New Insights and Treatments For VZV-induced Pain In a Rat Model of Postherpetic Neuralgia

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Varicella Zoster Virus is the causative agent of varicella (chickenpox) upon primary infection and zoster (shingles) upon reactivation from latency. Viral reactivation causes neurological complications, with pain being the most common. Almost all zoster patients are prescribed medication to alleviate pain and up to one third will develop a chronic pain state that lasts longer than 30-90 days known as Post-Herpetic Neuralgia (PHN). A majority of these PHN patients describe pain in one of three ways: a constant burning, intermittent stabbing or shooting, and lastly allodynia (pain from normally innocuous stimuli), which is the most common and debilitating. A rat model of VZV-induced pain was established in 1999 which mimics the pain seen in humans. The goal of this thesis is to utilize the rat model of VZV-induced pain to characterize the viral effects on the host, investigate mechanisms that result in chronic hypersensitivity, and to evaluate novel treatment strategies. We have discovered that viral infection of rat tissue ex vivo is limited, viral DNA persists at low levels early in infection, and viral RNA transcription is limited to immediate early and early expressed genes. We investigated host changes upon VZV infection which include a decrease in peripheral neurite innervation density and changes in whole dorsal root ganglia transcription with up-regulation of 86 genes and down-regulation of 114 host genes. We show that ORF47, a viral protein kinase, is both necessary and sufficient for the induction of hypersensitivity in rats, and this effect may be related to its phosphorylation of ORF9, an essential tegument associated protein. Interestingly, ORF47 may not be the only VZV protein involved in the induction of hypersensitivity, as
animals transduced with an HSV vector expressing IE62 also develop hypersensitivity to mechanical stimuli, suggesting a role for IE62 in VZV-induced pain. Lastly, we tested novel herpes simplex vectors that express pain relieving genes for their efficacy on VZV-induced pain. Vectors delivering human preproenkephalin, an endogenous opioid peptide, and soluble TNFα receptor alleviate hypersensitivity in our model. The rat model has enabled us to better understand and treat VZV-induced pain.
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LIST OF ABBREVIATIONS

ANOVA: Analysis of Variance
BAC: Bacterial artificial chromosome
cDNA: Complimentary DNA
CALCA: gene that encodes calcitonin gene relate product
CPE: Cytopathic Effect
DAMGO: [D-Ala\textsuperscript{2}, N-MePhe\textsuperscript{4}, Gly-ol]-enkephalin
DNA: Deoxyribonucleic Acid
dpi: Days Post Infection
DRG: Dorsal Root Ganglia
FBS: Fetal Bovine Serum
fLuc: Firefly luciferase
GFP: Green Fluorescent Protein
gp120: glycoprotein 120
HIGS: Heat Inactivated Goat Serum
HIV: Human Immunodeficiency Virus
hpi: Hours Post Infection
HSV: Herpes Simplex Virus
Kan\textsuperscript{r}: Kanamycin Resistance cassette
ICP: Infected Cell Protein
IE: Immediate Early
IHC: Immunohistochemistry
IL1β: Interleukin 1 β
IL7: Interleukin 7
MA: Mechanical Allodynia
NF200: Neurofilament 200
NMDA: N-Methyl-D-aspartic acid
NO: Nitric Oxide
NOS: Nitric Oxide Synthase
NPY: Neuropeptide Y
Ntrk2: Neurotrophic Tyrosine Kinase, Receptor, Type 2
ORF: Open Reading Frame
PBS: Phosphate Buffered Saline
PFA: Paraformaldehyde
PFU: Plaque Forming Units
PGP9.5: Protein Gene Product 9.5
PHN: Postherpetic Neuralgia
PML: Promyelocytic Leukemia Protein
pOka: Parent Oka strain
PRV: Pseudorabies virus
rLuc: Renilla Luciferase
RNA: Ribonucleic Acid
RTx: Resiniferatoxin

SCI: Spinal Cord Injury

SCID-hu: Severe Compromise Immunodeficiency in mice implanted with human tissue

SEM: Standard Error of the Mean

SNL: Spinal Nerve Ligation

sTNFR: Soluble TNF receptor

TH: Thermal Hyperalgesia

TNF: Tumor Necrosis Factor

Tnfrsf21: Tumor necrosis factor receptor superfamily member 21

TRADD: TNF Receptor Associated Death Domain

TrkB: Tyrosine Receptor Kinase B

TRPV1: Transient Receptor Potential Voltage cation channel subfamily V member 1

UL: Unique Long

US: Unique Short

UV: Ultraviolet

vOka: Vaccine Oka strain

VZV: Varicella Zoster Virus

wpi: Weeks Post Infection
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1.0 INTRODUCTION

Varicella zoster virus (VZV), an alphaherpesvirus, is a ubiquitous human pathogen that causes severe neurological complications upon its reactivation from a state of neuronal latency. Pain is the most commonly described complication from VZV reactivation. A subset of patients experience a chronic neuropathic pain state known as postherpetic neuralgia (PHN). PHN is often debilitating and can last for years after the rash resolves. Current treatments leave many of these chronic pain patients without adequate relief and often with unwanted side effects. The goal of this thesis project was to use a rat model of VZV-induced pain to investigate the underlying viral and host mechanisms contributing to pain and to evaluate novel treatment strategies.

1.1 CLINICAL CHARACTERISTICS AND DISEASE

1.1.1 Primary Infection and Clinical Presentation

Varicella zoster virus (VZV) causes two human diseases; varicella upon initial infection and herpes zoster upon viral reactivation from latency established in neurons of sensory and autonomic ganglia. Most varicella cases are reported in childhood, but may also occur in adults with often devastating consequences\(^1\). Varicella is almost always associated with a red,
vesicular, pruritic rash that covers most of the body.Prodromal symptoms associated with
varicella include fever, malaise, headache, and abdominal pain. The incubation period between
initial infection and eruption of a rash can last 10 to 21 days. VZV is spread predominantly by
aerosolized viral particles that are inhaled, but contact with infectious fluids and rash can also
spread the virus. Once inhaled, viral particles infect upper respiratory tract epithelial cells
around the Waldeyer’s ring, which include the adenoids and tonsils. The virus then infects
immune cells including various antigen-presenting cells such as dendritic cells that can traffic to
lymphoid organs and transmit the infection to other immune cells. Infection of immune cells,
particularly T cells, enables a blood-based viremia that allows for the efficient transfer of the
virus to the skin. At the skin, the virus replicates in the dermis and then epidermis where it
generates the characteristic rash. At the skin viral replication leads to lesions that form for 3 - 6
days and eventually heal and scab over. VZV infects and establishes latency in neurons of the
sensory and autonomic ganglia, which occurs in one of two ways: infection of axons innervating
the skin allow the virus to infect neurons via retrograde transport; or by VZV infected circulating
T cells that infiltrate into ganglia and infect ganglionic cells. The T cell based viremia likely
accounts for VZV establishing a latent state in all sensory and autonomic ganglia along the entire
peripheral neuraxis, as VZV nucleic acids are found in virtually every ganglia.

Contracting varicella in adulthood has serious implications. Fatalities, while rare, are
increased in adults and children over the age of 4. Complications associated with varicella
include secondary bacterial infection, varicella pneumonia, and neurological symptoms.
Contracting varicella during pregnancy, especially the first trimester, can be serious as VZV is
one of the few pathogens that can be passed between mother and child while in the womb.
causing birth defects. Vaccination and treatment with antiviral medication and antibodies reduce this risk \textsuperscript{11}.

VZV is a ubiquitous human pathogen that affects humans worldwide. Cases of varicella were as high as 4 million a year in the United States when vaccination was not required. However, epidemic varicella has largely been eradicated in this country and others that require vaccination, which has resulted in 82\% fewer varicella cases between 2000 and 2010 \textsuperscript{12}. In the United States and other temperate countries, 99.6\% of adults between the ages of 40 and 49 have antibodies directed at VZV suggesting prior VZV infection or vaccination \textsuperscript{13}. However, adults in tropical regions are far more susceptible to infection with as low as 50\% of adults seropositive for VZV antibodies \textsuperscript{14}.

The requirement for vaccination against varicella in the US and other countries has largely reduced epidemic spread of varicella and the associated morbidity and mortality. However the majority of countries do not require vaccination and therefore have endemic VZV, which can flare up as varicella epidemics. With increased vaccination, an increasingly aging population, and without the natural boost in immunity derived from epidemic VZV, zoster incidence is rising. VZV remains a public health concern that is largely unmet and in need of new treatments.

1.1.2 VZV Latency and subclinical disease

After primary infection, viral genomes remain latent with few apparent reactivation events over the lifetime of the host. VZV remains latent for the life of the host in neurons of the peripheral nervous system. Valuable information has been gained through the examination of post mortem tissue from VZV infected individuals. The burden of viral genomes in latently infected human
sensory ganglia varies widely with estimates of between 258±38 and 9,046±13,225 viral copies per 100,000 ganglionic cells in two studies\textsuperscript{15,16}. These numbers vary greatly due to small sample size n=18 and 17 respectively, and variations in the burden of infection between individuals in an outbred human population\textsuperscript{17}. Latent viral genomes exist as a circularized episome separate from the host genome that becomes associated with heterochromatin\textsuperscript{17-19}. This heterochromatinated state likely reduces viral gene expression to a select few genes that may be less chromatinated. Viral gene expression during latency has been controversial with early reports detecting viral transcripts (ORF21, 29, 62, 63, and 66) with ORF63 transcripts and protein expression proposed to be the hallmark of latency\textsuperscript{20-22}. These findings have recently become contentious as viral gene transcription is now recognized to increase with post mortem interval\textsuperscript{23} and ORF63 and other viral protein expression has also been called into question with the recent revelation that many of the antibodies used in these studies cross-react with blood group A antigens\textsuperscript{24,25}

There are several animal models of viral latency that recapitulate some of the hallmarks of viral latency in humans, but there are no models in which this latency can undergo reactivation. Rats, SCID-hu mice, guinea pigs and non-human primates have all been used as models of latency. These models have suggested a reduced number of viral transcripts with a proposed hallmark IE63 expression in innervating ganglia, although this may be questionable. Non-human primates have been used to model VZV infection and latency, using a close relative of VZV, simian varicella virus, which has many similarities to VZV, but has differences and has evolved with non-human primates. The requirements of VZV genes for latency has also been an important question investigated in cotton rats with 16 VZV gene deletion mutants tested for their ability to establish and maintain latency in cotton rats. Mutant viruses that lack the expression of ORFs 4, 29 and IE63 show impairment in the establishment of latency\textsuperscript{26}. This data is
contentious as the cotton rat model does not have detectable reactivation and may represent an abortive infection rather then true latency.

1.1.3 Reactivation and its subsequent disease

Viral reactivation from latency results in a disease known as herpes zoster. An estimated 32% of people will have herpes zoster during their lifetime, with the main risk factor being age 27. Other risk factors for zoster, besides age, include: cancer, as zoster is frequently reported among patients with leukemia, Hodgkin's and non-Hodgkin's lymphoma, and small cell carcinoma of the lung 1,28; transplantation and the associated immune suppression, HIV, and more recently stress from zero gravity 1,27,29. The incidence of zoster has also been documented to be lower in blacks than whites 27. Most of these risk factors involve decrease in immune function and therefore it is likely that the immune system plays a large role in containing VZV reaction. Herpes-zoster is characterized by a vesicular rash with surrounding erythema that encompasses 1 or 2 dermatomes and is often accompanied by pain and other neurological symptoms 30. The molecular events governing VZV reactivation are not well understood but are thought to follow, the closely related alphaherpes virus, HSV1. Viral reactivation is likely the result of a general derepression of chromatinated viral DNA, followed by expression of viral proteins and may or may not result in virus production. Zoster patients that have recently reactivated have been intranuclear inclusions, viral antigens, and virus particles in sensory ganglia 31,32. The large rash is a result of intraganglionic spread between neurons and surrounding cells during reactivation which results in the delivery of virions, en masse, to the periphery where viral infection of epithelial cells occurs with an associated inflammation 33-35. VZV reactivation is associated with pathological features that include: ganglionic inflammation.
and hemorrhagic necrosis with neuritis, possible localized meningitis, degeneration of related motor and sensory roots, necrosis or apoptosis of infected neurons, and demyelination likely resulting from mononuclear cell infiltration of microglial activation \cite{36,37}. Together this damage to neurons caused by reactivated VZV has effects on neurological functions and usually results in, at least, pain.

