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Functional Comparison of Two Virus Innexins

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FUNCTIONAL COMPARISON OF TWO VIRUS INNEXINS

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by
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ABSTRACT

Some Parasitoid wasps rely on Polydnavirus to determine suitable hosts and complete their parasitization. Ichnoviruses (IV), one subfamily of Polydnavirus, are associated with Ichneumonids, have one highly conserved gene family named *vinnexins*. It has been demonstrated *vinnexins* are homologues of insect gap junction genes (*innexins*). However, their functions to the virus, parasitoid wasp and secondary host are not fully understood.

In my thesis, I have focused on two *vinnexins, vnxG and vnxQ2*, to investigate their effects on insect cell line and caterpillar using recombinant baculoviruses. I expressed *vnxG* or *vnxQ2* in Sf9 cells and compared their influence on cell physiology. In addition, I expressed *vnxG* and *vnxQ2* in *Heliothis virescens* caterpillars and focused on their alteration to hemocytes. In the end, I will discuss potential mechanism by *vinnexins* may function and play a role. Both *in vivo* and *in vitro* works will serve as a starting point for future research.

DEDICATION

Mom and Dad

ACKNOWLEDGMENTS

This thesis was made possible by the generous guidance and support from my supervisor, Dr. Matthew Turnbull. I would also like to thank my committee members, Dr. Michael Caterino and Dr. Andrew Mount, for their advice and encouragement. I would like to acknowledge my colleagues in Entomology program and Turnbull's lab, who gave me indispensable help to complete my study and research. Finally, I would like to thank my parents and all my friends, for their understanding and love.

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CHAPTER ONE

INTRODUCTION

1. Parasitoid wasps and Polydnavirus

Parasitoids have a unique and successful strategy to complete their reproduction and early development, as they spend a significant portion of their life history intimately associated with a host in a relationship where the host will be depressed and ultimately killed. This special biological phenomenon has led to questions about the mechanisms by which they determine host range and manipulate host physiology.

Some subfamilies of ichneumonid and braconid parasitoid wasps have symbiotic viruses, Polydnavirus (PDVs), named as Ichnoviruses and Bracoviruses, respectively. The PDV genome is integrated in the wasp germ line, and transmitted vertically during wasp reproduction (Drezen, Chevignon et al. 2014). The virus genome is also excised, replicated, and packaged in wasp ovaries (Kroemer, Webb 2004), and during parasitization by the wasp of an insect (typically a caterpillar), virus is delivered into the host insect body cavity (hemocoel) (Strand, Burke 2013). Infection by the PDV results in numerous pathologies, including prevention of cellular encapsulation (Shelby, Webb 1999), the primary anti-parasitoid immune response (Beckage, Gelman 2004) while metabolic (Shelby, Webb 1997), developmental (Kaeslin, Pfister-Wilhelm et al. 2005), and antimicrobial immunity pathologies (Shelby, Webb 1999) also are observed.

Despite these commonalities, PDVs are a polyphyletic lineage: Bracoviruses have a distinct origin compared with Ichnoviruses (IVs) (Volkoff, Jouan et al. 2010). Given this, it is unsurprising that most of the commonalities are superficial, with few genes shared between the BVs and IVs, and distinct mechanisms of host manipulation and interaction (Webb, Strand et al. 2006, Bezier, Annaheim et al. 2009). As such, analysis of both BVs and IVs is warranted to understand better PDV evolution and mechanism of host determination.

2. Gap Junctions and Innexins

Gap junctions form intercellular channels, which connect adjacent cells and allow direct molecular exchange and cell-cell communication. This cell-cell structure allows intercellular transfer of molecules typically smaller than 1 kilo Dalton, like cAMP and Ca^{2+} (Scemes, Spray et al. 2009). This structure is vital for normal function of cells.

There are 3 families of gap junction proteins within the animal kingdom: the Connexins, the Pannexins and the Innexins. Vertebrates use proteins encoded by the *pannexin* and *connexin* gene families to assemble hemichannels (Su, Lau 2014). Connexin hemichannel can form functional gap junctions as well as non-junctional channels, while Pannexins form non-junctional channels that can function in paracrine signalling (among other functions). Protostomes use proteins encoded by the *innexin* gene family, which have similar functions to Connexins but are homologues of Pannexins (Hasegawa, Turnbull 2014). Structural analyses predict that Innexins have four

transmembrane domains, with three connective loops and intracellular N and C termini (Phelan, Starich 2001). Six Innexins form a hemichannel, and two hemichannels provided by two adjacent cells form a gap junction (Bauer, Löer et al. 2005).

The *innexin* gene family members of the fly *Drosophila melanogaster*, the nematode *Caenorhabditis elegans* (Phelan 2005) and more species have been identified. There are 8 *D. melanogaster innexins* and more than 20 *C. elegans innexins*, when most insects seem to have between 5-8 *innexin* members (Hasegawa, Turnbull 2014). In *C. elegans*, mutations in the *innexin unc-7* gene result in an uncoordinated phenotype (Starich, Xu et al. 2009), and in fly and worm, mutants show abnormal gap junction function (Phelan, Nakagawa et al. 1996). Gap junctions are essential for epithelial morphogenesis, as fly Innexin2 mutants display developmental defects in the embryo (Bauer, Lehmann et al. 2004). In *Anopheles gambiae*, loss of Innexin1 reduces anti-Plasmodium immunity (M. W. Li, Wang et al. 2014). These and more studies indicate essential and irreplaceable roles of *innexins* in many physiological systems.

3. *Campoletis sonorensis* Ichnovirus (CsIV) and Virus Innexin

The ichneumonid *Campoletis sonorensis* and its symbiotic virus, *Campoletis sonorensis* IV (CsIV), are commonly used as a model to study parasitization and PDV pathology (M. Turnbull, Webb 2002). The CsIV genome is dsDNA, ~250 kb in size, with 101 Open Reading Frames across 24 genomic segments (Webb et al. 2006). Genes of CsIV are expressed in infected host tissues and cause alterations in hemocyte population.

The expression of CsIV genes has an adverse impact on hemocyte attachment and spreading (M. W. Turnbull, Martin et al. 2004), although no virus replication occurs in the caterpillar host during this process (Theilmann, Summers 1988). Four Innexin homologue genes, named as virus Innexins (Vinnexins), are encoded by CsIV, with *vinnexins* (*vnx*) also identified in every other Ichnovirus genome sequenced to date (Hasegawa, Turnbull 2014).

Recent analyses hint at the functions of Vinnexins (Vnx) in the host. Morphological changes including relative decrease in cell area and length of longest axis, and relative increase of circularity, were observed following ectopic *vinnexin* expression in High Five caterpillar cell line (Hasegawa, Erickson et al. 2017), which indicates Vinnexins may be partly responsible for cell morphology alteration observed during CsIV infection (M. W. Turnbull et al. 2004). In addition, *vinnexin* expressions in *Xenopus laevis* oocytes leads to the formation of functional gap junctions differing in coupling strength and reliability (M. W. Turnbull, Volkoff et al. 2005, Marziano, Hasegawa et al. 2011). The GAL4-UAS system was used to ectopically express the four CsIV *vinnexins* in *D. melanogaster* embryos, revealing that the different Vinnexins may have different functions and influence hosts in different ways: global, constitutive expression of *vnxG* is embryonic lethal to *D. melanogaster*, while there was no observed effect of expressing the other three *vinnexins* (Hasegawa et al. 2017). Immunomicroscopy assays using epitope-tagged Vinnexins and Sf-Inx2 shows Vinnexins and Innexins appear at similar positions inside cells (Hasegawa et al., unpublished). Finally, in *X. laevis* oocytes expressing a combination of Vinnexin and Sf-Inx2, electric profiles were altered

(Marziano et al. 2011), indicating Vinnexins may alter cellular bioelectric processes. All these evidence suggest that Vinnexins can physically interact with host Innexins and potentially alter Innexin functions and disrupt host physiology.

4. Summary

Our knowledge about PDV function and pathology is still limited, but it has application value and potential on pest management, especially on lepidopteran pest control. Transgenic tobacco plants expressing TnBVANK1, which is encoded by *Toxoneuron nigriceps* BV, have insecticidal activity and can cause developmental arrest of *Spodoptera littoralis* larvae feeding on them (Di Lelio, Caccia et al. 2014). The CsIV *Cys-motif* protein gene family is an inhibitor of insect growth and development when expressed in transgenic plants (Fath-Goodin, Gill et al. 2006). Those results evidence the possibility to control lepidopteran pest by utilizing transgenic plants expressing PDV virulence genes. To take the fullest advantage of PDVs, we need to know this unique virus family better. A deeper insight into the pathology may unravel the mechanism of PDV gene function.

CHAPTER TWO

IN VITRO VINNEXIN EXPRESSION IN *SPODOPTERA FRUGIPERDA* CELL LINE

1. Abstract

Some parasitoid wasps are associated with Polydnaviruses, symbiotic viruses that encode virulence factors which are essential to successful parasitization by the wasp of a caterpillar host. Members of one group of Polydnaviruses, the Ichnoviruses, encode a multigene family known as Vinnexins. Vinnexins are homologues of insect gap junction genes, and form functional gap junctions that may affect host cell physiology. However, the role of Vinnexins in host pathology and the mechanism by which the Vinnexins affect their caterpillar host are largely unknown. In this paper, we generated recombinant baculoviruses to express *vinnexins* in *Spodoptera frugiperda* (Sf9) cells. To measure cell physiological changes caused by Vinnexins, cells were probed with a membrane potential-sensitive probe, DiBAC4(3), and a pH indicator, Carboxyfluorescein diacetate (CFDA). In addition, we utilized carbenoxolone and ouabain, respectively, to probe the role of gap junctions and hemichannels, and Na^+/K^+-ATPase in establishing membrane potential in studied cells. Our results indicate the Vinnexins induce cell membrane depolarization and cytoplasmic alkalization in a degree specific to each tested Vinnexin, and that neither Vinnexin hemichannels nor the Na^+/K^+-ATPase appear to underlie these effects directly. These results hint that members of the Vinnexin protein family may affect host bioelectrical phenomena to disrupt host cell physiology, and that the individual proteins of the family may differentially affect host physiology.

