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Intercellular Communication: the *Drosophila* Innexin Multiprotein Family of Gap Junction Proteins

Review

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Summary

Gap junctions belong to the most conserved cellular structures in multicellular organisms, from *Hydra* to man. They contain tightly packed clusters of hydrophilic membrane channels connecting the cytoplasms of adjacent cells, thus allowing direct communication of cells and tissues through the diffusion of ions, metabolites, and cyclic nucleotides. Recent evidence suggests that gap junctions are constructed by three different families of four transmembrane proteins: the Connexins and the Innexins found in vertebrates and in invertebrates, respectively, and the Innexin-like Pannexins, which were recently discovered in humans. This article focuses on the *Drosophila* Innexin multiprotein family, which is comprised of eight members. We highlight common structural features and discuss recent findings that suggest close similarities in cellular distribution, function, and regulation of *Drosophila* Innexins and vertebrate gap junction proteins.

Gap Junctions Were Originally Identified in Invertebrates

Direct intercellular communication is accomplished in almost all tissues and organs by gap junctions. Gap junctions are composed of clusters of membrane channels between adjacent cells, thereby allowing the rapid exchange of ions and metabolites of up to 1 kDa in size. In the 1950s, studies performed by Furshpan and Potter provided convincing evidence for direct communication between cells [1, 2]. They showed that action potentials pass directly from the giant interneurons in the nerve cord of the crayfish into the giant motoneurons. A similar experiment was conducted by Watanabe in neurons of the lobster cardiac ganglion [3]. The identification of the structures associated with direct intercellular signaling was made using electron microscopy. Specialized intercellular junctions were found that form a characteristic nexus, or "gap," between two adjacent cell membranes of two neighboring cells, and were therefore named gap junctions [4, 5, 6].

Although gap junctions were first studied in invertebrate species, the genes encoding their structural protein components were first isolated in vertebrates [7, 8]. These genes were named *connexins* in accordance with the morphological description of gap junctions as having a "nexus" structure. Connexins comprise a multigene family of integral membrane proteins, with 20 Connexin isoforms identified in mice and 21 in humans [9]. These proteins are characterized by two extracellular domains, four membrane-spanning domains, and three cytoplasmic domains, consisting of an intracellular loop, and amino- and carboxy termini. Six Connexin transmembrane protein units form a hemichannel, which is termed the Connexon (see [10] for review). In mammals, two hemichannels form a gap junction channel, with each hemichannel provided by one of the two neighboring cells. These two hemichannels dock head-to-head in the extracellular space to form a tightly sealed, double-membrane intercellular gap junction channel. Gap junctions allow diffusional exchange of ions, such as Ca^{2+}, and metabolites, such as inositol phosphates and cyclic nucleotides (see [11] and [12] for review). Gap junction channels are arranged into tightly packed plaques that may consist of hundreds or thousands of channels, forming gap junctions of several microns in diameter.

In the late 1990's, mutant analysis and molecular characterization in the fruit fly *Drosophila melanogaster* and the worm *Caenorhabditis elegans* lead to the identification of a *connexin*-analogous multigene family, the *innexins* (invertebrate *connexin* analogs) [13]. Genome sequencing projects unraveled 8 fly and 25 worm *innexin* genes (see [14] for review). No *connexin* genes were identified, however, consistent with earlier studies. Remarkably, Innexin and Connexin proteins share virtually no similarity in the primary amino acid sequence. In contrast, the structure and function of Innexins seem very similar to those of Connexins (see below). Meanwhile, *innexin* genes were identified in various other protostomal species, including the grasshopper, *Schistocerca americana* [15], the mollusk, *Clione limacine* [16], the flatworm, *Giardia tigrina* [17, 18], the polychaete annelid, *Chaetopterus variopedatus* [19], the leech, *Hirudo medicinalis* [20], the protochordate, *Ciona intestinalis* [21], and the cnidarian, *Hydra vulgaris* [18], suggesting that *innexins* may encode gap junction proteins in all invertebrates. In contrast, *connexin* genes seem to be restricted to vertebrates only [9]. Recently, an *innexin*-like gene family, consisting of three members in humans and mice, has been identified: the *pannexins* (pan: all, throughout; and nexus: connection, bond) [17, 22]. Pannexin proteins share the same overall membrane topology with Innexins and Connexins, and they form functional gap junctions when expressed in *Xenopus* oocytes [23]. The evolutionary relationship between Innexins, Pannexins, and Connexins is still under debate (see below).

Genomic Organization and Expression of *Drosophila* Innexin Family Members

The complete sequencing of the *Drosophila* genome has revealed eight *innexin*-encoding loci [24] (Figure 1A and Table 1). *innexins 1*, *2*, and *7* are clustered on chro-

