporate Inxs from more distantly related species, the numbers of absolutely conserved residues has diminished. The pentapeptide YYQWV close to, or at, the beginning of the second TM domain (Fig. 2) was originally proposed [47] as a signature sequence of Inx (then referred to as OPUS) proteins. It transpires that this motif is absolutely conserved only in the insect Inxs. The sequence YY(X)W(Z), where X represents the amino acids Q, R, M, E or S and Z, the amino acids V, M, A, I, S or T, is found in all invertebrate and viral Inxs except *Ce*-INX-22 (FYMWV) and *Hd*-VNX1 (YYQLC). This putative innexin signature motif is not found in the vertebrate Panx proteins (Fig. 2). Two other residues that are absolutely conserved (N in EC2 and W in TM4) and a number of residues that are strongly conserved (Fig. 2) in the invertebrate and viral proteins are not present in the vertebrate Panxs.

The alignment of all available innexin and pannexin sequences reveals an absolutely conserved P-X-X-X-W motif in the predicted second TM domain (Fig. 2). The

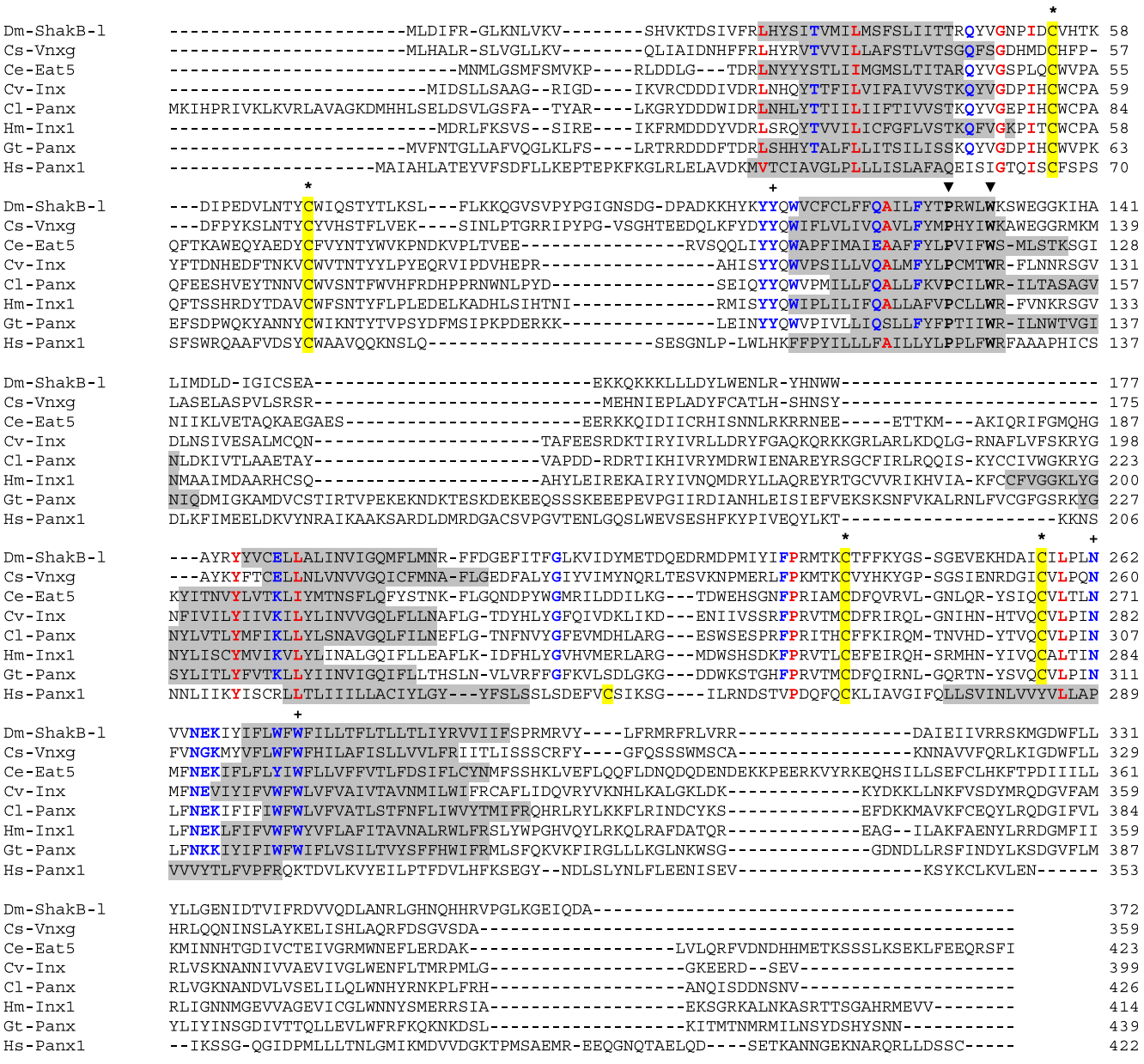


Fig. 2. Pileup of selected sequences from a multiple alignment of innexins. The sequences shown were selected from a multiple alignment of 61 innexins to illustrate conserved amino acid residues. The complete alignment (Supplementary material, Fig. S1) was generated in ClustalW [43] from the 61 sequences represented in Fig. 1. Transmembrane domains, predicted by HMMTOP [51], are shaded in grey. Two conserved cysteine residues in each of the predicted extracellular loops are shaded in yellow and marked by asterisks. A proline motif (bold font and marked by arrowheads) in the second transmembrane domain is strictly conserved in all known innexins and pannexins. Residues in blue font are absolutely (marked by a plus symbol) or strongly conserved in invertebrate sequences but are not found in any of the vertebrate pannexins. Residues in red font are strongly conserved in all sequences including the vertebrate pannexins. Note some residues that may appear to be well conserved in this alignment of eight sequences are not highlighted, as they are not conserved in the alignment of all available sequences (Supplementary material, Fig. S1). Abbreviations as in Fig. 1.

proline residue represents a potential correspondence between Inxs and Cxs; a proline in the same relative position is strictly conserved in the Cx family. Studies of Cxs suggest that this residue is part of a molecular hinge motif that kinks to bring about conformational changes in response to voltage [48,49] (reviewed in Ref. [50]). The presence of a corresponding residue in all known innexins suggests that this proline-based conformational switch is strictly conserved in gap-junction proteins.

Apart from the proline motif, the only other residues absolutely conserved in innexins and pannexins are a pair of cysteine residues in the predicted first EC loop (Fig. 2). The spacing between these residues is as follows, where X indicates the numbers of intervening residues: C-$X_{12–14}$-C in insect and Ichnovirus sequences and C-$X_{17}$-C in all other sequences, except *Ce*-INX-14 (C-$X_{19}$-C). A pair of cysteine residues is also conserved in the predicted second EC loop (Fig. 2). These residues align perfectly in all invertebrate

and viral sequences with similar spacing across phyla: C-$X_{16–18}$-C in all sequences except *Ce*-INX-22 (C-$X_{24}$-C). However, the pair of conserved cysteine residues in the predicted EC2 loop of the vertebrate Panxs (C246 and C265 in *Hs*-Panx1; Fig. 2) does not align with the cysteine pair in the invertebrate and viral sequences, although the inter-cysteine spacing is similar (C-$X_{18–20}$-C). Multiple alignments of all available sequences show complete alignment of one cysteine residue in the predicted EC2 loop (Fig. 2; Supplementary material, Fig. S1). This, however, is likely to be spurious since the residue at this position in the vertebrate Panxs (C265 in *Hs*-Panx1; Fig. 2) is the second cysteine of the pair aligned with the first cysteine of the conserved pair in the invertebrate sequences.

Positioning of the EC2 loop conserved cysteines is one of a number of striking differences, highlighted in Fig. 2, between the first and second halves of the invertebrate and vertebrate sequences. The other major differences are in the positioning of the TM domains and the length of the extracellular loops. The predicted first and second TM domains align very well in all sequences; the extracellular loop connecting these domains is similar in length in the Inx and Panx sequences averaging approximately 60 amino acids. By contrast, the predicted third and fourth TM domains of the vertebrate Panxs show only partial alignment with predicted TM3 and TM4 of the invertebrate sequences. The predicted second EC loop of the vertebrate Panxs is significantly shorter than that of their invertebrate homologues. Average numbers of amino acid residues in predicted EC2 are approximately 66 in the invertebrate sequences as opposed to 38 in the vertebrate pannexins. Interestingly, this means that the EC2 loops of the vertebrate pannexins are much more similar in size to the EC loops of Cxs (average 35 and 39 residues in EC1 and EC2, respectively) than those of Inxs. However, despite this apparent structural similarity, there is no sequence similarity between Cxs and Panxs.

