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Virus Innexins induce alterations in insect cell and tissue function

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ABSTRACT

Polydnaviruses are dsDNA viruses that induce immune and developmental alterations in their caterpillar hosts. Characterization of polydnavirus gene families and family members is necessary to understand mechanisms of pathology and evolution of these viruses, and may aid to elucidate the role of host homologues if present. For example, the polydnavirus vinnexin gene family encodes homologues of insect gap junction genes (innexins) that are expressed in host immune cells (hemocytes). While the roles of Innexin proteins and gap junctions in insect immunity are largely unclear, we previously demonstrated that Vinnexins form functional gap junctions and alter the junctional characteristics of a host Innexin when co-expressed in paired *Xenopus* oocytes. Here, we test the effect of ectopic vinnexin expression on host cell physiology using both a lepidopteran cell culture model and a dipteran whole organism model. Vinnexin expression in the cell culture system resulted in gene-specific alterations in cell morphology and a slight, but non-statistically significant, reduction in gap junction activity as measured by dye transfer, while ectopic expression of a lepidopteran *innexin2* gene led to morphological alterations and increase in gap junction activity. Global ectopic expression in the model dipteran, *Drosophila melanogaster*, of one *vinnexin* (*vinnexinG*) or *D. melanogaster innexin2* (Dm-*inx2*) resulted in embryonic lethality, while expression of the other *vinnexin* genes had no effect. Furthermore, ectopic expression of *vinnexinG*, but not other *vinnexin* genes or Dm-*inx2*, in *D. melanogaster* larval gut resulted in developmental arrest in the pupal stage. These data indicate the *vinnexins* likely have gene-specific roles in host manipulation. They also support the use of *Drosophila* in further analysis of the role of Vinnexins and other polydnavirus genes in modifying host physiological processes. Finally, our findings suggest the *vinnexin* genes may be useful to perturb and characterize the physiological functions of insect Innexins.

1. Introduction

Polydnaviruses are mutualistic viruses associated with certain taxa of parasitoid wasps in the families Ichneumonidae and Braconidae. Polydnaviruses (PDVs) associated with ichneumonid wasps are known as Ichnoviruses (IVs), and those with braconid wasps are known as Bracoviruses (BVs) (Webb and Strand 2005). The segmented, dsDNA genomes of both IVs and BVs are vertically transmitted as proviral integrated loci in the wasp nuclear genome (Belle et al., 2002; Desjardins et al., 2007; Volkoff et al., 2010). The integrated virus is excised, replicated, and encapsidated in the calyx region of female ovarian tissue and transmitted to an insect host during parasitization (Webb and Summers 1992; Volkoff et al., 1995; Webb 1998). Although virus replication is not observed in the infected (parasitized) host (Theilmann and Summers 1986), expression of PDV genes is essential to successful parasitization. Ichnovirus and Bracovirus genomes encode multimember gene families that share little overlap in gene composition, reflecting the paraphyletic nature of PDVs (Bezier et al., 2009; Volkoff et al., 2010). Ichnovirus genes evolve rapidly, including duplication within gene families, resulting in variation in gene content, and reflecting variations in host range and pathologies among closely related viruses (Webb et al., 2006; Tanaka et al., 2007; Djoumad et al., 2013). However, despite data suggesting functional diversification within IV gene families (Rasoolizadeh et al., 2009; Marziano et al., 2011; Gill and Webb 2013), relatively little direct evidence exists to test this hypothesis (Fath-Goodin et al., 2006; Kroemer and Webb 2006; Fath-Goodin et al., 2009).

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Although Ichnovirus replication is asymptomatic in the wasp, gene expression in the parasitized larval lepidopteran (caterpillar) host results in disrupted immunity and delayed or halted development (Kroemer and Webb 2004; Luo and Turnbull 2008). Lepidopteran immunity is broadly separated into two interconnected aspects of humoral and cellular immunity. Humoral immunity primarily is carried out by antimicrobial peptides produced by the fat body (Shelby and Webb 1999), whereas cellular immunity is primarily carried out by mobile hemocytes, particularly granulocytes and plasmatocytes (Pech and Strand 1996; Lavine and Strand 2001; Lavine and Strand 2002). Ichnoviruses disrupt the production of antimicrobial peptides (Doucet and Cusson 1996; Shelby et al., 1998), while *Campoletis sonorensis* IV (*Cs*IV) infection induces alterations in hemocyte populations and reduces hemocyte attachment and spreading, but does not alter phagocytosis (Davies et al., 1987; Davies and Vinson 1988; Turnbull et al., 2004). Much but not all of the effect of hemocyte attachment and spreading appears to be the result of *Cs*IV-encoded cys-motif proteins (Li and Webb 1994; Cui et al., 1997; Turnbull et al., 2004; Fath-Goodin et al., 2006). An emergent result of this cellular pathology is a significant reduction in cellular encapsulation, the primary cellular immune response to multicellular invaders. During encapsulation, a population of granulocytes recognizes and binds to a foreign object as a single layer, recruiting numerous layers of plasmatocytes, before a single layer of granulocytes terminates capsule formation (Pech and Strand 1996). Capsule morphogenesis involves extensive cellular adhesion and remodeling (Gupta 1991; Schmidt et al., 2008; Strand 2008). Regulation of encapsulation requires paracrine signaling (Clark et al., 1997; Strand and Clark 1999). Gap junctions observed between capsular and migrating hemocytes also have been hypothesized to serve a regulatory function (Grimstone et al., 1967; Baerwald 1979; Gupta 1991; Churchill et al., 1993).