2. Introduction

Polydnaviruses (PDVs) are large dsDNA viruses associated with certain lineages of endoparasitoid wasps (Drezen, Leobold et al. 2017). The PDV genome is integrated in the wasp germ line, and transmitted vertically during wasp reproduction (Strand, Burke 2015). The virus genome also is excised, replicated, and packaged in wasp ovaries, and during parasitization by the wasp of an insect (typically a caterpillar), virus is delivered into the host insect body cavity (hemocoel) (Strand, Burke 2015). PDV infection is associated with numerous pathologies, including prevention of cellular encapsulation (Strand, Burke 2015, Huw Davies, Strand et al. 1987), the primary anti-parasitoid immune response, disruptions of metabolic and developmental processes (Pruijssers, Falabella et al. 2009, Dover, Davies et al. 1988, Shelby, Webb 1997), and reduced antimicrobial immunity (Shelby, Webb 1999, Thoetkiattikul, Beck et al. 2005).

Intriguingly, given these commonalities, PDVs are a polyphyletic lineage: PDVs associated with wasps in the braconid wasp family, known as Bracoviruses, have a distinct origin relative to those associated with the ichneumonid wasps, known as Ichnoviruses (IVs) (Bezier et al. 2009, Volkoff et al. 2010). However, there are few genes shared between the BVs and IVs, and distinct mechanisms of host manipulation and interaction (Lapointe, Tanaka et al. 2007, Webb et al. 2006). Additionally, in general, the expression of BV and IV genes is required for successful parasitism (Cui, Soldevila et al. 2000, Strand, Dover 1991). Thus, there is value in examining both lineages for

mechanisms associated with successful infection pathology, as ultimately, these mechanisms to a large extent likely drive evolution of the associated wasp groups.

The *Campoletis sonorensis* Ichnovirus (CsIV) is the PDV associated with the ichneumonid wasp, *Campoletis sonorensis*. *C. sonorensis* has a broad host range, capable of successfully parasitizing more than two dozen lepidopteran species in the lab (Lingren, Guerra et al. 1970). CsIV infection and expression are essential to parasitoid emergence (Cui et al. 2000), although the viral genetic basis of many pathologies remain incompletely known. Virus gene expression in infected host tissues reduces humoral and cellular immunity, including encapsulation (Shelby, Webb 1999). CsIV encodes several multi-gene families including *cys-motif*, *vankyrins*, *rep*, *N*, and *vinnexins* (Webb et al. 2006). CsIV cys-motif proteins have been implicated in disruption of cellular immunity (X Li, B A Webb 1994, Cui et al. 2000), but functions of the other gene families are less clear.

There are four *vinnexin* loci in the CsIV genome (Webb et al. 2006), and *vinnexins* have been identified in every sequenced IV genome to date (Hasegawa, Turnbull 2014). The *vinnexins* are homologues of insect *innexins*, the latter of which encode the structural components of gap junctions. Gap junctions form between contiguous cells, allowing selective small-molecule exchange between cytoplasms, and facilitating multicellular behaviours (Hasegawa, Turnbull 2014). The CsIV *vinnexins* have been demonstrated to form functional gap junctions that exhibit Vinnexin-specific channel characteristics in paired *Xenopus laevis* oocytes, including coupling reliability and strength, and ability to form gap junctions in combination with a host caterpillar

Innexin protein, Sf-*inx2* (M. W. Turnbull et al. 2005, Marziano et al. 2011). The CsIV Vinnexins also were demonstrated, in a Vinnexin-specific fashion, to induce morphological changes in an insect cell line, while global expression of CsIV *vinnexinG* (*vnxG*), but not the other *vinnexins*, in transgenic *Drosophila melanogaster*, is embryonic lethal (Hasegawa et al. 2017). Thus, results support that the *vinnexin* gene family may encode pathology factors that differentially interact with different hosts, tissues, or physiological systems within hosts, as has been hypothesized for the *cys-moti* and *vankyrin* gene families.

The pathways by which Vinnexins affect host cell physiology remain unknown. Here, to address this, we generated recombinant baculoviruses expressing the CsIV *vinnexin*-genes, *vnxG* and *vnxQ2*. We observed Vinnexin-specific effect on cell viability, cell membrane potentials and intracellular pH relative to controls. By using pharmacological inhibitors, we performed initial tests of possible mechanisms by which Vinnexins may lead to changes in cell physiology. Our findings suggest Vinnexins, in a protein specific fashion, impact host bioelectric physiology, representing a novel mechanism of virus-induced pathophysiology in an insect host.

3. Methods

Cell culture and virus generation

The Sf9 cell line is a clonal isolate from the parental fall armyworm *S. frugiperda* (Noctuidae) cell line, a host to CsIV. We maintained Sf9 cells with Sf-900 Ⅲ Serum Free

Medium, supplemented with 1% Penicillin-Streptomycin. Cultures were passaged as needed and discarded at high passage number.

Recombinant viruses were generated using the Bac-to-Bac vector system (Invitrogen). *VnxG* and *vnxQ2* were PCR amplified from pIZT/*vnxG*-V5-His and pIZT/*vnxQ2*-V5-His (Hasegawa et al. 2017), respectively, using the primer pairs TCTAGAATGTTGCACGCTTTGCGGTC and AAGCTTTCAATGATGATGATGATGATGAGCATCCGAAACACC, TCTAGAATGTTTAACATTCTAAGTTCTTTGCGTGG and AAGCTTTCAATGATGATGATGATGATGaacgtccgcatctgactc (underline= 6x-His epitope). The Melanocortin-4-receptor (Mc4r) was isolated from cDNA from the brain of the sailfin molly, *Poecilia latipinna* (gift of Dr. Margaret Ptacek), using the primers GGATCCatggactacaaagacgatgacgacaagAACTCCACGGCTCAGCAAGGCT and aagcttTCACAGAAAGCTAATACACGAGAGAGAGCCT (underline= FLAG epitope). PCR products were digested and ligated into the pFastBac Dual plasmid and sequenced, then transformed into dH10Bac cells and selected with Kanamycin, Gentamicin, and Tetracycline. Positive colonies were cultured, miniprepped, and subjected to PCR and restriction digest verification. Recombinant bacmid DNA was transfected into High Five cells to generate P1 virus. Viral titers were determined by plaque assay, and all experiments were performed at a multiplicity of infection (MOI) of 10.

Immunoblotting

Infected cells were resuspended in lysis buffer (25mM Tris-HCl, pH 7.6; 150Mm NaCl; 1% NP-40; 0.5% TritonX-100; 0.1% SDS). Bradford assay was used for protein concentration determination. Equal amounts of protein were diluted in 4X loading buffer, incubated for 30 min at 37 ^{o}C and separated on 10% polyacrylamide gels and were transferred to PDVF membrane. To block the blot, 5% fetal bovine serum (FBS) in PBT (PBS + 0.02% Tween-20) was applied. Blots were probed with rabbit anti-His antibody (Thermo Fisher Scientific) at 1:1,000 in blocking solution at 4 ^{o}C overnight, and probed with polyclonal donkey anti-rabbit HRP-antibody (Invitrogen) at 1: 5,000 in blocking solution at room temperature for 1 h. After washing, blots were treated with ECL substrate (Thermo Fisher Scientific), developed, and visualized on X-Ray film.

Immunomicroscopy

Infected cells were washed in PBS between every step, fixed with 4% formaldehyde in PBS at room temperature for 15 min, permeabilized with PBST (PBS + 0.2% TritonX-100) at room temperature for 10 min and blocked with 5% dry milk in PBST at room temperature for 60 min. Primary antibody (mouse anti- GP64 plus rabbit anti-His or rabbit anti-FLAG) (Thermo Fisher Scientific) and secondary antibody (anti-mouse Alexa Fluor 488 plus anti-rabbit Alexa Fluor 594) (Jackson ImmunoResearch) were diluted in blocking solution, respectively, at 1:200 and 1: 1,000. DAPI counter staining was performed to visualize nuclei. Plates were imaged on a Nikon TE2000 epifluorescence microscope with NIS Elements BR 2.3 software.

Cell viability analyses

MTT proliferation assay was conducted in 96 well plate with 104 cells seeded and infected. Three days post-infection (pdi), 20 µl MTT solution (5mg/mL MTT in PBS) was added to each well, and then the plate was incubated for 3.5 hours at 37 °C. Media was then replaced by 150 µl MTT solvent (4mM HCl, 0.1% Nondet P-40 in isopropanol) and the plate was incubated for 15 min on an orbital shaker in the dark. Absorbance was read at 590 nm. Each treatment was done in triplicate.

Cell physiology analyses

Cells at 3 dpi were used for cell physiology analyses. Bis-(1,3-Dibutylbarbituric Acid) Trimethine Oxonol (DiBAC4(3)) was used to measure cell membrane potential. DiBAC4(3) was firstly diluted in cell culture media, and then applied to cells at a final concentration of 1 µg/ml. After incubating 5 min at room temperature in dark, cells were imaged with DS-Qi1Mc camera on Nikon TE2000 epifluorescence microscope using FITC filters. Intensity collections were performed by haphazardly selecting individual cells and manually outlining the cells to generate a region of interest in NIS Elements BR 3.0 software. For each treatment, 180 cells from 6 images were analyzed per replication. In some experiments, control and infected cells were treated with DiBAC4(3) and imaged, then media removed and replaced with media containing 10 µM ouabain or 10

μM carbenoxolone (CBX) with 1 μg/ml DiBAC4(3). Cells were incubated with the inhibitor for 10 min in dark and then imaged as described above. The mean DiBAC4(3) fluorescence intensity for each cell was normalized to background fluorescence and ambient light, by method adapted from published work (Adams and Levin, 2012).

$$Normalized\ Cell\ Intensity = \frac{Cell\ Mean\ Intensity - Darkfield\ Intensity}{Background\ Intensity - Darkfield\ Intensity}$$

Darkfield Intensity image was captured using the same exposure conditions but the excitation shuttered, and Background Intensity represented the mean intensity of 5 randomly selected areas lacking cells from same images that used to measure cells.

A working concentration of 4 μg/ml Carboxyfluorescein diacetate (CFDA) was used in this paper. Incubation, imaging and normalization were performed as above. All experiments were performed in triplicate.

Statistical analyses and figures

All the statistical analyses were performed in R x64 3.4.3. Graphs were generated in DataGraph V4.2.1 (Visual Data Tools Inc.)