The analysis above raises the question, addressed in a number of reports [7,12,13,40] of whether the vertebrate Panxs are true innexins. Excepting the lack of the YY(X)W(Z) motif, the first half of these proteins (predicted TM1, TM2 and the intervening loop) shows strong similarity to the invertebrate Inxs. Thereafter, similarity decreases resulting in poor alignment of predicted TM3, TM4 and the conserved cysteine residues in the predicted second extracellular loop. The simplest explanation is that the proteins belong to the same superfamily but have undergone significant sequence divergence during the evolution of chordate lineages.

## 4. Innexins co-operate to form functional channels in vitro

Gap junction hemichannels are classified as homomeric or heteromeric, composed of one or more than one protein species, respectively. Intercellular channels are referred to as homotypic if the two hemichannels are identical and heterotypic if the hemichannels differ in molecular composition. Thus, four possible intercellular channel types may be assembled from proteins expressed in neighbouring cells: homomeric/homotypic, homomeric/heterotypic, heteromeric/homotypic and heteromeric/heterotypic. For simplicity, the first two will be referred to as homotypic and heterotypic, respectively, and the latter two, which are impossible to distinguish in expression studies, will be referred to as heteromeric.

In heterologous systems, the vast majority of Cxs form homotypic channels and several of the proteins selectively interact to assemble heterotypic and heteromeric channels (reviewed in Ref. [22]). To date, 12 of the 61 known Inxs have been tested for ability to form channels in paired *Xenopus* oocytes. Of these, only six, the *Drosophila* proteins Shak-B(lethal) [32] and Inx2 [34], *C. elegans* INX-3 [33], *H. medicinalis* (leech) Inx1 and Inx2 [9] and mammalian Panx1 [12], form homotypic channels. Some of the proteins that fail to form homotypic channels are functional in heteromeric or heterotypic configuration. *Drosophila* Ogre and Inx3 both form heteromeric channels with Inx2 [34] (Holcroft C., Phelan P., unpublished data) and Inx4 interacts with Inx2 to form heterotypic channels [52]. Likewise, the two identified leech Inxs form heterotypic channels [9] and rat Panx1 and Panx2 form heteromeric channels [12]. No functional partners have yet been identified for *Drosophila* Shak-B(neural), *C. elegans* EAT-5 or the *C. variopedatus* Inx, shown to be incapable of forming channels independently [32,33,46]. The searches have not been exhaustive and the likelihood is that compatible partners will be identified for these proteins. An alternative possibility to consider is that some Inxs may not be components of functional channels but instead act as negative regulators of intercellular communication. This is not entirely unprecedented; one member of the Cx family, Cx33, which does not appear to form functional channels, specifically inhibits Cx37 channel formation when the two proteins are expressed in the same cells [53]. The physiological significance of this is not known but it is proposed to provide a means of selectively coupling different cell types in the testis [53].

While one cannot assume that any heterologous system faithfully reflects the situation in a whole organism, it is satisfying to note that there is, in general, good correspondence between in vivo expression patterns of innexins and their functional interactions in vitro. For example, in *Drosophila* Inx2, Inx3 and Ogre co-localize in several embryonic tissues (epidermis, gut and trachea) [15,34] and in the larval CNS (Holcroft C., Phelan P., unpublished data) in keeping with their ability to form heteromeric channels in *Xenopus* oocytes. Inx2 and Inx4 are expressed in adjacent cells in developing egg chambers ([15,54]; Section 5.5), raising the strong possibility that they engage in heterotypic interactions in the fly as in vitro. Expression patterns should continue to be useful in directing functional expression

studies to determine the possible extent of Inx channel diversity in a given organism.

As is the case with Cx-based gap junctions, the molecular composition of Inx channels influences their properties. This is exemplified by the differential voltage sensitivity of homotypic and heteromeric channels containing *Drosophila* Inx2. Homotypic Inx2 channels are sensitive to transjunctional and transmembrane voltage; Inx2–Inx3 heteromeric channels show significantly less sensitivity to transmembrane voltage while Inx2-Ogre heteromeric channels are voltage insensitive [34] (Holcroft C., Phelan P., unpublished data). Voltage sensitivity is likely to be particularly important in regulating gap junctions in excitable cells; asymmetric voltage sensitivity of heterotypic channels is proposed to underlie rectification at some electrical synapses [55,56]. There is some evidence to suggest that membrane potential differences between neighbouring cells may also influence intercellular communication in nonexcitable tissues [57]. So far, other properties of Inx channels, such as size and charge selectivity, have not been examined; however, based on Cx studies [58,59], it is reasonable to assume that the molecular make-up of a channel will determine its permeability to ions and signalling molecules. Regulated exchange of signalling molecules is likely to be important during development. For example, during the process of epidermal patterning in *Drosophila* and other insects, gap junctions within and between segments differ in size selectivity, thereby establishing communication compartments [60–62]. Inx2, Inx3 and Ogre are expressed in an overlapping pattern along either side of the segment borders [15,34] and may play an important part in this process. However, even in such a relatively simple system, the task of identifying the signalling molecules and elucidating precisely how the unique properties of molecularly diverse channels regulate cellular communication is a formidable one.

## 5. Genetic analyses of innexin function reveal diverse roles of gap junctions

Studies in invertebrates, traditionally, have made important contributions to gap-junction biology. Indeed, the phenomenon of electrical coupling was first described in an invertebrate [63]. In the past, the attractiveness of these systems lay in their relative simplicity and amenability to cell biological and electrophysiological studies. The identification of innexins has provided the necessary tools for genetic studies of gap junctions in invertebrates and, thereby, has greatly enhanced the potential of these organisms as models for functional studies of cell–cell communication. Below, I review progress, to date, in the genetic analyses of innexin function in *Drosophila* and *C. elegans*. Significantly, several of the functions that these studies ascribe to Inxs in invertebrates are reminiscent of the roles of Cxs in vertebrates. Thus, despite the lack of sequence similarity between the two gene families, genetic manipulation of innexin function, increasingly, is likely to provide valuable insights into the fundamental functions, and mechanism of action, of gap junctions.

### *5.1. Mutations in Drosophila shak-B and C. elegans unc-7 and unc-9 interfere with transmission at electrical synapses*

#### *5.1.1. Viable shak-B mutants have defective escape behaviour*

The *shak-B* locus is alternatively spliced to give rise to three protein products designated Shak-B(lethal) [19,20], Shak-B(neural) [18] and Shak-B(neural+16) [21]. Five common 3′ exons encode the region from the start of the predicted second TM domain to the C-terminus and, therefore, all Shak-B proteins are identical in these regions. A unique complement of 5′ coding exons is responsible for N-terminal sequence differences. Shak-B(neural) and Shak-B(neural+16) are 96% identical, differing only by the lack or presence, respectively, of an N-terminal tail of 16 amino acids. Shak-B(lethal) shares approximately 88% identity with the other Shak-B proteins with sequence differences scattered throughout the N-terminus, first TM domain and first EC loop.

*shak-B* transcripts are dynamically expressed in the developing nervous system. *shak-B(neural+16)* is found in the central and peripheral nervous system of late stage embryos, roughly coinciding with the period of neuronal differentiation [15,21]. The functional significance of this early expression is unclear. There are no obvious defects in the larval nervous system of *shak-B*$^{2}$ mutants that lack both *shak-B(neural)* and *shak-B(neural+16)* transcripts [18,21] (reviewed in Ref. [5]), and these flies develop normally. *shak-B(lethal)* is strongly expressed throughout the brain and ventral CNS between 40 and 65 h of pupation, the period when many postembryonic neurons are establishing connections. The consequences of loss of *shak-B(lethal)* function at this stage have not been examined; mutations in this transcript are embryonic lethal making analyses of postembryonic function difficult. In the adult nervous system, *shak-B* transcripts are confined to discrete groups of neurons, including those of the Giant Fibre System (GFS) and a haltere-to-flight motoneuron circuit [18,19,21,25,31,64]. The gene is required in these neurons for the formation and function of electrical synapses [25,26,31,64].