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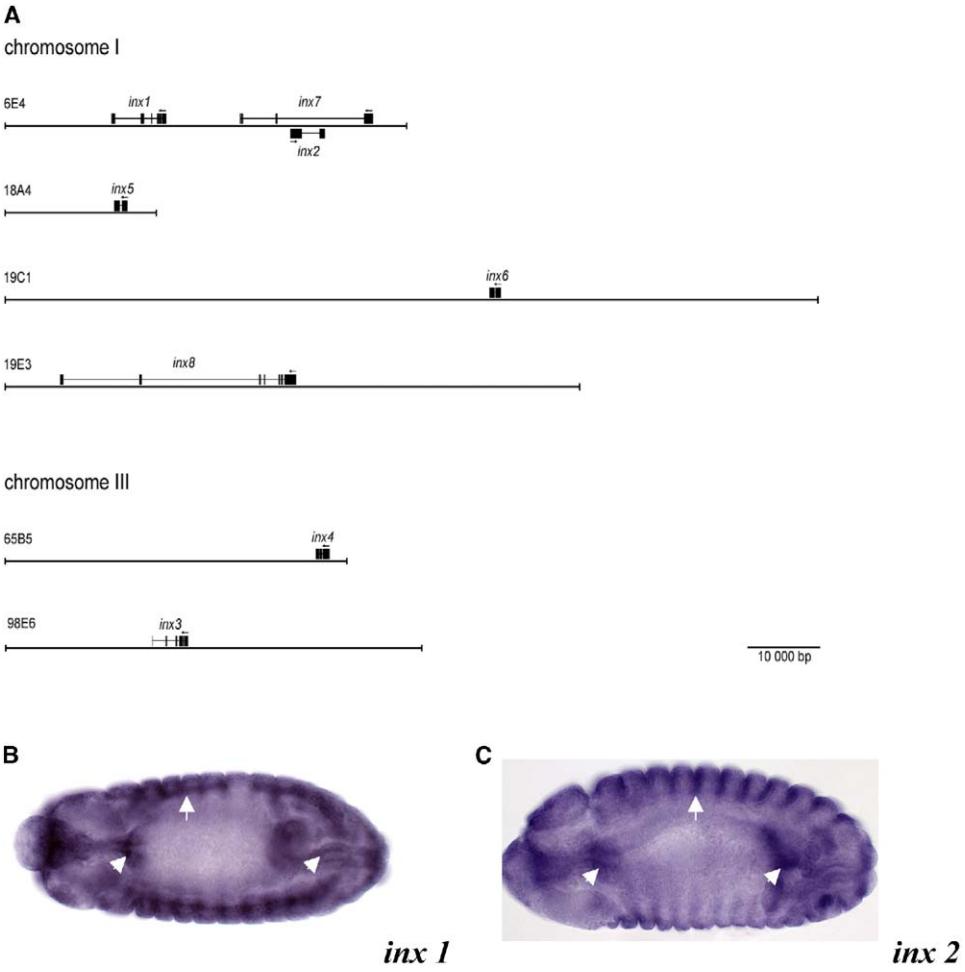

Figure 1. Chromosomal Locus of the *Drosophila innexin* Genes, and Overlapping *innexin 1* and *2* mRNA Expression Patterns in *Drosophila* Embryos

(A) The genomic locus and intron-exon structure of the 8 *Drosophila innexin* genes are depicted in a scheme that was assembled according to Flybase data. Note the clustering of *innexins 1*, *2*, and *7* on chromosome I.

(B and C) The mRNA expression patterns of *innexins 1* and *2* show overlapping segmental expression along the anterior-posterior axis (white arrow) and in the fore-and hindgut (white arrowheads).

mosome I within chromosomal region 6E4. *innexins 5*, *6*, and *8* are also located on chromosome I; however, they are dispersed across the chromosome and reside in regions 18A4, 19C1, and 19E3, respectively. The remaining *innexins*, *3* and *4*, are found on chromosome III, located at position 98E6 and 65B5, respectively. Clustered chromosomal localization is a phenomenon that has also been described for mouse and human *connexin* genes [25]. The more than 20 *connexin* genes in mice and humans share 40% sequence identity. Most of them show a common structural organization, and it has been demonstrated that some clustered *connexin* genes are expressed in the same tissue [9]. Whether clustered *innexins* are commonly regulated is not yet known.

In *Drosophila*, mutants have been isolated for some of the *innexin* family members and functions have been assigned to *innexin 1* (*ogre*) in the adult visual system [26, 27, 28], and to *innexin 8* (*shaking B*) in electrical transmission processes in the giant fiber system and in the visual system [29, 30, 31, 32, 33]. *Innexin 2* (*kropf*) is involved in embryonic epithelial organization and morphogenesis [34, 35], and *innexin 4* (zero population growth) in germ cell differentiation processes [36, 37]. No mutants are available so far for *innexins 3*, *5*, *6*, or *7*, and the function of these gap junction channel genes is still unknown.

In situ hybridization experiments revealed that most of the *Drosophila innexin* genes are expressed in a complex and, in most tissues, overlapping temporal and spatial profile [38, 39]. *innexins 1*, *2*, *3*, and *7* show a high degree of overlapping expression during oogenesis within the follicle cells; deposition of *innexin 2* mRNA into the oocyte was shown to be required for proper organization of embryonic epithelia [35]. These four genes also display overlapping expression domains in the embryo (see Figure 1B for *innexins 1* and *2*); they are found in segmental, reiterated, and overlap-

Table 1. Properties of *Drosophila* Innexins

Gene	Chromosome	Mutant	mRNA (bp)	Protein	pI	MW (kDa)	NT (aa)	TM1 (aa)	EL1 (aa)	TM2 (aa)	CL (aa)	TM3 (aa)	EL2 (aa)	TM4 (aa)	CT (aa)
inx1	I	*optic ganglion reduced* (*ogre*)	1089	Inx1	9.11	42.58	25	20	62	23	49	21	66	21	75
inx2	I	*kropf*	1104	Inx2	5.88	42.48	25	20	62	23	49	21	66	21	80
inx3	III		1188	Inx3	8.74	45.35	31	20	61	23	50	21	66	21	102
inx4	III	*zero population growth* (*zpg*)	1104	Inx4	9.13	42.78	25	20	63	23	50	21	66	21	78
inx5	I		1260	Inx5	8.93	48.97	25	20	115	23	50	21	66	21	78
inx6	I		1446	Inx6	7.79	55.66	25	20	97	23	50	21	66	21	158
inx7	I		1317	Inx7	9.18	50.66	27	20	63	23	66	21	63	21	134
inx8	I	*shak-B lethal*	1119	Inx8	8.91	44.35	25	20	63	23	49	21	66	21	84
mCx43	X		1146	Cx43	8.97	43.004	23	23	30	23	55	23	31	23	151

The designation of *innexin* mutants is listed next to the gene and the chromosomal locus columns. For comparison, Connexin 43 was added. aa, amino acid; pI, isoelectric point; MW, molecular weight; characteristic protein domains: CL, cytoplasmic loop; CT, carboxy terminus; EL, extracellular loop; NT, N terminus; TM, transmembrane domain.