Gap junctions are present in nearly all tissues of metazoans ranging from cnidarians to humans (Hasegawa and Turnbull 2014). They facilitate coordinated cellular responses to direct (Goodenough and Paul 2009) and secreted (Lechner et al., 2007; Levin 2007) signals from other cells by mediating the exchange and uptake of small metabolites. Gap junctions are comprised of two apposed hemichannels, contributed by neighboring cells, forming a connection between the cells; hemichannels also may be unapposed, allowing transfer between the extracellular environment and the cytoplasm. Hemichannels are multimers comprised by members of a protein family, with both homomeric and heteromeric combinations, the composition of which affects channel characteristics (Curtin et al., 2002; Weber et al., 2004; Ayad et al., 2006). In insects, gap junctions are encoded by Innexins (Phelan 2005), which are homologous to mammalian Pannexins (Abascal and Zardoya 2013). Innexin homologues termed Virus Innexins, or Vinnexins, have been isolated from multiple Ichnoviruses (Turnbull et al., 2005; Tanaka et al., 2007; Volkoff et al., 2010). Vinnexins are expressed in host lepidopteran tissues including hemocytes following infection, and form functional gap junctions as shown by electrophysiology in paired *Xenopus laevis* oocytes (Turnbull et al., 2005; Marziano et al., 2011). Additionally, Vinnexins are capable of forming heterotypic junctions (comprised by two different hemichannels) and possibly heteromeric hemichannels with host Inx2 protein, altering the junctional characteristics from those of channels composed of Inx2 alone (Marziano et al., 2011). Concurrently, Inx2 orthologues are expressed broadly in insects, including in hemocytes of parasitized individuals (Turnbull et al., 2005; Barat-Houari et al., 2006).

The role of Vinnexins in possibly altering host caterpillar physiology has proven difficult to assess due to the lack of molecular and genetic tools to readily manipulate both PDVs and caterpillars. To elucidate their roles, we describe here the effects of ectopic expression of Vinnexins in lepidopteran cell culture on cellular physiology. Additionally, we have expressed Vinnexins in the model dipteran, *Drosophila melanogaster*, to assess alterations in tissue and organismal physiology. Our results provide evidence for a possible role of the Vinnexins in altering hemocyte physiology, and further support the use of *Drosophila* to understand how Vinnexins alter cellular and tissue physiology of host insects.

2. Materials and methods

2.1. Synthesis of insect cell line expression constructs

The CsIV *vinnexins* were amplified from existing constructs (Turnbull et al., 2005; Marziano et al., 2011) for cloning into the pIZT/V5-His vector (Life Technologies) using the following primers incorporating restriction sites (underline), Kozak consensus motif (lower case 'g'), and removing the stop codon to produce C-terminal fusion to the V5-His epitopes: *vnxD*: 5′-AAAGGTACCA TGgTGCACGTAA and 5′-AATACTAGTCTTAACGTTTGCAAC; *vnxG*: 5′-GGTACCACGATGgTGC and 5′-CACCGCGGTAAAGCATCC; *vnxQ1*: 5′-TCTAGAATGGTCAAGATTTT and 5′-CCGCGGATAAGCATCTGCAA; *vnxQ2*: 5′-AAATCTAGAATGgTTAACATTC and 5′-AATCCGCGGTAAA-ACGTCCGCATC. Sf-*inx2* was amplified from cDNA synthesized from Sf9 RNA, using the primers 5′-GAATTCGCCATGgTTGAC and 5′-TCTAGAGTCAACACACTGTCCTTC. Clones were sequenced (Clemson University Genomics Institute) to ensure accuracy.

2.2. Caterpillar and cell line maintenance

Spodoptera frugiperda were maintained on pinto bean diet at 27 °C, 12 h photoperiod according to standard protocol (Perkins 1979). Larvae were staged by head capsule slippage for use in experiments. High Five cells (Life Technologies), which exhibit several characteristics of lepidopteran granulocytes (Beck and Strand 2003), were maintained as adherent cells at 27 °C in TnMFH media (Mediatech) supplemented with 5% FBS. Transfections were performed with Cellfectin II Reagent (Life Technologies) according to manufacturer's instructions. Stable transfections were established and maintained by zeocin selection (Life Technologies).

2.3. Cell spreading

Stable High Five cell lines (empty pIZT/V5-His vector, pIZT/Sf-*inx2*-V5-His, pIZT/*vnxG*-V5-His, pIZT/*vnxQ1*-V5-His, pIZT/*vnxQ2*-V5-His) were seeded at 3×10^4 cells per well of a four-well plastic Permanox chamber slide (Thermo Scientific), and incubated for 24 h under normal conditions. Five images were taken at a random field of view for each cell type and measurements of cell area, circularity, and elongation were performed using Nikon Elements Basic Research 3.0 software. The procedure was repeated three times to obtain three biological replicates. Image data were pooled per replicate, and mean area, circularity, and elongation were analyzed by Mann-Whitney test.

2.4. Dye transfer

Empty vector, Sf-*inx2*-V5, *vnxG*-V5, and *vnxQ2*-V5 stable High Five cell lines were seeded in 60 mm plastic dishes (Falcon) and allowed to adhere for 36 h. Thin wall glass capillaries (1.0 mm; World Precision Instruments) were heat pulled with a Flaming/Brown Micropipette puller (Sutter Instrument Co. P-97), and tips were broken with forceps to generate needles. Needles were backfilled with 1 mg/ml Lucifer Yellow warmed to 50 °C. Cells were microinjected with a Narishige IM300 microinjector while visualizing with a Nikon Eclipse TE2000S microscope until a fluorescent

exposure below 1 s was achieved. Starting from this point, dye transfer was assessed every 30 s for up to 2 m, which allowed accurate and rapid assessment of dye transfer between cells. At least five replicates were performed for each cell type, with a minimum of 10 injections per replicate. Data were analyzed by ANOVA with Fisher LSD to determine statistical significance.