During reactivation virions are not only delivered to the periphery but also to the central nervous system where they can cause severe but rare complications including myelitis, aseptic meningitis, encephalitis, and even vasculopathies leading to stroke \cite{38}. Viral reactivation causes neurological complications that can become chronic including: itch, paresthesia, loss of sensation, and most commonly pain \cite{34}. Herpes-zoster can be predicted by prodromal signs include: paresthesia, numbness, itch, and importantly pain (preherpetic neuralgia), which can precede the eruption of the rash by as much as 100 days \cite{39}. Zoster associated pain can occur before, during or after resolution of the vesicular rash and is an extremely common result, with up to 90\% of unvaccinated patients prescribed medication to alleviate pain \cite{30,40}. The prevalence of zoster patients that develop chronic pain long after the rash has resolved is estimated to be between 10 to 30\%, with incidence increasing with age \cite{41,42}. This subset of zoster patients experience a chronic neuropathic pain state known as postherpetic neuralgia (PHN), which is defined as pain lasting longer than 30-90 days after resolution of the herpes zoster rash.

Models for viral reactivation have been largely nonexistent, as the virus has proved to be difficult to reactivate. Prolonged culturing of excised latently infected human ganglia does not result in viral reactivation and there is no release of detectable infectious viral particles. There are two reports of experimental reactivation in guinea pig and rat latently infected excised
neurons, but both have yet to be validated in other laboratories. Overall, there is still much to discover about VZV reactivation that has been hampered by the lack of a small animal model.

1.1.4 Vaccines

In the US there are two commercially available vaccines, one for the prevention of varicella (Varivax) and one for the prevention of herpes zoster (Zostavax). Both vaccines utilize live-attenuated strain(s) developed in Japan after serial passage in guinea pig cells, followed by passage in human cells. Both vaccines are well tolerated with limited adverse events and few breakthrough VZV infections and both vaccines generate broad humoral and cellular immunity. Varivax is up to 85% effective at reducing varicella and has largely eradicated VZV epidemics in the countries that require vaccination. However, breakthrough varicella has been reported, although vaccinees have reduced burden of primary disease and adverse effects. As the Varivax is an attenuated virus, the vaccine virus can still establish latency, reactivate, and cause neurological disease but at reduced incidence compared to wild type VZV.

The herpes-zoster vaccine (Zostavax) has an increased dose, 14 fold higher, compared to the varicella vaccine. Zostavax is much less effective, with only a 51% reduction in the incidence of herpes-zoster and 66.5% reduction in burden of illness including postherpetic neuralgia. Only 27% of patients older than 60 years of age are vaccinated, largely from unwillingness of the target population to receive the vaccine, lack of knowledge of the disease, and medical professionals not recommending the vaccine. This leaves a substantial portion of vaccinated patients that can experience vaccine or wild-type VZV reactivation and the associated pain. This will no doubt become an increasing public health concern as our population ages and lives longer.
1.2 VZV BIOLOGY

Our knowledge of initial infection by varicella-zoster virus has greatly improved with the development of new in vitro and in vivo models. However our knowledge of viral latency and reactivation are still inadequate and require new models to further our understanding of these phenomena. The study of VZV is hampered by a number of complications of working with the virus. First, VZV is highly human specific and does not readily infect non-human cells or animals, unless adapted for growth in those cells. Secondly, in vitro generated VZV is highly cell associated and yields very low levels of cell free virus, which affects our ability to achieve synchronous infections therefore limiting our knowledge of the kinetics of viral expression. Lastly, viral reactivation from latency has been hard to experimentally reproduce. With these drawbacks, much of what we hypothesize about VZV is assumed from a certain degree of homology between VZV and the more easily studied Herpes Simplex Virus (HSV).

1.2.1 Classification and genome.

Varicella Zoster virus (VZV) belongs to the herpesvirinae family, subclassified as an alphaherpesvirus. VZV is a double stranded DNA virus with a 125-kilobase genome, which can be separated into unique long and unique short sections both flanked by repeat regions (Figure 1). Unique short flanking repeats of VZV are large, but unique long repeats much smaller, only 88.5bp \(^1\). The genome is packaged in two predominant isomers with a fixed UL orientation and two different orientations of the US region, dictated by packaging sequences in the left-hand end of the UL region \(^57\).
Figure 1: Schematic of HSV and VZV genomes. The HSV1 genome is about 148 kilobases whereas the VZV genome is only about 125 kilobases. Both alphaherpesviruses have repeat regions that flank unique regions. The genomes to be represented as: terminal repeat long (TRL), unique long (UL), internal repeat long (IRL), internal repeat short (IRS), unique long (US), and terminal repeat short (TRS). VZV has smaller unique long flanking repeats (as small as 83.5 base pairs) than HSV.

VZV is closely related to Herpes Simplex Virus (HSV) with much of VZV biology based on its similarity to HSV. Overall the gene order and direction of orthologous VZV and HSV genes is the same, such that they are colinear and positionally the same. VZV encodes 70 open reading frames with all but six (ORFs 1, 2, 13, 32, 57 and S/L) having orthologs in HSV. VZV encodes its own DNA polymerase (comprised of ORFs 28 and 16), which is sensitive to acyclovir once the drug is processed by the viral thymidine kinase (ORF36). VZV also encodes a ribonucleotide reductase (comprised of ORFs 18 and 19) that converts ribonucleotides to deoxyribonucleotides. At least 2 DNA binding proteins ORF29, which binds single stranded DNA, and ORF51, which binds to the viral origin of replication, are encoded by VZV. The virus has two protein kinases, ORF47 and 66, which will be discussed in further detail in chapter 3. VZV has seven glycoproteins: gB, gC, gE, gH, gI, gK, gL, gM and gN.
1.2.2 Viral Particle and in vitro Lifecycle

The virion itself is made up of a capsid that surrounds one copy of the genome; a proteinacious tegument and an envelope studded with viral glycoproteins. The mature, infectious enveloped VZV particle is pleomorphic to spherical in shape and up to 200-400 nm in diameter. The capsid is made up of 162 capsomeres in a 5:3:2 axial symmetry. The viral proteins that make up the capsid have not been defined but are presumed to be the VZV homologs of the genes that make up the HSV capsid. The tegument surrounding the capsid allows the virion to transport proteins that help establish viral infection and these include ORFs 4, 10, 47, 62, 63, and 66. The capsid is assembled in the nucleus and is transitionally enveloped as it exits from the nucleus at the nuclear membrane. The initially budded viral particles can de-envelop into the cytoplasm and undergo secondary envelopment at the Golgi. Particles can also capture membrane from the rough endoplasmic reticulum (ER), cytoplasmic vesicles, and the cell surface. Our understanding of viral egress is far from complete and would likely be better understood once synchronized infections with cell-free virus are achieved.

Initial virion attachment is mediated by cell type specific receptors including insulin degrading enzyme and mannose-6-phosphate. After initial attachment the virion enters the cell by fusion of the viral envelope with the cytoplasmic membrane or is taken up by endocytosis. Once the cell is infected, viral tegument associated proteins are hypothesized to be transported with the uncoated capsid to the nucleus, where they likely promote efficient viral gene transcription and productive infection. Immediate early messenger RNAs (mRNAs) are transcribed in the nucleus, transported to the cytoplasm, translated, and the proteins they encode are brought back to the nucleus. Immediate early proteins allow for the efficient transcription of early mRNAs that are again transcribed in the nucleus, translated in the cytoplasm, and some are
transported back to the nucleus, where they facilitate viral DNA replication. After viral DNA replication has started late genes are transcribed and translated, with some of the proteins transported back to the nucleus while others become associated with the host membranes, cytoplasm and cytoplasmic structures. Capsids form in the nucleus, which have viral DNA loaded into them through a portal structure. These completed and filled capsids are transported out of the nucleus through an envelopment/de-envelopment process at the nuclear membrane.

The viral tegument, which is made up of ORFs 4, 10, 47, 62, 63 and 66, and viral glycoproteins are acquired as the virion becomes re-enveloped at the transgolgi network. Some of these virions will reach the plasma membrane and bud off, but others will be diverted to lysosomes and are degraded. This lifecycle results in the release of infectious virions between 8-16 hours post infection with viral proteins detected as early as 4 to 6 hours after infection.

In VZV and HSV, viral gene expression is carefully regulated during infection and separated into three categories (Figure 2). Genes transcribed without other viral gene expression or in the presence of translation blocking drugs such as cyclohexamide, are known as immediate early genes; genes expressed after immediate early genes but before viral DNA synthesis (or in the presence of a viral DNA synthesis inhibitor such as phosphonoacetic acid), are termed early genes; and those genes that are expressed maximally after viral DNA replication, are defined as late genes. In general immediate early genes are responsible for turning on early genes, which in turn are responsible for replicating viral DNA, which once started allows late genes to be expressed; which encode structural proteins that include those involved in capsid formation and DNA packaging. VZV gene expression is thought to largely follow the pattern defined for HSV,
but has not been experimentally determined because synchronous infection with VZV is currently not possible.

**Figure 2: Schematic of the temporal cascade of viral gene expression in human alphaherpes viruses.**

The temporal expression cascade of viral genes is highly regulated and separated into immediate early, early, and late gene expression patterns. This expression pattern allows for the detection of a possible block in replication in non-permissive cells.

VZV has four putative immediate early genes based on their homology to HSV IE genes: IE62, IE61, IE63, and IE4 all of which have been shown to affect viral transcription events.

Of the immediate early genes, IE62 is arguably one of the most important and is required for viral replication *in vitro* and *in vivo*. The protein is found in the tegument of virions and in the nucleus of infected cells, however phosphorylation of IE62 by the two viral protein kinases, ORFs 47 and 66, affects its subcellular localization. IE62 is the homolog of ICP4 in HSV1 and can inefficiently complement an ICP4 deficient mutant virus. It is the major viral transactivator, responsible for promoting gene transcription of early and late genes. In transient expression assays, IE62 activates expression of all three temporal gene classes. IE62 is used to boost expression of viral genes *in vitro* and to increase the infectivity of viral DNA. IE62 activates diverse promoters by interacting with various cellular transcription factors including SP1, TATA-binding proteins, TATA-binding protein complexes, and the mediator complex.
IE63 encodes a tegument-associated phosphoprotein. IE63 has homology to HSV1 US1.5 protein, which is collinear with HSV ICP22. Work from one group suggests IE63 is required for infectivity, while another group reports IE63 deficient viruses can replicate, albeit inefficiently. IE63 has been suggested to be essential for the establishment of latency in cotton rats. Transcription and protein expression of IE63 in latently infected ganglia has long been used as a hallmark for viral latency in humans, but as reported earlier, this has come into question. During infection, IE63 primarily localizes to the nucleus but at early time points it is found in the nucleus. Cellular kinases, Casein kinases I and II, and Cyclin dependent kinase 1 and 5 can phosphorylate the C terminus of IE63. Phosphorylation of IE63 is necessary for viral growth in skin, but not T cells in the SCIDhu model.

Another immediate early gene associated with the tegument is IE4. IE4 is the ortholog to HSV ICP27, but does not complement an ICP27 deficient mutant virus. IE4 can transactivate immediate early, early, and late viral genes but does not share the transrepressor activity of ICP27. IE4 has been shown to interact with many proteins involved in transcription including: TFIIB (the TATA binding protein), two subunits of NF-κB; p50 and p65, and IE4 also binds to IE62. IE4 may have posttranscriptional regulatory activities. Deletion of IE4 impairs viral latency in cotton rats, with fewer latently infected animals and lower viral burden in those animals.