The GFS (Fig. 3A) is a relatively simple neural circuit that mediates a ‘jump-flight’ escape behaviour in the fly. The cell bodies of the pair of large bilaterally symmetrical Giant Fibres (GFs) are located in the brain and receive input from the visual system. The GF axons extend from the brain to the ventral CNS where they synapse directly with the tergotrochanteral (jump) muscle motoneurons (TTMns) and indirectly, via the peripherally synapsing interneurons (PSIs), with the motoneurons of the dorsal longitudinal flight muscles (DLMns). The right and left GFs are interconnected

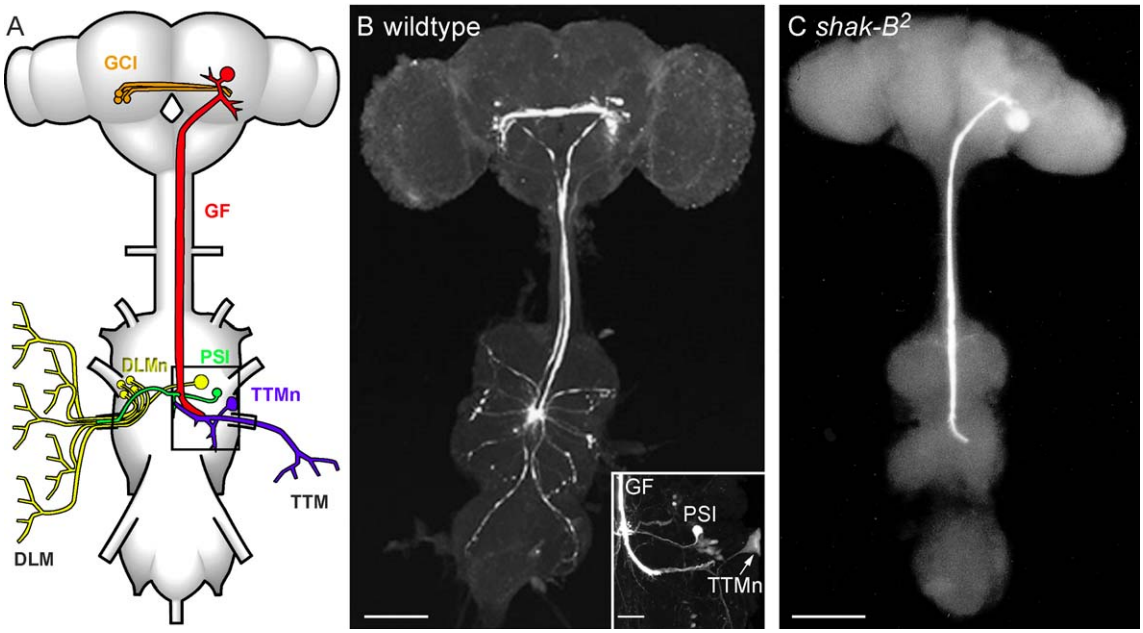


Fig. 3. *shak-B* is required for dye coupling in the *Drosophila* GFS. Schematic drawing of the GFS in the adult fly CNS (A). Only one side of the bilaterally symmetrically pathway is shown. The Giant Fibre (GF) axons extend from the brain to the ventral CNS and form electrical or mixed electrochemical synapses with the motoneurons (TTMn) of the tergotrochanteral muscle (TTM) and with the peripherally synapsing interneurons (PSI), which synapse with the motoneurons (DLMn) of the dorsal longitudinal flight muscles (DLM). The right and left GFs are interconnected in the brain by a bundle of GCIs. The small fluorescent dye Lucifer Yellow, injected into one of the GF axons, diffuses into the GCIs, the contralateral GF, the TTMns, PSIs and several unidentified neurons in the thoracic ganglion in wild-type flies (B). Inset in B corresponds to the boxed region in A, and shows GF coupling to the PSI and TTMn in the thoracic ganglion. No dye coupling is observed in the GFS of *shak-B*$^2$ mutants (C) that lack functional *shak-B(neural)* and *shak-B(neural+16)* transcripts. (A) Adapted from a drawing by J. Blagburn [31]; (B–C) reprinted from Ref. [25] by copyright permission of the Society for Neuroscience. Scale bars: B, C, 100 μm; inset (B), 20 μm.

by a bundle of large interneurons, the Giant Commissural Interneurons (GCIs), which cross the midline of the brain. In common with other escape circuits in invertebrates and lower vertebrates, many of the GFS neurons are electrically coupled. The central GF-TTMn, GF-PSI and GF-GCI synapses are electrical or mixed electrochemical synapses [25–27,31,65–68]. Comparative studies of wild-type and *shak-B*$^2$ mutant flies, which have normal GF morphology [69] but a defective escape behaviour [70], demonstrate that loss of *shak-B* function specifically disrupts electrical transmission at these synapses. Stimulation of the GFs in the brain of *shak-B*$^2$ mutants fails to evoke a response from the flight muscles (DLMs) and the response of the jump muscles (TTMs) is delayed compared to wild-type [70,71]. The response latency of the GF-TTMn synapse suggests that loss of *shak-B* function interferes with fast electrical transmission while sparing a slower chemical component of transmission. This is further supported by the observation that motoneuron to muscle transmission, which is exclusively chemical, is unaffected [70]. More direct evidence for a role for *shak-B* at electrical synapses comes from dye-coupling studies. Small gap-junction permeant molecules such as Lucifer Yellow, cobalt and neurobiotin diffuse freely through the GFS in wild-type flies but dye coupling is completely eliminated in s*hak-B*$^2$ mutants (Fig. 3B–C) [25,26]. Finally, conventional EM studies reveal a dramatic reduction in the numbers of gap junction profiles at the GF-TTMn and GF-PSI synapses in *shak-B*$^2$ mutants. Chemical synaptic contacts between the same pre- and post-synaptic partners, on the other hand, are unaffected [31].

The mutation *shak-B*$^2$ lies in an exon common to *shak-B(neural)* and *shak-B(neural+16)* [18,21] and, therefore, causes loss of function of both of these proteins. At the time the studies described above were carried out only Shak-B(neural) was known to exist. Reasonably, therefore, it was assumed that this protein was required for the formation and function of electrical synapses in the GFS. An anomaly was that, despite its seemingly essential role in vivo, the protein failed to form homotypic channels in *Xenopus* oocytes, although the possibility of functional interactions with other Inxs could not be ruled out ([32]; Section 4). The identification of *shak-B(neural+16)* transcripts by Zhang et al. [21] and their demonstration that these, rather than *shak-B(neural)* transcripts, are expressed in the GFS strongly suggest that the N-terminally extended Shak-B(neural+16) protein is the major player at the GFS synapses.

One or both of the *neural shak-B* transcripts are also required for electrical transmission between haltere sensory neurons and their postsynaptic targets [64]. The halteres are small hindwings that act as mechanosensory organs to maintain balance during flight. Haltere afferents form mixed electrochemical synapses with an identified motoneuron, B1, which innervates the first basalar flight muscle, and

with several haltere interneurons. Electrical and dye coupling between these neurons is abolished in *shak-B*$^2$ mutants. As in the GFS, chemical transmission between the haltere sensory neurons and B1 is unaffected [64]. Other less well-defined neural circuits, such as those involved in grooming and feeding behaviours, are disrupted in *shak-B*$^2$ mutants [72,73] suggesting that these circuits use electrical synapses and highlighting, perhaps, a general role for *shak-B* in the regulation of electrical transmission.

#### *5.1.2. unc-7 and unc-9 mutants have a very similar uncoordinated phenotype*

A complete map of electrical and chemical synapses in the *C. elegans* nervous system has been constructed from EM data [74]. The worm's 302 neurons are interconnected by about 560 gap junctions and about 5000 chemical synapses. At least seven innexins are likely to be responsible for the establishment and maintenance of the electrical connections. Six genes, *unc-7*, *inx-1*, *inx-3*, *inx-4*, *inx-10* and *inx-11*, are expressed in neurons [16] although the identity of cells expressing specific *inx* genes has not been documented. So far, mutations in one of these, *unc-7*, have been shown to interfere with electrical connections [75]. *unc-9* mutants display an uncoordinated phenotype identical to that of *unc-7* mutants [76,77] suggesting that this innexin, for which no expression data is available, also acts in neurons.

In wild-type worms, muscle contraction-relaxation is coordinated in the dorso-ventral and anterior–posterior planes so that the animals move in a smooth sinusoidal manner. Coordination is lost in *unc-7* and *unc-9* mutants causing the body to kink or bend irregularly as the worm moves forward [75–77]. Precisely how *unc-7* and *unc-9* coordinate locomotion is not known. Consistent with its pattern of expression, *unc-7* appears to be required in neurons, possibly motoneurons, rather than in muscles. Selective loss of function from the AB.p cell, the progenitor of most of the body wall motoneurons and some interneurons, but not from the AB.a cell, which gives rise to other interneurons, or from the P1 muscle progenitor [78,79] reproduces the *unc-7* phenotype [75]. Analysis at the cellular level, so far, has been limited to a single *unc-7* mutant. In this worm, ectopic gap junctions were observed between interneurons and motoneurons. In wild-type worms, AVB interneurons form electrical synapses with the DB and VB motoneurons, which drive forward locomotion; the AVA interneurons are electrically coupled to the DA and VA motoneurons, involved in backward locomotion [80]. In the *unc-7* mutant nerve cord examined, AVA neurons formed connections with their usual motoneuronal targets, DA and VA, but also formed inappropriate connections with the DB and VB neurons (White J., Southgate E., Thompson N., unpublished data cited in Ref. [75]). The mutation studied, *unc-7(e5)*, approximates a null mutation (Starich T.A. and Shaw J.E., unpublished data cited in Ref. [5]) hence one cannot invoke altered expression to explain the appearance of ectopic junctions. One can only speculate at present as to why a loss-of-function mutation in a gap-junction gene would lead to formation of ectopic gap junctions. Possibilities are that *unc-7* has a developmental role in establishing the precise pattern of connectivity; the gene is expressed in neurons from early stages of development [16,75] and studies in *Drosophila* support a similar function for the innexin *ogre* ([81]; Section 5.3.2). Alternatively, and perhaps more likely, UNC-7 may be a component of heteromeric channels in the DB and VB cells; in its absence its normal functional partner(s) may interact with other Inxs in the AVA neurons to form inappropriate synapses. Given the similarities of the mutant phenotypes, UNC-9 is a strong candidate for functional interactions with UNC-7. However, it is not known at present whether it is expressed in the motoneurons affected in *unc-7* mutants, nor is it known whether, or how, these proteins interact with one another, or with other Inxs, to form channels.

