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Review

Recent findings in evolution and function of insect innexins

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ABSTRACT

The past decade has seen significant advances in the field of *innexin* biology, particularly in the model invertebrate organisms, the nematode *Caenorhabditis elegans* and the fly *Drosophila melanogaster*. However, advances in genomics and functional techniques during this same period are ushering in a period of comparative *innexin* biology. Insects are the most diverse metazoan taxa in terms of species number, as well as in developmental, physiological, and morphological processes. Combined with genomics data, the study of *innexins* should rapidly advance. In this review, we consider the current state of knowledge regarding *innexins* in insects, focusing on *innexin* diversity, both evolutionary and functional. We also consider an unusual set of *innexins*, known as *vinnexins*, that have been isolated from mutualistic viruses of some parasitoid wasps. We conclude with a call to study insect *innexins* from a broader, evolutionary perspective. Knowledge derived from such comparative studies will offer significant insight into developmental and evolutionary physiology, as well as specific functional processes in a taxon that has huge biomedical and ecological impact on humans.

1. Introduction

Gap junctions and their structural genes have been identified in nearly all metazoan taxa. In the 1980s, connexin genes were identified as the molecular basis for gap junctions in rats and other mammals [1,2], although numerous studies failed to identify connexin genes in invertebrates such as the nematode *Caenorhabditis elegans* and the fly *Drosophila melanogaster*. Genetic screens and heterologous functional studies with these two model invertebrates identified the *innexin* genes and confirmed that they form gap junctions [3,4]. Sequence analyses later identified genes that were similar to *innexins* in chordate genomes, termed pannexins [5]; subsequently, phylogenetic analyses have supported that *innexins* and pannexins are evolutionarily homologous, supporting their evolutionarily common origin, while connexins are unrelated [6–8].

Insects (and the remainder of the phylum Arthropoda) demonstrate incredibly diverse structure and function. Insects initially appear in the fossil record approximately 400 million years ago [9], and arthropods more than 525 million years ago [10]. Arthropods account for almost 85% of animal species described [11], and exhibit a wide range of morphologies, physiologies, and niche inhabitance. Insects alone account for more than 75% of known metazoan species [12], and exhibit tremendous morphological diversity, ranging from major variations on the insect body plan, to incomplete and complete metamorphosis (that is, minor to major morphology differences through ontogeny), to subtler changes including polyphenisms [13,14]. Insects colonize, inhabit, and alter essentially all niches on Earth, with the exception of the deep ocean, and as such demonstrate a wide range of physiological adaptations. They (particularly flies, or the Diptera) also exhibit faster genomic divergence rates than mammals and other vertebrates [16]. Innexins and gap junctions have been hypothesized to play major roles in contributing to the morphological and physiological variation [15], although to date little systematic analysis examining this relationship has been performed in this major taxon. Rather, the overwhelming majority of work in insects on *innexins* and gap junctions has been performed in *D. melanogaster*, due to the genetic, genomic, and molecular tool chest available for this model organism. Given the long evolutionary history, the breadth of morphological and physiological diversity, and the rate of genome evolution, our current understanding of the diversity of insect *innexins* is likely a very limited representation of the diversity that is present. Recent work in numerous non-model insects supports this observation [17–22].

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Beyond simple diversity, there are other reasons to take a comparative approach to studying insect *innexins*. As stated, insect species, morphological, and even genomic diversity outstrips that of other phylogenetic lineages. The i5K initiative, which proposes to leverage community resources to sequence and annotate the genome of 5000 insect species representing the breadth of insect biodiversity [23], will generate tremendous amounts of genomic data. These data will facilitate a range of comparative genomics projects, and eventually comparative functional genomics. Relevant to *innexins*, this project initially will facilitate identification of the genomic complement of insect *innexins*; that is, the increasing availability of genomic sequences will permit the identification of the core *innexin* genes of insects. In parallel, genome sequences will permit identification of genomic novelty in the *innexin* gene family – sequences that exist in certain lineages and not in others. As the complement of *innexins* is determined for insect lineages, characterization studies will permit inferences to be drawn regarding the role of Innexins in functional diversification.