The putative immediate early gene is IE61 encodes a phospho-protein that localizes to the nucleus during infection. Unlike the other immediate early genes, IE61 is not associated with the tegument and is therefore not brought in with the virus particle during infection. IE61 can either activate or repress IE4 and IE62 in plasmid transfection studies depending on cell type and transfection conditions. IE61 is the ortholog to HSV1 ICP0 and can complement an ICP0
deletion virus suggesting they exhibit similar function\textsuperscript{91}. IE61 is not necessary for viral growth in culture, but does effect syncytia formation, and deletion of IE61 does not effect establishment of viral latency \textsuperscript{92,93}. Recently a role for IE61 in counteracting an intrinsic antiviral defense has been uncovered in which, promyelocytic leukemia protein (PML) cages that envelope incoming viral DNA is partially counteracted by ORF61 protein \textsuperscript{94,95}. Like HSV1 ICP0, ORF61 possesses a RING finger domain, and localizes to nuclear domain 10 bodies to affect their structure. However, while some intrinsic cellular defenses are degraded by both ICP0 and ORF61, the latter does not degrade PML.

This virus encodes two early expressed, serine/threonine kinases, ORF47 and ORF66. Both virally encoded protein kinases are dispensable for viral growth \textit{in vitro}, but both are known to be necessary for different stages of \textit{in vivo} spread. The ORF47 kinase is acidophilic with a consensus phosphorylation motif similar to human casein kinase II. A deduced motif of: S/T-X-D/E-D/E has been identified with a preference for acidic amino acids -1 or +1 \textsuperscript{173}. The kinase appears to have orthologs in all herpesviruses, and is similar to the UL13 kinase of HSV1 The targets for ORF47 have not been fully elucidated, but it is known to phosphorylate several viral proteins including: itself, IE62 (the major transactivators of viral gene expression), ORF9 (a tegument protein), and ORF32 \textsuperscript{131,174,175}. The only known host target is IRF3, but it seems likely there are others\textsuperscript{176}.

The second viral kinase, encoded by ORF66, is a serine/threonine kinase that is the VZV ortholog to HSV1 and PRV US3, a kinase that is conserved among the alphaherpesviruses. ORF66 is a basophilic kinase with a target serine preceded by lysines and/or arginines \textsuperscript{177}. ORF66 has several known targets including itself, IE62, Matrin 3, and HDAC 1 and 2 \textsuperscript{128,178-180}. 
The target motif of ORF66 overlaps with protein kinase A. Viruses deficient in ORF66 exhibit reduced growth in T cells and corneal fibroblasts in vitro \(^{128,181}\).

Both kinases are necessary for growth in certain cells in vivo. ORF47 is necessary for productive viral growth in T cells and skin in the SCID-hu model and an ex vivo model of human T cell VZV infection, as well as for transferring infection from dendritic cells to susceptible fibroblasts \(^{158,181,182}\). Loss of ORF47 kinase function results in aberrant capsid formation that results in decreased cell free spread in vivo \(^{67,182,183}\). ORF66 is needed for efficient viral replication in T cells in a SCID-hu model but has only a slight reduction in growth in skin \(^{184,185}\). Therefore, while both kinases are dispensable for viral growth in vitro, but clearly affect virulence in vivo.

ORF9 is a late expressed, tegument-associated gene that is essential for viral growth \(^{186,187}\). During infection, transcripts from the ORF9 gene are readily detected and when quantified, are the most abundant viral transcripts \(^{188,189}\). ORF9 is the ortholog of HSV1 UL49 and shares homology with the UL49 encoded protein, VP22 of HSV1. ORF9 interacts extensively with viral proteins including IE62, gE, gI, gH, gN, ORF15, ORF23, ORF38, and ORF47 \(^{186,190-193}\). These extensive interactions likely make ORF9 important in proper tegument and virion formation, as ORF9 hyperphosphorylation (mediated by ORF47 at Serine 84) influences virion formation and egress \(^{193}\).

1.2.3 Animal models of varicella zoster virus latency

Small animal models of VZV infection and latency are largely hindered by the inability of VZV to replicate in most non-human tissues. Viral latency has been reported in several small animals.
including laboratory rats, cotton rats, and guinea pigs, but these models have been criticized, as they do not support viral growth. Other groups have modeled VZV infection using explanted human fetal tissue into SCID mice. While this has enabled the study of factors affecting initial viral infection and the innate immune system’s role in controlling VZV infection they lack neural connectivity and an adaptive immune system. There are no reproducible models of viral reactivation in animals, with the possible exception of two reports\textsuperscript{26,43,44,96}. The models include:

1.2.3.1 Guinea Pigs

The vaccine was passaged in guinea pig cells, which provide some permissivity to VZV growth. These animals have also served as a model for varicella zoster virus infection and latency. Animals inoculated with a guinea pig adapted virus can transmit the virus between animals and exhibit some of the hallmarks of human infection including a monocyte associated viremia\textsuperscript{97,98}. Animals may develop a rash in rare cases. VZV proteins and nucleic acids have been detected in sensory as well as enteric ganglia in animals, and the virus can become latent\textsuperscript{44,99}. Recently this model has been expanded to investigate the effects of VZV infection on enteric neurons. Interestingly, when dissociated enteric neurons are exposed to cell-free VZV (that was not adapted for growth in guinea pigs) the virus infects neurons and expresses viral genes, but does not cause cytopathic effect until superinfected with Adenovirus vectors expressing ORF61 or its HSV homolog ICP0, at which time cytopathic effect and viral reactivation were reported\textsuperscript{44}. As these animals are fully immunocompetent, this model enables the study of immune responses to VZV with infected animals seroconverting and exhibiting a T cell response to infection\textsuperscript{100-102}. The major disadvantage of this model is its need for adapted virus, which may have characteristics and mutations that make extrapolation of the data to human disease more difficult.
1.2.3.2 SCID-hu Mice

The lack of wild type VZV replication in virtually all small animal models led to the development of the SCID-hu model for VZV. Severely compromised immune deficient mice are implanted with human fetal DRG, skin, or thymus/liver tissues, which are accepted, as there is no adaptive immune system and cannot be rejected by the host. This model has revolutionized some of our understanding of primary VZV infection and has led to interesting data regarding viral persistence. Cell associated VZV injected into DRG xenografts results in immediate changes to the tissue, including denucleation of neuronal cell bodies, cytoplasmic inclusions, and production of quantifiable infectious progeny virus $^{103}$. During initial infection, IE63 immunoreactivity is readily detected in neuronal and satellite cells. VZV infection also results in polykaryon formation between neurons and support cells that have assembled capsids, but produces no appreciable changes to cellular organelles. These changes suggest full viral replication without the accompanying cell death $^{104}$. By 8 weeks post infection the acute viral lifecycle changes into a state of persistence or latency within the DRG in which only IE63 RNA is detected but at low levels $^{105,106}$. Few IE62, 63 and ORF31 transcripts are detected during this stage and no viral proteins are detected during persistence, as seen for recent data in humans $^{105,107}$. Interestingly SCID-hu DRG mice infected with the live attenuated vaccine strain have similar numbers of persisting/latent genomes and similar replication in neural tissue as wild-type virus $^{103}$. These data confirm the ability of the vaccine to enter latency, as seen in vaccinated patients that have zoster at the site of vaccination.

This model added another potential route of viral infection of ganglia in which infected T cells, injected into the tail vein of mice, traffic to and infect implanted neural tissue $^{106}$. This
finding has changed the way we think about how VZV reaches the ganglia and may explain how VZV can reach ganglia along the entire neuraxis.

While we have learned a great deal about VZV pathogenesis from this model, it is not without its drawbacks. Mice are not a natural host for VZV and therefore this model is highly artificial. Secondly, these animals have no adaptive immune system and therefore the study of the immune responses to VZV is out of the question and may change the way the host interacts with the pathogen. Lastly, the model relies upon fetal tissue that may be hard to acquire and may not behave like more mature, post natal ganglionic tissue.

1.2.3.3 VZV Latency in Rats and other rodents

VZV infection of dissociated rat neurons led the same research group to inoculate laboratory rats with VZV injections along the spine. Viral proteins and nucleic acids were detected in nervous tissue at 6 and 9 months post infection, respectively. Interestingly, an initial report suggested that viral nucleic acids are present in both neurons and non-neuronal cell types in ipsilateral DRGs when the dorsal root was severed prior to VZV inoculation along the spinal cord. This may suggest limited hematogenous spread of the virus.

Other groups have inoculated rats at the footpad and skin with VZV nucleic acids detected in innervating ganglia by in situ hybridization. VZV DNA was detected in both ipsilateral and contralateral ganglia in both non-neuronal and neuronal cell types. Viral DNA and RNA have been reported to persist in rats for up to 18 months post infection possibly indicating viral latency in these animals. While genomes persist, there is no evidence that it is infectious or capable of reactivation. We argue viral infection of rat tissue represents an abortive infection. However, rats exhibit chronic hypersensitivity to mechanical and thermal stimuli after
VZV infection, to date this is the only small animal model for VZV-induced pain\textsuperscript{111}. This model of VZV-induced pain will be further discussed below.

Other rodent models that have been reported include newborn rats, corneal injections in mice, and a latency model in cotton rats. Newborn rats inoculated with VZV intraperitoneally had viral DNA and RNA present to 5-6 weeks post infection\textsuperscript{112}. Mice infected in the cornea had disseminated infection/latency with viral DNA found in the trigeminal ganglia as well as the brainstem, kidneys, spleen, and liver at 1 month post infection, although this has never been reproduced\textsuperscript{113}. Viral RNA was also detected in the trigeminal ganglia and spleen\textsuperscript{113}. Lastly, cotton rats infected with VZV establish a latency-like state when injected along the spine\textsuperscript{114,115}. The cotton rat model was used to investigate which viral genes are necessary for “latency”, but suffers from the same arguments regarding what the latency state is.

While these models have allowed us to gain new insights in VZV pathogenesis and latency, they leave a lot to be desired. Again the major drawback for all of these models is that VZV is a human pathogen that is highly restricted to cells of human origin and infection of rodents does not fully recapitulate any of the signs of human disease. Therefore the relevance of these models and how they translate to human disease remains to be firmly established.

\subsection{The VZV-induced pain model in Rats}

There is only one model for VZV-induced pain, in which Wistar or Sprague Dawley rats inoculated with VZV at the footpad develop quantifiable chronic hypersensitivity to mechanical and thermal stimuli that lasts from 6-14 weeks. This phenomenon was first reported in 1999 and has since been expanded upon by several groups including ours\textsuperscript{111,116,117}. Rats inoculated with VZV develop hypersensitivity in the ipsilateral but not contralateral paw, and sham, vehicle, and control-cell inoculated animals showing no biased responses on either the ipsilateral or
contralateral paws. Severity of hypersensitivity is related to the viral dose in the inoculum, with lower doses having a smaller effect on hypersensitivity \(^{118}\). It has been reported that the same animals that have hypersensitivity to stimuli also exhibit anxiety-like behaviors in field paradigm tests \(^{117}\). Importantly, hypersensitivity in the rat is reduced by UV (which I report here) or heat inactivation of VZV \(^{116,119}\) but is not affected by antiviral administration \(^{116,120}\). These data suggest that full, ongoing viral replication is not required to induce hypersensitivity in animals but that viral infectivity/ transcription is required.

Viral gene expression and nucleic acids are detectable in rat nervous tissue out to 6 months post inoculation in the model, and IE63 and IE62 immunoreactivity have been observed in ipsilateral ganglia. IE62 expression appears to colocalize with neuropeptide Y (NPY), neurofilament 200 (NF200) and peripherin suggesting peripheral sensory neurons are infected with VZV \(^{111,117,118}\). However, these data have been called into question as the immunoreactivity described in these studies do not mimic what is seen \textit{in vitro} and may be the result of anti VZV antibodies exhibiting cross-reactivity with blood group antigens \(^{24}\). We report that viral DNA and RNA are observed in innervating ganglia by quantitative PCR and by others using in situ hybridization indicate VZV persists up to 9 months post infection \(^{43,109}\).