*unc-7* is also implicated in electrical connections between the extrapharyngeal and pharyngeal nervous systems mediated by the RIP and I1 neurons [82]. The RIP-I1 connection is involved in mechanosensory, for example tail tap, modulation of pharyngeal pumping. The tail tap response is abrogated in *unc-7* mutants [83]. Loss of RIP-I1 connections may also account for the insensitivity of *unc-7* and *unc-9* mutants to the anthelmintic drug ivermectin [75,77], an agonist of glutamate-gated chloride channels, which prevents feeding by hyperpolarizing the pharyngeal musculature [84]. Two pathways have been proposed to explain how the drug brings about its effects. In the first, it acts directly on the muscle, opening chloride channels in the membrane to hyperpolarize the cells. In the second, it opens glutamate-gated chloride channels in an extrapharyngeal neuron; hyperpolarization of the neuron spreads via the RIP-I1 connection to the muscles of the pharynx. The second pathway is consistent with the loss of ivermectin sensitivity in *unc-7* and *unc-9* mutants [85].

### *5.2. C. elegans eat-5, inx-3 and inx-6 synchronize pharyngeal muscle contraction*

The worm pharynx is a neuromuscular organ that is responsible for ingesting and partially digesting food. Functionally, the pharynx is divided into three regions each composed of specific muscle cells surrounding a central lumen. From anterior to posterior, these are the corpus, which is further subdivided into an anterior procorpus and a more posterior metacorpus, the isthmus and the terminal bulb (Fig. 4). Synchronized contractions of the muscles are responsible for sucking food into the lumen of the corpus and transferring it, via the isthmus, to the terminal bulb where a grinder breaks it down before passing it to the intestine [82]. This rhythmic muscle contraction, although not dependent upon neural stimulation, is regulated by neurons of the pharyngeal nervous system [86].

As outlined in Section 5.4.2, morphogenesis of the pharynx, during embryogenesis, requires the function of

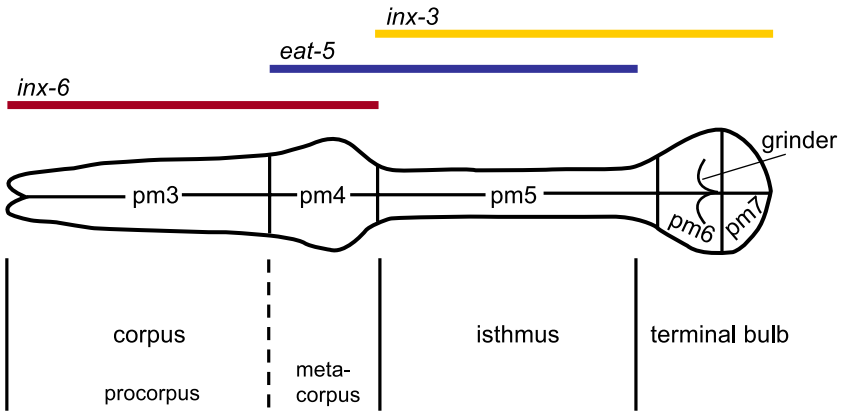


Fig. 4. Anatomy of the *C. elegans* pharynx. The pharynx is divided into three functional units: the corpus, which is subdivided into an anterior procorpus and a posterior metacorpus, the isthmus and the terminal bulb. The pharyngeal muscles (pm), specific to each region, are indicated. The coloured bars delimit expression domains of *inx* genes. Adapted from Ref. [28].

*inx-3*. Pharyngeal expression of INX-3 persists in adult worms; it is concentrated in the isthmus and terminal bulb (Fig. 4) where it overlaps the expression domain of *Ce-inx-2* [16,24,28]. At least two other innexins are also found in the pharynx (Fig. 4). *eat-5* is expressed in the muscles of the metacorpus and isthmus at larval and adult stages [28]. *inx-6* is first detected during embryogenesis in presumptive pharyngeal precursors. During differentiation of the pharynx, it localizes to the corpus muscles and the marginal cells of the isthmus, where expression persists in adults [29].

Consistent with their adult expression patterns, *inx-3*, *eat-5* and *inx-6* are required for synchronized contraction of the pharyngeal musculature. This pharyngeal pumping phenotype has not been extensively analyzed in *inx-3* mutants, the majority of which die as embryos. In those mutants that hatch, the gross anatomy of the pharynx is highly abnormal because of the earlier morphogenetic defects (Section 5.4.2). Nonetheless, the pharyngeal muscles do contract; the corpus muscles, which do not normally express INX-3, contract in synchrony whereas contraction of the muscles of the terminal bulb, which express INX-3 in wild-type animals, is asynchronized [24]. Embryonic development is normal in *eat-5* and *inx-6* mutants and there are no observable defects in the morphology of the pharynx of newly hatched larvae. However, mutant larvae are small and starved in appearance [28,29,87]. This phenotype can be attributed to a loss of the normally near-synchronous contractions of anterior and posterior pharyngeal muscles that are essential for feeding. In *eat-5* mutants, the muscles of the metacorpus and isthmus are electrically and dye (carboxyfluorescein) uncoupled so that, although the individual regions of the pharynx contract normally, corpus and terminal bulb contractions are asynchronized [28]. *inx-6* mutations interfere with electrical coupling in the procorpus. The contraction–relaxation cycle of these muscles, consequently, is abnormal and they are dye-uncoupled from the muscles of the metacorpus, which contract normally and in synchrony with more posterior muscles [29].

The regional-specific expression (Fig. 4) and action of *inx-3*, *eat-5* and *inx-6* in the pharynx suggest that gap junctions composed of these innexins have distinct properties. This is supported by the finding that *eat-5* and *inx-6* are not fully functionally interchangeable. Expression of *eat-5* under the control of the *inx-6* promoter only partially rescued the *inx-6* mutant phenotype whereas expression of *inx-6* restored wild-type function [29]. Direct comparisons of the properties of pharyngeal gap-junction channels are not yet possible. INX-3 has been shown to form homotypic channels in paired *Xenopus* oocytes [33] (Section 4). EAT-5 alone does not form functional channels in the same system, nor does it interact with INX-3 to form heterotypic or heteromeric channels [33] (Section 4). INX-6 has not yet been tested in heterologous systems but genetic studies in the worm suggest that it is competent to form channels. Ectopic expression of the gene throughout the pharyngeal musculature causes hypercontraction consistent with the formation of ectopic gap junctions [29]. Clearly, INX-6 may form homomeric channels and/or, based on the expression patterns in Fig. 4, assemble heteromeric or heterotypic channels with EAT-5. Heterotypic INX-6/EAT-5 channels, conceivably, could regulate communication at the procorpus–metacorpus and metacorpus–isthmus boundaries.

### *5.3. ogre and shak-B are required for postembryonic development of the Drosophila nervous system*

#### *5.3.1. Numbers of postembryonic neuroblasts are reduced in ogre mutants*

*ogre* (*optic ganglion reduced*) is expressed during embryogenesis in the epidermis, gut, tracheal system, salivary glands, cardioblasts and the nervous system [15,88–90]. During larval and pupal stages, the gene is found predominantly in the CNS and in the imaginal disks, epithelial pouches that give rise to adult specific structures such as legs, wings and retina [88]. Expression in the adult is confined to the follicle cells of the ovary and the cardia (gut proventriculus) [15,88]. At all stages of development,

and in most tissues, expression at least partially overlaps that of *inx2* and *inx3* [15,34,91].

The functional significance of the embryonic and adult expression of *ogre* is unclear. *ogre* mutant embryos show no obvious phenotype [92] although, clearly, there may be subtle defects in individual tissues. So far, the gut has been examined but, in contrast to *inx2* mutants (Section 5.4.1), these studies failed to reveal any morphogenetic abnormalities [93]. Adult phenotypes have not been examined because the majority of *ogre* mutants die as larvae or early pupae. Consistent with its expression pattern in larvae and pupae, *ogre* is essential for normal postembryonic development of the CNS.