Currently, the insect *innexin* field is dominated by knowledge and studies in *D. melanogaster*. Eight *Drosophila innexin* loci have been isolated and transcript patterns analyzed through development [24], with reverse-genetic approaches allowing targeted analysis of Innexin function. A subsequent section of this review briefly will consider knowledge regarding function of *innexin* orthologues, recognizing that although the majority of these data stem from *Drosophila*, there is still much to learn from this model insect. But, the i5K project will greatly expand the possibilities for functional genomics, beyond *Drosophila*, in addition to evolutionary genomics. There is tremendous heterogeneity across insect taxa in regards to manipulability for functional studies, including logistics (e.g., rearing), tool development, and biological susceptibility to manipulation. For example, RNAi functions well, if in varied fashion, in Diptera (flies), Coleoptera (beetles), and Isoptera (termites) [25,26], but generally very poorly in Lepidoptera (moths, butterflies) [27]. As genomic resources relative to *innexins* increase, organisms that are more amenable to (or interesting for) functional studies will be identified. This will permit comparative functional studies, allowing the development of insight into conservation and divergence of *innexin* orthologues, their interactions with cellular partners, and so forth.

In this review, we will discuss the current state of understanding regarding the phylogenetic pattern of insect *innexins* relative to other *innexin* lineages. From this, we infer what appears to be the basal complement of *innexin* genes in insects. From there, we identify what appear to be unique clades of insect *innexins*. In considering these *innexin* clades, we briefly review the roles associated with Innexins, particularly in considering the potential for conservation and divergence within insect evolutionary lineages. Finally, we discuss *innexin* homologues within the Polydnaviridae, a family of mutualistic insect viruses. Together, these data point to a rich future for *innexin* work, promising many exciting insights into both gap junction roles and the pathways underlying many physiological processes in insects.

2. Phylogeny of insect innexins

Gap junction genes have now been identified in the genome of all Eumetazoa that have been examined, with the exception of echinoderms, and at least one *innexin* is encoded by the genome of the parasitic dicyemid mesozoans, *Dicyema japonicum* and *Dicyema koshidai* [28]. However, in line with previous reports, a BLAST search of the Placazoa and the Parazoa (Porifera) revealed no *innexin* homologues (BLAST search, December, 2013), as is expected given the absence of intercellular junctions in the Porifera. The pattern of *innexin* genes reflects deep evolutionary relationships within metazoans, implying that the pattern may be useful in phylogenetic studies and that *innexins* possibly play a role(s) in major evolutionary advances [28]. As previously reported [6,7,28], and demonstrated in Fig. 1, *innexins* exhibit phylum-specific diversification. It appears that *innexins* originated early in metazoan evolution, predating the divergence of the Lophotrochozoa (including the phyla Annelida and Mollusca) and Ecdysozoa (the molting phyla, including the phyla Nematoda and Arthropoda), which would account for the occurrence of pannexins in Deuterostomia (including mammals and other vertebrates). Following this initial genesis, *innexins* have undergone diversification within the phyla, including Arthropoda (including insects), Nematoda, Mollusca, and others represented in Fig. 1. The basis of this diversity is unclear. Based on conservation of only a single site across ecdysozoan lineages, *innexin* diversification was proposed to be the result of genetic drift [6]. However, the results of systematic selection analyses have not been reported, thus selection for functional variation cannot be ruled out. Indeed, alignments of insect *innexins* demonstrates multiple conserved sites, suggesting selection may vary at different phylogenetic levels.

The majority of physiological evidence of the role of Innexins comes from *D. melanogaster*, a member of the order Diptera (flies) (see below). Genomic analysis identified eight members of the *innexin* gene family in *D. melanogaster* [29]. Mutants have been identified for many members of the gene family, associating function with specific *innexin* lesions or alterations. Sequences of insect genomes are now permitting the identification of many more *innexin* loci, allowing for the development of a more robust insect *innexin* gene tree. Upon examination of the arthropod-specific clades of the tree (Fig. 1, "Insect" branches), several patterns emerge. Chiefly, the evolutionary pattern within orthologues (e.g., Inx2) largely is congruent with organismal patterns (Fig. 2). The Inx2 proteins form distinct organismal order-level clades, including the holometabolous Hymenoptera (bees, wasps, and