ping patterns within the epidermis and in other epithelial tissues, such as the developing foregut, the hindgut, and salivary gland tissues [34, 40, 41]. *innexins 2* and *3* are also coexpressed in the embryonic nervous system. A striking feature is the localization of *innexin 1* and *innexin 2* mRNAs to apical sites of epithelial cells, suggesting that these mRNAs may interact with specific factors for mRNA transport and/or localization [34, 41]. The functional significance of this localization, however, is not yet known. The expression of *innexin 4* is restricted to the male and female germ line, and the *innexin* genes *5* and *6* appear to be expressed at low levels during embryogenesis [39]. All *innexin* genes, with the exception of *innexin 4,* show expression in imaginal discs and in the CNS of newly formed white pupae [39]. In mammals, most cells and tissues also express more than one *connexin* gene allowing the formation of heteromeric Connexin channels (Connexin subunits differ within one Connexon) and heterotypic gap junctions (two Connexons each consisting of different Connexins) (see [10] and [42] for review). Variation in Connexin stoichiometry provides a basis for the selectivity of channels to metabolites such as ATP and allows cells to dynamically regulate their intercellular communication properties [43, 11]. Similarly, it has been shown for *Drosophila* Innexins, including Innexins 2 and 3, and for the mammalian Pannexins 1 and 2 in mice, that they form heteromeric and heterotypic gap junctions in the heterologous *Xenopus* expression system [40, 23]. Molecular and functional studies with *innexin 1* and *innexin 8* in the visual system further suggest that proper physiological functions of gap junctions are dependent on specific combinations of Innexin family members [27].

Protein Structure of *Drosophila* Innexins

Innexins display the same overall topology as Connexins and Pannexins (Figure 2). These proteins have two extracellular loop (EL) domains (EL1 and EL2), four hydrophobic transmembrane (TM) domains (TM1–TM4), and three cytoplasmic domains, including an intracellular loop (IL) and amino- and carboxy termini (NT and CT, respectively).

The size of the *Drosophila* Innexin proteins ranges from 42 kDa to 55 kDa, with Innexin 2 being the smallest (42.58 kDa) and Innexin 6 the largest (55.66 kDa) member of the protein family (Table 1). The differences in size are moderate in comparison to the murine Connexin protein family, with the largest being more than twice the size of the smallest (range, 26–57 kDa,). Similar to Connexins, the number of amino acids (aa) of the predicted four TM domains is very similar among all Innexin proteins, and alignments indicate high sequence homology within these domains. Especially conserved are a five amino acid stretch (YYQWV) and a proline residue within the second TM (Figure 3A). Interestingly, a proline residue is found at a similar position within the second TM of Connexins. Proline residues are thought to mediate conformational changes associated with voltage gating [44, 45]. The function of the YYQWV amino acid stretch in Innexins is still unknown. Notably, the YYQWV motif, which was once described as a signature sequence of Innexins (Figure 3A) [46, 14], is not found in vertebrate Pannexins (Figure 3B, second TM in yellow). Additionally, the amino acid residues preceding and following the four TM domains of *Drosophila* Innexins show some obvious similarities to Pannexins (Figure 3A, aa in gray and blue). In Connexins, the first transmembrane domain is required for membrane insertion [47], and it is also reported to be a crucial regulator of Connexin oligomerization [48, 49]. The selectivity signal regulating subunit compatibility might be located in the amino terminal portion (NH_2-terminal, first TM and/or first EC domain) [10]. Corresponding regions are not conserved in Innexins.

The ELs, EL1 and EL2, are also very similar among the *Drosophila* Innexins (Figures 2 and 3A). Exceptions are Innexins 5 and 6, with long EL1s of 97 amino acids for Innexin 5 and 115 amino acids for Innexin 6 (Figures 2 and 3, Table 1). Size differences within the ELs may

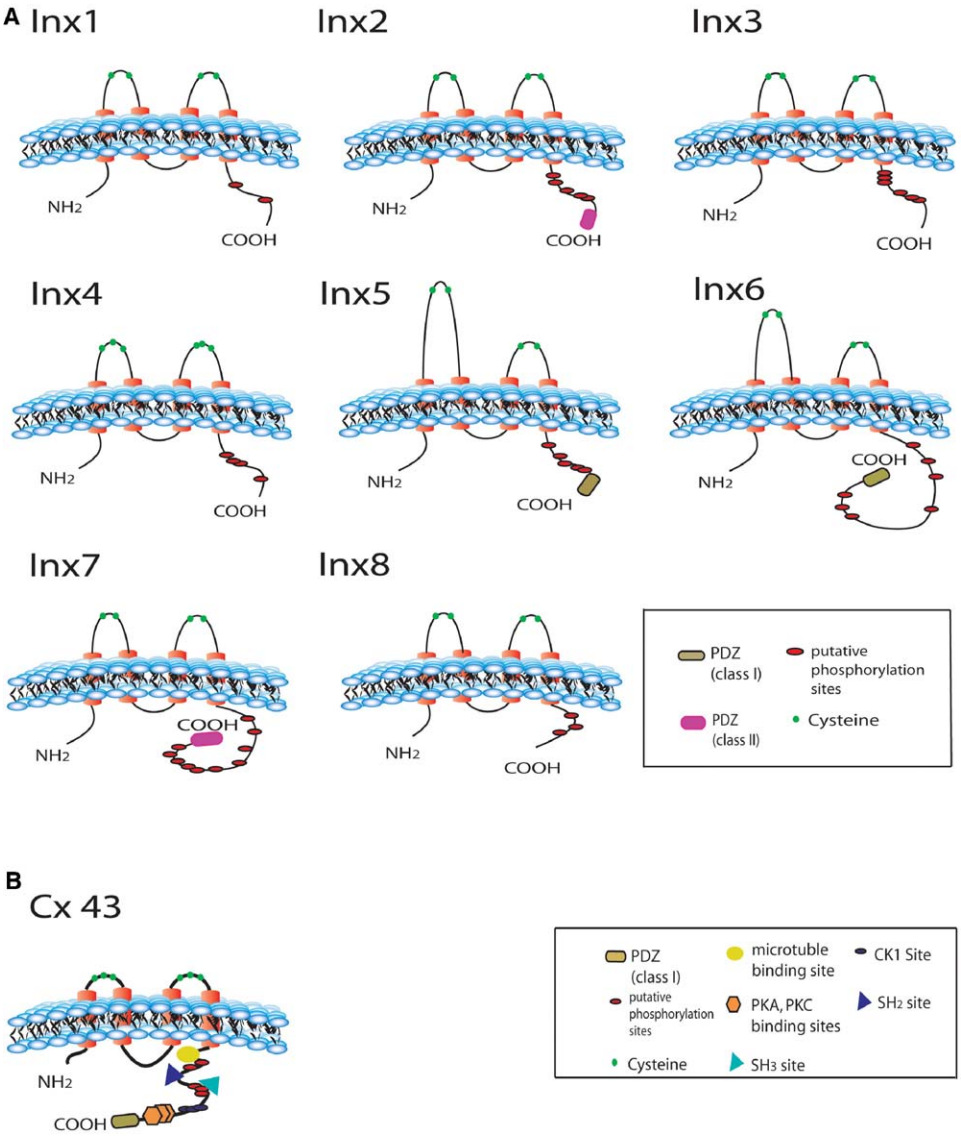

Figure 2. Membrane-Spanning Structure of the *Drosophila* Innexin Proteins and of Connexin 43