2.5. Lepidopteran immunity assays

Encapsulation assays were performed to test the role of gap junctions and Innexins in lepidopteran immunity. *In vivo* assays were carried out by injection and assessment of targets in the presence and absence of the gap junction inhibitor carbenoxolone. Four independent replicates, each consisting of three 4th instar *Spodoptera frugiperda* larvae, were performed per treatment. Larvae were anaesthetized and injected using a Hamilton syringe with approximately 30 sterile DEAE Sephadex beads (dry size range 40–125 μm; Sigma) pre-stained with Congo Red to aid in recovery; pre-wetted, sterilized beads were suspended in 10 μl of carbenoxolone (Sigma; final intrahemocoelic concentration approximately 10 mM) or 10 μl of sterile water. Hemocytes from the three larvae per treatment replicate were pooled by collection on ice in anticoagulant buffer (Pech et al., 1994). Cells were centrifuged for 5 min at 500*g*, resuspended in PBS (pH 7.0), and an aliquot analyzed by Trypan blue exclusion assay to assess viability. Each larva was dissected 5 h post-injection, sufficient to allow multiple layers of cell adhesion, and recovered beads imaged and scored. Data were analyzed by *t*-test.

For *in vitro* encapsulation, High Five cells were suspended at 1.2×10^5 cells per 200 μl TnMFH + 5% FBS in a PCR tube with approximately 30 DEAE Sephadex beads, and incubated with slow end-over-end rocking for 16 h at 27 °C. Beads were placed in a 96-well plate and scored for encapsulation. Encapsulation was scored on a scale of 0–2, with 0 = no adherent cells, 1 = single layer or incompletely surrounded bead, and 2 = multiple layers of cells surrounding the bead (Beckage 1998). Data were analyzed by ANOVA.

2.6. Generation of transgenic Drosophila melanogaster

To ectopically express the *vinnexin* genes in *Drosophila*, complete cDNAs for *vnxQ1* (NCBI Accession Number AY197487) and *vnxQ2* (AY197488) were cloned into the BglII site of the pUAST-attB plasmid (Bischof et al., 2007); the cDNAs for *vnxD* (AY197485) and *vnxG* (AY197486) were cloned into a derived pUAST-attB vector encoding a C-terminal 3X HA epitope. In addition, the Dm-*inx2* cDNA (LD22570, *Drosophila* Genomics Resource Center) was cloned into the BglII site of pUAST-attB. Constructs were confirmed by DNA sequencing. To generate transgenic lines, constructs were injected into embryos containing a source of ΦC31 integrase and an *attP* landing site (51D) on the second chromosome (Bischof et al., 2007). The *P{Act5c-GAL4}25FO1/CyO* (Ekengren et al., 2001) driver stocks and UAS-GFP control responder stock were obtained from the Bloomington *Drosophila* Stock Center, Indiana University. The *NP1-GAL4* (Hayashi et al., 2002) driver stock was obtained from Kyoto Stock Center (DGRC). All flies were maintained at 25 °C on standard yeast/agar media.

2.7. Western blotting and immunomicroscopy

Immunological techniques were based on previously published methods (Turnbull et al., 2005). For western blots, cells were resuspended in lysis buffer (25 mM Tris-HCl, pH 7.6; 150 mM NaCl; 1% NP-40; 0.5% TritonX-100; 0.1% SDS + protease inhibitors), total cell lysate diluted in 4X reducing loading buffer, boiled for 10 min at 95 °C, and separated on 10% polyacrylamide gels. Proteins were transferred to PVDF membranes and blocked with 4% dry milk in PBT (PBS + 0.02% Tween-20). Blots were probed with mouse anti-V5 antibody at 1:2500 (Life Technologies) in PBT, washed, and probed with goat anti-mouse HRP-antibody at 1:10,000 (Jackson Immunoresearch) in PBT. Blots were processed for ECL substrate (Pierce) and visualized on film. For immunomicroscopy, cells were fixed 36–60 h post seeding in PBS plus 3.7% paraformaldehyde, permeabilized with PBST (PBS plus 0.02% TritonX-100), and blocked with 4% dry milk in PBST. Primary mouse anti-V5 (Life Technologies) and secondary anti-mouse Alexa Fluor 598 (Life Technologies) were diluted in PBST at 1:200 and 1:1000, respectively. *Drosophila* embryos were collected and prepared using standard protocols (Narasimha and Brown, 2006) before probing as above with mouse anti-HA antibody (Thermo Scientific Novex). Imaging was performed using a Nikon Eclipse TE2000S fluorescent microscope and processed with NIS Elements BR 3.0 software.

2.8. Statistical analyses

Statistical analyses, as noted above, were performed using StatPlus:mac (AnalystSoft) on data exported into Microsoft Excel.

3. Results

3.1. Ectopic expression of Sf-inx2 and CsIV vinnexin genes in High Five cells

The *Spodoptera frugiperda inx2* orthologue (Sf-*inx2*) and all four CsIV *vinnexin* genes were cloned into the pIZT-V5-His vector for stable expression under an IE promoter in High Five lepidopteran cells. We generated stable lines with the empty vector, Sf-*inx2*, and each of the four *vinnexin* genes. Expression was verified by anti-V5 western blot (Fig. 1A) and immunomicroscopy (Fig. 1B). VnxD expression was not observed by immunoblotting, although it was detected at similar levels to the other Vinnexins in immunomicroscopy. Multiple solubilizing, transfer, and running conditions failed to reliably enable detection of VnxD-V5 by western blot (data not shown), possibly indicating poor transfer, reduced protein stability, or inaccessibility of epitope. All gap junction proteins localized primarily to the cytoplasm, but were observed at appositional membranes, as well, as expected.