The *Drosophila* nervous system develops in two phases. The larval neurons are born and establish connections during embryogenesis. A population of quiescent embryonic neuroblasts is reactivated during larval life; these divide to produce adult-specific neurons that form synaptic connections during metamorphosis and, together with persistent or remodelled larval neurons, form the adult CNS [94]. The optic lobes arise from postembryonic neuroblasts (pNBs) that form two distinct proliferation centres in the larval brain. The outer proliferation centre gives rise to neurons of the first and second optic ganglia, the lamina and medulla, the targets of retinal photoreceptors; the third neuropil, the lobula complex, arises from cell divisions in the inner proliferation centre [95]. Scattered pNBs give rise to postembryonic neurons of the brain and ventral CNS [96]. Ogre is expressed by pNBs with the strongest expression in the outer optic proliferation centre [88]. In *ogre* mutants, there is a significant decrease in the numbers of pNBs and, consequently, mutant nervous systems are much smaller than those of wild-type and the patterning of cells is severely disrupted [92]. The effects are most marked in the optic lobes that develop almost exclusively from pNBs. In hypomorphic mutants, which survive to late pupal or adult stages, the regular arrays of neurons that are characteristic of wild-type optic lobes are barely discernable. *ogre* null mutants die, as larvae or early pupae, well before postembryonic neuron differentiation is complete.

The most plausible explanation for the *ogre* neurogenic phenotype is that Ogre-containing gap junctions regulate proliferation and/or survival of pNBs. Comparisons of wild-type and *ogre* mutant nervous systems labelled with thymidine [97], or its analogue, bromodeoxyuridine (BrdU; Fig. 5) indicate that the numbers of S phase cells are

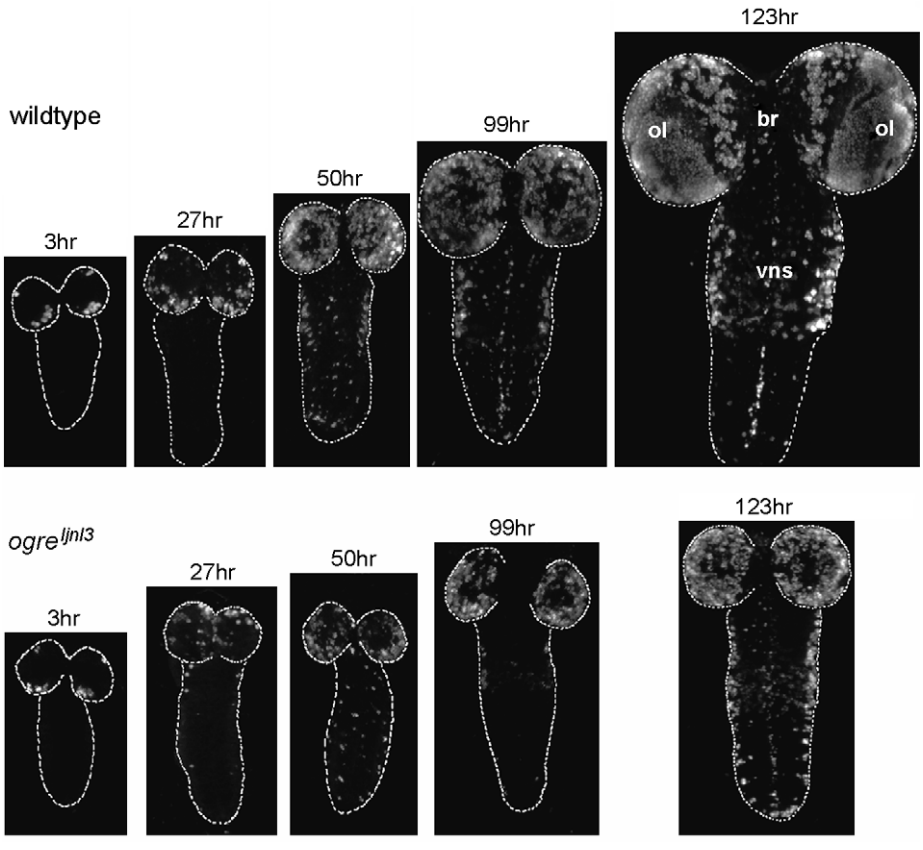


Fig. 5. *ogre* is required for cell proliferation in the developing *Drosophila* CNS. S-phase cells in the CNS of wild-type (upper panel) and $ogre^{ljnl3}$ mutant (lower panel) larvae were detected by bromodeoxyuridine (BrdU) labelling. Nervous systems were dissected at the indicated times after larval hatching (ALH), immediately following a 3-h pulse of BrdU, and labelled with an anti-BrdU antibody. The pattern of labelling is identical in wild-type and mutant at 3 h ALH. At later stages of development, fewer S-phase cells are evident in the mutant and the CNS is significantly smaller than that of wild-type. ol, optic lobe; br, brain; vns, ventral nervous system.

reduced in the mutant CNS. This suggests that reduced or delayed cell proliferation is likely to be a significant factor in the loss of pNBs. Some role for cell death in reducing the pool of pNBs cannot be ruled out. Histological analysis [97] revealed an increase in what appeared to be degenerating cells in mutant nervous systems. It is not known, however, whether the dying cells are pNBs or whether cell death is a secondary consequence of the defect in neurogenesis. The loss of pNBs in *ogre* mutants suggests that gap junctions regulate the availability of proliferative or survival signals for these cells. Whether such putative signals are exchanged between the pNBs or passed to the pNBs from surrounding glial cells is not known nor is the exact composition of the gap junctions involved. Ogre fails to form homotypic channels in *Xenopus* oocytes so it is likely to require a partner Inx to form functional channels in the CNS. Inx2 is a strong candidate; it co-expresses with Ogre in the optic proliferation centres and these proteins form functional heteromeric channels in *Xenopus* oocytes (Holcroft C., Phelan P., unpublished data; Section 4).

Several members of the Cx family have been implicated in neurogenesis by virtue of their expression patterns (reviewed in Ref. [98]) but, so far, there are no reports of neurogenic defects in Cx knockout mice. However, knockdown of Cx43 in the embryonic chick retina, using antisense oligonucleotides, results in a reduced eye phenotype (reviewed in Ref. [99]). Consistent with the studies of the *ogre* phenotype in *Drosophila*, the loss of retinal cells in the chick appears to be due primarily to reduced cell proliferation rather than death of neural precursors.

#### *5.3.2. Loss of ogre or shak-B function during development disrupts chemical synaptic transmission in adults*

In contrast to its essential role in the optic lobes (Section 5.3.1), *ogre* does not appear to be required in the eye during larval development. The *ogre* mutant eye is morphologically normal. The photoreceptor cell axons grow into the optic lobes as usual exhibiting no significant pathfinding errors although, because of the optic lobe defects, the patterning of the axon terminals in the lobes is abnormal [81,92]. Ogre is required, however, in the photoreceptors later during pupal development when these cells rearrange and establish their final synaptic connections with their optic lobe targets. R1–R6 photoreceptors synapse with L1 and L2 lamina monopolar neurons; photoreceptors R7 and R8 synapse with neurons of the medulla. Hypomorphic *ogre* mutants have abnormal electroretinograms (ERGs) [81,92,100]. The receptor potential (the presynaptic response to light) is normal; the 'on' and 'off' transients that measure the postsynaptic response of the lamina monopolar cells to activation of R1–R6 [101,102] are missing. This phenotype is rescued by expression of *ogre* specifically in the photoreceptor cells and replicated in genetic mosaics with loss of *ogre* function in the eye, but normal function in the optic lobes [81,100]. *ogre* is not expressed in late pupal or adult photoreceptors [15,88] and, thus, the ERG phenotype must reflect a requirement for the gene in these cells during pupal development.

The 'on' and 'off' transients of the ERG similarly are attenuated in *shak-B*$^{2}$ mutants, which are genetically null for both *shak-B(neural)* and *shak-B(neural+16)* [81,100,103]. *shak-B(neural)* expression in the lamina during pupal development is sufficient to rescue the phenotype whereas expression in adults only is ineffective [81,100]. Thus, *ogre* and *shak-B(neural)* appear to be required during pupal development in the retinal photoreceptors and lamina monopolar neurons, respectively, for normal synaptic connectivity between these cells. Photoreceptor to monopolar neuron transmission is mediated by conventional chemical synapses in adults; alterations in ERG transients similar to those in the *inx* mutants are observed in mutants that disrupt neurotransmitter synthesis or release [104,105]. Therefore, the loss of ERG transients in *ogre* and *shak-B*$^{2}$ mutants suggests that gap junctions have a developmental role in establishing or fine-tuning the pattern of chemical connectivity. This is consistent with reports in other invertebrate [106], and in vertebrate [107,108], systems of transient gap junctions between cells destined to form chemical synapses. In the fly visual system, it is not known whether transient junctions form between photoreceptors and monopolar cells or whether photoreceptors and monopolar neurons couple to neighbouring R and L cells, respectively. Whichever cell types are interconnected, additional Inxs are likely to be involved since neither Ogre nor Shak-B(neural) form homotypic channels, or heterotypic channels with one another, in *Xenopus* oocytes [32] (unpublished data cited in Ref. [100]; Section 4). More detailed analysis of Inx expression patterns during the period of synaptogenesis in the visual system is required to identify potential functional partners for Ogre and Shak-B(neural).