4. Results

Recombinant protein expression. Recombinant baculoviruses were generated encoding *vnxG* or *vnxQ2* with 6x-His C-terminus epitope, under regulation of the polyhedrin promoter. A baculovirus encoding sailfin molly fish (*P. latipinna*) *Mc4r*, a G protein-coupled receptor, with N-terminus FLAG epitope, was generated to serve as a recombinant virus control. Sf9 cells were infected with the viruses at MOI =10 and assayed by western blot and immunomicroscopy for protein expression. Anti-His western blot (Fig. 1A) demonstrated a band at ~40 kDa for the Vinnexins, in accordance with previous expression data (Hasegawa et al., 2017; Turnbull et al., 2005); anti-FLAG western blot verified FLAG-Mc4r expression (data not shown). Expression kinetics were visualized by anti-epitope western blot and peak expression observed at 3-4 dpi for vnxG and vnxQ2 (data not shown). Anti-His immunomicroscopy demonstrated extensive membrane localization of VnxG and VnxQ2 (Fig. 1B), as well as more diffuse cytoplasmic presence, similar to results with plasmid based expression (Hasegawa et al., 2017); anti-FLAG immunomicroscopy verified FLAG-Mc4r membrane localization (data not shown). These results indicated a successful NPV infection and vinnexin expression for subsequent analysis. They also support the use of NPV-FLAG-Mc4r as an appropriate virus and transmembrane protein control.

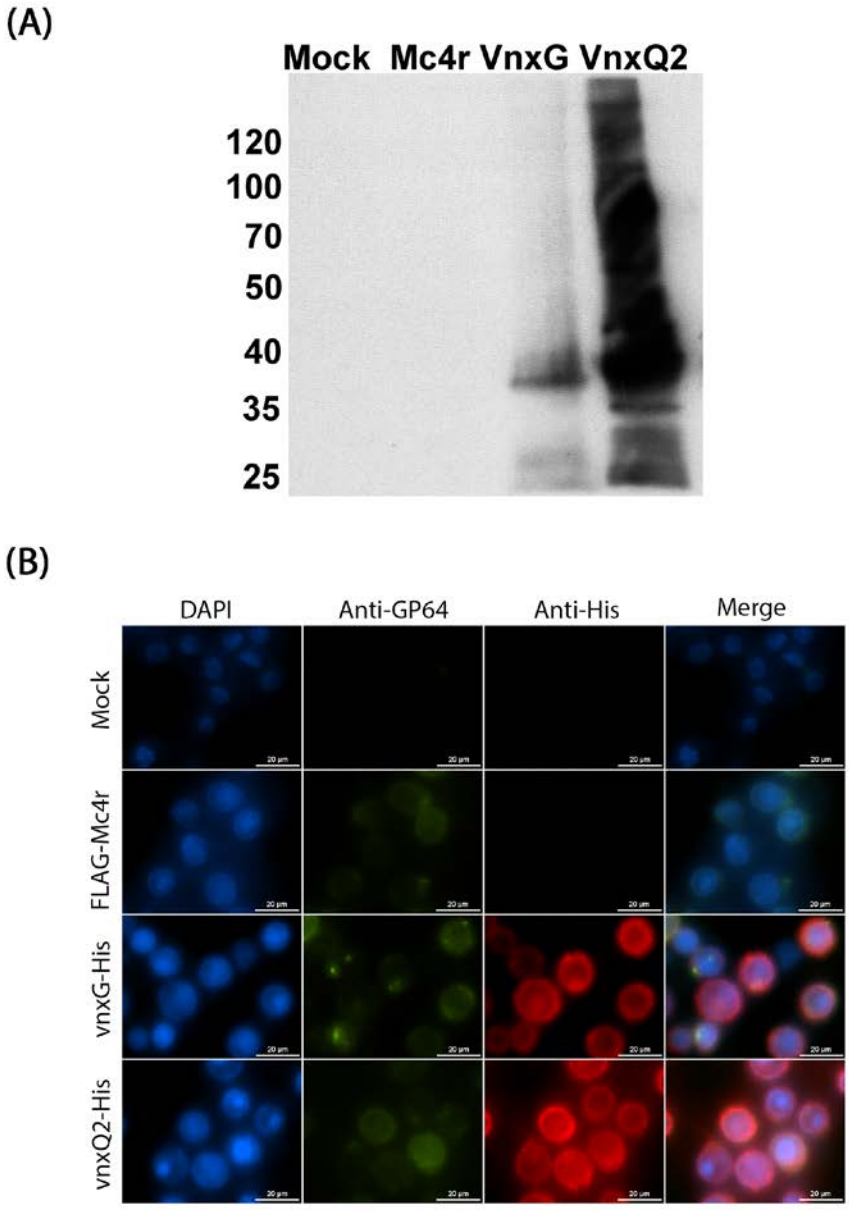

Fig. 1. *Vinnexins* were expressed by recombinant baculoviruses and localized at cell membrane. Sf9 cell were infected with recombinant baculoviruses, FLAG-Mc4r as virus control, *vnxG*-His and *vnxQ2*-His for *vinnexin* expression at MOI= 10 or Mock treatment and were screened 3 dpi. (A) Expression of *vinnexins* were verified with western blot. M: Mock, V: NPV-FLAG-Mc4r, G: *vnxG*-His, Q2: *vnxQ2*-His. (B) Presence of GP64 (anti-GP64) confirmed baculoviruses infection. Localization of VnxG and VnxQ2 was examined by antibody to the C-terminus 6x-His epitope. DAPI was used at a working concentration of 1 µg/ml for nuclear staining. Cells were imaged using identical exposure conditions.

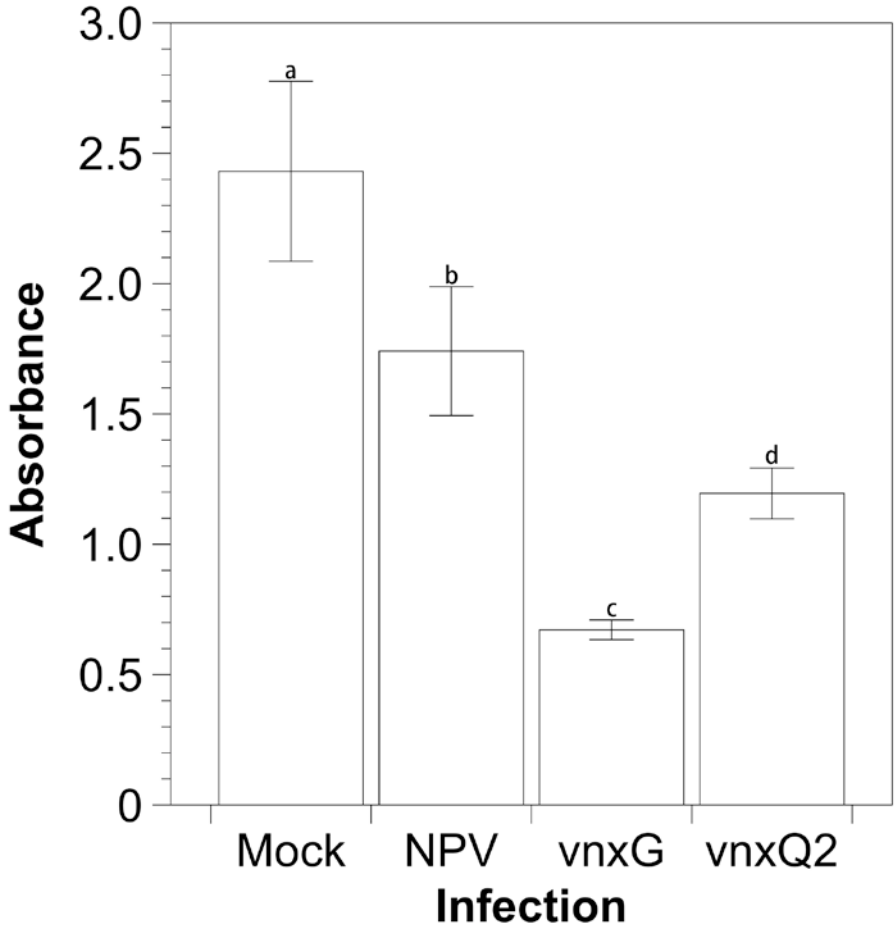

Fig. 2. Expression of *vnxG* and *vnxQ2* results in a decline of cell viability. To measure the change in cell viability caused by expression of *vinnexins*, 10^4 cells were seeded and left untreated (Mock) or infected with control NPV (Mc4r) or *vinnexin* (*vnxG*, *vnxQ2*) recombinant AcMNPV. At 3 dpi, cells were subjected to MTT assay. Bars with different letter indicate significant difference at $p < 0.05$. Error bars show standard deviation.

Vinnexin expression affects cell viability. The effect of infection on the cell viability with the recombinant virus protein expression was measured at 3 dpi by MTT assay (Fig. 2). A significant effect was found by ANOVA (F (3,26) =161.7) and compared by Tukey multiple comparisons. Sf9 cells infected with the FLAG-Mc4r virus showed a significant

decrease of viability compared with uninfected cells (p <0.001), and both *vnxG* (p <0.001) and *vnxQ2* (p <0.001) recombinant virus infection significantly decreased cell numbers compared with FLAG-MC4r. Moreover, *vnxG* expression significantly decreased viability relative to the expression of *vnxQ2* (p <0.001).

Vinnexin expression depolarizes cell membranes. Membrane potential (Vmem) of uninfected, control virus (*Mc4r*), and experimental virus (*vnxG* and *vnxQ2*) infected cells were examined. Cells were incubated with the fluorescent membrane potential sensitive probe DiBAC4(3) (Fig. 3B). A higher number reflected stronger fluorescence intensity, signifying cell depolarization. Vmem was significantly altered by infection (ANOVA, F (3,1146) =383). Relative to uninfected cells, infection with *Mc4r* had no effect on Vmem (p =0.498). Cells infected with the *vnxG* recombinant had significantly higher DiBAC4(3) values than uninfected (p <0.001) or *Mc4r* infected (p <0.001) cells. Similarly, *vnxQ2* infection resulted in significantly more intensely fluorescing cells than uninfected (p <0.001) or FLAG-Mc4r infected (p <0.001) cells. *VnxG* infected cells had significantly greater DiBAC4(3) intensities than *vnxQ2* (p <0.001), as well. Together, our data suggest that vinnexin expression leads to depolarization of cell membranes.