3.2. Expression of Vinnexins broadly affects cell morphology

Following establishment of stable lines, cells were examined for alterations in morphology reflective of CsIV infection (Turnbull et al., 2004). Work focused on VnxG, VnxQ1, and VnxQ2, as electrophysiological analyses demonstrated these three proteins exhibit distinct characteristics in *Xenopus* oocytes (Marziano et al., 2011), and western blots suggested reduced detectable quantities of VnxD (Fig. 1A); we also examined *Sf*-Inx2, as a control for ectopic alteration of gap junction presence. We observed significant alterations in spreading ability of High Five cells stably expressing a *vinnexin* gene, relative to control cells stably transfected with the empty pIZT-V5-His vector. Cells expressing either *vnxQ1* or *vnxQ2* exhibited a relative decrease in cell area (Fig. 2A) and length of longest axis (Fig. 2B), and a relative increase of circularity (Fig. 2C). Expression of *vnxG* did not affect cell area, circularity, or elongation, relative to control cells. Expression of Sf-*inx2* resulted in an alteration in area similar to those of cells expressing *vnxQ1* or *vnxQ2*. Cells expressing Sf-*inx2* closely resembled those expressing *vnxG* in regards to elongation and circularity, exhibiting significant changes in cell morphology relative to control, empty vector-transfected cells. Ectopic expression of the Inx2 or Vinnexins did not induce any visible signs of apoptosis. Cell doubling time was tested in parallel and no significant differences observed between

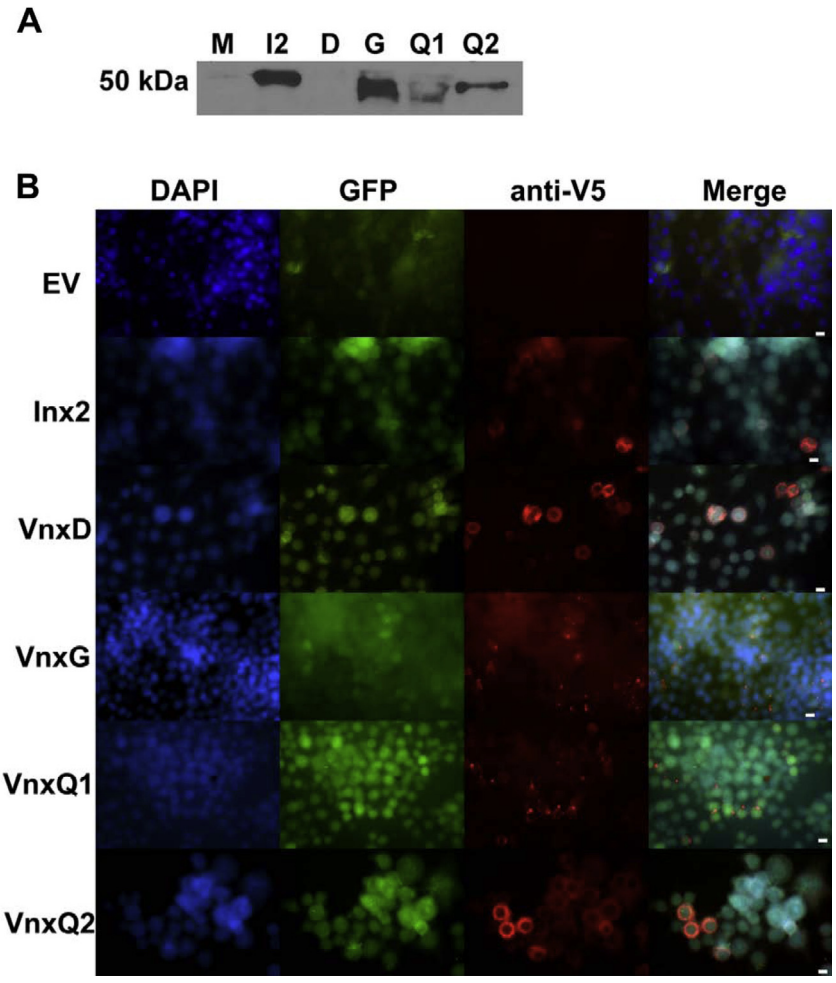

Fig. 1. Ectopic expression of CsIV *vinnexins* and host Sf-*inx2* in High Five cell line. High Five cell lines stably transfected with empty pIZT-V5-His vector (EV), Sf-*inx2*, or a *vinnexin* were examined by (A) western blotting or (B) immunofluorescence of V5 epitope 60 h following plating. Scale bar in B is 10 μm.

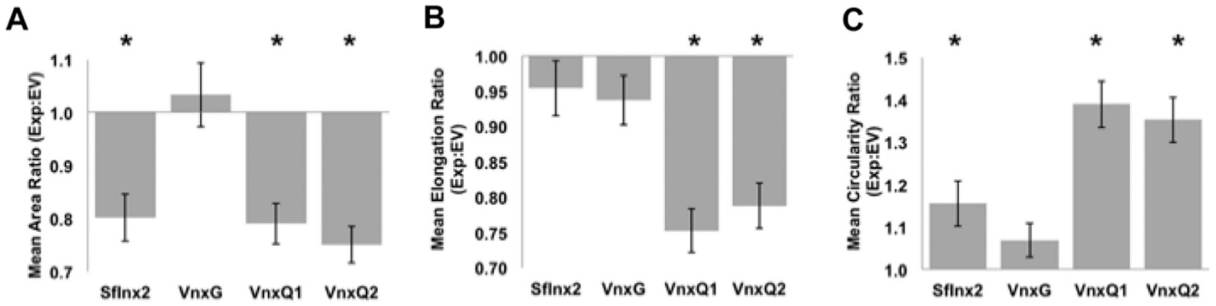

Fig. 2. Ectopic expression of insect or virus *innexins* induces morphological alterations to High Five cells. Cell area, elongation, and circularity were quantified 24 h after plating for High Five lines stably expressing Sf-*inx2*, *vnxG*, *vnxQ1*, and *vnxQ2*; values are presented as a ratio to High Five cells stably transfected with pIZT-V5 empty vector (EV). (*) Significant difference from High Five transfected with empty pIZT-V5 vector, as tested by Mann-Whitney. Error bars represent s.e.m.

cells stably transfected with the empty vector, VnxG, or VnxQ2 [ANOVA, $F(2,5) = 0.317$, $p = 0.742$]. Viability was unaffected by ectopic Vinnexin or Inx2 expression relative to mock transfected cells as determined by Trypan blue staining for three days post-transfection [ANOVA, $F(4,56) = 0.904$, $p = 0.468$]. Therefore, while cell viability did not appear to be altered by ectopic Inx2 or Vinnexin expression, ectopic expression of Inx2 and some, but not all, Vinnexins affects cell morphology.