(A) The predicted structures of the eight *Drosophila* Innexin proteins are based on the data derived from Innexin alignments and data listed in Table 1. Striking features of the different Innexins, such as varying length of the extra or intracellular loop, varying length of the C terminus, and putative PDZ domains, are depicted using different symbols and colors.

(B) Scheme of the vertebrate gap junction protein Connexin 43. Consensus interacting protein modules and putative phosphorylation sites [55] are shown as symbols.

influence the width of the gap and, thereby, the quality of molecules that pass through the gap junction channel. A characteristic feature of all *Drosophila* Innexins are two cysteines within EL1 and EL2 (Innexin 4 is an exception, as it contains a third cysteine within both ELs; Figures 2 and 3, open circles). The spacing between the two conserved cysteines (C) is $CX_{13/14}C$ for EL1 and $CX_{17}C$ for EL2. Moreover, the amino acids around the second cysteine within EL1 and around the cysteine pair within EL2 are rather conserved between the different Innexin family members. Particularly within EL2, a stretch of 35 amino acids is identical or displays only conservative amino acid changes in most cases (Figure 3A, aa in gray and blue). Pannexins also contain two cysteine residues within EL1 and EL2; however, the spacing of the cysteines in EL2 is not conserved compared to those in Innexins (Figure 3B, cysteins in green). Three cysteine residues within each EL are characteristic for Connexins (see [9] for review). The distances between the cysteine residues are also conserved for Connexins and Pannexins [14, 18] (Figures 2, 3A, and 3B). It has been shown in the case of Connexins that the disulphide-linked ELs are crucial for the docking of the two hemichannels to generate a gap junction channel [50, 51, 52]. Therefore, the high degree of sequence homology within EL1 and EL2 of gap junc-

tion proteins may reflect evolutionarily conserved sequences necessary for efficient and specific docking of hemichannels.

The N termini and the intracellular loops are less variable among Innexins (Figure 3A and Table 1). An exception to this rule is Innexin 7, with a cytoplasmic loop of 66 amino acids compared to the 49–50 amino acids for the other *Drosophila* Innexins. It has been shown for Connexins that the N-terminal domain is required for membrane insertion and oligomerization [49] and that the cytoplasmic loop is involved in voltage gating [53, 42].

The carboxyterminal tails of Innexins show the highest variability in length. Innexin 6 and Innexin 7 display the largest C-terminal domains, with 158 and 134 amino acids, respectively (Figure 3A and Table 1). A small stretch of amino acids within the C-terminal tail starting with the amino acid sequence GDW is common to all Innexin proteins (Figure 3A, aa in gray and blue). Only Innexin 7 lacks this sequence motif. A closer look at the C-terminal region shows putative PDZ binding domain motifs for a number of Innexins (Figures 2 and 3A). These motifs are often present as a C-terminal tetrapeptide, and can be subdivided into different classes. Class I motifs contain the consensus sequence X-S/T-X-V and class II contains sequence X-ϕ-X-ϕ, where ϕ represents a hydrophobic amino acid and X represents an unspecified amino acid [54]. Whereas these putative PDZ domains under the above mentioned classification are within the extreme C-terminal region of Innexin 5 and 6 (class I) and Innexins 2 and 7 (class II), functional data for these domains are still lacking. Interestingly, PDZ domains are also found at the C terminus of several Connexins (Figure 2B) (see [55] for review). It has been shown that functional differences between Connexins reside within the carboxyterminal tail, which is subject to phosphorylation and is a target of several protein-protein interactions (see [55] for review). Connexin 32 contains both a calmodulin binding domain and a short juxtamembrane region with a crucial gap junction-targeting motif [42]. The carboxyl tail of Connexin 43 is modified posttranslationally by phosphorylation (see [56] for review) and interacts with other associated proteins, such as microtubules and zonula occludens (ZO)-1 [55, 57, 58]. Furthermore, there is extensive evidence that it interacts with the intracellular loop via a "ball and chain" mechanism, allowing chemical gating of the channel [59]. Other studies also indicate an involvement in the formation of heteromeric Connexons [49] and contributions to the gating characteristics of heteromeric channels (see [12] for review). The ability to form homotypic and heterotypic channels provides greater complexity in the regulation of gap junction communication. We recently demonstrated the direct molecular interaction between Innexins 2 and 3 via their C-terminal domains (Lehmann et al., personal communication). This is consistent with the coupling experiments in *Xenopus* oocytes, in which Innexin 3 forms functional gap junction channels only in the presence of Innexin 2, thus providing strong evidence for heteromeric and heterotypic Innexin channels [40]. A high-resolution model of Connexin 43 gap junction channels had been described [53]. Similar structural and functional data are still lacking for Innexins.

Innexin Protein Distribution and Cellular Dynamics

To study the dynamics of Innexin proteins, antibodies have been raised against several Innexins, including Innexin 1, 2, 3, and 4 [36, 41, 60, and our unpublished data]. In most cases, peptide sequences of the C terminus or the intracellular loop domain were used for antibody generation. Using confocal microscopy of whole-mount embryos and *Drosophila* tissue culture cells, it was determined that Innexin proteins are expressed in a punctuate pattern in the plasma membrane of the epithelial cells (Figure 4A); Innexins 1, 2, and 3 are found within the membranes of cells in the epidermis, the salivary glands, and the fore- and the hindgut (Figure 4A) [34, 35, 41, 60]. Furthermore, punctuate staining was observed in the cytoplasm, most likely reflecting intracellular protein stores. Similarly, Innexin 4 (zero population growth) was shown to localize to the membranes of germ cells and surrounding somatic cells [37]. Notably, a punctuate protein distribution is also characteristic of Connexins, which are contained within intracellular stores in the ER-Golgi interfacial regions, and within the membrane at sites of cell-to-cell contact in gap junctions [61].