### *5.4. Drosophila inx2 and C. elegans inx3 are required for epithelial morphogenesis*

#### *5.4.1. Morphogenesis of the gut and other epithelial tissues is disrupted in Drosophila inx2 mutants*

Inx2 is expressed during embryogenesis in the epidermis and in the developing gut, tracheal system and salivary glands, overlapping expression domains of *inx3* and *ogre* [15,34,90,109]. Phenotypic analyses of *inx2* mutants demonstrate an essential role for the gene in the morphogenesis of these epithelial tissues.

*inx2* mutants were originally identified in a screen for genes involved in gut development [109]. The locus, and associated mutations, was designated *kropf* (German=craw, crop) presumably reflecting the engorged appearance of the oesophagus of those mutants (a minority) that survive to the first larval instar. In *kropf* mutant larvae food becomes trapped in the foregut because a valve-like structure called the proventriculus that regulates its passage from foregut to midgut fails to form [109]. The *Drosophila* foregut

(pharynx and oesophagus) and the hindgut (small and large intestines and rectum) are derived from ectoderm; the midgut is endodermal in origin. The proventriculus develops from the ectoderm at the foregut–midgut boundary by a series of morphogenetic movements that convert a simple tube into a three-layered valve (Fig. 6). The initial event is the evagination of the foregut epithelium to form a bulge called the 'keyhole' (Fig 6a). The 'keyhole' epithelium then folds back anteriorly over the oesophagus forming a cup-like structure (Fig. 6b). Finally, the midgut epithelium moves anteriorward causing the posterior keyhole to involute over the anterior lip of the developing proventriculus; at the same time, the posterior oesophagus inserts into the endodermal pouch of the anterior midgut (Fig. 6c) [110,111]. In wild-type embryos, *inx2* is initially detected throughout the foregut ectoderm and the endoderm of the anterior midgut; coincident with keyhole formation, its expression localizes to the primordium of the proventriculus [93,109]. In the absence of *inx2* function, in *kropf* mutants, the 'keyhole' cells fail to evaginate at the appropriate stage preventing further development of the proventricular valve. Interestingly *ogre*, although partially co-localized with *inx2* during the period of proventricular development, appears not to be essential for this process. The proventriculus develops normally in *ogre* mutants [93].

The functions of *inx2* are by no means restricted to the gut. The majority of *kropf* loss-of-function mutants die as embryos in which case lethality cannot be attributed to a feeding defect. Mutant embryos are typically smaller than wild-type and exhibit a range of epithelial tissue defects. The Malpighian tubules, salivary glands, trachea and hindgut, organs that, like the foregut, arise by invagination of primordia of ectodermal origin, are significantly reduced in size or absent. There are holes in the head epidermis, intermittent defects in the segmented epidermis and in extreme cases the cuticle, normally secreted by the epidermis, is missing [90]. The severity of the defects is greater in mutants lacking both maternal and zygotic *inx2*; in these embryos, extensive cell death is observed in epithelial tissues. Overexpression of *inx2* in early embryos, likewise, results in excessive apoptotic cell death [90]. Thus, cell survival and morphogenesis depend on tightly regulated levels of *inx2*.

Some progress has been made in identifying both molecules that act upstream of *inx2* and molecules that interact with the protein at the cell membrane to maintain epithelial integrity. The expression of *inx2* during proventriculus development appears to be regulated by the conserved *wingless* (*wnt1*) signalling cascade that is known to act both at the foregut–midgut, and the midgut–hindgut, boundary to regulate gut morphogenesis [112,113]. In *wingless* (*wg*) mutants, *inx2* transcripts are not detected in the primordium of the proventriculus and, in the more posterior domain of expression, at the midgut–hindgut boundary levels are reduced relative to wild-type. As a corollary, ectopic expression of *wg* in mesoderm or endoderm results in ectopic expression of *inx2* [109]. *inx2* mRNA is up-regulated in *Drosophila* S2 cells (an embryo derived cell line) transfected with *armadillo* (*arm*). The cytoplasmic pool of Arm protein, the *Drosophila* homologue of vertebrate β-catenin, transduces the Wg signal to the nucleus [114]. These data suggest that Wg acts via Arm

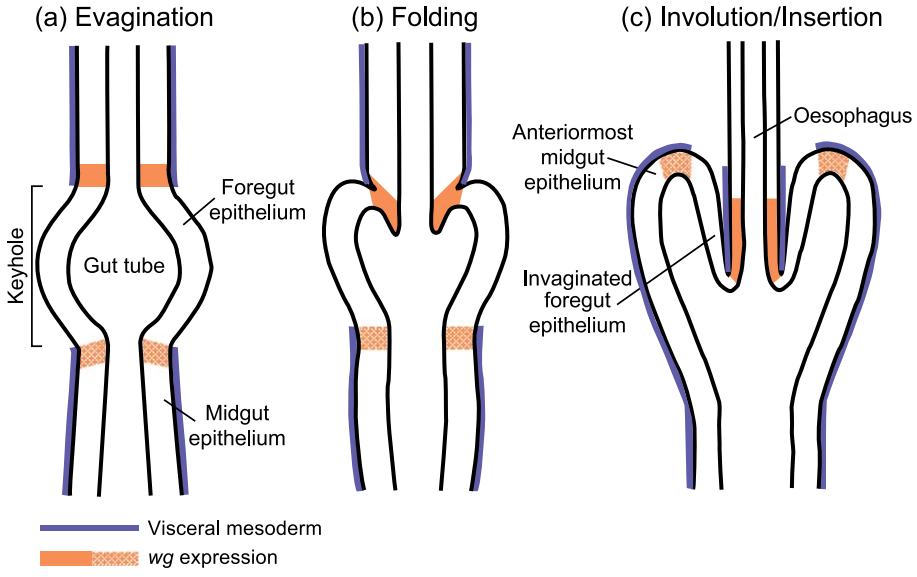


Fig. 6. Morphogenesis of the proventriculus of the *Drosophila* gut. Development of the proventriculus occurs during late embryogenesis. The ectoderm at the foregut–midgut boundary evaginates to form a structure called the 'keyhole' (a). The 'keyhole' epithelium folds back anteriorly over the foregut (b). The midgut epithelium, just posterior to the keyhole, moves anteriorward and the posterior keyhole involutes; the keyhole cells elongate as the posterior oesophagus inserts into the endodermal pouch of the anterior midgut (c). Domains of *wingless* (*wg*) expression anterior (orange) and posterior (patterned orange) to the keyhole are shown to clarify the cell movements. The gut is surrounded by visceral mesoderm (blue) except in the region of the keyhole. Drawn according to Ref. [111].

to regulate, directly or indirectly, *inx2* transcription in the gut.

Several lines of evidence support an interaction between Inx2 and the adherens junction proteins, *Drosophila* E-cadherin (DE-Cad) and membrane-associated Arm. In the membrane of epidermal cells, Inx2 is concentrated in the apico-lateral domain overlapping expression domains of DE-Cad and Arm. Mutations in the genes encoding either of these proteins disrupt plasma membrane targeting of Inx2 so that the protein accumulates in the cytoplasm. Likewise, in *kropf* mutants, DE-Cad and Arm are mislocalized. Over-expression of *inx2* in alternate epidermal parasegments of the embryo, under the control of the pair-rule gene *paired*, results in accumulation of Arm specifically in the Inx2 overexpressing stripes. Finally, DE-Cad and Arm co-precipitate with Inx2 from *Drosophila* embryo extracts, suggesting that these proteins are components of the same membrane complex [90]. Similarly, in other polarized epithelia, Inx2 is concentrated in discrete, albeit different, domains along the apico-basal axis: baso-laterally in salivary glands and laterally in the hindgut where expression overlaps that of the septate junction-associated protein Coracle, a homologue of vertebrate protein 4.1. Genetic studies are consistent with an interaction between Inx2 and Coracle [90] although, as yet, there is no evidence that these two proteins physically interact.

Consistent with the interactions of Inx2 in *Drosophila*, studies in vertebrate systems have provided evidence, first, that expression of two members of the Cx family, Cx43 [115,116] and Cx30 [117], are regulated by Wnt proteins. Secondly, Cxs frequently co-localize in the cell membrane with cadherin and catenin proteins and these proteins have been shown to influence the assembly of gap junctions in some cell types [118–122] (reviewed Ref. [123]). Thus, despite the lack of sequence homology between Inxs and Cxs, the mechanisms by which these proteins are regulated at nuclear and cellular levels are likely to be conserved.