3.3. Ectopic gap junction protein expression alters gap junctional intercellular communication

We next examined the ability of ectopically-expressed insect and virus Innexins to alter gap junction intercellular communication. Paired cells stably-transfected (identified by pIZT plasmid-encoded GFP-expression under IE promoter) with the empty vector or one of the gap junction genes were injected with the gap

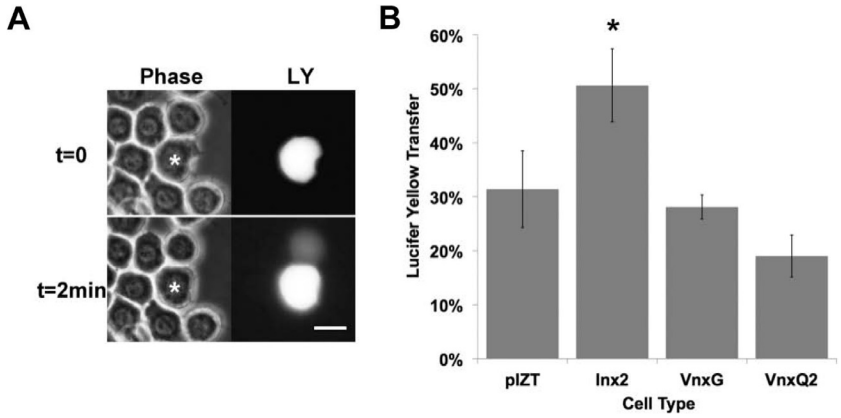

Fig. 3. Vinnexin expression minimally reduces intercellular dye transfer. (A) Representative image indicating injected cell (*) and transfer of Lucifer Yellow (LY) to adjacent cell within two minutes. (B) Ectopic expression of Sf-*inx2* resulted in a significant increase in intercellular dye transfer, relative to empty vector control cells (pIZT), while ectopic expression of *vnxG* and *vnxQ2* did not affect dye transfer. * in B indicates significant difference from High Five transfected with empty pIZT-V5 vector, as tested by Mann-Whitney. Error bars represent s.e.m.

junction-permeant dye Lucifer Yellow (LY), and intercellular dye transfer observed (Fig. 3A). Approximately 31% (n = 77) of cells stably transfected with the empty vector exhibited LY transfer to one or more neighboring cells. Cells stably transfected with *vnxG* or *vnxQ2* exhibited similar dye transfer rates to empty vector-transfected cells (Fig. 3B; *vnxG*: 28%, n = 62, p = 0.69; *vnxQ2*: 19%, n = 65, p = 0.12), while cells stably transfected with Sf-*inx2* exhibited a significant increase (51%, n = 86, p = 0.03) in LY transfer relative to empty vector control. Eight percent of Sf-*inx2* injected cells exhibited secondary LY transfer (transfer from the recipient cell to a cell not adjacent to the injected cell), while those expressing a *vnxG* or *vnxQ2* never exhibited this behavior. Transfer to more than one neighboring cell also was frequently observed between sf-*inx2* cells (7.0%), but rarely among empty vector (3.9%), *vnxG* (3.2%), or *vnxQ2* (0%) transfected cells.

3.4. Cellular encapsulation is disrupted by carbenoxolone but not vinnexin expression

Given the effects on morphology and dye transfer, we tested how Vinnexins might affect hemocytic encapsulation. We established a bioassay in caterpillars to provide initial insight into whether gap junction intercellular communication is necessary for encapsulation. DEAE-Sephadex chromatography beads were injected into the hemocoel of caterpillars in the absence or presence of carbenoxolone (approximately 10 mM final concentration), an inhibitor of gap junction intercellular communication and hemichannel activity in High Five and Sf9 cells (Luo and Turnbull 2011; Dahl and Muller 2014). In control (water-injected) caterpillars, 92% of beads were fully encapsulated by hemocytes (Fig. 4A), while only 49% of beads were encapsulated in caterpillars co-injected with carbenoxolone (Fig. 4B, p = 0.021). Neither hemocyte viability (Fig. 4B, p = 0.289) nor number of hemocytes recovered (p = 0.114) differed between treatments, supporting that carbenoxolone inhibits encapsulation by interfering with Innexin activity, rather than by killing hemocytes.

We next tested the ability of granulocyte-like High Five cells and stable *vinnexin*-transfected High Five cells to participate in encapsulation of chromatography beads. Control High Five cells adhered to and surrounded beads in a thin layer (Fig. 4C), mimicking granulocyte behavior (Pech and Strand 1996). *Vinnexin*-expressing cells exhibited no difference from untransfected High Five cells in full encapsulation of chromatography beads (p = 0.90). Intrahemocoelic *vinnexin* expression in caterpillars was attempted with baculovirus- and plasmid-based techniques, but resulted in insufficient differentiation from non-recombinant virus and low transfection efficiency, respectively, to deduce an effect on encapsulation (data not shown).