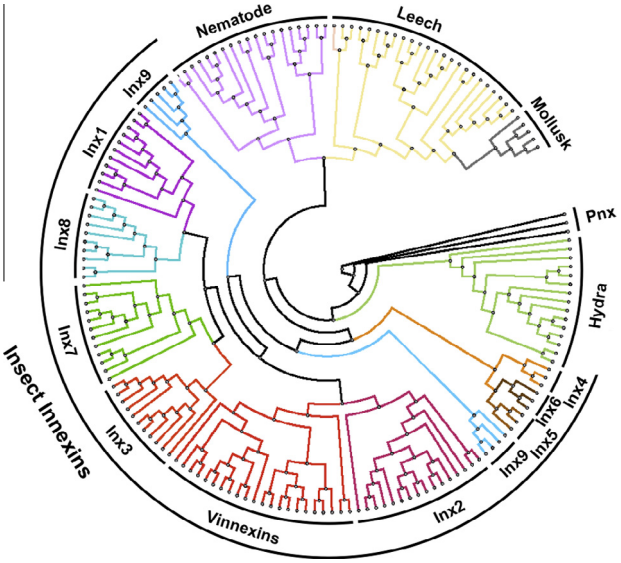

Fig. 1. Phylogenetic tree of conceptual Innexin and Pannexin translation products demonstrates phylum-specific diversification of the gene family. *Innexin* and pannexin sequences were downloaded from Genbank. Innexins were analyzed from phyla included Arthropoda (insects), Annelida (leech), Nematoda, Mollusca, and Cnidaria (Hydra). Pannexins representing mouse, rat, and human were included. Conceptual translated products were aligned using MUSCLE and a Neighbor-Joining tree created and visualized in Unipro UGENE. Innexins form phylum-specific clades, supporting gene diversification following, rather than preceding, diversification of phyla.

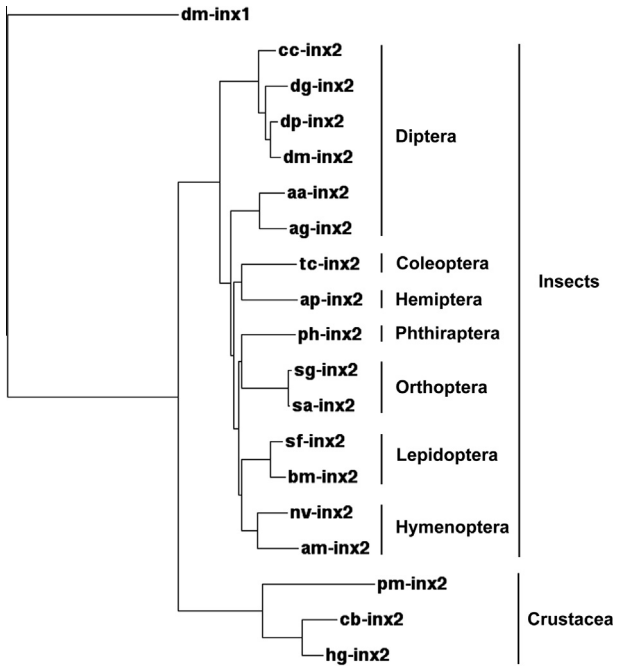

Fig. 2. Innexin2 orthologues demonstrate diversification within arthropod lineages. Conceptual translations of insect and crustacean *inx2* orthologues were aligned using CLUSTALW, and a Neighbor-Joining tree created and visualized in DNAStar Lasergene.

ants), Lepidoptera (butterflies and moths), and Diptera (flies), and the hemimetabolous Orthoptera (locusts), Phthiraptera (lice), and Hemiptera (bugs); somewhat surprisingly, Inx2 orthologues of holometabolous insects (those that undergo full metamorphosis) are not distinct from those of hemimetabolous insects (those that undergo gradual metamorphosis). Additionally, non-insect arthropod *innexins* [from the lobster *Homarus gammarus* [30], crab, and shrimp] nest with their insect orthologue, although as outliers to the insect orthologues. This observation, albeit based on limited data, supports diversification of the arthropod *innexins* prior to the divergence of major arthropod taxa, followed by subsequent evolution within taxa. Not all *innexins* demonstrate such predictable patterns, though: *inx2* is highly conserved relative to other insect *innexins* (*inx2* orthologues in Fig. 1 are an average of 81% similar, while those of *inx1* are 67%).