#### *5.4.2. Several events in embryonic morphogenesis are disrupted in C. elegans inx-3 mutants*

INX-3 is first detected in *C. elegans* embryos at the two-cell stage and continues to be expressed by essentially all cells during the period of cell division and gastrulation. During the comma stage expression localizes to specific tissues, notably the epidermis (hypodermis), pharynx and intestine. Epidermal expression is transient, declining to undetectable levels shortly after hatching, whereas expression in the alimentary tract persists into adulthood. Post-embryonically, INX-3 is found transiently in motoneurons and the progenitors of body wall, sex (uterine and vulval) muscles. Expression continues throughout larval life in all developing sex muscles but is restricted to the vulval muscles in adults [16,24].

Surprisingly, given its expression pattern, INX-3 appears not to be required for early embryonic development; cell division, body axis specification and gastrulation, the first major morphogenetic movement, occur normally in *inx-3* mutants [24]. Subsequent steps in embryonic morphogenesis are disrupted leading to a range of phenotypes and culminating in embryonic or early larval lethality [24]. The second major morphogenetic movement in *C. elegans* is ventral enclosure, during which the epidermal sheet that covers the dorso-lateral surface of the developing embryo is pulled from either side to the ventral midline and sealed to enclose the embryo. This is followed by elongation during which epidermal cells undergo dramatic shape changes as the ovoid embryo transforms into an elongate worm [124]. The first observable defect in *inx-3* mutants is an anterior protrusion of presumptive neuronal cells prior to epidermal enclosure. In the majority of these animals, enclosure occurs normally but some of the protruding cells, which detach from the embryo, are lost in the process. Occasionally, the epidermis does not completely enclose the animal or ruptures at later stages of development probably because of defects in epidermal cell positioning or cell–cell contacts. Loss of *inx-3* function has more marked effects on the process of elongation; the entire body or discrete regions may fail to elongate fully resulting either in short, dumpy worms or slender worms with midbody bulges [24]. Successful elongation requires both epidermal cell-shape changes, driven by actin microfilament contraction, and contraction of the underlying body wall muscles [124]. Thus, *inx-3* may be required for either, or both, of these processes. Defects in elongation are also observed in the pharynx, specifically in the corpus and isthmus that are frequently shorter than normal. In addition, the anteriormost region of the pharynx, the buccal capsule, fails to attach to the nose in mutant embryos possibly due to malformation of the nose caused by the earlier loss of anterior cells. Attachment of the pharynx is required for pharyngeal elongation hence there may be a causal relationship between these two phenotypes [24].

Interestingly, two of the phenotypes observed in *inx-3* mutants, the detachment and extrusion of anterior cells and the failure of pharyngeal attachment, are suggestive of adhesion defects. One may speculate, therefore, that gap junctions interact with adherens junctions in coordinating these morphogenetic events in *C. elegans*. This is entirely consistent with the observed interactions between the components of adherens junctions and gap junctional Inxs during epithelial morphogenesis in *Drosophila* (Section 5.4.1).

### *5.5. Drosophila inx4 (zpg) is required for survival of differentiating germ cells*

In contrast to the widespread tissue distribution of many *Drosophila* innexins, the expression of *inx4*, also known as *zero population growth* (*zpg*), is restricted to germ-line cells [15,54]. Mutations at the *zpg* locus, as the name suggests,

disrupt germ cell development leading to infertility in both males and females [54]. The gonads of *zpg* mutants are very small compared to those of wild-type flies because of a dramatic reduction in the numbers of germ cells.

Fig. 7 summarizes the development of egg chambers and spermatocytes in *Drosophila*. In the female ovarioles (ovarian compartments), germ-line stem cells are located in the anterior germarium, surrounded by somatic terminal filament, apical cap and inner-sheath cells. The germ-line stem cells divide asymmetrically to produce a stem cell and a cystoblast. Cystoblasts undergo four rounds of cell division, with incomplete cytokinesis, to produce 16-cell cysts that eventually give rise to egg chambers; one of the cyst cells becomes the oocyte while the remaining cells differentiate into (germ-line) nurse cells. As the egg chambers mature, they are enveloped in somatic follicle cells and bud off from the germarium posteriorly [125]. Similarly, in the germinal centre at the apical tip of the testis, male germ-line stem cells divide asymmetrically to produce a stem cell and a gonialblast (primary spermatogonium). Gonialblasts divide to produce an array of 16 interconnected secondary spermatogonial cells that ultimately differentiate into spermatocytes. As they develop, the spermatogonia are in close contact with two types of somatically derived cells: cyst cells, which arise from division of two somatic cell progenitors that enclose the original stem cell, and the apical hub cells of the testis [126].

Loss of *zpg* function halts germ cell development prematurely. Stem cells are present and appear to divide normally but many of the later stages of germ cell development are not represented in mutant gonads. In females, 99% of ovarioles lack cystoblasts; in males, gonialblasts may be present but spermatogonia and spermatocytes are missing [54,127]. Two lines of evidence support the conclusion that early germ cells die as they begin to differentiate. Cells with morphology intermediate between gonialblasts and spermatogonia are observed in *zpg* mutant testes [54]. Female *zpg* mutant germ cells die when induced to differentiate by expression of a *bamC* (*bag of marbles*) transgene that promotes cystoblast differentiation in wild-type flies [127]. Thus, *zpg* is required for survival of germ cells at very early stages of differentiation. Loss of gene function may lead to cell death, directly, by depriving the cells of a survival or growth factor or, indirectly, by depriving them of a factor that promotes differentiation once initiated.

As yet, there are no clues to the identity of the factor(s) required to support germ cell development. The source of this putative factor, or the signalling molecules that regulate it, is also not known but it is likely to be the

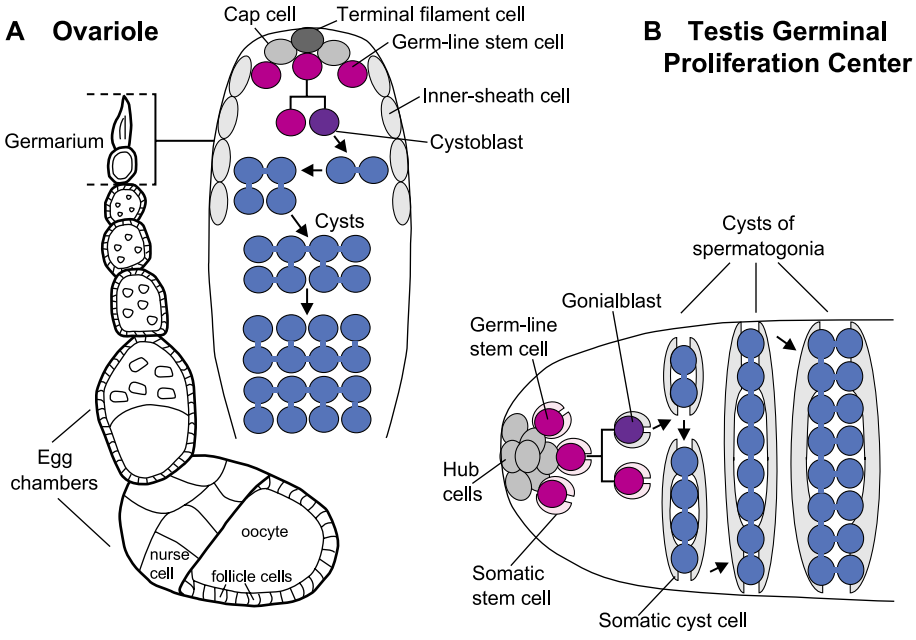


Fig. 7. Germ cell development in *Drosophila*. Female ovariole (A). Cell division occurs in the germarium at the anterior tip of the ovariole. The germ-line stem cells (pink) are closely associated with somatic terminal filament (dark grey), apical cap (mid grey) and inner-sheath (pale grey) cells. Asymmetric division of each germ-line stem cell yields a daughter stem cell and a cystoblast (purple). Cystoblasts undergo four rounds of cell division with incomplete cytokinesis to produce a cyst of 16 interconnected cells (blue). One of the two cyst cells with four ring canals differentiates into an oocyte and the other 15 cyst cells become nurse cells. As they develop, egg chambers, surrounded by somatic follicle cells, occupy progressively more posterior positions in the ovariole. Male germinal proliferation center at the apical tip of the testis (B). Each germ-line stem cell (deep pink) and a pair of associated somatic stem cells (pale pink) divide asymmetrically. Germ-line cell division produces a stem cell and a gonialblast (purple). Division of the two somatic cells produces two daughter somatic cells that envelop the daughter germ-line stem cell, and two cyst cells (grey) that envelop the gonialblast. Four rounds of gonialblast division, with incomplete cytokinesis, gives rise to a cyst of 16 interconnected spermatogonia (blue). The somatic cyst cells do not divide again but grow in size to encase the spermatogonial cyst. A (left panel), adapted from Ref. [125].