3.5. Ectopic expression of vnxG or Dm-inx2 disrupts Drosophila melanogaster development

The lack of tools to manipulate gene expression and function *in vivo* in PDVs and lepidopterans led us to test the effects of

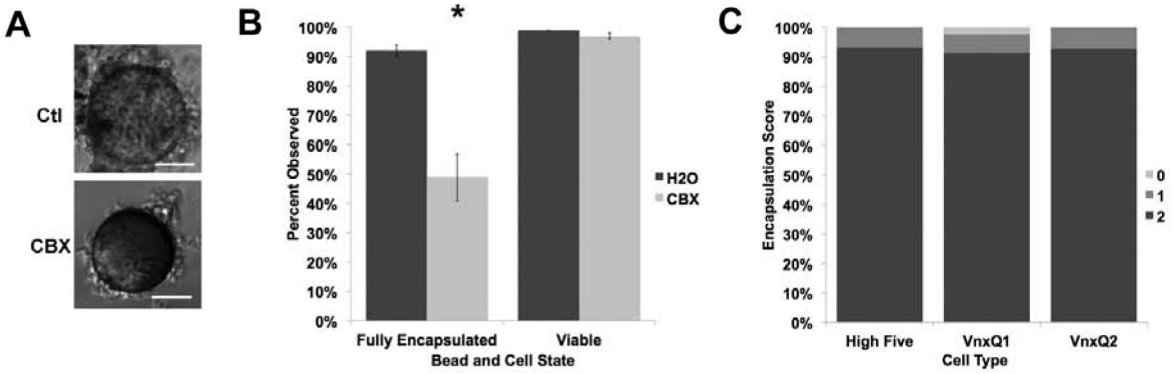

Fig. 4. Carbenoxolone reduces encapsulation by hemocytes, but Vinnexin expression does not alter encapsulation in cultured cells. Fourth instar *S. frugiperda* larvae were injected with DEAE-Sephadex beads in the presence and absence of the gap junction inhibitor, carbenoxolone (CBX). (A) Recovered bead from caterpillar co-injected with water (Ctl) or carbenoxolone (CBX) (scale bar = 10 μm). (B) Intrahemocoelic encapsulation of beads is reduced 5 h post-injection in the presence of carbenoxolone (~10 mM hemocoelic concentration) relative to water injections, while cell viability did not differ significantly between water control and carbenoxolone injected larvae; error bars are s.e.m., and * indicates significant difference at $p < 0.05$ from control. (C) Encapsulation did not vary between control High Five cells and those transfected with *vnxQ1* or *vnxQ2*.

ectopic expression of Vinnexins in the model insect, *Drosophila melanogaster*. We utilized the GAL4-UAS system, in which offspring of a parent carrying a GAL4 driver under the regulation of a tissue-specific enhancer and a parent with a UAS-responder gene are examined for an effect on phenotype (Duffy 2002). We first tested the ability of ectopically-expressed Innexin or Vinnexin to disrupt organismal viability. The ubiquitously-expressed actin driver line P{Act5C-GAL4}25FO1/CyO (Ekengren et al., 2001) was crossed to UAS-*gfp*, UAS-Dm-*inx2*, UAS-*vnxD*, UAS-*vnxG*, UAS-*vnxQ1*, or UAS-*vnxQ2* responder lines. Offspring were examined for Curly (Cy) versus non-curly wing phenotype, and departure from the expected 50% non-Curly wing phenotype in offspring was considered evidence of lethality due to the product of the gene under UAS regulation. The offspring of GFP crosses did not deviate from 50% expectation (χ^2 analysis, $p = 0.43$), indicating that transgene expression alone was insufficient to induce lethality. Similarly, offspring of P{Act5C-GAL4}25FO1/CyO × UAS-*vnxD* ($p = 0.86$), P{Act5C-GAL4}25FO1/CyO × UAS-*vnxQ1* ($p = 0.40$), and P{Act5C-GAL4}25FO1/CyO × UAS-*vnxQ2* ($p = 0.60$) all were indistinguishable from 50% (Table 1). However, global expression of either Dm-*inx2* ($p < 0.01$) or *vnxG* ($p < 0.01$) invariably was lethal, indicating that these two gene products were sufficient to disrupt normal cellular function and ultimately organismal viability. Immunomicroscopy verified that *vnxD* and *vnxG* were expressed (Fig. 5), supporting that loss of viability was due to differences in protein-specific function and not absence of transgene product in the *vnxD* line.

Drosophila melanogaster inx2 and *inx3* are essential to epithelia and gut development (Bauer et al., 2001; Lehmann et al., 2006), and the gut epithelial lining is an important barrier to infection in *D. melanogaster* (Davis and Engstrom 2012), while *inx1* plays a role in *Anopheles* gut immunity (Li et al., 2014). To determine the

Table 1
Global expression of *vnxG* and Dm-*inx2* in *Drosophila melanogaster* disrupt viability. UAS responder flies carrying a *Cs*IV-*vinnexin* or *Dm-inx2* transgene were crossed to Act5C-GAL4/CyO flies and the ratio of Curly and Non-curly offspring analyzed for departure from expected 50% distribution by Chi-Square test. [1]-Significance (S) at $p < 0.05$.