Membrane integration of Connexins takes place in the endoplasmatic reticulum (ER) where the transmembrane topology of Connexins is achieved (see [10] and [42] for reviews). After integration into the ER, Connexins form hexameric Connexons, possibly via dimeric and tetrameric intermediates [62]. Connexin-fluorescent proteins have enabled the life cycle of gap junctions to be studied in live cells in real time. Connexons reside in highly mobile vesicular carriers that move to and from the gap junction. Vesicles of <0.5 μm traffic toward the plasma membrane and larger vesicles, of <0.5–1.5 μm, may correspond to internalized gap junctions [63]. Insertion of Connexons into the plasma membrane occurs over large areas of the cell surface where they diffuse laterally, joining the periphery of preexisting gap junction plaques (see [10] and [63] for reviews). The picture emerging is that new channels are continuously added to the edge of the gap junction plaque and older, paired Connexons are removed from its center area [64]. Degradation is accomplished by proteasomal and lysosomal pathways. Proteasomal degradation is a general mechanism for disposing of mutational, faulty, or overproduced Connexins, and occurs in the secretory pathway [65]. Degradation may be regulated by phosphorylation. For instance, PKC and MAP kinase are involved in TPA-induced degradation of Connexin 43 and inhibition of gap junction intercellular communication [66]. There are no data available yet on how trafficking and degradation of Innexins occurs in cells.

Interactions of Innexins with Other Cell Junction Components

Interactions of Innexins with Core Components of Adherens Junctions

In *Drosophila*, *D*E-cadherin and armadillo, the vertebrate β-catenin homolog, are core components of adherens junctions. Cadherins are part of a major family of transmembrane glycoproteins known to play important roles in the regulation of cell adhesion and cell

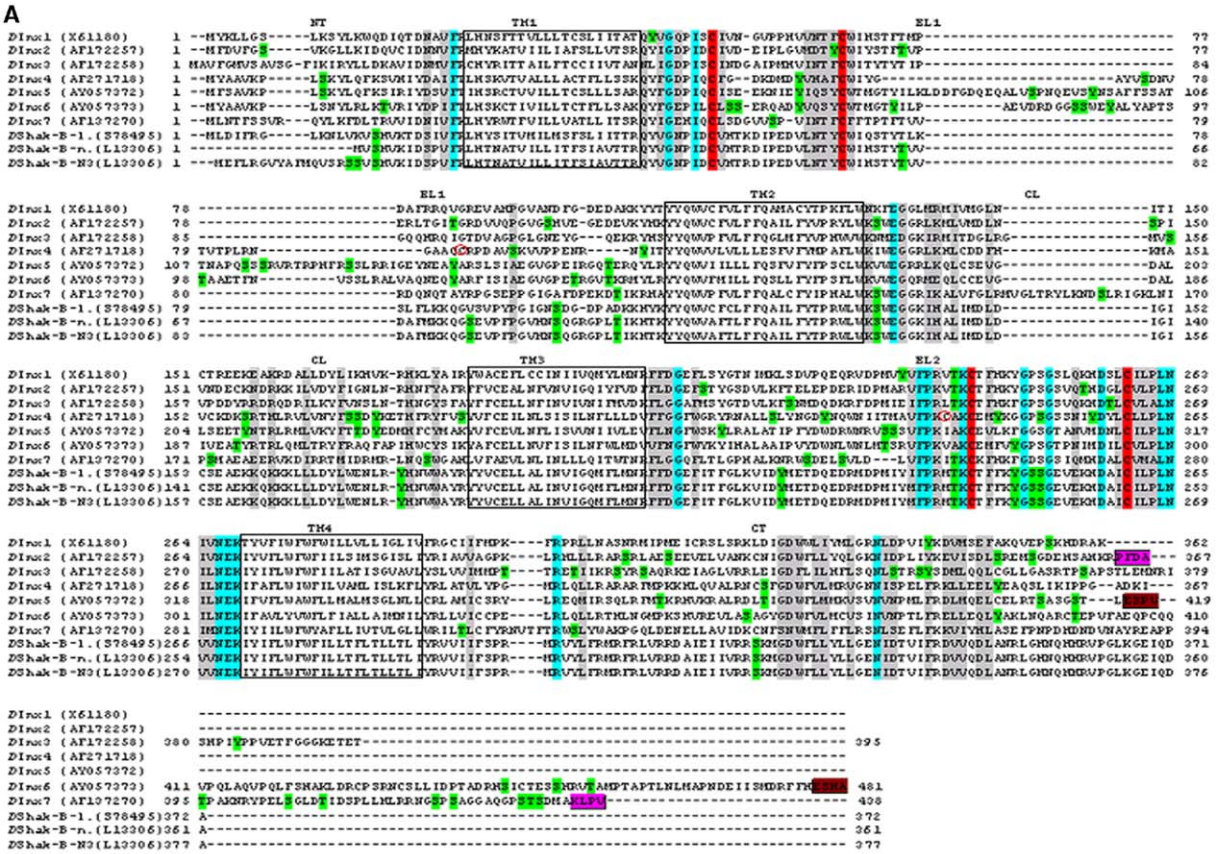

Figure 3. Amino Acid Alignment among the Eight *Drosophila* Innexin Proteins and among *Drosophila* Innexin and Murine Pannexin Proteins