The host changes in the ganglia induced by VZV infection in rats have not been well studied. Animals infected with VZV have increased expression of proteins known to be up regulated in some chronic pain states including NPY, galanin, activating transcription factor 3, the voltage-gated calcium channel subunit α2δ1, and the sodium channels Na\(_v\)1.3 and Na\(_v\)1.8 when compared to control animals \(^{118}\). The observed up-regulation of voltage-gated calcium channels may partly explain why some pain responds to gabapentin, which blocks these channels, as gabapentin is often prescribed to alleviate zoster pain. Another group investigated
central mechanisms that may be mediating the chronic hypersensitivity and found increased immunoreactivity of GFAP but not OX42 in the spinal cord of VZV infected animals. This has been further assessed by intrathecal administration of astrocyte toxin L-α-amino adipate (LAA), which reduced VZV-induced nocifensive behaviors and action potentials, although responses did not fully return to baseline. Again, a microglial toxin (minocycline) did not have the same effect. Increases in iNos and IL1β expression were also observed in the spinal dorsal horn of the VZV infected animals. They concluded that increased astrocyte activation but not microglia might be mediating the chronic hypersensitivity. Taken together, these data suggest there is immune activation after VZV inoculation, particularly of astrocytes in the spinal cord, which may have a role in VZV-induced pain in this model.

This model has extensively been used as a platform to test treatments for their effect on VZV-induced pain. Compounds that reduced VZV-induced hypersensitivity in the rat model of VZV-induced pain are also in use in human patients include morphine, amitriptyline, gabapentin, and ibuprofen. Other novel compounds have been tested in VZV-infected rats that relieve hypersensitivity include: a cannabinoid receptor antagonist (Win55212-2), sodium channel blockers mexiletine and lamotrigine, astrocyte toxin LAA, as well as iNOS inhibitors (L-NIL), NO scavengers (PTIO), IL1 receptor antagonist, the cytokine inhibitor pentoxifylline, the NMDA receptor antagonists AP5 and (R)-CPP, and the non-competitive NMDA receptor antagonist MK801, and lastly histamine H3 receptor antagonists GSK189254 and GSK334429. As this is the only model of VZV-induced pain, it remains the only platform to test the efficacy of treatments.
1.3 PAIN AND POSTHERPETIC NEURALGIA

Pain is a complex phenomenon that is defined by the International Association for the Study of Pain as “an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage.” Painful stimuli are sensed in peripheral skin tissue by innervating neurons that are classified by their conduction velocities into the fast conducting $A\beta$, slightly slower $A\delta$, and unmyelinated, slowly conducting $C$ fibers. These fibers have various molecular and biochemical characteristics and can be further subdivided based on these criteria. However, not every cutaneous innervating neuron responds to painful stimuli, as only subsets are able to respond to stimuli in the noxious range. Primary afferents synapse onto second order neurons in the spinal dorsal horn where pain signaling is transmitted to higher centers in the brain.

1.3.1 Human Postherpetic Neuralgia and zoster associated pain

Both chronic and acute zoster associated pain are classified as neuropathic pain. Neuropathic pain occurs after an insult to the nervous system, in this case the reactivation of VZV from a state of neuronal latency. Zoster associated pain often precedes the characteristic rash and can last long after the reactivation resolves. Neuropathic pain can arise from multiple etiologies that can be influenced by genetic predispositions and environmental factors making treatment of neuropathic pain difficult. Zoster associated pain and postherpetic neuralgia have no universal treatment, with a significant portion of PHN patients unable to achieve relief with the current armamentarium.
1.3.2 Acute Zoster Pain

Greater than 90% of zoster patients are prescribed medication to mitigate pain. This pain can be apparent before, during or shortly after onset of the rash. Itch, paresthesia, and dysesthesia can also accompany VZV reactivation. Most zoster patients experience some discomfort and pain, which are thought to be the result of viral replication in the skin and ganglia and an associated inflammation. Treatment for acute zoster pain includes antivirals, non-steroidal anti-inflammatory drugs, opioids, gabapentin, lidocaine patches and tricyclic antidepressants. However, in most cases, acute zoster pain resolves with or shortly after resolution of the rash.

1.3.3 Postherpetic Neuralgia

Pain is the most common complication associated with herpes zoster. Up to 30% of zoster patients will develop a chronic pain state classified as postherpetic neuralgia, which can be defined as pain lasting longer than 30 days although it may last for months to years. PHN patients describe having either spontaneous/on-going pain, stimulus evoked (allodynia) pain, or evoked or spontaneous intermittent lancinating pain. Acute zoster associated pain is receptive to antiviral and corticosteroid treatment, however these treatments have no effect on the chronic pain associated with PHN unless administered within 72 of clinical disease. PHN can have many disparate consequences affecting quality of life, depression, and health care costs. PHN patients have even gone to extreme lengths to avoid pain including withdrawal from society and in extreme cases suicide. Several mechanisms, which are all the result of viral replication and the associated damage and cell death, have been proposed to explain the development of PHN including irritable nociceptors, deafferented nonallodynic subtype, and deafferented alldyonic
In patients that have intact peripheral nociceptors, PHN may be a result of abnormal nociceptor activity and central sensitization (irritable nociceptor subtype). Patients that have lost peripheral C-nociceptor function but have preserved tactile Aβ fibers, may have allodynia that is the result of increased and/or incorrect Aβ fiber synapse onto dorsal horn neurons not normally receiving those inputs (deafferented allodynic subtype). PHN patients that have lost peripheral Aβ nociceptors and do not have mechanical allodynia, may have chronic pain derived from central sensitization (deafferented nonalldynic subtype). In a study of PHN patients 25% were categorized as resulting from irritable nociceptors, 50% deafferented allodynia, and 25% deafferented without allodynia.

Treatments for PHN include first line NSAIDs and antivirals, which have limited efficacy when administered past 72 hours post clinical disease; second line antidepressants: amitriptyline, desipramine and nortriptyline, and anticonvulsants such as: gabapentin, pregabalin; with the last line including topical capsaicin/lidocaine, opioid analgesics, or surgical removal of the affected area of skin. However, a portion of patients receiving any/all of these interventions will see no relief from pain. Leaving a need for new treatments that can address the multiple etiologies proposed to explain VZV induced chronic pain. This is not surprising, as PHN results of multiple etiologies including neural changes, neuronal death, and ganglionic and peripheral inflammation. Importantly, PHN needs to be better understood in order to deliver better treatments. We propose this rat model will allow for the investigation of VZV induced pain including its mechanisms.
2.0 STATEMENT OF RESEARCH QUESTION AND SPECIFIC AIMS

Zoster associated pain, particularly the chronic pain state, postherpetic neuralgia, can be debilitating and have many consequences as zoster affects the aging population. Current treatments do not meet the needs of all Zoster and PHN patients. The goal of this thesis project is to better understand the mechanisms underlying VZV-induced pain in a rat model of PHN, and to test novel treatment strategies involving the ganglionic expression of pain modulating genes from HSV vectors.

The mechanisms contributing to PHN are complex; more importantly, any current treatment may be relatively ineffective in a substantial portion of patients. New treatments have been difficult to develop, as VZV species specificity has prevented the animal modeling of latency/reactivation cycle. However, the rat model for VZV-induced sensitivity can potentially reveal new approaches to treat pain in human patients and uncover the mechanisms underlying VZV-induced pain. We show here that Sprague-Dawley rats infected with VZV exhibit behaviors indicative of pain upon mechanical and thermal stimulation, which peaks between 4-6 weeks and spontaneously resolves by 8-10 weeks. My overall hypothesis is VZV-induced nocifensive behaviors are due to viral infection and viral gene expression in the corresponding dorsal root ganglia. Understanding the role of VZV infection and gene expression in the rat model will help define the contribution of the virus to the mechanisms underlying VZV-induced pain. Viral gene and protein expression, as well as viral burden have
not previously been determined in this model. In addition, we hypothesize that viral gene expression is at least partially responsible for the induction of nocifensive behaviors. Identification of these genes could lead to second-generation vaccines that, when reactivated, do not induce pain and serve as novel targets for pharmaceutical intervention. Third, the rat model provides a platform to test new treatment strategies for PHN that can be translated to human patients. My specific aims are as follows:

Specific Aim 1 will correlate viral parameters and changes in host gene transcription affecting nocifensive behaviors with its resolution in vivo. VZV is known to be highly host restricted, and previously published data indicate that replication of viral DNA is not necessary to induce nocifensive behaviors. My results indicated there is a need for viral gene expression to induce pain, given UV-inactivated VZV does not generate chronic nocifensive behaviors. We investigated viral gene expression, protein localization, and genome copies in the ganglion of this model at various time points post inoculation. We have also investigated changes in host gene transcription and peripheral nerve innervation. These studies were used to address the question, is viral gene expression required to induce pain pathology in this model, and what are the effects of viral infection on host gene expression and physiology?

Specific Aim 2 will evaluate candidate VZV gene deletions to obtain a virus that does not generate nocifensive behaviors. We discovered that a virus lacking one of the viral kinases, ORF47, does not induce nocifensive behaviors, while knockout of the other viral protein kinase, ORF66, did not have an effect on pain. Interestingly, ORF47 delivered to and expressed in the DRG using a replication deficient HSV delivery system was shown to induce chronic nocifensive behaviors. We have also shown that the kinase activity of ORF47 is necessary for both of these phenomena. This strongly suggests kinase activity of ORF47 plays a key role in
VZV-induced pain. These studies will indicate the role of viral kinases in pain induction in the model. This could establish new targets for treatment of VZV induced pain.

Specific Aim 3 will assess the use of different treatments for their effect on VZV-induced nocifensive behaviors. While the rat model has been successfully employed to test the effect of pharmaceutical interventions on PHN, we utilized a different strategy and used it to evaluate the potential for gene delivery and expression approaches as treatments. We utilized replication incompetent HSV vectors to deliver and express modulators of pain as treatments for VZV induced pain. We demonstrated that a vector expressing human preproenkephalin (HPPE) ablates nocifensive behaviors induced upon VZV infection, in a dose dependent manner. Prophylactic inoculation with the HPPE expressing vector also blocked the development of nocifensive behaviors post VZV inoculation. This novel treatment could serve as a new, long-term treatment for human patients suffering from PHN.
3.0 GENERAL MATERIALS AND METHODS

**Cells and Viruses.** VZV Parent of Oka (pOka) is a low-passage clinical varicella isolate used at less than 12 passes following receipt (kind gift from Dr. M. Takahashi (Osaka University, Japan)). VZV was grown in the human melanoma cell line MeWo (ATCC, Manassas, VA) using Modified Eagles Media (MEM: Life Technologies Inc., Grand Island, NY) supplemented with 10% fetal bovine serum (FBS) and antibiotic / antymycotic mixture (Sigma, St. Louis, MO). High titer VZV was prepared on fresh monolayers of MeWo cells infected at ~0.1 plaque forming units (PFU)/cell and harvested at 48-72 h post infection by trypsin digestion when cells showed approximately 70-80% cytopathic effect (CPE). Analyses of the stock virus by immunostaining or flow cytometry for VZV surface proteins revealed >90% of the cells were infected. VZV viability and infectious titer were maintained by slow-freezing infected cells as aliquots under conditions to maintain cell viability (MEM containing 20% FBS and 10% Dimethyl sulfoxide (Sigma)) that were stored under liquid nitrogen. Uninfected cell equivalents used as controls were treated similarly. VZV titers were determined by plaque assay on MeWo cells.\(^{128}\)

Uninfected cell equivalents used as controls were treated similarly. VZV titers were determined by plaque assay on MeWo cells.\(^{128,129}\) Luciferase expressing virus was generated by inserting a firefly luciferase (fLuc) cassette with a Zeocin resistance marker onto the C-terminal end of IE63 in the GS1783 pOka BAC, using techniques previously described.\(^{130}\) Bacterial colonies were
selected on Chloramphenicol and Zeocin and, correct insertion of the cassette was verified by DNA sequencing.