Given the above, it would not be surprising to see taxon-specific exploration of sequence space within the insect *innexins*. It appears, for example, that *inx4*, *inx5*, and *inx6* are limited in taxonomic distribution to the Diptera. They occur in the respective genome of all *Drosophila* spp.; *inx4* appears to be the ancestral locus, and an orthologue is identifiable in the genome of the mosquito *Aedes aegyptii* (XM_001652124). However, these genes appear to lack conserved orthologues in other insects or arthropods (based on BLAST searches with the *D. melanogaster inx4, inx5,* and *inx6* coding sequences, December, 2013). This leads to the question of whether the physiological roles of these Innexins are carried out by paralogous Innexins in different insect orders, or whether the functions of Inx4, Inx5, and Inx6 are gene family novelties limited to the Diptera. The possibility that *inx4*, *inx5*, and *inx6* orthologues exist in other orders, but have not yet been identified, is also a possibility.

While the *inx4/5/6* clade seems to be a novelty arisen in dipterans, there also appear to be novel *innexins* in other insect orders. The model beetle, *Tribolium castaneum*, encodes a molecule (XM_967885) which groups with other predicted Innexins from the orders Lepidoptera (the moth *Bombyx mori* and butterfly *Danaus plexippus*), Hymenoptera (the ants *Acryomyrmex echinatior* and *Camponotus floridanus*, and the wasp *Nasonia vitripennis*), and Phthiraptera (the body louse, *Pediculus humanus*). To date, these molecules (“Inx9”) remain putative Innexins, as neither expression nor functional characterization have been reported. However, if demonstrated to be functional molecules, it will be interesting to observe whether they perform roles overlapping those of the fly Inx4/5/6 proteins, novel ones, or a combination thereof. Additionally, it remains to be seen how broadly orthologues of this molecule are conserved, and whether they represent a loss in Diptera, as would be predicted by the current taxonomic distribution. Alternatively, the fly *inx4/5/6* clade may represent a highly divergent *inx9* locus in flies, in which instance many of the same functional questions remain relevant.

Finally, our lab and others have described homologues of *innexins* in the genome of polydnaviruses [31–34]. Polydnavirus *innexins*, or *vinnexins*, have been described from the genomes of *Campoletis chloridae* and *Campoletis sonorensis* Ichnoviruses, *Hyposoter didymator* and *Hyposoter fugitivis* Ichnoviruses, and *Tranosema rostrale* Ichnovirus, with *innexin* gene family numbers ranging from four to more than 15. Innexin sequence analysis supports the hypothesis that the *vinnexins* arose from capture of an ancestral insect *innexin2* locus (Fig. 1), followed by duplication and diversification of the *vinnexins* within the polydnavirus lineage. Greater consideration of the *vinnexins*, including their evolution, is below.

3. Roles associated with specific Innexins

In considering the evolution of Innexins and their function, we briefly describe reported roles associated with paralogues of the family. Primarily, the data which associate a member of the family with a specific role stem from the fly, *D. melanogaster*. However, where possible, we consider orthologues from other insects.

3.1. Innexin1

inx1 transcripts have been detected in embryonic insects from numerous taxa [21,24,35–37]. Primarily, Inx1 has been ascribed developmental roles, particularly in the nervous system. Developmental expression of *D. melanogaster-innexin1* (*dm-inx1*, *optic ganglion reduced, ogre*) in embryonic and post-embryonic neuroblasts is necessary for optic ganglion and retinal development in pupae [37]: Ogre is specifically required during pupal stage, along with *Dm*-Inx8 [specifically, the ShakB(Neural) isoform], to generate proper retinal photoreceptor (Ogre) connections to laminal neurons [ShakB(Neural)] [38]; this highlights the potential for stage-specificity of role, given metamorphic changes in structure. Ogre also was demonstrated to overlap in expression with *Dm*-Inx2 in glial cells of the larval nervous system [39]. Knockdown of glial cell Ogre resulted in reduced larval CNS size, and in adults, defective behaviors and reduced viability. Inx1 has been implicated in non-developmental roles, as well, including direct electrical coupling of neurons in the CNS of locusts [21] and the stomatogastric nervous system of lobsters [30]. Ogre also likely forms heteromeric Inx1/Inx3 gap junctions between ovarian follicle cells in flies, although the role of these junctions is unclear [37,40].