Testing possible channels underlying Vmem changes. Given the observed changes in Vmem, we hypothesized that unapposed Vinnexin-hemichannels might provide a mechanism for ion translocation across the membrane. We tested this using the gap junction and hemichannel inhibitor carbenoxolone (CBX) in combination with

DiBAC4(3) staining. Uninfected and infected cells at 3 dpi were incubated with DiBAC4(3), imaged, and media removed. Media containing 10 µM CBX and 1 µM DiBAC4(3) was added and cells were imaged. DiBAC4(3) intensity increased in all pre/post-CBX comparisons (Fig. 4A). However, the ratios of post-CBX to pre-CBX showed no comparable difference among treatments (Kruskal-Wallis, p =0.123), which indicated no apparent difference in responsiveness to CBX from Vinnexin-expressing cells, and suggested that unapposed Vinnexin hemichannels are not directly responsible for the depolarization.

We replicated this approach with the Na^+/K^+-ATPase pump inhibitor, ouabain. As expected incubation of cells in 10 µM ouabain again led to significantly greater DiBAC4(3) values relative to pre-treatment in pre-/post-ouabain addition comparisons (Fig. 4B). Similar to CBX results, when comparing the ratio of post-ouabain to pre-ouabain, there was no apparent response differential induced by *vinnexin* expression relative to the two controls (Kruskal-Wallis, p =0.554). In both cases, significant change between pre- and post- was observed in each treatment and was verified by t-test (data not shown).

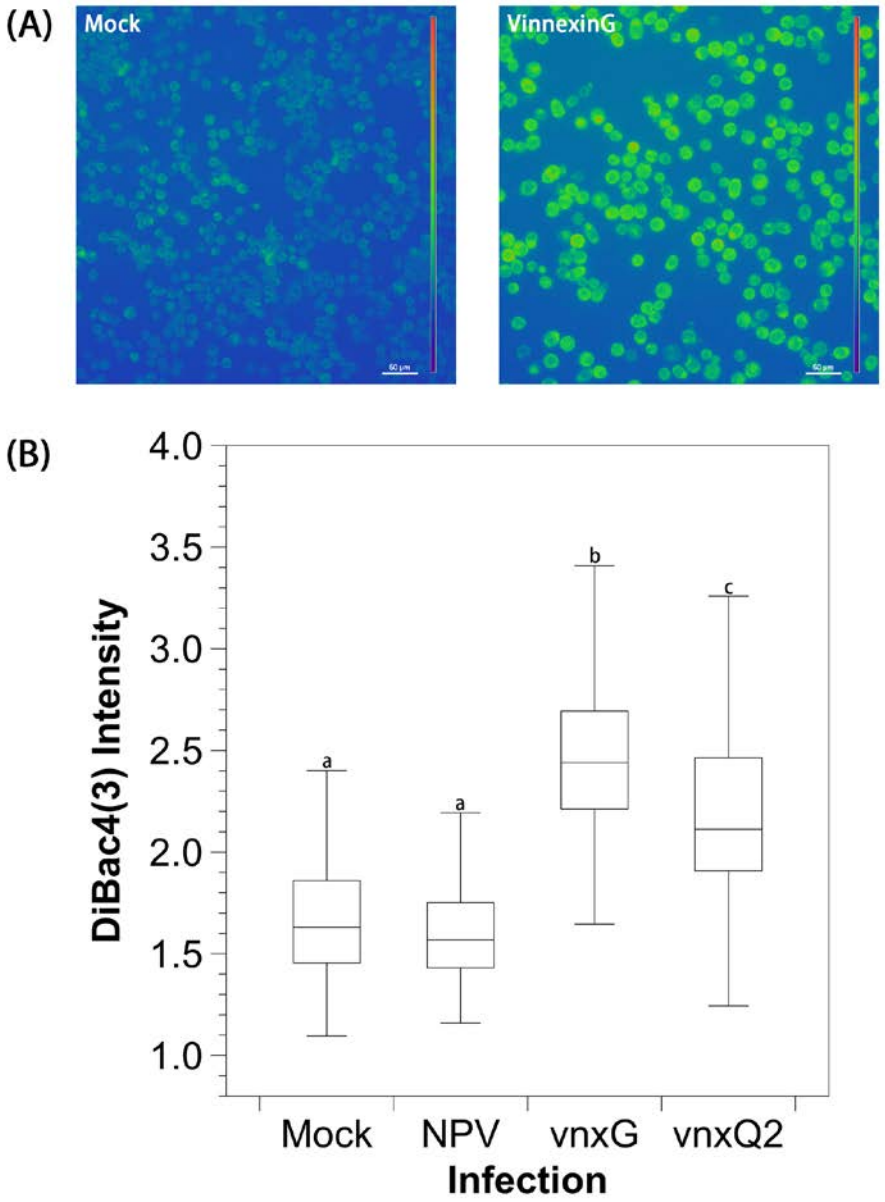

Fig. 3. The expression of *vnxG* and *vnxQ2* causes depolarization of Sf9 cells. Infected and uninfected cells were incubated with media containing 1µg/ml DiBac4(3) at 3 dpi. (A) Representative image of DiBac4(3) stained cells pseudocolored to indicate polarization: blue is hyperpolarized to depolarized red. (B) *Vinnexin* expression (*vnxG*, *vnxQ2*), but not mock treatment and control NPV (Mc4r), induces membrane depolarization. Superscript letters indicate significant difference at $p < 0.05$.

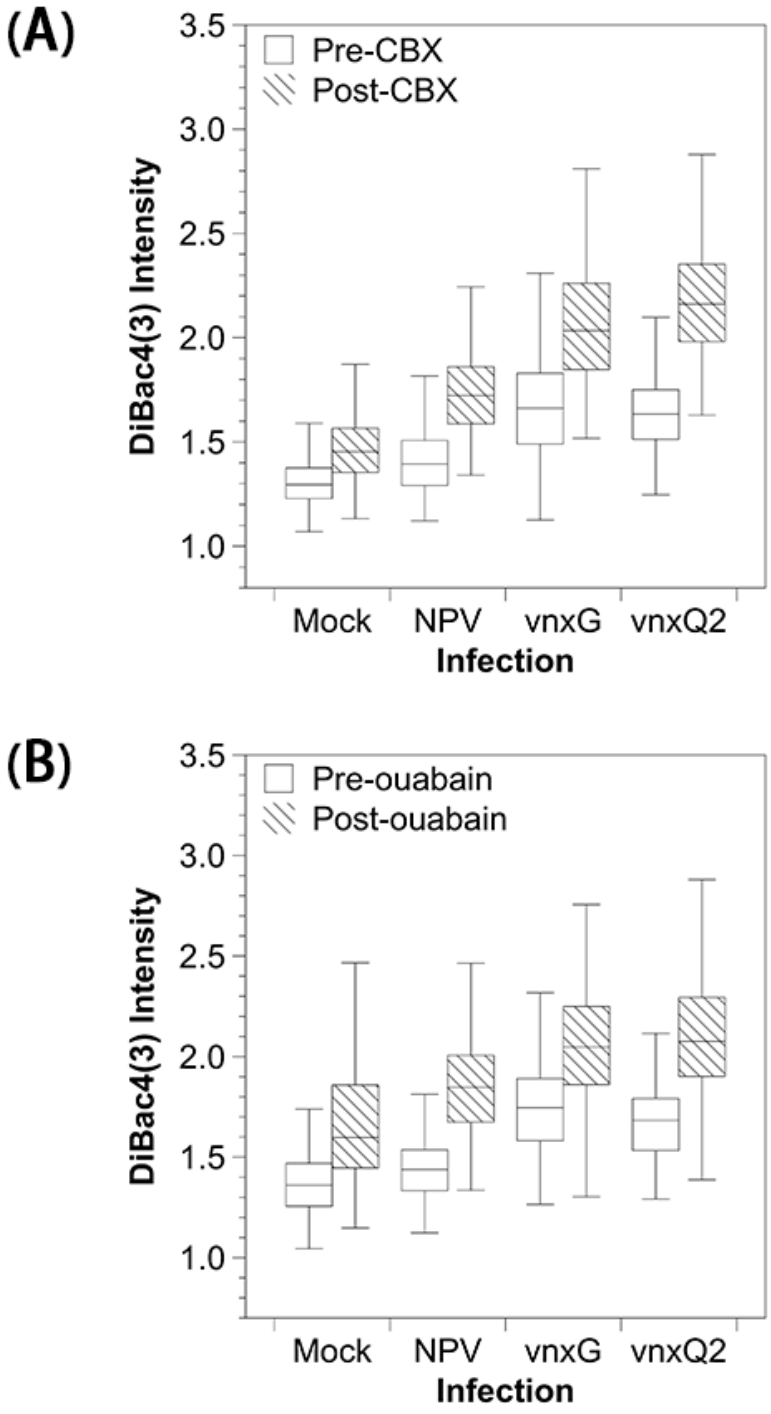

Fig. 4. Inhibiting hemichannels and Na^+/K^+-ATPase does not affect depolarization. Cells were incubated with DiBac4(3) and imaged to measure initial intensity (pre-). Media was replaced by fresh media containing DiBac4(3) and (A) carbenoxolone (10 μM) or ouabain (10 μM).

Vinnexin expression affects intracellular pH. Cells were incubated with 4 µM CFDA and imaged to determine cytoplasmic pH (Fig. 5). Expression of *vnxG* and *vnxQ2* resulted in a significantly greater CFDA intensity, relative to controls (ANOVA, $F(3,1227) = 67.93$, $p < 0.001$). The mock treatment cells showed no significant difference to the Mc4r infected cells ($p = 0.8$), while *vnxG* and *vnxQ2* significantly differed from both mock and NPV virus control ($p < 0.001$) and each other ($p = 0.044$). As increased CFDA intensity indicates higher pH, our results supported that vinnexin expression results in alkalization of cytoplasm.