ORF66Stop was described previously $^{128}$, ORF47stop was a kind gift from Dr. J. Cohen $^{131}$, and ORF47 kinase mutants (47ΔCterm, 47D-N, 47P-S) were a kind gift of Dr. A. Arvin $^{67}$. Viral ORF47 and ORF9 mutations were generated as described previously using Red mediated recombination in bacterial artificial chromosomes containing the entire VZV genome $^{128,130}$. A kanamycin cassette was used to disrupt the open reading frame of ORF47 and generate point mutants E85R and S84A/E85R by recombination. The primers listed below (Table 1) were used to PCR amplify the kanamycin resistance cassette (Kan') from the plasmid pEP-kanS2 and add ORF47 and ORF9 flanking sequences required for homologous recombination. The gel-purified PCR products were then used to transform the Escherichia coli GS1783 (a kind gift from Dr. G. Smith (Northwestern University, Chicago, IL)) containing the pOka VZV BAC and imparts chloramphenicol resistance by electroporation. Recombinants were selected by plating on LB agar containing 30μg/ml kanamycin and 30μg/ml chloramphenicol and grown at 32°C. AvrII or HindIII digestion of DNA from individual colonies was used to map the correct insertion of the cassette for ORF47 and ORF9 mutants, respectively. In order to make the ORF47 rescuant and complete the generation of the ORF9 point mutants, a second round of recombination was induced to remove the Kan' through homologous recombination of repeated sequences flanking the Kan' cassette. This was achieved through the induction of the restriction enzyme, I-SceI, in liquid cultures containing 1% arabinose, and heated to 42°C for 15 min. Colonies grown at 32°C on LB agar containing chloramphenicol and arabinose were checked for loss of the Kan' cassette by replica plating individual colonies onto plates containing kanamycin. The BAC DNAs were
transfected into MeWo cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA), and VZV plaques were visible after 3 to 5 days of incubation at 35°C.

Growth kinetics of new viral mutants were completed as detailed in 128. Briefly, 6 well plates seeded with MeWo cells were infected with 400pfu/well. Cells from each well were dissociated by trypsin digestion and titrated in duplicate onto fresh MeWo cells at each day post infection. Plates were stained with gentian violet solution enabling the visualization of plaques and syncytia under a dissection scope and the resulting titer calculated.

UV inactivation of VZV was carried out in 1mL aliquots of virus spread over a 35-mm diameter well of a 6-well plate and irradiated using parameters determined empirically from UV inactivation kill curves. The light source (Spectronics, Westbury NY) was a 2 x 15W unfiltered source emitting UV-C wavelength with intensity of 1550 Joules at 10 inches. Exposures (<15 min) were timed to result in a reduction of virus titer by >100 fold. Cells were then washed and treated for rat inoculation.

**HSV Vectors.** HSV-1 vectors used here (vHG, HSV47VZV, and HSV47VZV D-N) and vHPPE and vHG (detailed previously132) are based on HSV-1 strain KOS 321 and rendered replication incompetent by deletion of the essential ICP27 gene and both gene copies of the essential ICP4 protein. The vector also contains alterations of the promoters of the immediate early ICP22 and ICP47 genes so that they are expressed as early or β-genes. The entire VZV ORF47, 62, 63, and 66 genes were cloned by PCR, ligated into the vector SASB3, and subsequently transfected with vHG infectious viral DNA. HSV47VZV D-N viruses were generated by overlap PCR methods that mutated the aspartate of a DYS motif at position 282 in the ORF47 kinase to asparagine. Control HSV virus vHG contains two copies of a CMV IE promoter driven enhanced green
fluorescent protein (eGFP) gene, followed by a bovine growth hormone polyadenylation signal at each ICP4 locus. HSV vector vHPPE was generated from vHG by standard co-transfection-homologous recombination methods so that the EGFP genes were replaced with cDNA for human PPE. Viruses were prepared and titrated in Vero 7b complementing cells expressing the ICP4 and ICP27 genes in trans. Control HSV virus vHG contains two copies of a CMV IE promoter driven enhanced green fluorescent protein (EGFP) gene, followed by a bovine growth hormone polyadenylation signal at each ICP4 locus. HSV vector 47VZV and 47VZV D-N were generated from vHG by standard co-transfection-homologous recombination methods so that the EGFP genes were replaced with either ORF. HSV vectors were prepared using Vero 7b cells grown in infected at an MOI of 0.001. Virus was grown for 24 hr at 37°C followed by subsequent growth at 33°C until ~80% CPE. Virus was released from the surface of cells by adjusting the entire harvest to 0.45 M NaCl for 3 hr at room temperature. Cellular debris was removed by low-speed (3,000 rpm for 5 min) centrifugation and the supernatant filtered using 0.8µm-filtration units (Thermo-Fisher, Pittsburgh, PA). Virus in the filtrate was concentrated by high-speed centrifugation (19,000 rpm for 45 min) and resuspended in 1xPBS (Sigma) containing 10% glycerol, aliquoted and stored at ~80°C. All HSV vector stocks were verified to lack replication competent virus by titration on non-complementing Vero cells, and verified for VZV protein expression by western blot.
### Primers:

#### Table 1: Primers used to generate mutant viruses

<table>
<thead>
<tr>
<th>Virus</th>
<th>Primer</th>
<th>Sequence</th>
</tr>
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<tbody>
<tr>
<td>47Kan</td>
<td>Forward</td>
<td>CCACTCGAGTAATCGGAAACTATAGCTAGTAACATTAATACG</td>
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<tr>
<td></td>
<td>Reverse</td>
<td>GTACTAGCTATAGTTTTCCGATTACTGCAGTGTTATTCTCAACG</td>
</tr>
<tr>
<td>HSV47 VZV</td>
<td>Forward</td>
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#### Table 2 qPCR primers

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Animal Inoculation. Animals were housed and experimental manipulations on animals performed in ABSL-2 facilities approved by the Association for the Accreditation of Laboratory Animal Care (AALAC). All procedures were approved by the University of Pittsburgh Institutional Animal Care and Use Committee (IACUC). Male 200-250g Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA) were acclimatized to housing for 1 week and then assessed for baseline mechanical allodynia (MA) and thermal hyperalgesia (TH) responses, as detailed below, to obtain baseline measurements and identify pre-existing atypical responses. Following isoflurane-induced anesthesia, $2 \times 10^5$ PFU of cell-associated VZV (or uninfected cell equivalents) were injected subcutaneously beneath the plantar surface of the glabrous skin on the right hind footpad. Rats were monitored for recovery and return to normal activity. Administration of VZV-infected cells, uninfected cells or the maximal dose of HSV vector did not induce changes in grooming, self-maintenance and feeding habits or weight loss. After 24h, we were unable to observe any significant indication of swelling, inflammation or general redness in the ipsilateral injected footpad as compared to the contralateral non-injected hindpaw.

Behavioral Testing. MA was determined using one set of calibrated von Frey filaments (Stoetling, Wood Dale, IL) and the “up down” method established previously $^{133,134}$. Briefly, animals were allowed to equilibrate on a wire grid-based platform apparatus (IITC Life Sciences, Woodland Hills, CA) for 15 min. Starting with a von Frey filament of 10g weight of force, each filament was applied for 6s with sufficient force to cause bending against the paw when applied to one foot at a time. A positive response was defined as rapid withdrawal and/or licking of the paw upon application of the stimulus, and was followed by application of the next lower weight
von Frey filament. A negative response was followed by testing with the next higher weight von Frey filament. A total of 6 measurements were recorded for both left contralateral (uninoculated) and right ipsilateral (inoculated) footpads. We imposed a cut off of 150g during the analysis. Since some animal to animal variation of sensitivity was occasionally found, MA sensitivity variations from animal to animal were minimized by representing responses as a ratio of the calculated mean ipsilateral/contralateral response for each animal. MA testing preceded TH measurement on the same animal. TH was determined using a Hargreaves apparatus (IITC) according to established methodology. Briefly, rats were allowed to equilibrate on a glass stage heated to 34°C for at least 15 min. A calibrated focused radiant heat source was placed at a set distance under one paw at a time, starting with the contralateral paws, and the time recorded for animals to remove their paws in response to the heat source was recorded as the latency withdrawal period with a cut-off of 25 seconds. Paw withdrawals due to locomotion or weight shifting were not counted, and the trials were repeated. Animals were given 1-2 minute rest between measurements and were tested at least three times per paw.

**Drug Treatments.** DAMGO ([D-Ala², N-MePhe⁴, Gly-ol]-enkephalin) and naloxone hydrochloride were purchased from Sigma. Rats were first assessed for VZV-induced behavioral indicators of pain by MA. Animals then received 20µL injections containing DAMGO administered subcutaneously into the footpad at 1 and 10µg per 20µL. Animals were assessed for MA measurement at 5, 20 and 60 min post drug administration using von Frey filaments. Naloxone treatment studies used animals showing a VZV-induced pain response that had been alleviated by HSV vHPPE. These rats were subjected to footpad administration of 5 and 50µg total per 20µL of naloxone, followed by similar MA behavioral testing at 5, 20, 60 min and at 24
hr post administration. In both studies, control animal groups received each compound without prior VZV inoculation, or received PBS into the ipsilateral or contralateral footpad following VZV inoculation. All animals underwent identical treatments and handling.

**Nucleic acid extraction, purification and quantification.** Animals were euthanized at stated time points with CO2 and subsequent cardiac puncture. DRGs corresponding to L4,5 were removed and snap frozen in liquid nitrogen and placed at -80°C until processing. For DNA, DRGs were left in buffer ATL (Qiagen) + proteinase K (Qiagen) solution overnight at 55°C. DNA was extracted using the DNeasy kit (Qiagen) according to the manufacturers protocol. To extract RNA and DNA from the same samples, DRGs were mechanically homogenized in TRIzol reagent (Life Technologies Inc., Carlsbad, CA) on a Kinematica PT1200E homogenizer (Kinematica, Bohemia, NY) for 7 seconds at ~75% power. Nucleic acids were extracted according to manufacturers protocols. DNA pellets were resuspended in Tris EDTA buffer (6.8), and RNA in nuclease-free H2O. Viral DNA copies were determined by comparing Ct values from tissue to a standard curve spiked with equivalent amount of rat spleen DNA. RNA was converted to cDNA using a High Capacity RNA/cDNA Kit (Life Technologies Inc.) following manufacturers protocol and subsequently used for qRT-PCR. Gene expression was determined by the 2^-DeltaDeltaCt method comparing (i) TRADD, Ntrk2, and TRPV1 to β-Actin by SYBR Green; and (ii) Calca (Life Technology, Rn00569199_m1), human preproenkephalin (Hs00175049_m1 Applied Biosystems), or VZV genes (IE62, IE63, ORF47, ORF29, gC, ORF21) to GAPDH (Life Technology, Rn01775763_g1) by hydrolysis probes.

RNA for Gene Array analyses was further purified RNeasy Mini kits (Qiagen, Valencia, CA). All nucleic acids were quantified using a Nanodrop Spectrophotometer (Thermo-Fisher,
Pittsburgh, PA). Between 150 and 180ng of extracted RNA was processed using a 3’ IVT Express Kit (Affymetrix Inc., Santa Clara, CA: catalog # 901228) to yield 49 ± 6 μg of amplified RNA (mean ± SD, n = 6). Between 19 and 20 μg of amplified RNA was hybridized to Rat 230 2.0 microarrays (Affymetrix, catalog #901228), which were then developed and scanned using an Affymetrix GeneChip 3000 Array scanner. Raw data were processed using GCOS v. 1.4 software (Affymetrix) using software defaults, except that for change calls the perturbation value was changed from 1.1 to 1.05, and the p value from 0.0002 to 0.005. Data exported to Microsoft Excel for examination included transcript detectable (Present/Absent) calls and pairwise transcript change (Increase/Decrease /No change) calls. For 13,490 unique, unambiguously characterized genes all 9 pairwise comparisons were made between the three control and three experimental samples. A plausible pairwise change occurred if the sample with higher expression was detectable (called Present) and GCOS software made a change call (increase or decrease). For 1,674 genes with at least 4 of 9 plausible pairwise changes between groups all 6 values were ranked and 344 genes were selected where one group had a rank sum > 13. This condition requires that the group values were perfectly separated, or that the lowest value in the higher expressing group overlapped with the highest value in the lower expressing group. Finally, 181 genes were selected for which the mean of all valid pairwise changes was at least 1.3 fold. A further 19 genes fulfilled all the above conditions but showed some ambiguity in their annotations. Of the total of 200 genes, 86 showed increased expression with VZV while the remaining 114 showed decreased expression. 191 were mapped to the Ingenuity Pathway Analysis (IPA; build 321501M) database.
**Luciferase assay.** Assays were performed according to manufacturers recommendations (Promega Madison, WI). Briefly, cells were scarped off of plates and lysed using 1x freshly prepared Reporter Lysis Buffer with one freeze thaw cycle. Lysates were clarified by centrifugation at 12,000xg for 2 min at 4°C. Supernatants were stored at -80°C until use. Samples were thawed and 20 µL were added to 96-well plates followed by 100 µL of substrate and mixed. Luciferase expression was analyzed on 1450 Microbeta plate reader (Perkin Elmer, Shelton, CT). Wells containing only lysis buffer were used to establish baseline fluorescence and uninfected cells as negative controls. Data presented is normalized to negative controls.