3.2. Innexin2

The most widely characterized *innexin* in insects is *inx2*, having been analyzed in Lepidoptera [18,19,41], Orthoptera [21,35], and Diptera [39,42–45], as well as in the non-insect lobster (*H. gammarus*) [30]. *Dm*-Inx2 is required broadly for embryonic epithelial morphogenesis [40,42,43,45–48]. *Dm*-Inx2 is required for

embryonic gut formation [42], acting downstream of Wingless signaling [42,48]; *Dm*-Inx2 activity upstream of *wingless*, *hedgehog*, and the Notch ligand, *delta*, is a rare demonstration in insects of the interdependence of paracrine and gap junction communication [48]. *Dm*-Inx2 also was demonstrated to interact with adherens and septate junction proteins [43] and *Dm*-Inx3 [45], reciprocally affecting junctional distributions; specifically, it appears that *D*E-Cadherin plays a role in proper trafficking of *Dm*-Inx2 channels [49]. Outside of embryonic development, *Dm*-Inx2, as noted above, interacts with Ogre in larval CNS glial cells, and knockdown results in reduced size of larval CNS and failure of flies to eclose (i.e., adults to emerge from pupa) [39]; the authors suggest this may occur due to a requirement for gap junctional communication between glial cells, and/or between glial cells and neurons. In the ovary, *Dm*-Inx2 junctions between follicle cells and oocytes are necessary for proper oogenesis [40].

Two novel junctional roles in insects have been reported recently for *Dm*-Inx2.

Following epidermal damage to the *Drosophila* embryo, calcium waves are triggered and transferred from neighboring cells via gap junctions to induce an inflammatory response. Transmission, although not initiation, was reduced in *inx2* mutant fly lines, leading to a reduced inflammatory response [50]. *Dm*-Inx2 gap junctions also were demonstrated to mediate intercellular transfer of GDP-L-fucose, a substrate for *O*-fucose modification, in the wing imaginal disc [51].

3.3. Innexin3

Dm-Inx3 is mutually dependent with *Dm*-Inx2 to localize properly, at least in some circumstances [40,45], and channel heteromerization is critical for epithelial tissue morphogenesis and polarity [44,45]; heteromeric Ogre/Inx3 channels may form and be required for proper oogenesis, as well [40]. *Dm*-Inx3 is involved in dorsal closure of the *Drosophila* embryo, as well as in the stability of other Innexins and *D*E-Cadherin, through the formation of a complex [52]. Data from the Sf9 and Spl221lepidopteran cell lines were taken to implicate *Spodoptera litura*-Innexin3 (as well as *Sl*-Inx2) in regulation of apoptosis, as overexpression resulted in hallmarks of apoptosis [19]. However, a mechanism has not been demonstrated to conclusively support a role for Innexins in apoptosis, at this time.

3.4. Innexin4, Innexin5, and Innexin6

Limited data exist for these three genes. The *zero-population growth* (*zpg*) mutant was identified to have gap junction deficits in the germ line, resulting in failure to synthesize mature germ cells in both males and females [53]; to date, this is the only reported *inx4* mutant or associated phenotype. No functions have been ascribed to *inx5*, while *Dm*-Inx6 has been shown to be necessary in heterotypic (with *Dm*-Inx7) gap junction formation in the mushroom bodies for the formation of memories [54].

3.5. Innexin7

The use of RNAi has demonstrated that *Dm*-Inx7 is essential to axon guidance and embryonic nervous system development [55]. As already noted, *Dm*-Inx7 additionally is required in heterotypic Inx6/Inx7 channels in specific nerves of the mushroom bodies for the formation of memories [54]. *Dm*-Inx7 exhibits different subcellular localization patterns in the developing central nervous system (nuclear) and epithelial tissue (cytoplasm and membrane), suggesting dependence on tissue-specific regulation [55].

3.6. Innexin8

Three *dm-inx8* (*shakB*) splice variants are expressed in confined regions of the central nervous system, including those of the Giant Fiber System. Loss of *Dm*-ShakB function disrupts electrical transmission, resulting in defective escape behavior [56–58]. Electrophysiological studies in *Xenopus* oocytes demonstrate *Dm*-Shak-B(Lethal), but not *Dm*-Shak-B(Neural), can form homotypic channels and furthermore, differential voltage gating of heterotypic channels containing *Dm*-Shak-B(Lethal) and *Dm*-Shak-B(Neural + 16) is required for rectification of electrical synapses in the Giant Fiber System [3,59]. Similarly, *Dm*-Ogre and *Dm*-Shak-B(Neural) are required at the pre- and post-synapse to maintain retina-lamina neural transmission, and this specific complex is largely irreplaceable by other gap junctions [60].