Table 2
Innexin mutant phenotypes

| Gene | Mutant phenotype | References |
|---|---|---|
| *Dm-shak-B(neural)* and/or *shak-B(neural+16)* | Loss of electrical synapses in several neural pathways including the GFS | [25,26,30,31,64] |
| | Behavioural defects in escape (due to loss of GFS synapses), grooming and feeding | [70,72,73] |
| | Defects in development/function of chemical synapses in the visual system | [81,100,103] |
| | Delayed development of $K^+$ currents in larval somatic muscle | [136] |
| *Dm-shak-B(lethal)* | Embryonic/early larval lethality, cause unknown | [19,20] |
| *Dm-ogre* | Small size of the adult CNS, particularly the optic lobes, due to reduced numbers of postembryonic neuroblasts | [88,92,97] |
| | Defects in development/function of chemical synapses in the visual system | [81,100] |
| | General developmental delay; late larval/early pupal lethality | [92] |
| *Dm-inx2* | Defects in morphogenesis of the gut, Malphigian tubules, salivary glands, trachea and epidermis; embryonic/larval lethality | [90,93,109] |
| *Dm-inx4* | Male/female infertility due to defects in germ cell development | [54,127] |
| *Ce-unc-7* and *unc-9* | Uncoordinated movement, possibly due to aberrant electrical connections between neurons | [75–77] |
| | Loss of mechanosensory modulation of pharyngeal pumping, reduced drug (anthelmintic) sensitivity, possibly due to loss of electrical connections between the pharyngeal and extrapharyngeal nervous systems | [77,83] |
| | Egg-laying defect | [75] |
| *Ce-eat-5* | Feeding defects due to asynchronized contraction/loss of electrical coupling of pharyngeal muscles | [28,87] |
| *Ce-inx-6* | Feeding defects due to asynchronized contraction/loss of electrical coupling of pharyngeal muscles | [29] |
| *Ce-inx-3* | Morphogenetic defects: protrusion/loss of cells, partial failure of ventral enclosure and elongation, Feeding defects (in surviving worms) due to asynchronized contraction of pharyngeal muscles | [24] |

*Dm*, *Drosophila melanogaster*; *Ce*, *Caenorhabditis elegans.* For summaries of innexin expression patterns, see Refs. [15,16].

somatic cells that surround developing germ cells. Gap junctions have been observed between germ cells and somatic cells by EM and dye coupling in *Drosophila* and other insects [54,128–130]. In both males and females, Zpg protein localizes to the surface of germ cells and is concentrated at sites of germ cell-somatic cell apposition [54]. In the ovariole, Zpg is concentrated in the membrane of germ-line stem cells, dividing cyst cells and developing egg chambers at sites of contact with somatic apical cap cells, inner-sheath cells, and follicle cells, respectively. In the testis, the protein is found in spermatogonia and early spermatocytes at points of contact between these cells and somatic cyst cells [54]. Somatic cells, on the other hand, do not express detectable levels of Zpg. This suggests that germ cell-somatic cell junctions consist of heterotypic channels formed by the docking of Zpg (Inx4) hemichannels in germ cells with somatic cell hemichannels composed of a different Inx. Inx2 is good candidate; *inx2* mRNA is expressed in the somatic follicle cells of developing egg chambers [15] and the protein has been shown to form heterotypic channels with Inx4 in paired *Xenopus* oocytes [52] (Section 4). *ogre* and *inx3* transcripts are also found in follicle cells [15] but it is not known if Ogre and Inx3 proteins functionally interact with Inx4 and, thus, whether they are likely to participate in germ cell-follicle cell coupling. Both proteins form functional heteromeric channels with Inx2 in paired *Xenopus* oocytes [34] (Holcroft C., Phelan P., unpublished data; Section 4) and may, therefore, be components of gap junctions between neighbouring follicle cells.

The role of innexins in germ cell development in *Drosophila* has parallels with the role of connexins in gonad development in mammals. In the ovary, Cx37 is expressed in the oocyte and is a component of gap junctions that couple the oocyte to the surrounding somatic granulosa cells [131]. Cx43 couples the granulosa cells to one another [132,133]. Knockout of either gene in mice interferes with ovarian follicle growth and oocyte maturation [131,132,134]. The testes of Cx43 knockouts or knock-ins, in which Cx43 is replaced with another Cx, are also smaller, with fewer germ cells, than the testes of wild-type mice [134,135]. Thus, in mammals, as in flies, gap junctions are required for germ line development in both sexes.

## 6. Summary and perspectives

Identification of the multigene family of innexins has opened an interesting debate about the molecular evolution of gap junctions. More importantly, it has made it possible to apply genetic approaches in simple model invertebrates to unravel the diverse roles of direct cell–cell communication. To date, analyses of Inx function in *Drosophila* and *C. elegans* have confirmed the classic roles of gap junctions in coordinating electrical activity in differentiated neurons and muscles and provided genetic evidence to support their roles in neural development, epithelial morphogenesis and germ cell differentiation (Table 2).

A picture emerging from the genetic analyses to date is that, in many cases, multiple *inx* genes are required for a given biological process. This is consistent with the overlapping patterns of tissue distribution of these genes and the encoded proteins. Some degree of functional redundancy among *inx* genes is suggested by the fact that loss-of-function mutations do not always yield an obvious phenotype in tissues known to express the mutated *inx*. However, studies that have directly addressed the question of genetic redundancy suggest that innexins are highly specific in their roles so that they rarely are completely functionally interchangeable. Curtin et al. [100] tested several *inx* genes for ability to rescue the loss of ERG transients in *ogre* and *shak-B* mutants, a phenotype that reflects the requirement for these genes in establishing photoreceptor-lamina neuron synapses during pupal development (Section 5.3.2). *shak-B(neural)*, *shak-B(lethal)*, *inx2* and *inx7* failed to rescue the transients in *ogre* mutants although *ogre* itself was fully effective in the same experimental paradigm. Similarly, *shak-B(neural)* rescued the ERG phenotype in *shak-B* mutants whereas *ogre* failed to do so and *inx7* only partially restored function. In the *C. elegans* pharynx, *inx-6* failed fully to substitute *eat-5* function in synchronizing contractions of anterior and posterior muscles [29]. Failure of one *inx* to substitute for another could be because the substituted protein fails to form channels independently or with the endogenous Inxs. Partial rescue, on the other hand, clearly indicates that channels are assembled and suggests that the properties of these channels are sufficiently different from wild-type channels as to be unable fully to compensate. Attempts to replace one Cx with another, similarly, have led to the conclusion that these genes, by and large, are not functionally interchangeable [137]. Such specificity of action of gap-junction genes coupled with the diversity of their roles explains why organisms have so many Cx or Inx genes.

In vivo studies of Inx function have, so far, largely been conducted in the fly and worm. Inxs have been isolated from several other invertebrates including grasshopper, leech and mollusc (Section 2). While the impetus for these studies has been, in part, to address questions about gap-junction gene evolution, the potential of these organisms for functional studies should not be overlooked. The power of *Drosophila* and *C. elegans* lies in their genetic tractability. A drawback is that the cells in these tiny organisms are relatively small. Even the so-called GFs in *Drosophila* are fairly modest in size compared to some of the large neurons of leeches and molluscs. These cells are highly accessible and lend themselves particularly well to cell biological and electrophysiological studies [138–140]. By injecting *inx* mRNA, as in the recent report from Kelmanson et al. [141], or interfering dsRNA, it should now be possible to manipulate or knockout gap junctions in individual cells. Combining cell-specific gene knockout with electrophysiology and cell biology provides unique opportunities to investigate in situ how changing the cellular complement of gap-junction genes influences the permeability properties of the intercellular channels and how this, in turn, affects cell physiology.

The presence of innexin homologues in the genomes of insect viruses is intriguing. The innexin encoding polydnaviruses are symbiotic proviruses of wasps that parasitize lepidopteran larvae. Viral gene expression in the infected lepidopteran host is required to disrupt host immunity and development thereby allowing the parasite egg to hatch and develop [10,11,142]. At least some viral Inxs are expressed in haemocytes, the major immune cells of insects, suggesting that they facilitate immunosuppression by interfering with normal cell–cell communication between these cells [10,11]. Further studies in this novel system will be interesting to shed light on the mechanisms by which gap-junctions regulate insect immune responses.

Perhaps the most surprising finding in recent years is that *inx* homologues persist in vertebrate genomes [7,12,13]. While knockout of these *panx* genes is required to demonstrate conclusively that they function in vivo, their ability to induce formation of intercellular channels in vitro and their tissue specific expression patterns suggest that this is likely to be so. In the brain, *panx* transcripts are found in neurons, such as the pyramidal cells of the hippocampus, that are known to be electrically coupled but, unlike other hippocampal neurons, do not require Cx36, the major neuronal Cx [12,13]. Remarkably, this raises the possibility that neighbouring cells in the brain, and probably in other tissues, use different gene families to assemble their intercellular channels. Whether this is functionally significant or a quirk of evolution remains to be determined.

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## Note added in proof

Since this review was submitted innexins have been identified in *Hydra*. Connexins appear not to be present in

this simple coelenterate suggesting that innexins are the primordial gap-junction proteins [143].

## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbamem.2004.10.004.

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