Transgene	Cy	Non-Cy	n	% Cy	Sig.[1]
GFP	56	48	104	54%	NS
Dm-*Inx2*	239	0	239	100%	S
vnxD	65	63	128	51%	NS
vnxG	72	0	72	100%	S
vnxQ1	50	42	92	54%	NS
vnxQ2	48	43	91	53%	NS

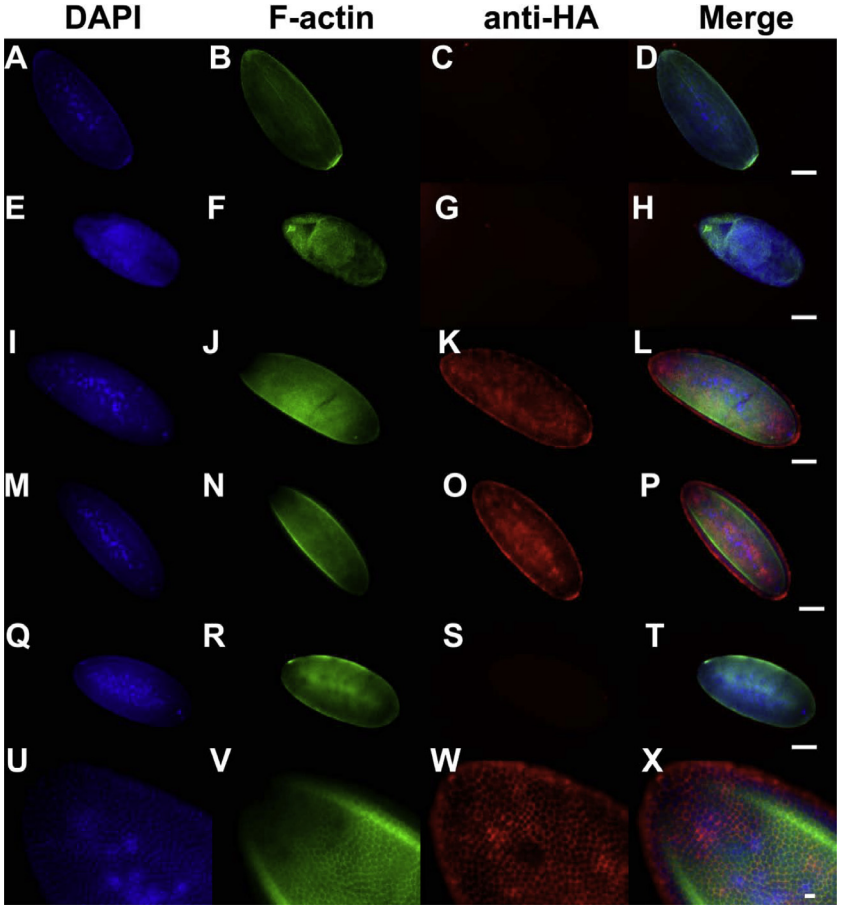

Fig. 5. Immunofluorescence microscopy verifies expression of HA-tagged *vinnexin* transgenes. Prepared embryos were immunoprobed with anti-HA antibody and imaged by epifluorescence microscopy to verify expression of the UAS-transgene. A–D: Driver line (P{Act5C-GAL4}25FO1/CyO, or Act5C-GAL4) offspring; E–H: Act5C-GAL4 × UAS-*Dm*-Inx2 offspring; I–L: Act5C-GAL4 × *VnxD-HA* offspring; M–P: Act5C-GAL4 × *VnxG-HA* offspring; Q–T: Act5C-GAL4 × *VnxQ2-HA* offspring; U–X: Act5C-GAL4 × UAS-*VnxG-HA* embryo at higher magnification. A–T scale bar = 100 μm; U–X scale bar = 10 μm.

effect of vinnexin expression and consequentially altered gap junction biology in intestinal tissue, we crossed the NP1-GAL4 intestinal driver line to UAS-*gfp*, -*Dm-inx2*, -*vnxD*, -*vnxG*, -*vnxQ1*, and -*vnxQ2* responder lines. All crosses produced viable adult progeny except for the cross to UAS-*vnxG*, which generated no viable adult progeny (data not shown). All NP1-GAL4 × UAS-*vnxG* progeny arrested at late pupal stage, with pigmentation, bristles, and meconium visible. Individuals dissected from arrested pupae did not display any gross morphological defects in the gut.

4. Discussion

Coordination of host activity is essential both in response to a parasite and in parasite manipulation of host physiology. Considering the absence of PDV replication in the infected caterpillar, PDV gene expression in the host is likely associated with the latter. Thus, characterization of virus gene products that play integral roles in this manipulation should recapitulate the mechanisms by which polydnaviruses carry out their mutualistic function, and may also elucidate host pathways involved in anti-parasite immunity. The Vinnexins are situated in an interesting nexus in this consideration: their host homologue Innexins coordinate multicellular activities, although their role in insect immunity is largely unclear, while Vinnexins form functional gap junctions in a heterologous system, but with an unclear role in the infected host. Here, we have characterized the physiological consequences of ectopic *vinnexin* expression, and observed altered cellular morphology and intercellular transfer, but not an alteration in hemocytic encapsulation. We also have demonstrated that ectopic expression of *vnxG*, but not the other *vinnexins*, in *D. melanogaster* results in organismal lethality, supporting future work with this system both to identify Vinnexin mode of action and to identify motifs important to interacting with host physiology.

The observed morphological differences in cells ectopically expressing a Vinnexin mirrors pathologies observed in *Cs*IV-infected caterpillars: hemocytes from *Cs*IV-infected hosts fail to spread fully, as do cells that are exposed to plasma from infected caterpillars (Davies et al., 1987; Turnbull et al., 2004). Connexins, the vertebrate gap junction protein family, have roles in adhesion, migration and morphogenesis, and volume regulation (Quist et al., 2000; Elias et al., 2007; Cotrina et al., 2008; Prochnow and Dermietzel 2008; Naus and Laird 2010), as well as the release of, and response to, cytokines (Oviedo-Orta et al., 2001; Eltzschig et al., 2006; Pelegrin and Surprenant 2009). Although a role has been inferred for caterpillar Innexins in hemocyte migration and cytokine uptake and release (Liu et al., 2013), this role has yet to be empirically demonstrated. However, *Dm*-Inx2 and *Dm*-Inx3 physically interact with the *Drosophila* homologues of the cell adhesion molecule *D*E-Cadherin and β-catenin (Lehmann et al., 2006; Giuliani et al., 2013), suggesting the potential for integration of adhesion, signaling, and cell communication. Overexpression of Sf-*inx2* altered cellular morphology, indirectly supporting this postulate. That expression of *vnxG* did not alter morphology is surprising, but this may indicate that the different Vinnexins interact with different host molecules, resulting in non-identical pathophysiology. Testing of Vinnexin interactions with these pathways is underway, and should aid in clarification of the mechanisms by which both Vinnexins and Innexins affect these physiological activities.