4. Numerous gap junction roles have not been ascribed to specific Innexin(s)

In addition to the above, numerous studies have demonstrated gap junction roles in systems across insect taxa without identifying the relevant Innexins. For example, neuronal networks in the brain of cockroaches and grasshoppers are maintained by gap junctions [61,62]; although *inx1* and *inx2* have been identified in the brain of two locust species [21,35], their relevance to these specific circuits is unclear. Electrical coupling of Malpighian tubule principal cells via gap junctions facilitates transepithelial ion transport for primary urine production in the mosquito, *Aedes aegypti* [17]. However, although transcripts for *inx1*, *inx2*, *inx3*, and *inx7* were detected in the Malpighian tubules, the Innexins which comprise the junctions have not been reported. Numerous studies of the wing imaginal disc of *D. melanogaster* have shown the presence of gap junctions, which may act to form discrete compartments during development [63,64]. *In situ* hybridization demonstrates all eight *innexins* are transcribed in the wing disc [24]. However, while studies suggest that intercellular communication by gap junctions may underlie certain morphogenetic signaling cascades in the wing disc [65–67], the respective roles of the various Innexins are, as of yet, unknown.

As holometabolous insects undergo molting and eventually metamorphosis, they can be excellent models of the role of gap junctions in regulating tissue growth, remodeling, and morphogenesis. Gap junction patterns and coupling vary through inter- and intramolt stages between caterpillar epithelial gut cells [68,69]. Similarly, junctional coupling in the cuticular epidermal cells of the beetle *Tenebrio molitor* varies with molt stage [70], presumably under regulation of the molting hormone β-ecdysone [70,71] at a post-translational level [72]. As well, numerous observations have been recorded, from several orders of insects, of gap junctions forming between hemocytes, particularly during the cellular immune response of encapsulation [73,74]; junctional coupling between these hemocytes has been reported [75–77]. However, similar to instances of cellular networks and compartmentalization, the associated Innexins regulating these morphogenetic processes are unknown.

5. Vinnexins of polydnaviruses

The genome of certain members of the Polydnaviridae family of viruses have recently been demonstrated to encode functional Innexins, which have been termed *vinnexins* (*virus innexins*) [32,33]. Polydnaviruses (PDVs) are an intriguing virus taxon, associated with certain subfamilies of wasp parasitoid. The segmented dsDNA genome of PDVs is integrated into the genome of certain