(A) The eight *Drosophila* Innexins, Innexin 1–7, and Innexin 8 designated as *shak* B, according to the known mutants and its identified splice variants. TM domains are marked by boxes, and a characteristic pair of cysteine residues within each EL is shown in red. An exception is

motility [67]. They mediate cell-cell adhesion through Ca^{2+}-dependent homophilic interactions between their extracellular domains. The intracellular domains of Cadherins are known to interact with armadillo, which, in turn, is linked to α-catenin; α-catenin binds to F-actin, thereby recruiting the actin cytoskeleton to the adherens junction complex [68]. Mutations in *D*E-cadherin ("shotgun" mutants) and armadillo disrupt cellular polarity and lead to severe epithelial defects [67]. In *innexin 2* (*kropf*) mutants, epithelial development and gut morphogenesis are disrupted as well, and it could be demonstrated that correct levels of Innexin 2 are required for the organization and the survival of epithelial cells [35]. In the embryonic epidermis, Innexin 2 was found to accumulate in the apicolateral membrane domain, colocalizing with armadillo and *D*E-cadherin (Figure 4B). Immunoprecipitation analysis, yeast two hybrid studies, and loss- and gain-of-function analyses demonstrated direct molecular interactions between Innexin 2 and the *D*E-cadherin/β-catenin adherens junction complex [35]. Noteworthy is the observation that, in the hindgut, Innexin 2 accumulates in the lateral domain and in the basolateral position in the salivary gland epithelial cells. These data suggest that the localization of Innexin 2 along the apicobasal axis of epithelial cells is controlled by tissue-specific membrane localization factors that are still unknown (Figures 4A and B). In contrast, Innexin 1 was shown to accumulate in the basolateral domain of both salivary glands and the hindgut [60] (Figure 4A). In polarized thyroid epithelial cells, Connexin localization was detected in distinct regions of the lateral plasma membrane [69]. Therefore, the differential localization along the apicobasal axis of epithelial cells seems to be a feature of Connexins as well.

Connexin proteins have also been shown to interact with a diverse array of proteins to form multiprotein complexes [55], especially with components of other intercellular junctions. A number of studies have indicated a close association between gap junctions and adherens junctions. Inhibition of Cadherin function can disrupt gap junction formation and inhibit cell-cell coupling, suggesting that localization of Cadherin to cell-cell contact sites may be a prerequisite for gap junction formation [70, 71]. Conversely, inhibition of Connexin 43 can block adherens junction formation [72]. Moreover, the interaction extends not only to the core proteins of adherens junctions, but also to associated proteins, such as α-catenin and $p120^{ctn}$ [73]. It has been suggested that Connexin 43 is assembled as part of a multiprotein complex that could regulate coordinated assembly of adherens and gap junctions [71].

Interactions of Innexins with Core Components of Septate Junctions

Septate junctions (SJ) are positioned basal to adherens junctions in the lateral membrane of *Drosophila* epithelial cells. Similar to other intercellular junctions, SJ have been proposed to play a role in formation of transepithelial diffusion barrier [68]. Components of SJ identified in *Drosophila* are, for example, the Claudin-like protein megatrachea (Mega), the integral transmembrane protein neurexin (Nrx), and coracle (Cor), a scaffolding protein of the 4.1 protein family [74, 75, 76] (Figure 4B). All of these components seem to interact and are essential for barrier function. Whereas Mega and Nrx are located in the SJ exclusively, Cor reveals additional localization along the basolateral membrane [75]. Claudins, the structural membrane components of septate junctions, display a similar overall membrane topology as Innexins [77]. Evidence for an interaction of Innexin 2 and Cor in *Drosophila* embryos was provided by immunoprecipitation experiments and loss- and gain-of-function analyses [55], suggesting that Innexin 2 not only interacts with core components of adherens junctions but also with proteins located at SJ (Figure 4B).

In vertebrates, Claudins are core components of tight junctions, which are analogous barrier-forming structures to SJ in *Drosophila* (Figure 4C, compare with 4B). It has been demonstrated that Connexin 43 interacts with its carboxyterminal tail with the ZO-1 protein, which is a tight junction-associated PDZ domain-containing protein in vertebrates [55, 78]. ZO-1, which is a member of the family of membrane-associated guanylate kinase (MAGUK) proteins, acts as a peripheral membrane scaffolding protein that is specifically enriched at tight junctions of epithelial and endothelial cells [79]. It functions to tether TM proteins to the actin cytoskeleton [79]. Moreover, ZO-1 is not only a tight junction component, but is also associated with adherens junctions [55] (Figure 4C). Wu and colleagues have demonstrated that the Connexon 43/ZO-1/β-catenin complex is required for the targeting of Connexon 43 to the plasma membrane [80]. In summary, these data suggest that Innexins and Connexins may interact with a similar set of evolutionarily conserved key components of adherens and septate/tight junctions.

WNT Signaling as a Regulator of *innexin* and *connexin* Transcription

The WNT/wingless signaling cascade is an evolutionarily conserved signaling pathway that plays important roles in directing cell fates and cell behavior during development and disease [81]. WNT/wingless proteins encode a large family of secreted cysteine-rich glycoproteins that can influence cell-to-cell communication, both during embryonic development and in adult life. WNT1 associates to the Frizzled receptor, which, in turn, recruits the intracellular protein Dishevelled, leading to a downregulation of glycogen synthase kinase 3β (GSK3β) activity. GSK3β inactivation results in an in-

Innexin 4, having an additional cysteine within both ELs (red circles). Amino acid residues that are identical among all Innexins are in blue and conservative amino acid changes are depicted in gray. Putative phosphorylation sites of the Innexin proteins are shown as green letters. According to Sheng and Sala [54], class I and II PDZ motifs are depicted in brown and purple, respectively. Dashes were introduced to preserve sequence overlap. CL, cytoplasmatic loop; CT, C terminus; EL, extracellular loop; NT, N terminus; TM, transmembrane domain.
(B) Comparison of *Drosophila* Innexins (*D*Inx) and murine Pannexins (mPanx). TM domains are depicted in yellow and the YYQWV-peptide in *Drosophila* Innexins is marked by a box. The YYQWV amino acid stretch is missing in Pannexins.
Alignments were performed using Clustal W software [99].