**Primary rat tissue culture.** Rat tissue was excised from euthanized naïve animals. Lung, kidney and glabrous hindpaw skin were removed and placed on ice. Tissue was minced with scissors and added to a dissociation solution containing 0.2% Trypsin (Gibco), 0.1% Collagenase (Sigma), and 400 mg/mL DNase-I (Qiagen) in Hank’s Buffered Salt Solution-CMF (Gibco). Cells were dissociated at 37°C for 30 min, then centrifuged at 5000xg for 5 min at 4°C. This process was repeated on pelleted tissue. Dissociated primary cells were plated on 6-well plates coated in 0.1% gelatin in Dulbecco’s Modified Eagles Media supplemented with (MEM: Gibco-Life Technologies Inc.) supplemented with 10% fetal bovine serum (FBS) with antibiotic and antimycotics mixture (Sigma). Media was changed the following day to remove contaminating red blood cells.

**Sections.** Animals were euthanized and perfused with formalin. Glabrous footpad skin was removed at 10 days post infection and placed in formalin overnight at 4C. Samples were then switched from fixative to sucrose and left overnight at 4C. Samples were then positioned in OCT
compound (Sakura, Torrance, CA), frozen in liquid nitrogen, and stored at -80°C until sectioning. Blocks were removed from -80°C and sectioned into 30µm sections using a cryostat at -20°C.

**Immunofluorescence.** Cells and sections were stained using established protocols. Briefly, all samples were fixed in 4% PFA for 15 minutes at room temperature, washed twice with PBS, and permeabilized with 0.2% TritonX-100 for 5 min at room temperature. All samples were blocked in 10% Heat Inactivated Goat Sera (HIGS) for 1 hr at room temperature. Primary antibodies: anti Ku-86 [mouse monoclonal 1:50 dilution (Santa Cruz, Santa Cruz, CA)] and anti PGP9.5 [rabbit polyclonal 1:1000 dilution (AbD Serotec, Raleigh, NC)] were diluted in 10% HIGS and incubated with tissue overnight at 4°C on a rocking platform. Samples were washed three times with PBS + 0.1%Tween-20, secondary antibodies conjugated to Alexafluor fluorophores [1:800 dilution (Life Technologies Inc.)] for 1hr at room temperature and subsequently washed three time with PBS. Cultured cells were imaged on a Nikon Eclipse TE2000-E microscope equipped with a 20x air objective. Nuclei were stained using NucBlue Live Cell Stain (Life Technologies Inc.). Sections were imaged on Olympus IX81 FluoView 1000 confocal microscope (Olympus, Central Valley, PA) with a 20x oil (refractive index 0.85) objective. Images were processed in Fiji is ImajeJ (FIJI, open source), where lengths were converted from pixel lengths to millimeter. All PGP9.5+ neurites that reached from the dermis past the stratum basale layer of the epidermis were counted.

**Cell lysates and western blot.** Cells were scraped into lysis buffer (50mM Tris, pH7.4, 150mM NaCl, 5mM EDTA, 1% Triton X-100) and sonicated for 5 seconds, and finally boiled for 10min. Lysates were stored at -20°C until needed. 10μl of lysates were run on a 7.5% acrylamide gel at 70V for 2.5 hours. Protein was transferred to a PVDF membrane (Millipore, Billerica MA) at
100V for 1 hour at room temperature. Membrane was then blocked in Odyssey blocking buffer (LI-COR, Lincoln NE) overnight at 4C on a rocking platform. Blots were probed for ORF9 phosphorylated species using an anti ORF9 antibody (rabbit polyclonal diluted to 1:2000 in Odyssey blocking buffer) for 1 hour at room temperature on a rocking platform. Membranes were then washed three times with 1x Tris buffered saline with 0.1% Tween 20. A secondary antibody (goat anti rabbit conjugated to IRDye 800CW (LI-COR) at 1:20,000) was used to detect bound primary antibody. Membranes were then washed three times with 1x Tris buffered saline with 0.1% Tween 20 and imaged on an Odyssey Imager (LI-COR).

**Statistical Analysis.** All statistical tests were performed on GraphPad Prism 5 software. All curves were plotted and the area under the curve (AUC) calculated for each animal. Mean Area Under the Curve was then compared between groups using a One-Way ANOVA with Tukey’s multiple comparison test comparing all groups to each other. Individual time points were compared using One-Way ANOVA with Dunnett’s multiple comparison test to the specified control group.
4.0 ANALYSIS OF VZV INFECTION AND GENE EXPRESSION AND EFFECTS VZV INFECTION ON THE HOST IN A RAT MODEL OF VZV INDUCED PAIN

The majority of this chapter will be used as the basis of a manuscript for submission, authored by: Jean-Marc G. Guedon, Michael B. Yee, Mingdi Zhang, Stephen A. Harvey, William F. Goins, and Paul R. Kinchington.

TNF soluble receptor data is from an accepted review in the journal Molecular Pain authored by Jean-Marc G. Guedon, Joseph C. Glorioso, Paul R. Kinchington, and William F. Goins.

This chapter was written by JGG and PRK with edits from SAH and WFG. All animal data was collected and analyzed by JGG with the help of WFG, MZ, and PRK. JGG performed all immunohistochemistry and microscopy along with the analysis of that data. MBY generated the luciferase and fluorescent viruses, dissociated and analyzed viral infection of primary tissue. SAH performed gene array analysis.
4.1 ABSTRACT

Reactivation of the herpesvirus Varicella Zoster Virus (VZV) from a neuronal latent state results in herpes zoster, for which the most common complication is pain. Pain associated with acute zoster progresses in a significant fraction (>30%) of patients to a more debilitating and protracted pain state termed post herpetic neuralgia (PHN). Insights into treatment for pain and PHN have been gained from a rat pain model, in which VZV induces prolonged nocifensive behavioral indicators of pain following VZV inoculation into the footpad. Here, we address aspects of rat permissivity to VZV, viral access to the sensory dorsal root ganglia (DRG) and host cell changes that result. VZV infected primary rat cultures from multiple organs show a post entry block in VZV replication with only partial viral gene expression. Nevertheless, rats inoculated with VZV show a reduction of peripheral PGP9.5 positive-neurite innervation of the injected ipsilateral paw skin not present in contralateral skin or in rats receiving uninfected cells. At the corresponding DRG, low levels of VZV DNA were detected, peaking at 5 days post infection and diminishing thereafter without detectable virus infectivity. Limited transcription from candidate immediate early and early genes was detected. Gene array analyses revealed that changes in host expression occurred in ipsilateral DRG of rats exhibiting hypersensitivity, with 84 up regulated and 116 down regulated genes. These included qRT-PCR validated transcriptional up regulation of TRADD, NTRK2, TRPV1, and down regulation of CALCA. With an observed increase in immune response factor we also who that VZV-induced hypersensitivity can be partly relieved by peripheral inoculation with an HSV vector expressing TNF soluble receptor. These data suggest that VZV inoculation into the rat may be abortive, that infection of cutaneous afferents
results in viral DNA and gene expression in the DRG soma, and that this expression results in peripheral, central, and immunological changes associated with pain signaling.
4.2 INTRODUCTION

Herpes zoster, a painful and debilitating disease most often seen in the elderly and immune compromised, is the clinical manifestation of reactivation of the human herpesvirus varicella-zoster virus (VZV) from a neuronal latent state that was established during the primary infection and disease, chickenpox. Zoster affects some quarter of the population in their lifetime with incidence rising with age and/or decline of cellular immune status caused by senescence, disease or its treatment 27,137. Of the numerous neurological complications associated with zoster, the most common and major cause of morbidity is pain. Almost all zoster patients suffer some acute pain near the timing of skin lesions, with >90% of unvaccinated individuals seeking pain alleviating medication 40,137. However, more problematic and difficult-to-treat chronic pain states develop in a third of zoster cases, termed post herpetic neuralgia (PHN), which often become severe as to lead to disparate secondary consequences that affect quality of life issues, such as depression, withdrawal from society, and even suicide. While there is a vaccine for zoster it is only partially effective, reducing zoster incidence by 51% and the “burden of illness” (which includes pain and PHN) by 66% 138. A significant patient fraction have been reported to gain little to no relief from any treatment strategy, often at the cost of unwanted side effects. Most of the adult population over 30 in the USA harbor wild-type VZV within their ganglia, and are thus at risk for the development of zoster and PHN. The incidence of zoster is increasing and therefore it remains an urgent public health need to find the cause of VZV-induced pain and PHN, and to develop more effective treatments 139,140.
Why zoster is so frequently complicated by pain is not clear, nor is it known why a third of zoster cases develop the more protracted pain of PHN. Studies of human patients have led to several theories. It is clear that reactivation of VZV results in extensive intraganglionic spread and involvement of many neurons and satellite cells, which permit the large scale delivery of virus to the periphery and the large lesions typical of zoster. The resulting inflammation occurs not only at the periphery, but also at the ganglia, resulting in a persistent ganglionitis. Examination of DRG from cadavers with zoster and PHN at the time of death has suggested that zoster results in changes in neuronal anatomy, physiology and circuitry. However, the understanding of the VZV-induced chronic pain has been difficult to experimentally investigate because the high degree of human species specificity of VZV precludes small animal modeling of latency, reactivation and zoster-like disease.

An exception to modeling of VZV induced disease is the infection of the rat, which has been used as a preclinical model of VZV-induced pain. Inoculation of live high titer cell-associated VZV into the glabrous region of the footpad induces measurable and quantifiable nocifensive behaviors of prolonged duration, which partially recapitulate mechanical and thermal hypersensitivity seen in many PHN patients. This model has permitted the evaluation of novel as well as established pharmacological agents for the treatment of VZV-induced pain, as well as treatment by ganglionic gene delivery and expression approaches. Virology in the model has not been extensively evaluated. We have previously reported that viral gene expression is required for the onset of pain behaviors, and expression of viral proteins in the corresponding DRG of the inoculated footpad have been observed. However, the level of expression and number of neurons expressing VZV proteins has varied extensively from a majority to only a small percentage. Intriguingly, two studies report the maintained
development of prolonged VZV-induced pain indicators in animals undergoing concurrent treatment with high dosing of the antiviral acyclovir from infection, which suggests that VZV triggers pain in the model without the need for ongoing viral genomic replication\textsuperscript{118,120}. This also suggests that primary viral infection may initiate changes in the host that are sufficient for the development of the prolonged pain response in this model.