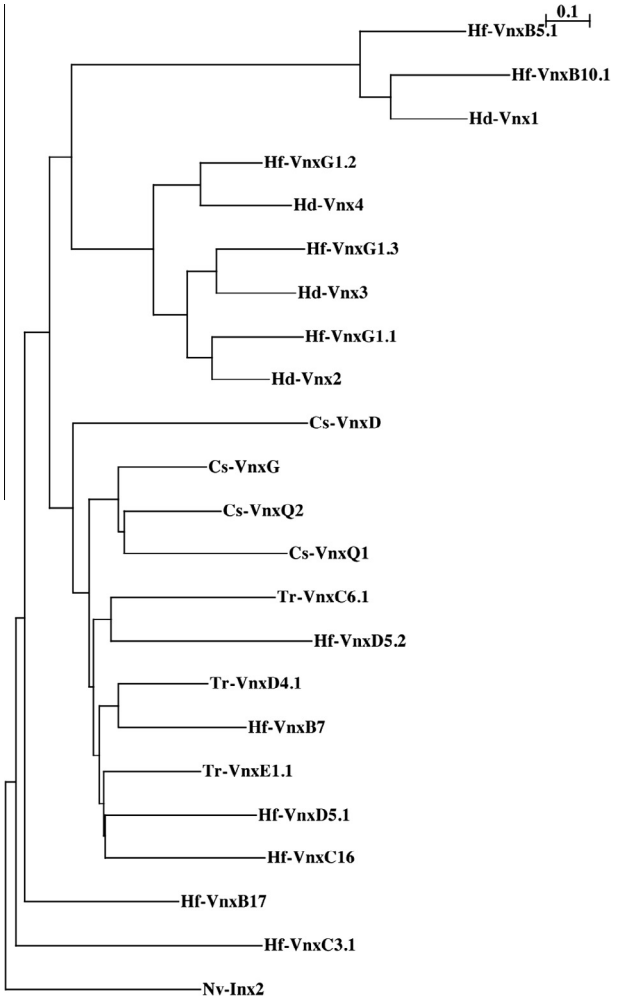

Fig. 3. The Vinnexins are a monophyletic lineage arising from an ancestral insect inx2 gene. The conceptual translation products of twenty-two *vinnexins* from four Ichnoviruses, representing three genera of virus (*Campoletis sonorensis* IV, *Hyposoter didymator* IV, *H. fugitivis* IV, and *Tranosema rostrale* IV), were aligned with ClustalW and a Neighbor Joining tree was generated in DNAStar Lasergene. The tree was visualized in NJPlot [106].

lineages of wasps in the families Braconidae and Ichneumonidae; viruses associated with wasps of either family are referred to as Bracoviruses and Ichnoviruses (IVs), respectively. The viruses are transmitted vertically as proviruses, and all members of infected species have the virus [78–80]. The PDV genome is replicated and encapsidated asymptomatically in the wasp ovaries, and delivered during parasitization into the hemocoel of a host insect (typically a juvenile lepidopteran, or caterpillar). Expression of virus genes leads to disruption of manifold physiological processes including abrogation of immunity [81–85] and altered endocrine profile and development [86–90], and virus gene expression is essential for successful parasitization [81]. Few virus structural genes have been identified in the encapsidated virus genome [91,92], and virus replication is not detected in the infected caterpillar [93,94]. The genome of every campoplegine-lineage IV analyzed to date has been found to encode at least four *innexin* loci [33]; IVs from the Banchinae subfamily lack *innexin* loci [95,96]. The *vinnexin* genes likely arose from integration of host wasp *inx2* into an ancestral IV genome, followed by significant gene duplication and diversification due to intra- and inter-segmental recombination and duplication (Fig. 3). Many of the highly conserved sites of insect Innexins are present in Vinnexins [31], although some are altered and may be useful in future studies of structure–function relationships in the Innexins.

The Vinnexins initially were hypothesized to disrupt hemocytic encapsulation, the primary anti-parasitoid immune response of caterpillars, presumably by inhibiting gap junctional intercellular communication [78,79]. Like many IV genes, *vinnexin* transcripts are broadly detected in host caterpillar tissues [31,97]. However, an antibody against the *C. sonorensis* IV (*Cs*IV) VinnexinQ2 (*Cs*-VnxQ2) only identified the protein in *Cs*IV-infected hemocytes of the caterpillar *Heliothis virescens*, where the protein localized to cellular membranes [31]. Results from paired *Xenopus* oocytes injected with *Cs*IV *vinnexin* cRNA surprisingly demonstrated that *Cs*-VnxD and *Cs*-VnxG were capable of forming functional gap junctions [31]. Further oocyte analyses of all four *Cs*IV Vinnexins and a host lepidopteran Innexin2 (*Spodoptera frugiperda*-Inx2) demonstrated that all four Vinnexins form homomeric channels, and, to varying degree, form heterotypic and possibly heteromeric channels with *Sf*-Inx2 [41]. Interestingly, co-expression of Vinnexins with *Sf*-Inx2 (i.e., potentially heteromeric channels) results in altered channel characteristics. Thus, heterologous expression studies support that Vinnexins may serve to subtly alter, rather than ablate, gap junction communication between infected cells. Studies of Vinnexin expression in lepidopteran cell culture (both Sf9 and High Five cells) supports this hypothesis of alteration rather than inhibition: Vinnexins co-localize with lepidopteran Innexins (Fig. 4), physically interact with Inx2 and each other, and are associated with changes in dye transfer and cell morphology (Hasegawa et al., in preparation). Both lepidopterans and polydnaviruses are difficult to manipulate, making functional analysis in the lepidopteran difficult. Ectopic expression in the lepidopteran using in vivo transfection of one or more *vinnexins*, as has