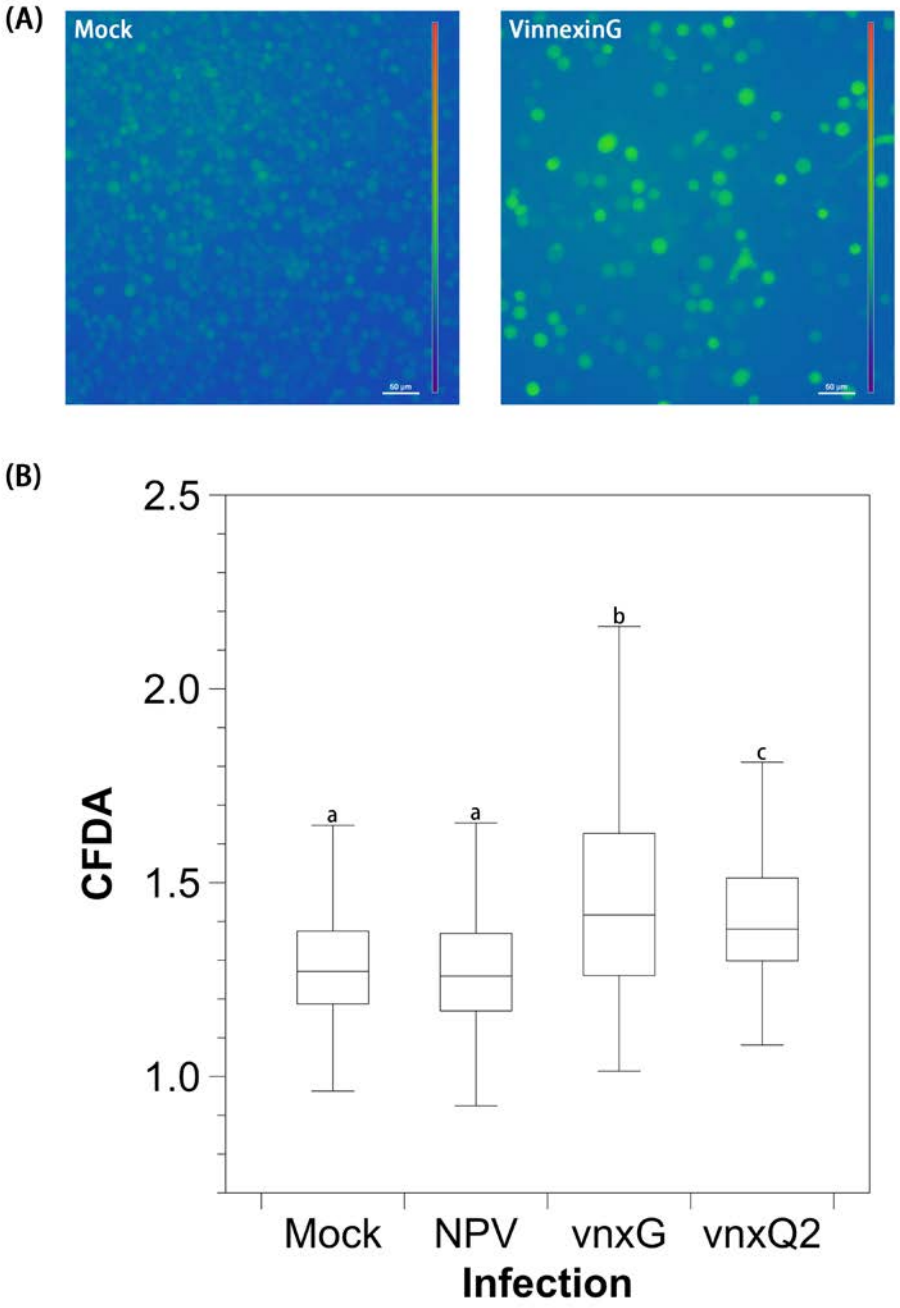

Fig. 5. The expression of *vnxG* and *vnxQ2* alkalinizes Sf9 cells. At 3 dpi, cells in all groups were incubated in media plus 4 μM CFDA for 5 min before imaging to determine the relative internal pH. (A) Representation CFDA stained cell. Alkalized cells had a pseudocolor from yellow to red. (B) *VnxG* and *vnxQ2*, but not mock treatment and control NPV, result in cell alkalization. Superscript letters indicate significant difference at p <0.05.

5. Discussion

Gap junction molecules (Innexins in invertebrates, and Pannexins and Connexins in chordates) form channels at cell membranes. Our previous work has demonstrated Polydnaviruses associated with ichneumonid wasps, the Ichnoviruses, encode multiple Innexin homologues, and that these molecules, known as Vinnexins, form functional gap junctions (Marziano et al. 2011, M. W. Turnbull et al. 2005). Like other gap junction molecules, Vinnexins are predicted to have four transmembrane domains between cytoplasmic N- and C-termini, and two extracellular loops. Six Vinnexin subunits together are predicted to form a hemichannel, and two connected hemichannels contributed by adjacent paired cells compose a functional gap junction. This in turn facilitates transmission of small metabolites from cell-to-cell through gap junction channels, or even between the cytoplasmic and the extracellular environment through unapposed hemichannels (Evans 2015, Luo, Turnbull 2011). The study of Vinnexin function hints this unique family contributes to altering host normal gap junction functions, such as developmental patterning (Richard and Hoch, 2015), bioelectrical patterning (Levin, Pezzulo et al. 2017) and immunity (M. W. Li et al. 2014). However, the mechanisms by which Vinnexins affect cell and ultimately host physiology remain a mystery. Due to the complexity of the IV transcriptome in infected caterpillars (Gill, Webb 2013, Webb et al. 2006), we have opted to develop tools to ectopically express *vinnexins* to examine the impact on cell physiology and begin to address role in host manipulation.

Previous functional studies with Vinnexins likewise have relied on non-native regulators. Weak promoters (e.g., the Baculovirus *Orgyia pseudotsugata* MNPV immediate early promoter) resulted in low levels of expression, possibly resulting in difficulty in observing subtle cellular physiological changes. However, use of stronger regulators, such as the constitutive enhancer Act5C from D. melanogaster, resulted in more observable changes, including embryonic lethality by *vnxG* (Hasegawa et al. 2017). Analysis of naturally parasitized *Heliothis virescens* indicates that all four CsIV *vinnexins* are transcribed at levels significantly greater than the host innexin, hv-*inx2*, although at significantly lower levels than the CsIV *cys-motif* genes (Turnbull et al., unpublished). We therefore reasoned that expression at higher levels under the polyhedrin promoter was more likely to result in reliable and observable phenotypes.

Vinnexin expression using the Baculovirus Expression Vector system (BEV), specifically under regulation of the Polyhedrin promoter, resulted in high levels of protein expression (Fig. 1). Vinnexin protein localization was qualitatively similar to that observed in transfected cells (Hasegawa et al. 2017), and CsIV-infected caterpillars (M. W. Turnbull et al. 2005).

CsIV infection induces alteration in hemocyte population and cell death (Davies, Preston 1987, Davies, Vinson 1988), while prior results with transfected cells suggested that *vinnexin* expression did not alter cell viability (Hasegawa et al. 2017). Our current results, however, indicate that overexpression of *vnxG* and *vnxQ2* does lead to loss of viability (Fig. 2). As infection with a *vinnexin*-encoding virus results in greater viability loss than the recombinant baculovirus encoding the membrane-localizing Mc4r protein,

we suggest that *vinnexin* expression leads to cell death. The basis for this increased loss of viability was not clear, so we tested cell physiological parameters to identify possible mechanisms by which Vinnexins may affect cell viability and function.

Vinnexin expression induced cell membrane depolarization. Cell membrane potential is a consequence of ion movement across membranes, and underlies numerous cellular behaviours (Levin 2014). Disruption of membrane potential can alter cell functionality, including ion exchange, signalling communication, ontogenesis and development (Levin et al. 2017). As a result of alteration in hemocyte membrane potential, hemocyte ability to participate in immune response could be negatively affected, through disruption of adhesion, chemo- and electro- taxis.

Previously, we observed localization of epitope-tagged Vinnexins at the membrane of solitary cells (Hasegawa et al. 2017) suggesting the prospect of an unapposed hemichannel. We reasoned that these hemichannels might provide a mechanism to transfer ions from the cytoplasm to the extracellular environment, and vice-versa, and thus be the mechanism underlying the observed membrane potential alterations. However, while carbenoxolone treatment induced depolarization in both controls and *vinnexin* expressing cells, there was no differential shift observed in *vinnexin* expressing cells relative to controls. Media change during applying inhibitors can explain the increased DiBAC4(3) intensity post-treatment (Lynch and Turnbull, unpublished). This suggests that ectopic hemichannels, formed by Vinnexins, are not providing ion transfer across the membranes to a scale resulting in the observed significant depolarization. Former studies have demonstrated association of insect Innexins with the

Na^+/K^+-ATPase pump (Kruger, Bohrmann 2015, Lautemann, Bohrmann 2016). We therefore postulated that the Vinnexins might colocalize with the pump, leading to either its mis-localization or reduced activity, either of which might result in depolarization. However, our lack of differential results between the controls and *vinnexin* expressing cells with the Na^+/K^+-ATPase inhibitor ouabain data suggest that Vinnexins do not (significantly) affect Na^+/K^+-ATPase activity. This leads us to hypothesize that Vinnexin activity may affect other ion channels, such as Na^+, K^+, or Cl^- channels, or possible ion or proton pumps. We began testing this last hypothesis by observing the cytoplasmic pH of cells with the pH-sensitive fluorescence dye. Our findings with CFDA indicate that *vinnexin* expression results in alkalization of infected cells. Together with our observations on membrane potential, we hypothesize that the Vinnexins may alter proton pump activity, such as an insect Na^+/H^+ exchanger. Such activity would result in membrane depolarization (Na^+ influx) and cytoplasmic alkalization (H^+ efflux). The Na^+/H^+ exchanger has been found and sequenced in *D. melanogaster* (Giannakou, Dow 2001), providing a model to test moving forward.

We hypothesize that Vinnexins may be altering cell physiology by modifying cell bioelectric profiles, thus altering cell functional physiology. The parasitoid wasp *Habrobracon hebetor* induces membrane potential shifts in hemocytes of the lepidopteran host (Kryukova, Chertkova et al. 2015). How bioelectrical patterns link to insect immunity is largely unknown, whereas in vertebrates, it has been demonstrated that both embryonic frog (Pare, Martyniuk et al. 2017) and mammalian (C. Li, Levin et al. 2016) macrophages utilize bioelectric signalling to respond to wounding and/or infection.

Thus, Vinnexins may modify hemocyte bioelectric reception and/or response pathways, altering hemocyte immune capability. This is currently under investigation by our lab.