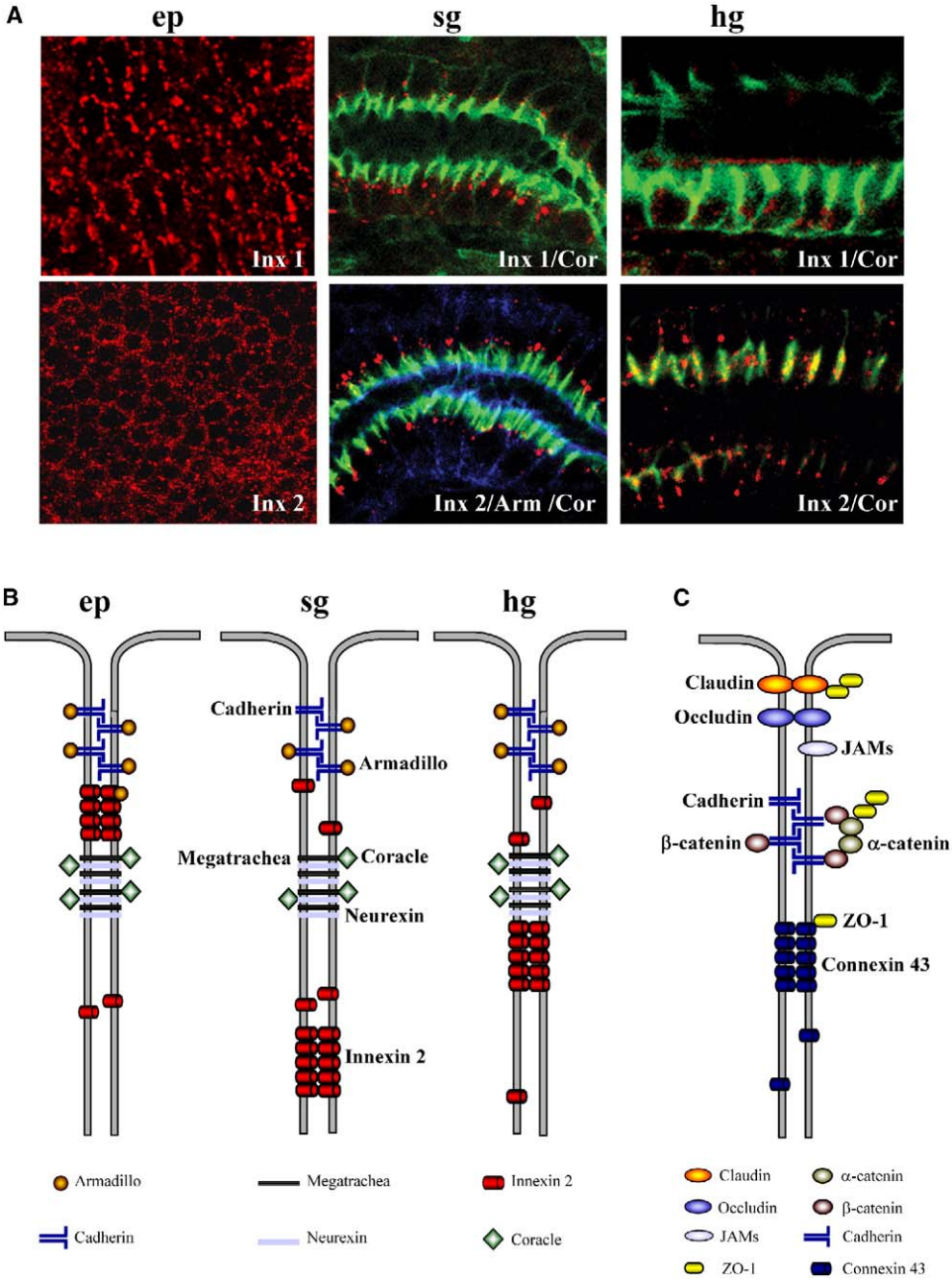

Figure 4. Confocal Images of Innexin Protein Localization in Developing Epithelia

(A) Innexins 1 and 2 are expressed in a typical punctuate pattern at the membrane and in the cytoplasm of epithelial cells (in red). Cellular distribution of Innexins 1 and 2 (in red) are shown for the epidermis (ep), the salivary glands (sg), and the hindgut (hg). The following markers were used: armadillo (blue) and coracle (green).

(B) This panel shows a scheme of Innexin 2 localization within epithelial cells of different tissues. Different symbols are used to show the core components of adherens and septate junctions. Innexin proteins are depicted as red barrels.

(C) Connexin 43 and putative interaction partners (modified according to Giepmanns [55]).

crease in cytosolic β-catenin by augmenting this protein's half-life. The resulting stabilization of nonphosphorylated β-catenin/armadillo promotes its entry into the nucleus, where the protein complexes with TCF transcription factors to modulate the expression of specific target genes (Figure 5) [82].

In *Xenopus* embryos, ectopic WNT1 expression induces gap junction communication [83, 84]. During murine brain formation, *wnt1* and *connexin 43* are coexpressed at the midbrain/hindbrain boundary, and interference with *wnt1* or *connexin 43* expression leads to severe brain defects [85]. Ectopic expression of *wnt1* in the limb mesenchyme of the mouse results in an increase of *connexin 43* transcription [86]. It was shown

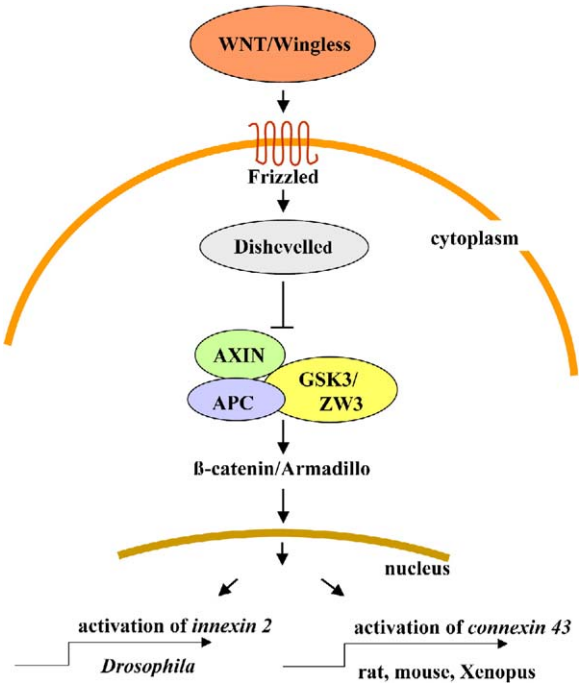

Figure 5. Transcriptional Regulation of *innexin* and *connexin* Genes in Response to the Conserved WNT/Wingless Signaling Pathway

innexin 2 transcription and *connexin 43* transcription are both positively regulated by the evolutionarily conserved WNT/wingless signaling pathway.