Here we further address this model by evaluating permissivity to VZV infection and the changes associated with VZV inoculation in rats and the development of pain-like behaviors. We show that primary cell cultures derived from multiple tissues of Sprague-Dawley rats are restrictive for full VZV replication, with the block occurring at a post entry stage and after the initiation of viral gene expression. VZV inoculation \textit{in vivo} at rat footpads that normally lead to the onset of pain behaviors result in low viral DNA delivery to the corresponding DRG and limited gene expression there. However, VZV induces peripheral neurite retraction at the skin and also induces changes in the host transcription in DRG that innervate the inoculated footpad. These include candidate genes induced in immunity. Accordingly, VZV-induced hypersensitivity was reversed in animals that received an HSV vector expressing soluble TNF receptor, but was not in those inoculated with a control vector. We conclude that pain in this model is unlikely to be the consequence of persistent viral replication in the DRG, but may be the result of prolonged changes triggered by abortive viral gene expression events occurring at the periphery and DRG upon infection.
4.3 RESULTS

4.3.1 A post-entry block to VZV permissivity in primary rat tissue cultures.

Animals inoculated with VZV develop hypersensitivity to both mechanical and thermal stimuli (Fig 3, thermal data not shown). While the rat model of VZV-induced PHN has been established as a preclinical model for testing novel analgesics and treatment strategies involving ganglionic delivery of pain modulators, no other sign of pathogenesis, skin disease or other external clinical sign develops. To our knowledge, there have been no reports of clinical signs (other than prolonged sensitivities) in rats inoculated with VZV. Attempts to detect viral replication in the inoculated footpad and corresponding DRG, as well as from PBMC fractions and spleen, have not proved fruitful (data not shown). We therefore asked if the rat was permissive for VZV infection, as it is generally understood that VZV is highly specific for its human host. Primary cultures were established from tissues from Sprague Dawley rats, the strain used in our VZV pain model studies. Cultures were established from glabrous paw skin, lung, kidney and DRG, grown to near confluence and infected with VZV by sparse overlay of highly infected MeWo cell associated stocks, at a ratio of approximately 1 infected MeWo cell per 300 uninfected rat primary cells. Primary cultures infected with wild-type VZV showed no plaque formation or visible centers of cytopathic effect over 10 days (data not shown). To more closely monitor infection, we infected primary cultures with a recombinant dual fluorescent VZV, in which both copies (ORFs 62 and 71) of the gene encoding the immediate early expressed IE62 transactivator (homologous to HSV ICP4) were tagged with mCherry, and the ORF23 late expressed gene encoding the capsid protein (homologous to HSV VP26) was tagged with green fluorescent protein (GFP). This dual fluorescent virus replicated in permissive (MeWo and
ARPE19) cells to levels indistinguishable from wild type VZV (data not shown). Live cell imaging of the same fields in primary cultures of lung fibroblasts (Fig 4) at 24hpi revealed the formation of small foci of RFP and occasionally GFP positivity involving 3-13 cells, suggesting VZV can infect rat cell primary tissue monolayers (Fig. 4A). However, these did not increase in size or spread further to other cells by 48 or 96h pi, and started to lose fluorescence by 120hpi. Cultures fixed at 96hpi under conditions to maintain reporter fluorescence were co-stained for the human specific Ku86 protein to identify input MeWo cells (Fig. 4A). This revealed that the majority of RFP/GFP positive cells in these “microplaques” were not input MeWo cells as they did not show Ku86 immunoreactivity, given that most fluorescent cells contained mCherry signal, indicative of IE62 gene expression, and only a fraction displayed detectable GFP signal, signifying ORF23 expression. We take this to indicate that primary rat cultures can be infected by VZV, and some viral gene expression is permitted, but productive replication is blocked and prevents further spread.

![Graph](image.png)

**Figure 3: Sprague-Dawley rats inoculated with VZV become hypersensitive to mechanical stimuli by 8 days post infection.** Animals were pretested prior to infection for baseline MA responses, and then inoculated (n=3 per group) at day 0 with 2x10^5 PFU of pOka (VZV) or uninfected cell equivalents (Control cells). Animals
were evaluated for mechanical allodynia (MA) scores depicted as the ratio of ipsilateral to contralateral responses. Mean±SEM plotted. This study is representative of multiple similar studies.

To support these data we developed two additional VZV reporter recombinant viruses: one in which firefly luciferase (fLuc) was expressed as a “fusion” protein to the ORF63 gene encoding the immediate early protein IE63, and a second VZV in which renilla luciferase (rLuc) reported the expression of the VZV ORF9 tegument protein. Our strategy used T2A ribosome skipping motifs to permit reporter expression from the native ORF loci and promoters, but as separated proteins. The T2A motif results in the VZV protein containing a small 23-residue addition to the C terminus, while only a single residue is added to the luciferase reporter, and did not affect its enzymatic activity. These viruses also showed efficient replication in VZV permissive cultures at levels similar to wild type virus (data not shown). In permissive MeWo and ARPE19 cells infected with these mutant VZV at 1 infected per 50 uninfected cells, fLuc activity reporting expression of VZV IE63 (Fig. 4C) increased over time and showed over a hundred fold increase between 4 and 72hpi, representing the amplification and spread of virus in the culture. In contrast, fLuc activity in non-permissive murine B6WT3 fibroblasts initially showed a burst of expression that only marginally increased over time (Fig. 4C). In rat primary cell cultures derived from rat lung and kidney, IE63-reporter activity was similar to that seen in B6WT3 cells, and did not undergo the rapid increase reflecting VZV cell-to-cell spread. A more dramatic reduction of luciferase activity was seen in rat cultures infected with VZV expressing ORF9-reporter. In VZV permissive lines, rLuc activity greatly increased over time, with slightly delayed kinetics compared to that for IE63 (Fig. 4D). However in rat cultures, ORF9 reporter activity at 18hpi remained at levels similar to input alone. Assessment of viral DNA copies in infected rat primary cultures indicated no increase above input, as seen in VZV permissive
cultures (Fig. 4E). We take these and the previous results to suggest that rat primary cultures support initial infection with some viral gene expression. Similar results were seen in cultures established from skin and other tissues (data not shown). These indicate that full productive infection is blocked in cells of rat origin, most likely at a stage after the initiation of IE gene expression and before viral DNA replication. Thus it appears the rat is not permissive to full VZV replication and virus production.
Figure 4: VZV exhibits infectivity, limited gene expression and no DNA amplification in primary rat tissue culture. Primary tissue was excised from naïve rats, dissociated and cultured. Cultures were infected with 500
pfu of VZV-63LucZeo and analyzed by live cell microscopy, immunofluorescence, and luciferase activity in cells harvested at the various time points shown. (A) VZV infection of primary rat skin fibroblasts. Primary rat tissue cultures were infected with 500 pfu of cell-associated VZV expressing fluorescent genes mCherry and GFP tagged to both copies of IE62 and ORF23, respectively, were assessed by live cell imaging of the same fields at 24 and 72hpi. At 96hpi, cultures were fixed and stained for human specific anti-Ku86 (purple) to identify human cell inocula under conditions that maintained GFP and mCherry fluorescence. (B) Diagram of luciferase expressing VZV. VZV IE63fLuc recombinant virus has firefly luciferase (fLuc) under the control of the native ORF63 promoter via a T2A ribosomal skipping sequence downstream of ORF63, and ORF9rLuc recombinant virus contains renilla luciferase (rLuc) under the control of the ORF9 promoter via a T2A ribosomal skipping sequence. Luciferase expression over time in permissive cell lines (MeWo and RPE), non-permissive mouse derived cell line (B6WT3), and primary rat tissue cultures (lung and kidney) infected with IE63fLuc (C) or ORF9rLuc (D) are shown. (E) Viral copies per 100ng of total extracted DNA from primary rat tissue cultures were determined by qPCR utilizing primers directed at ORF29. Input represents cell-associated virus alone, plated at the time of infection and harvest at 48hpi. These data are representative of two independent experiments.

4.3.2 Peripheral changes in rats showing pain include a reduced innervating axonal density at the glabrous paw skin.

We then addressed what changes occurred in the rat model after VZV infection that correlate with the hypersensitivity that develops. It has been reported that skin biopsies of patients suffering from severe PHN exhibit reduced peripheral innervation compared with non-PHN zoster patients \(^{141,142}\). Rats were therefore inoculated with VZV and allowed to develop MA responses as detailed previously, which normally develop within 1 week post inoculation and extend beyond 40 days post infection (Fig 3) \(^{30,119}\). Control uninfected cell inoculated rats or those receiving VZV and showing hypersensitivity were sacrificed at 9 dpi and the glabrous footpad skin was removed, preserved, cryosectioned at 30-\(\mu\)m and stained for the pan-neuronal
marker, PGP9.5. PGP9.5+ neurons that projected from the dermis past the stratum basale layer of the epidermis were counted from confocal images of Z-stack projections for each footpad and are presented as neurites per millimeter of stratum basale. We observed a reduction in PGP9.5+ neurites/mm of stratum basale between control cell inoculated animals (18.92±1.26) and VZV-infected animals (14.73±1.10) (Fig. 5) with the groups determined to be significantly different by the two-tail t-test (p=0.0203). These results were not seen in animals receiving VZV that has been inactivated by UV irradiation, which do not develop hypersensitivity pain responses. These findings indicate that VZV inoculation in the rat induces neurite retraction, similar to that reported in human PHN patients, and describes one potential consequence of VZV infection in the rat that may contribute to nocifensive behaviors in the rat.
Figure 5: VZV inoculation into rats results in reduced peripheral innervation at the ipsilateral footpad. Animals were inoculated with either control cells or VZV-infected cells. At 9 dpi animals were euthanized and glabrous hindpaw skin removed and cryosectioned into 30-µm transverse sections. (A) Sections were stained with antibody to PGP9.5+ (green) present on neurites and NucBlue live cell stain to identify nuclei (blue). Neurites that innervate from the dermis past the stratum basale layer of the epidermis (white line) were counted (white arrows). (B) Quantification of PGP9.5+ neurite density (n=3 animals, 4 sections counted /animal) per mm of stratum basale interface (length determined by FIJI). Statistical significance determined by two tail unpaired T-test with *p<0.05 [Mean±SEM plotted for B].
4.3.3 VZV DNA accesses the dorsal root ganglia and undergoes limited transcription.

We then examined changes in the DRG induced by VZV inoculation. In humans, VZV primary infection at the skin results in the infection of axonal termini infiltrating the skin, followed by retrograde transport of virus to the ganglia and the onset of a persistent state of latency. While our evidence suggests the rat is not fully permissive for VZV infection, we hypothesized that the post entry block to replication may still permit the infection of peripherally innervating axons from the primary inocula, which may result in changes in gene expression in neurons of the dorsal root ganglia. Rats have been shown to model some aspects of latency, particularly in the cotton rat, where VZV DNA is detectable in the corresponding DRG for some periods of time\(^{75,92,93,114,115,143,144}\). We first sought to address if VZV reach the DRG by assessing viral genome copy numbers in VZV inoculated animals at 2, 5, 7, and 9 days post infection (dpi). L4,5 DRG were harvested and nucleic acids extracted from the pooled ganglia. DNA was assayed by quantitative PCR for ORF29 and compared to standard curves of known concentrations of VZV BAC DNA to establish genome copy number. Viral genome copies could be consistently detected in a fraction of infected animals as early as 2 dpi and significant DNA lasted until 9 dpi (Fig. 6A). However, detectable genomes were not found above background levels (sensitivity of 200 genomes/DRG) at 14, 28, 56 dpi (data not shown). These results indicate that VZV does access the DRG, but that viral DNA reaching the DRG are low, may be cleared, and do not persist at levels above background. Thus the prolonged hypersensitivity seen in the rat following VZV inoculation may be the result of VZV induced events that persist after VZV has been cleared.