Manipulation of host cell bioelectric patterns by viruses has been previously described. Numerous viruses encode ion channels, rendering an electrochemically conducive environment to replication. For example, *Paramecium bursaria* chlorella virus 1 encodes small potassium ion channels, which is required for maintaining current kinetic (Kang, Moroni et al. 2004). Additionally, a large number of virus families have representatives that encode viroporins, molecules that are essential to facilitating virus release from infected cells by forming hydrophilic pores (Nieva, Madan et al. 2012). However, Vinnexins are not expressed in the wasp host (Turnbull et al., 2005) and Polydnaviruses, including the Ichnovirus CsIV, do not replicate in the infected caterpillar host (Bitra, Burke et al. 2016, Cui et al. 2000). Thus, bioelectric modification of infected cells is likely to play a role in other host pathways, with the most probable being immune dysfunction given that this is a primary effect of IV infection (Shelby, Webb 1999). This, in light of the above considerations on the role of bioelectric pathways in vertebrate immunity, suggests the Vinnexins may be affecting a largely underappreciated regulatory aspect of insect immunity, for example, bioelectric patterning, enhancing virus, and by extension, wasp host fitness.

In conclusion, we have expressed virus gap junction molecule homologues (Vinnexins) in a cell line derived from a virus host. These molecules induce significant alterations in cell membrane potential and cytoplasmic pH, both of which are components of the cell's bioelectric profile. We hypothesize that this alteration in cellular bioelectric

profile may alter the capacity of the cell to respond to the parasitoid wasp (and other immune challenges), which would represent a novel modality by which viruses may manipulate host immune physiology.

CHAPTER THREE

IN VIVO VINNEXIN EXPRESSION IN *HELIOTHIS VIRESCENS* LARVAE

1. Abstract

The expression of Polydnavirus (PDV) genes in parasitized host caterpillars is necessary for certain parasitoid wasp egg's development. PDV infected caterpillars show developmental stunting and a compromised immune response. The mechanisms by which PDVs alter host physiology and determine host suitability are complex and unclear. Evidence hints that the IV protein family, Vinnexins, may negatively affect host immune system. Here, we use recombinant baculoviruses to express *vinnexins* in newly molted 4th instar *Heliothis virescens* and investigate their function and influence on hemocyte count, host development and host mortality. We found that expression of *vinnexins* does not alter total hemocyte number, but induces significantly higher mortality and causes significant reduction in molting from 4th instar to 5th instar. Our findings suggest Vinnexins can enhance baculovirus virulence and cause physiological alteration to host caterpillar.

2. Introduction

Together with wasp's egg, the Polydnavirus (PDV) is delivered into secondary host during parasitization. The PDV subfamily Ichnovirus are reported from more than

20 species of ichneumonid wasps (M. Turnbull, Webb 2002). When females lay their eggs into host larvae, the IV is injected into the insect body cavity (hemocoel) and transported through the open circulatory system and tracheal system. Tissues exposed to insect blood, including muscle, fat body, Malpighian tubules and hemocytes, are found infected by IV after initial infection (Stoltz, Vinson 1979).

IV gene expression causes multiple pathogenic alterations to host immunity and development, such as a disruption of hemocyte adhesion (M. Turnbull, Webb 2002), alterations of cytoskeleton (M. W. Turnbull, Martin et al. 2004), disruption of cell signaling (Gueguen, Kalamarz et al. 2013) and compromised immune response (Shelby, Webb 1999, Glatz, Asgari et al. 2004). Studying IV pathology and mechanism is difficult because multiple gene families contribute to host physiological disruption, so it is necessary to investigate individual gene family function to obtain a specific view about IV gene function and viral protein interaction. In the past, IV protein functional analyses have mostly focused on the *cys-motif* family, especially its role in suppressing immune system and manipulating host transcript pattern (Gill, Webb 2013, Kim 2005). The *vinnexins*, virus homologues of insect gap junction gene, are highly conserved among IVs and have unclear functions. Previous studies about Vinnexins suggest their roles in disruption of host functional gap junction (such as interactions between hemocytes) and hint they have protein-specific functions (Marziano, Hasegawa et al. 2011).

To have a better understanding of the effects of Vinnexins on larval lepidopterans, we used recombinant baculovirus as the vector to express *vnxG* or *vnxQ2* in *H. virescens* larvae. Our results demonstrated using rec-NPV is a proper and efficient way to study

single IV gene family function, allowing discrimination of protein-specific pathology and providing reference for further studies on molecular mechanism of viral protein.

3. Methods

Insect rearing and virus injection

Third instar *H. virescens* caterpillars were purchased (Benzon Research, Carlisle, PA) and reared at 27°C. Newly molted 4th instar larvae, staged according to head capsule width (Strand et al., 1988), were used for recombinant virus injection, and were maintained at 27°C after treatment for data collection. Recombinant viruses were generated and titered as above.

Immunoblotting

To verify the expression of *vinnexins in vivo*, three 4th instar larvae per treatment, were immobilized on ice and injected with 5 µL mock treatment (Hink's TNM-FH Insect Medium), or 100 pfu recombinant virus (*Mc4r*, *vnxG* and *vnxQ2* in same volume of Hink's TNM-FH Insect Medium) containing 0.1% food dye by a Hamilton #701 needle. Three days post injection (dpi), treated caterpillars were immobilized on ice and bled into cold anti-coagulant buffer (0.098M NaOH, 0.186M NaCl, 0.017M EDTA, 0.041M Citric acid, pH 4.5). Samples were centrifuged for 5 min at 500 x g, room temperature. The

supernatant was discarded and the pellets were gently rinsed with cold PBS (pH 7.0) and resuspended in lysis buffer (25mM Tris-HCl, pH 7.6; 150Mm NaCl; 1% NP-40; 0.5% TritonX-100; 0.1% SDS). Equal volume of total protein sample was diluted in 4X reducing loading buffer (200mM Tris-HCl, pH 6.8; 40% glycerol (100%); 0.4% bromophenol blue; 8% SDS; 10% 2-mercaptoethanol), incubated for 30 min at 37ºC and separated on 10% polyacrylamide gels (Bio-Rad, Mini-PROTEAN® TGX™ Precast Gels). Proteins were transferred to PVDF membrane and blocked with 5% fetal bovine serum (FBS) in PBST (PBS + 0.02% Tween-20). Antibodies used were diluted in blocking buffer and were: rabbit anti-His antibody (Thermo Fisher Scientific) at 1:1,000 (40C, overnight), and polyclonal donkey anti-rabbit HRP-antibody (Invitrogen) at 1: 5,000 (room temperature, 1 hour). After thoroughly washing, the blot was visualized with ECL substrate and developed.

Hemocyte Count Determination

To test the effect of Vinnexins on hemocyte number, three Mock treated or infected caterpillars at 3 dpi were immobilized on ice and bled into cold PBS. Hemocytes were centrifuged for 5 min at 500 x *g*, room temperature, resuspended with PBS and cell counts were performed with hemocytometer. Each treatment was done in triplicate.

Caterpillar Survival Rate and Development Measurement

Thirty caterpillars of each treatment were checked daily for mortality for 10 days. Caterpillars were recorded as live, dead or pupated. Dead caterpillars were removed from diet tray after counting to avoid contamination. Death during the first three days was considered as caused by physical damage and was removed from analyses while pupated caterpillars after day 10 were considered as alive. The instar of each caterpillar was recorded at 3 dpi and the proportion of 4th instar caterpillar was calculated. Each treatment was replicated 3 times.

4. Results

Recombinant protein *in vivo* expression. Newly molted 4th instar *H. virescens* were injected with 100 pfu of recombinant viruses encoding *Mc4r*, *vnxG* or *vnxQ2*, or the same volume of media. Anti-His western blot verified vinnexin in vivo expression in hemocytes (Fig. 6A). These results demonstrated a successful virus injection, hemocyte infection and target protein expression. Anti-His antibody was also used in immunomicroscopy (Fig. 6B). *Vinnexin* expression was detected and the protein appeared to localize to the cell plasma membrane.

Vinnexin expression has no effect on total hemocyte counts (THC). Hemocytes were collected from three individuals and pooled, centrifuged, resuspended and counted. Values from 3 replicates were compared. There was no significant reduction in THC found in virus injected groups (Mc4r, VnxG and VnxQ2) relative to mock treated group

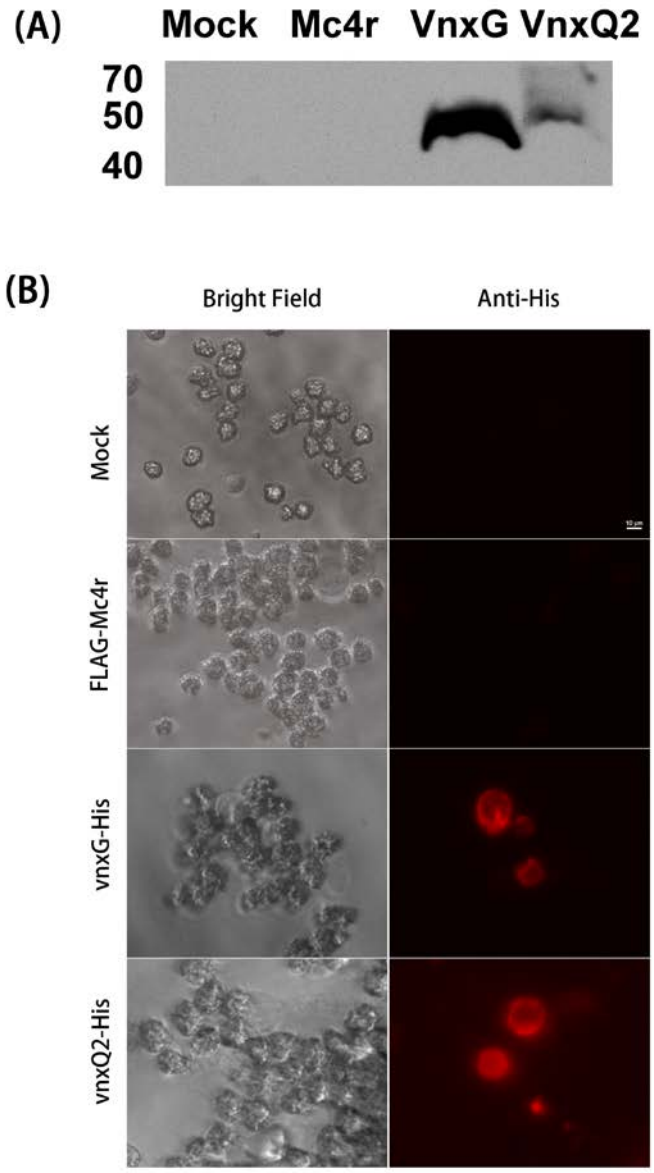

Fig. 6. *Vinnexins* are expressed by recombinant baculoviruses in *H. virescens* larval hemocytes. Newly molted 4th instar caterpillars injected with 100 pfu recombinant baculoviruses or media and were bled for hemocyte sample at 3 dpi. (A) Expression of *vinnexins* was verified with western blot. M: Mock, V: NPV-FLAG-Mc4r, G: *vnxG*-His, Q2: *vnxQ2*-His. (B) Anti-His antibody was used to verify *vinnexins* expression.