that the human, murine and rat *connexin 43* promotor contains several consensus TCF/LEF binding sites. Stably overexpressing *Xenopus* Wnt1 in PC12 cells resulted in increased *connexin 43* mRNA and protein levels leading to the formation of functional gap junctions [87]. Also, induction of *wnt1* expression in cardiomyocytes leads to an enhanced chemical coupling and increased Connexin 43 protein expression [88]. These data have shown that *connexin 43* acts as a functional target of WNT1-signaling, and *connexin 43* can be regulated by WNT1 at the transcriptional level. Similar results have been obtained with Connexin 30 [89].

The WNT/wingless signaling pathway was also shown to regulate the transcription of *Drosophila innexin 2* in epithelial precursor cells of the proventriculus [34]. Furthermore, *innexin 2* mRNA expression can be induced in tissue culture cells by cotransfecting β-catenin/armadillo, the mediator of the wingless signal (Figure 5) [34]. It is remarkable that, although Innexin and Connexin proteins share little sequence homology, both multigene families seem to be regulated by the same evolutionarily conserved signaling pathway, the WNT/wingless cascade (Figure 5).

Conclusions

Gap junctions of invertebrates and vertebrates are highly homologous on the structural level. Although their respective protein components, the *Drosophila* Innexins and the vertebrate Connexins, do not show significant homology at the primary amino acid sequence, intriguing similarities seem to exist on the structural and functional levels. Innexins display the same overall topology as Connexins. In both protein families, the carboxy-terminal tails show the highest variability in length and sequence composition, whereas other domains, such as the N termini and the intracellular loops, are less variable. Moreover, the TM domains and the ELs are highly conserved in both classes of gap junction proteins. Similarities exist in cellular distribution and in molecular interactions with the components of other cellular junctions. The finding that an Innexin 2-GPF fusion protein is transported and inserted into the membrane in heterologous HeLa cells (C. Lehmann, A.W., and M.H., unpublished data) further supports the hypothesis that some of the key interactions of Innexins and Connexins with other proteins may rely on common, conserved interaction partners in both invertebrates and vertebrates. Genetic screens in *Drosophila* may, therefore, identify new regulators and interaction partners that might also play crucial roles in vertebrate gap junction biogenesis. The conservation of structural motifs between proteins with primary amino acid sequence similarity has been observed in a number of cases. One example is the Shc phosphotyrosine binding domain (PTB). PTB adopts a structural conformation similar to that of the pleckstrin homology (PH) domains [90], although the extent of primary sequence conservation between these two domains is low. In the future, a high-resolution model for an Innexin channel might help to identify functional domains required for Innexin biogenesis.

The discovery of presumptive *innexin* homologs in humans and mice, named *pannexins* [17], raised the question of the evolutionary origin of gap junction proteins. Because an *innexin* gene was recently identified in the radially symmetric and diploblastic cnidarian *Hydra* [18], one of the earliest-diverging metazoan phyla (see [91] for review), a scenario has been proposed to address the problem of gap junction evolution [18]. In order to coordinate gap junction communication, *innexins* may have evolved in diploblasts, which represent an evolutionary grade below the deuterostome-protostome divergence. *innexins* were then inherited to both protostomes and deuterostomes. The *connexins* arose de novo in deuterostomes, which may have allowed the *innexins* to diverge and form a new subfamily, the *pannexins* [18]. However, there is still some debate as to whether *pannexins* represent *innexin*-like genes [17] or, rather, encode a different family of proteins with little similarity to Innexins [21].

The evidence that Innexin and Connexin family members may both interact with core components of adherens junctions, such as β-catenin, and the fact that several members of both gene families are transcriptionally regulated by the WNT signaling cassette, may reflect an evolutionarily conserved mechanism that ensures coordinated assembly of junctions in polarized epithelial cells. Cnidarians, such as *Hydra*, are among the simplest living metazoans. They consist of two body layers: an outer ectoderm and an inner endoderm, separated by an extracellular matrix (mesoglea), and represent the first animals with a defined body axis and a nervous system [91]. Ultrastructural analysis has shown the presence of both functional gap junctions and SJ,

important for intercellular adhesion and cell-to-cell communication [92, 93]. Recent molecular studies indicate the presence of several junction core components in *Hydra*, including the gap junction-forming Innexins [18], the ZO-1 homolog HZO-1 [94], and the *Hydra* β-catenin homolog, Hy β-catenin [95]. There is evidence that aggregation of *Hydra* epithelial cells closely resembles cadherin-mediated aggregation of vertebrate cells. It was suggested that not only β-catenin, but the entire cadherin/catenin-cell adhesion complex might have been established at the start of metazoan evolution [95]. β-Catenin has a dual role: it acts as a core component of adherens junctions and as a transducer of the WNT signal. Several components of the canonical WNT signal transduction pathway, including the homologs of Wnt, Dishevelled, GSK3, and Tcf, have been isolated from *Hydra*, in addition to β-catenin. Furthermore, it has been shown that WNT signaling is required for cell-to-cell communication during axis formation in *Hydra* [96]. It is thus possible that a regulatory protein-protein interaction network of adherens junction core components, together with Innexins and WNT-dependent regulation of *innexin* transcription, had evolved in *Hydra*. Subsequently, this network was used by evolving gap junction genes, including the *connexin* genes in deuterostomes. Evidence for the functional conservation of *cis*-regulatory control regions and entire regulatory networks has been shown in many cases (see [97] for review), most prominently for *pax6/eyeless* in various animal phyla (see [98] for review) and for *Brachyury* regulation and function (see [97] for review). Future studies will be needed to determine whether there is molecular evidence for the conservation of common regulatory networks during the evolution of gap junction gene families.

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