Previous work demonstrated that the proteins tested here are capable of forming homomeric and homotypic gap junctions (Turnbull et al., 2005; Marziano et al., 2011). In caterpillar cell lines, ectopic Sf-*inx2* expression increased dye transfer, while ectopic expression of *vnxG* or *vnxQ2* did not affect rates of dye transfer between cells. Electrophysiological data implicate the Vinnexins in altering *Sf*-Inx2 channel characteristics, including conductance (Marziano et al., 2011). Thus, the Vinnexins may primarily act in host cells to modify channels, rather than forming *de novo* ones; this possibility remains to be tested. Alternatively (or in addition), the Vinnexins may function via the activity of unapposed hemichannels, providing a mechanism for cell transfer of molecules to and from the environment. Such unapposed hemichannel activity has been demonstrated in uninfected lepidopteran hemocytes (Luo and Turnbull 2011), although the functions of such activity are currently unknown. Finally, subtle but significant effects on small molecule transfer may not be captured by the dye transfer assay. While the lack of knowledge concerning the breadth of biologically relevant transferred molecules in insects imposes limits on clarifying how Vinnexins may affect gap junction activity *in vivo*, testing of candidate molecules is currently underway.

Anopheles gambiae Innexin1 was demonstrated recently to mediate gut resistance to *Plasmodium* in mosquito, likely by a Toll-mediated pathway (Li et al., 2014), while global knockdown of Dm-*inx5* and Dm-*inx6* increased mortality from orally-acquired *Serratia* in *Drosophila* adults (Cronin et al., 2009). Innexins and gap junctions thus appear essential to cellular and humoral immune responses to gut invaders in insects. Our data represent the first demonstration of the necessity of Innexins or gap junctions to intrahemocoelic immunity and the encapsulation response. The ability of IV-infected cells to engage in encapsulation is severely reduced, and several reports have implicated various IV gene families in disrupting encapsulation including cys-motif (Li and Webb 1994; Cui et al., 2000; Gill and Webb 2013), rep (Rasoolizadeh et al., 2009), and viral ankyrin (Kroemer and Webb 2005) members. Images of encapsulation in IV-infected larvae (e.g., (Barat-Houari et al., 2006)) suggest that initial stages of encapsulation may occur, with intermediate to late stages inhibited. These latter stages of encapsulation are those in which gap junctions have been noted to occur (Grimstone et al., 1967; Baerwald 1975). Our *in vitro* encapsulation assay with the granulocyte-like High Five cell line thus may be inadequate to test the role of Vinnexins in disrupting immunity. That is, if Vinnexins interfere with capsule morphogenesis through altering granulocyte-plasmatocyte or plasmatocyte-plasmatocyte interactions (e.g., recruiting, adhesion, and spreading), then lack of these cells in the line may preclude testing of this behavior. Testing of gap junction activity in cultured hemocytes transformed or transfected with a *vinnexin* remains a possibility.

Given the dearth of testable emergent properties with cell lines, we turned to the model fly, *D. melanogaster*. *Drosophila melanogaster* has been used previously to examine an ankyrin-domain containing protein of the polydnavirus *Cotesia congregata* Bracovirus (Duchi et al., 2010), linking the protein to endocrine alterations associated with *Cc*BV infection (Valzania et al., 2014). The power of the *D. melanogaster* system was extended to test and demonstrate differential ability of *Cs*IV ankyrin gene family members to interfere with aspects of fly hematopoiesis and immune signaling (Gueguen et al., 2013). The roles of Innexins in *D. melanogaster* physiology have not been elucidated to the degree of NF-kB, although over- and under-expression of various *innexins* generally has negative consequences, including disrupted morphogenesis, organogenesis, and cell physiology (Curtin et al., 2002; Lehmann et al., 2006; Giuliani et al., 2013). Thus, the loss of viability associated with over-expression of Dm-*inx2* and *vnxG* are not surprising, given both exhibit strong junctional conductances in paired *Xenopus* oocytes (Stebbings et al., 2000; Turnbull et al., 2005; Marziano et al., 2011; Holcroft et al., 2013); failure of the other *vinnexins* to affect viability was surprising, since they also form homomeric-homotypic junctions in paired oocytes (Marziano et al., 2011). However, *vnxG* exhibited the highest proportion of heterotypic and heteromeric junctions in paired *Xenopus* oocytes when tested

with Sf-*inx2* (Marziano et al., 2011). VnxG also is most similar to Inx2 orthologues from lepidopterans and *D. melanogaster* (Hasegawa and Turnbull 2014), possibly suggesting a greater potential for successful interactions with cellular machinery to form functional units. Failure of the remaining *vinnexins* to affect viability may thus reflect inability to interact with or affect junctions and junctional complexes in *Drosophila* and the tested lepidopteran systems.

We have described significant variation in phenotypic effect of ectopic expression of *Cs*IV *vinnexins*. Systematic analysis of the roles of Innexins in multiple tissues, including hemocytes, and in additional physiological systems including multiple components of immunity is necessary to better understand this gene family and provide greater context for the Vinnexins. The use of conditional expression of the *vinnexins* in *Drosophila*, using the repertoire of tools available in that system, will facilitate dissection of the signaling and cellular physiology associated with *vinnexin* expression. Comparing results from ectopic *vinnexin* expression in *Drosophila* will also enable analysis of the significance of the diversity within this multi-gene family. This in turn should provide insight into the role of Vinnexins in manipulation of Ichnovirus-infected insects, as well as the functions of Innexins in those insects.

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