(ANOVA, F (3,8) =0.207, p =0.889). The expression of a vinnexin did not show effects on THC compared with control virus (p >0.9), and VnxG and VnxQ2 did not differ (p >0.99) (Fig. 7).

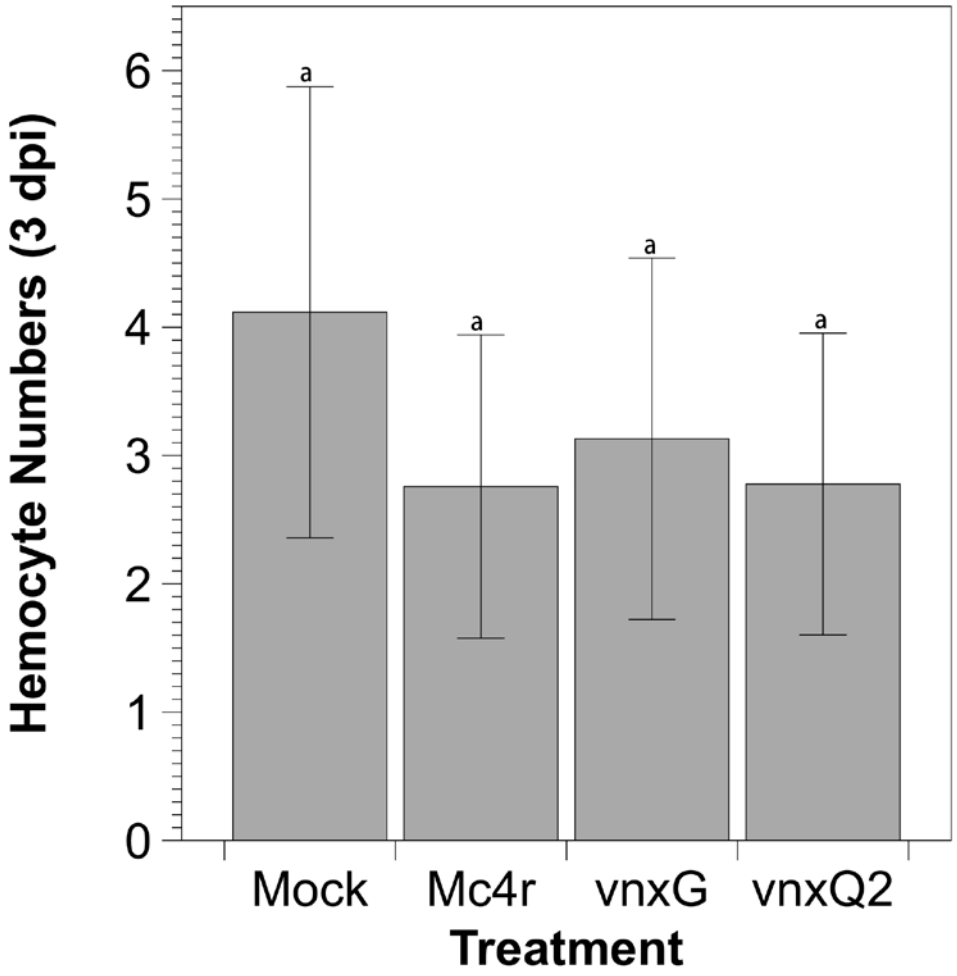

Fig. 7. *Vinnexin* expression does not affect total hemocyte count of *H. virescens* larvae. Hemocytes were collected at 3 dpi from 3 caterpillars and were counted with hemocytometer. The unit of cell count is ten million per milliliter. Bars with different letter indicate significant difference at p <0.05. Error bars show standard deviation.

Infection with vinnexin recombinant baculoviruses increases caterpillar mortality.

H. virescens is a permissive host to wild type AcMNPV, the baculovirus we used for

recombinant virus generation. Mortality due to the virus and vinnexin expression was examined and compared to uninfected control in a 10-day observation (ANOVA, F (3,8) =32.78, p <0.01). Vinnexin expression was significantly associated with a higher mortality (p <0.01). In addition, the VnxG did not differ from VnxQ2 (p >0.9).

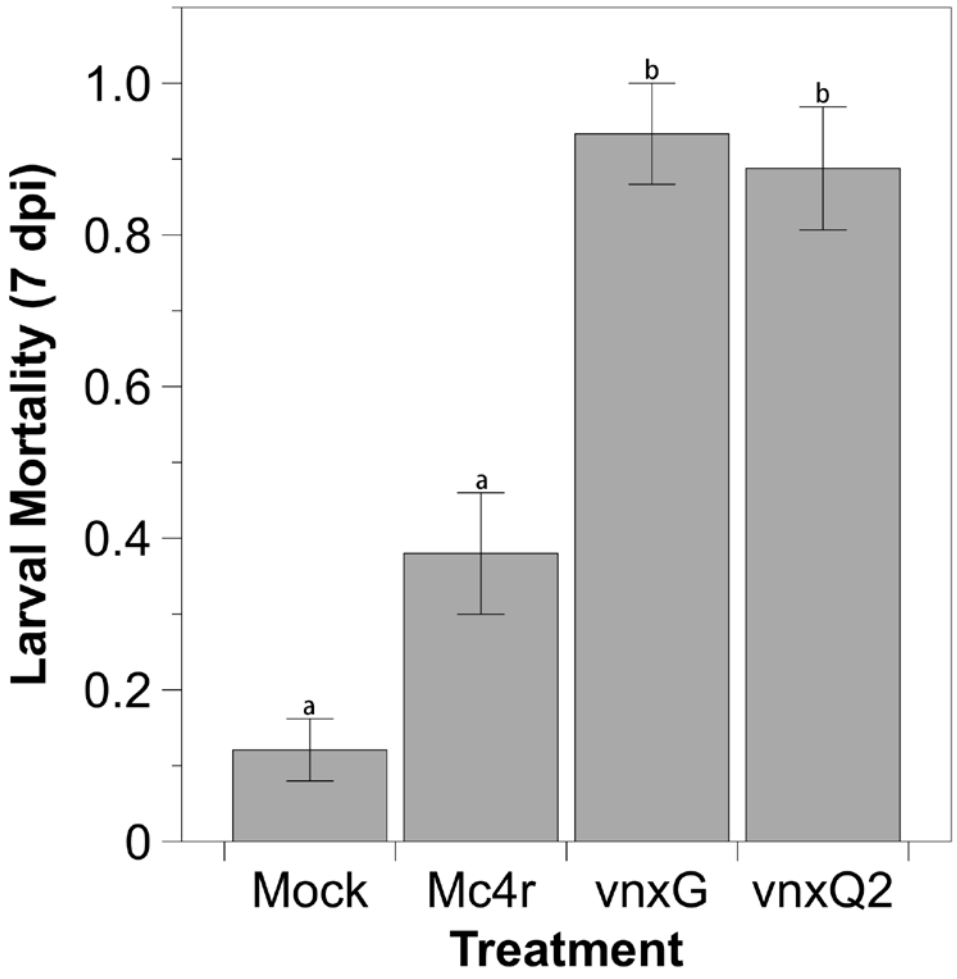

Fig. 8. *Vinnexin* expression leads to a higher mortality during NPV infection. Young 4th instar larvae were injected with 100 pfu recombinant viruses or media and maintained at room temperature. Infected caterpillars were inspected daily and mortality was recorded for ten days. The caterpillar mortality for each treatment was compared. Superscript letters indicate significant difference at p<0.05. Error bars show standard deviation.

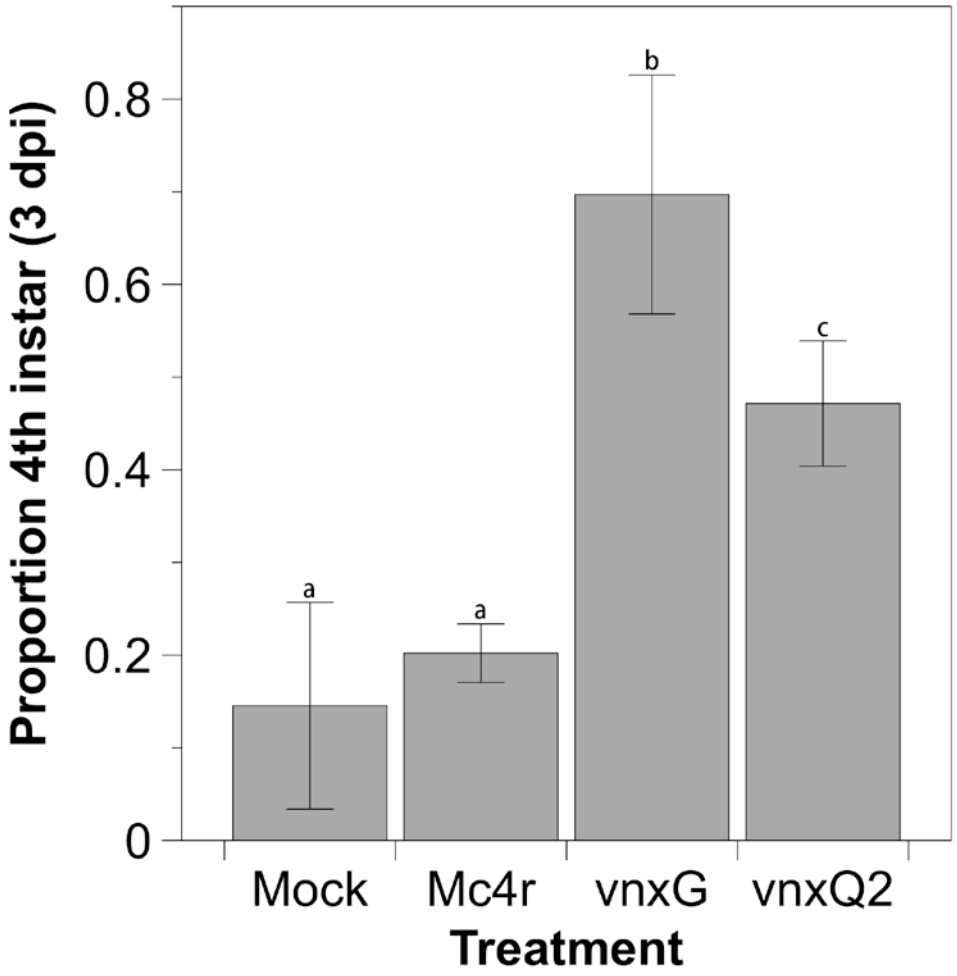

Fig. 9. *Vinnexin* expression delays caterpillar development. Mock and NPV infected caterpillars were maintained and observed for 3 dpi. The developmental stage for individual caterpillar was recorded daily as 4th instar or 5th instar. The proportion of 4th instar was calculated and compared. Superscript letters indicate significant difference at $p < 0.05$. Error bars show standard deviation.