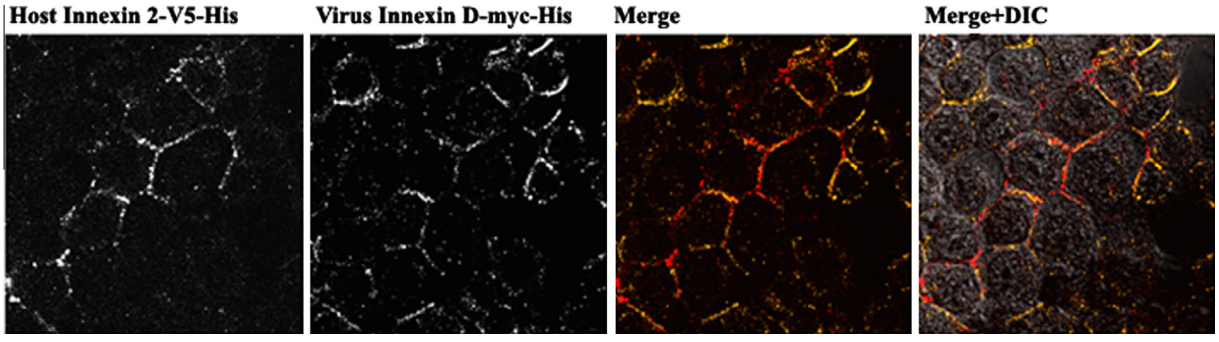

Fig. 4. Vinnexin cellular distribution overlaps with a host Innexin. C-terminal epitope-tagged *Cs*IV VnxD and *Spodoptera frugiperda* Inx2 were transiently expressed in High Five cells by plasmid transfection. Epitope-tagged proteins were detected at 2d post-transfection by confocal immunomicroscopy using antibodies against V5 (*Sf*-Inx2) and myc (*Cs*-VnxD).

been used to analyze protein tyrosine phosphatases of the *Cotesia plutellae* Bracovirus [98], and heterologous expression in *Drosophila* using the GAL4-UAS system, as was used to examine a PDV IKB-like molecule [99], may facilitate experimental testing of Vinnexin function.

6. Future directions for insect innexins

Insects are an incredibly diverse animal group which contribute significantly to human biology. They are intrinsically of interest due to their diversity, their roles in human misery and infrastructure costs (e.g., via transmission of pathogens and agricultural damage), and even their benefits (e.g., via pollination and silk production). They also have played, and continue to play, important roles in our understanding of human physiology and biomedical processes. However, it is necessary to develop reliable phylogenetic relationships to maximize comparative studies, whether to use insects as models for biomedical processes, or to compare the regulation of developmental processes across insects. The phylogenetic tree examining the insect Innexins, therefore, provides an initiation point for elucidation of the roles of orthologues and paralogues across insect taxa. Examining the evident patterns from this phylogeny suggests several studies for future work in insect Innexins.

Immediately, the sequence separation of insect *innexins* and mammalian pannexins, and even other invertebrate *innexins*, supports that *innexins*, and insect gap junctions, may serve as interesting targets for regulation of physiological processes in pest insects. For example, *Dm*-Inx2 is required for proper gut morphogenesis in *Drosophila* [42,46], and gap junctional communication correlates to midgut proliferation during molt in the caterpillar *Manduca sexta* [100,101]. It may be possible to target *inx2*, or another *innexin*, utilizing RNAi to disrupt gut development. Indeed, several studies have demonstrated the utility of orally delivered dsRNA in gene knockdown and subsequent disruption of physiological processes in insects [102–105].

The current diversity of *innexins* and the promise of even more data resulting from the i5k genomics projects is most exciting, though, from an immediate comparative perspective. As noted above, the majority of data regarding both *innexin* genes and Innexin proteins has been derived from the model fly, *D. melanogaster*. Flies repeatedly have been shown to be evolutionarily divergent, raising a need for more comparative studies of *innexins*. The identification of novel *innexin* paralogues, such as those that we have termed here *inx9*, begs the question of function; they also raise the question of how many additional, novel *innexin* loci there may be among insects. Given the large number of taxa and the potential for Innexin roles in developmental processes, coupled with diversity of developmental processes in insects, it may be a long time before characterization of novel *innexins* and functions dries up. Similarly, as touched on above, there are myriad physiological processes in insects in which gap junctions have been demonstrated, in which the relevant Innexin has not been isolated. Gap junctions appear poised to be recognized as playing significant parts in highly important events like anti-parasite immunity, toxin-induced gut turnover and modeling, and metamorphosis. Isolation and characterization of the way that the relevant Innexin(s) contribute to these physiological processes in the most diverse group of animals on the planet promises to provide interesting insight for years to come.

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