Infection with vinnexin recombinant baculoviruses induces developmental stunting of host caterpillar. Caterpillars were examined at 3 dpi and the percentage of 4th instar for each treatment was compared with Mock treatment (χ^2 (3, N =4) =58.21, $p < 0.01$). The control virus showed no effect (p =0.2446), but a significant increase in the

proportion of 4th instar in VnxG (p <0.01) and VnxQ2 (p <0.01) groups was found when compared with Mock treatment. Additional, there was a significant difference between VnxG and VnxQ2 (p =0.0229).

5. Discussion

We hypothesize that Vinnexins may play a role of disrupting host cell-cell communication and increasing virus pathology. To test protein specific role *in vivo*, a series of recombinant baculoviruses, encoding *vinnexins* or *Mc4r*, were generated, verified and used for injection in newly molted 4th *H. virescens* caterpillar, which is fully permissive to the NPV vector, AcMNPV. As virus injection can cause physical damage, we firstly optimized injection system to minimize unpredicted mortality below 10%.

During initial stage of hemocoel infection, wild type and recombinant NPVs infect hemocytes (Granados, Lawler 1981) and tracheal system and induce systematic infection (Engelhard, Kam-Morgan et al. 1994). Hemocytes serve as immune cell of caterpillar, they mediate cellular defense response, including phagocytosis and encapsulation (Lavine and Strand, 2002). However, NPV-infected caterpillars show an impaired immune response (Andersons, Gunne et al. 1990). In addition, the phenoloxidase activity, part of the humoral immune response, is suppressed by NPV (Trudeau, Washburn et al. 2001). Previous work showed co-infection by AcMNPV and PDV causes rapid death to caterpillar (Gill, Fath‐Goodin et al. 2006) as PDV increased virulence of AcMNPV (Washburn, Haas-Stapleton et al. 2000). In our experiment, we

opted to use rec-baculovirus to express and study individual Vinnexin. Increased mortality suggested Vinnexins enhance NPV virulence. One possible mechanism to be verified is that Vinnexins co-disrupt hemocyte functions, like communication, recruitment and anti-viral defense, and alter cell membrane susceptibility to NPV, rendering systemic infection. These, considering CsIV infection pathologies, suggest Vinnexin may affect hemocyte functions.

Ichnovirus infection disrupts both cellular and humoral immune response. Upregulated cell death (Lapointe, Wilson et al. 2005, Djoumad, Dallaire et al. 2013, Zhang, Wang 2003), hemocyte inactivation (Glatz et al. 2004), cytoskeleton degradation (X Li, B A Webb 1994), encapsulation disruption (Glatz et al. 2004) and melanization inhibition (Shelby, Webb 1999) are reported. In part, IV-induced cell mortality is conducted by the inhibition of translation initiation factors (Kim 2005) or by inhibition of hemocytic RNA translation because of *cys-motif* protein (Kim, Webb 2003). However, our hemocyte count data supported Vinnexins do not cause cell lose that is induced by CsIV.

Ichnoviruses also cause host caterpillar developmental arrest, failure to grow, molt and pupate (Shelby, Webb 1997). In our tested Vinnexin groups, two times more caterpillars remained at 4th instar at 3 dpi and those delayed caterpillars died eventually. It suggests expression of *vinnexins* may affect caterpillar growth rate. Generally, two major hormones that responsible for regulating growth, molting and metamorphosis are ecdysteroid and JH (Staal 1975). In nonparasitized caterpillars, the levels of JH drop from high amount to trace amount through larval development while ecdysteroid also changes

through ontogeny. However, studies demonstrated those hormones can be altered by IV during parasitization (Cusson, Laforge et al. 2000). For example, in parasitized caterpillars, the JH was altered to maintain at a high level to arrest metamorphosis (Shelby, Webb 1997) or the 20-hydroxyecdysone titer was depressed (Dover, Davies et al. 1987), in both cases molting and pupation are compromised -it allows a longer time for wasp development inside the host (Lawrence and Lanzrein, 1993). One hypothesis is that the reduction of molting rate is associated with an unnormal distribution/transportation of hormone via Vinnexin affected gap junctions, forcing caterpillars to remain as larvae, but we do not have data to support this hypothesis so far.

In summary, we successfully used recombinant baculovirus to express detectable Vinnexins in *H. virescens* caterpillars, which enables follow up *in vivo* work. We tested the effects of Vinnexins on host caterpillar physiology in several ways, seeing a significant increase in mortality and significant developmental delay, but no significant change in hemocyte counts. The particular role that Vinnexins play to infected hemocytes is under investigation in our lab, like alteration of immune response. Our preliminary results about Vinnexin pathology provide some support that they enhance the virulence of IVs, and we hypothesize it is by disrupting hemocyte behavior and facilitated virus systemic infection.

CHAPTER FOUR

CONCLUSION

Symbiotic Polydnaviruses are essential for some parasitoid wasps for successful parasitization. During wasp evolution, all the PDV genes including *vinnexin* have been integrated in the wasp genome. Virus gene expression in the parasitized host is obligatory for a wasp egg to develop, hatch and repeat the life cycle. The mechanisms of wasp host determination and host manipulation are highly associated with PDV evolution, a link that is poorly understood. Although the genome of some PDV species has been sequenced and identified (Choi, Roh et al. 2005, Chen, Gao et al. 2011, Desjardins, Gundersen-Rindal et al. 2008, Djoumad, Stoltz et al. 2013, Espagne, Dupuy et al. 2004, Jancek, Bezier et al. 2013, Tristan Dorémus, François Cousserans et al. 2014), the virus pathology and single gene family function are still largely unrevealed. One protein family, the Vinnexins, is found highly conserved in Ichnoviruses. Their functions are predicted to relate to gap junction as they are homologues of insect gap junction protein (Innexin). One example of IV vinnexins, CsIV vinnexins gene family, has four members to date: *vnxD, vnxG, vnxQ1* and *vnxQ2*. All of them are predicted to be transmembrane protein with four transmembrane regions, but they appear to have protein-specific roles and functions based on previous studies (Hasegawa, Erickson et al. 2017, M. W. Turnbull, Volkoff et al. 2005).

To narrow down the target and investigate functions of individual Vinnexin, we opted to choose Baculovirus Expression System as a tool to express vinnexins ectopically in hosts. When we expressed vinnexins in Sf9 cells, there was a reduction of cell

proliferation rate (Fig. 2), which is not observed with infected *H. virescens* hemocyte (Fig. 8). Vinnexins also affected several Sf9 cell physiologies, including membrane potential (inducing depolarization) (Fig.3) and internal pH (inducing alkalization) (Fig 5). Our results suggested that gap junction hemichannels and Na^+/K^+-ATPase are not directly responsible for ion flow (Fig 4), but imply H^+ relevant pump, like H^+ exchanger and H^+/K^+ pump, may be the key component by which Vinnexins alter host cell physiology. How vinnexins affect caterpillar hemocyte physiology and function are under study now. Our preliminary data suggests that vinnexin-`expressing hemocytes also have depolarized membrane (data not shown).

The effect of Vinnexins on organism physiology was tested in *H. virescens* caterpillars. *Vinnexin* expressing caterpillars had an increased mortality which hints Vinnexins can enhance AcMNPV virulence (Fig. 8). This possibility could be tested by expressing *vinnexins in viv*o with other vectors and determining host mortality due to Vinnexins. The effect of *vinnexin* expression on infected hemocyte is complex but may have a negative impact on host immune system and facilitate systemic infection by the NPV. Wild type CsIV-infected caterpillars show developmental delay (Fath-Goodin, Gill et al. 2006) while a significant developmental delay was found in our observation (Fig 9), which hints Vinnexins could play a role of regulating host feeding, digestion, energy balance, growth and molting.

Recombinant baculoviruses are commonly used for heterologous gene expression. Using rec-NPV to study Vinnexins has several advantages over other expression vector systems: 1) High levels of recombinant gene expression regulated by several strong

promoters; 2) The recombinant protein expressed at the predicted location in the cell; 3) The protein can be studied individually (Vinnexins or Mc4r); 4) Suitable for not only cell culture but also many species of larvae. However, *vinnexins* and *Mc4r* are regulated by the polyhedrin promoter, so there is a chance of overexpression, and the overexpressed protein may cause artificially significant difference relative to wild type protein. We are unware of any study that demonstrates the polyhedrin promoter may introduce significant variation. Overexpressed gap junction proteins may result in increased formation of functional gap junctions but not significantly change protein function (Suzuki, Brand et al. 2001). Use of Mc4r control virus helps us address the suitability for using NPV vector. In future work, there are some options to improve our baculovirus expression system, like introducing a natural *vinnexin* promoter to more accurately affect *vinnexin* expression, to reduce overexpression artifacts (Soldevila, Webb 1996).

This project focuses on the function of Vinnexins and compares their influences on insect cells and host caterpillar. Our findings provide reference for further research about the mechanism of Vinnexins and PDV, and have potential application for modifying and using NPVs to control lepidopteran pests.

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