We next sought to determine if any viral gene expression can be detected at the rat DRG. Some groups have suggested there is viral gene expression in DRG of rats by \textit{in situ}
hybridization and immunohistochemistry\textsuperscript{43,109,116,117} at various times post infection including the products of VZV immediate early (IE) gene 63, IE62 and IE4 in rat ganglia have been reported. However, reports of VZV protein expression in latently infected human ganglia have recently been clouded and even refuted by indications of cross reactivity of many antibodies with blood group antigens\textsuperscript{24,145}. In the rat, both IE62 and IE63 protein have been reported in very high fractions of DRG neurons, which is not consistent with the low numbers of viral genomes detected in our work. We thus examined VZV RNA expression of IE62 and IE63 at 2, 5, 7, and 9 dpi, which represent the two genes that may be expressed in non-permissive rat cultures. No-RT controls of the same RNA samples run in parallel showed only minimal DNA contamination, establishing that any increase in signal observed were the consequences of amplification of VZV RNA. We observed detectable increases in IE62 and IE63 expression at early time points compared to control animals and to the reference gene GAPDH. We also detected some expression of candidate VZV early genes, from VZV ORF47 (UL13) and ORF29 (ICP8). However, expression of two predicted VZV late genes, including from VZV ORF14 (gC) and ORF21 (UL37) were not detectable (Fig 6B). These data indicate that VZV inoculation of the rat, most likely results in an abortive axonal infection and the delivery of low levels of viral DNA to the DRG. These transmitted genomes undergo a limited level of short-term transcription that includes the transcripts for the viral immediate early and possibly early genes.
**Figure 6: VZV genome copy number and transcription from ipsilateral innervating dorsal root ganglia (DRG) in rats exhibiting VZV induced hypersensitivity.** DRG were isolated from naïve animals or animals infected with VZV at 2, 5, 7, 9 days post infection (dpi). (A) Viral copy number was determined by SYBR green qPCR to ORF29 and back calculated to display copies per L4,5 DRG (n=12 controls, n=8 experimental groups). Viral gene expression of genes corresponding to the different kinetic classes including immediate early (ORF62, 63), early (ORF47, 29), and late (ORF14, 21) (B) were determined by Taqman qPCR using specific primer-probe sets for each gene displayed as $2^{-\Delta\Delta Ct}$ to the GAPDH reference gene (n=3). No RT controls were used.
4.3.4 Host gene transcription changes at the ganglia induced by VZV infection.

Given that our data indicates that VZV inoculation of the footpad results in limited virus access the DRG, limited level of gene transcription and induced changes at the periphery, we next addressed the possible changes in host gene transcription due to VZV inoculation. Animals inoculated with VZV-infected cells were verified for VZV-induced mechanical and thermal hypersensitivity at day 10 dpi, that was not seen in animals receiving control-uninfected cells (Fig. 7A). We performed gene array analyses of whole DRG using total RNA from these same control and VZV-inoculated animals at 10 dpi. Animals were sacrificed and L4,5 DRGs were removed, and RNA extracted from animals was pooled. Each group represents 2 animals that were MeWo injected or VZV-inoculated, with 3 replicates per group. Analysis of the data reported over 200 genes were significantly (p>0.001) differentially regulated in the ipsilateral DRG of VZV inoculated rats compared with control cell-inoculated animals. A selection of differentially expressed genes are shown in figure 7B and C. VZV infection resulted in 86 genes to be up regulated (Fig. 7B) and 114 genes to be down regulated compared to control cell-inoculated animals (Fig. 7C). Of 13,490 unique, unambiguously characterized genes surveyed by the microarray, only 200 satisfied our requirements for ≥ 1.3 fold mean change that was statistically viable over the 3 replicates (Fig. 7B and C). Of interest, several genes related to immune function and pain were differentially regulated by VZV infection and validated by qRTPCR (Fig. 8A,C,E,G) including the TNF receptor associated death domain (TRADD, Control Cells 173.5±11.2 and VZV 214.7±1.1 with a fold change of 1.23), neurotrophic tyrosine
kinase receptor type 2 (NTRK2 or TrkB, Control Cells 402.6±4.2 and VZV 512.1±29.2 with a fold change of 1.27), transient receptor potential cation channel, subfamily V, member 1 (TRPV1, Control Cells 370.8±19.9 and VZV 458.6±28 with a fold change of 1.24), and calcitonin-related polypeptide alpha (CALCA or CGRP, Control Cells 7699±205.6 and VZV 7024±91.4 with a fold change of 0.87). These fold changes were also confirmed by qRT-PCR to the respective sequences to validate gene array analyses (Fig. 8B,D,F,H). Moreover, the observed differential regulation of these specific genes sheds light onto the potential mechanisms of how VZV might induce pain and the resulting behavioral changes observed in VZV-inoculated animals, since select genes that overlap those seen here have been shown to be differentially regulated during VZV infection in vitro and in rats, as well as in various other pain models and are well known pronociceptive genes\textsuperscript{146-148}. We conclude that VZV induces considerable anatomical and transcriptional changes in neuronal cells that correlate with the onset of hypersensitivity associated with the development of pain.
Figure 7: Host DRG gene transcription is affected by peripheral VZV inoculation. Animals were injected with control cells or VZV-infected cells. (A) At 10 dpi animals infected with VZV had significant changes in their mechanical (gram, left panel) and thermal (seconds, right panel) nocifensive paw withdrawal thresholds consistent with pain sensation. Affymetrix array analysis heat-map from RNA isolated from these DRGs displays genes that were (B) up regulated (red) or (C) down regulated (blue) by VZV infection. Statistics used are two tailed t-test *=p<0.05, ***=p<0.01 [Mean±SEM plotted for A and B].
4.3.5 VZV inoculated animals treated with a HSV vector expressing TNFα soluble receptor exhibit reduced hypersensitivity.

Since TNFα, IL-1β and IL-6 are the main pro-inflammatory cytokines secreted by various activated immune and glial cells in other models of inflammatory and neuropathic pain. Their alteration in VZV infected DRG suggested a potential role for TNFα signaling in VZV-induced pain. This provided a new target for intervention of VZV-induced pain. The significant increase in immune signaling genes included TRADD and IL7 receptor, further suggesting immune signaling during VZV-induced hypersensitivity (Fig. 7). To clarify the role of TNFα in VZV-induced pain, we tested HSV vector-mediated expression of the human TNFα soluble receptor (sTNFR) for its effects on VZV-induced hypersensitivity. Animals inoculated with VZV at day 0 rapidly established robust indicators of hypersensitivity (Fig. 9B and C). Animals were treated at day 21 with either control HSV vector (T0ZH) or HSV expressing sTNFR (T0TNFαsR) (Figure 9) at a dose of 10^8 pfu. Animals inoculated with HSV expressing sTNFR showed a rapid and sustained decrease in hypersensitivity within days following T0TNFαsR administration, while control vector inoculated animals did not. Mechanical paw withdrawal thresholds increased as early as 7 days after HSV sTNFR-expressing vector inoculation and lasted until 23 days post HSV vector inoculation (Figure 9B) or 44 days post the introduction of VZV-infected cells. However, after 35 days post HSV vector treatment the mechanical hypersensitivity began to spontaneously resolve in VZV-infected and untreated animals, as we
and others have seen previously. Compared to the effects of vector-mediated sTNFR on mechanical pain, thermal relief took longer to respond to sTNFR HSV gene therapy, but the response lasted about the same length of time. With the thermal pain response relief started at 23 days post HSV sTNFR-expressing vector inoculation (44 days post the introduction of VZV-infected cells) and lasted till 41-dpi after HSV vector injection (Figure 9C). Taken together these results indicate that TNFα plays an role in VZV-induced pain and opens up a new target for analgesic relief of VZV-induced pain.
Figure 9: Alleviation of VZV-induced hypersensitive nocifensive behaviors by administration of TNFα soluble receptor (sTNFR) expressed from a replication defective HSV vector. A diagram of the vectors used in this study. All vectors lack ICP27 and ICP4, and contain an expression cassette in the UL41 locus (A). All animals (n=4) were infected with VZV at day-0 (B). At 21 days post VZV infection (denoted by arrowheads),
animals were inoculated with either HSV sTNFR vector (TNFαsR) or control HSV vector (T0ZHG). Animals were monitored for hypersensitivity to (B) mechanical (gram weight) and (C) thermal sensitivity (ratio of ipsilateral/contralateral paw withdrawal latencies) and the data is plotted as Mean±SEM. Mean area under the curve was calculated for the region represented by brackets for each animal with the Mean±SEM plotted. Statistics determined by two-tailed T-test (*=p<0.05, **=p<0.01).

4.4 DISCUSSION

The most common descriptive of chronic pain that follows a third of VZV reactivated disease and zoster is allodynia, is defined as hypersensitivity and pain resulting from normally innocuous stimuli that does not usually evoke a pain response. Patients develop intense and debilitating pain from stimuli such as touch by clothing, hair or gust of wind, that may remain painful even after removal of the stimuli. A smaller fraction of PHN patients show increased sensitivity to heat or cold. These hypersensitivities are mimicked in rats inoculated with VZV at the footpad, which are manifested as measurable decreased paw withdrawal threshold responses to mechanical and thermal stimuli. The rat model has been developed in strains Wistar and Sprague-Dawley, and have been extensively used to test treatments for alleviation of the chronic hypersensitivity. However, there is a paucity of experiments characterizing the virology in the model and what neuronal changes occur that correlate with, and might lead to, hypersensitivity after VZV inoculation. Intriguingly, the data presented here suggest that rats are not permissive for VZV productive infection, but that the block in viral replication appears to be at least partially a post entry event. VZV is generally considered to be highly human specific, and most non-primate cells do not support VZV replication. Likewise, and in contrast to HSV-1, most small animal models, with the exception of pain in rats and guinea pig reactivation model,
show no detectable consequences of VZV infection, disease, or permissivity. The mouse is highly refractory to VZV and infection of SCID mice harboring human tissues results in VZV only replicating in the human tissue implants\textsuperscript{156-158}. In the rat, the only sign of infection is the prolonged hypersensitivity, so we considered it important to assess rat permissivity to VZV infection.

While primary cultures established from several organs and tissues of the rat did not support productive VZV replication or plaque formation, our newly developed reporter viruses clearly established that VZV could enter rat primary cells and initiate viral gene expression. We showed this by both the production of fluorescent proteins and by the expression of quantifiable enzyme activity (Fig 4). However, while VZV may enter and express VZV IE genes in rat cells, the work suggests late gene expression minimal or absent. In particular, the promoter activity of the late gene encoding the ORF9 tegument protein is remarkably inactive in rat cells (Fig 4D). These data suggest the block is perhaps at the stage of viral DNA replication, which would effectively limit the maximal expression of VZV late genes. Indeed, we found no increase in genome copies after VZV inoculation. This block is reminiscent of VZV in cells of mice and hamster origin, where VZV may enter but not efficiently express late proteins\textsuperscript{159}. The post entry block would also be consistent with our work showing that inoculation of animals with UV irradiated virus (which would interfere with gene expression) inhibits the development of pain indicators\textsuperscript{119}, but treatment of VZV inoculated rats with high dose acyclovir to block viral DNA replication does not stop pain development\textsuperscript{116,120}. While primary cultures might not fully reflect the behavior of VZV in the rat, we have not succeeded in obtaining evidence \textit{in vivo} of VZV replication. Infectious VZV has not been obtained from any rat tissues within 48hr post VZV inoculation. Thus, we consider the hypersensitivity that develops to be consequence of those rat
host cells that are primarily infected by the human cell-associated input in the inoculum. This would almost certainly include infection of peripherally innervating neuronal termini, and is supported by our finding that VZV DNA reaches the ipsilateral DRG rapidly after footpad inoculation, and undergoes limited gene transcription (Fig 6). Quantitative PCR indicated low viral genome copy numbers in the DRG, and RT PCR detected expression of immediate early genes 62 and 63 as well as candidate early VZV genes. Other groups have also found VZV DNA in rat DRG following peripheral VZV inoculation, although quantification was not undertaken. Sadzot-Delvaux et al (1995), found VZV DNA in rat sensory ganglia using in situ hybridization out to 7 dpi, similar to our finding of VZV DNA out to 9 dpi \(^\text{109}\). However our data suggests total genome load is low, at approximately 300 copies per L4,5 DRG. Similar levels are also found in the cotton rat “latency” model at 40-50 copies per 500ng of DNA \(^\text{115,144}\), within the magnitude seen in our studies (DNA yield from rat L4,5 DRG is approximately 3.5-4µg). Regarding viral gene expression, we detected quantifiable immediate early gene transcription, which would be observed even with a post entry block of viral replication. IE62 and 63 transcripts have been found in rat DRG by in situ hybridization and the proteins have been found using immunohistochemistry (IHC) by other groups \(^\text{43,109,111,117,118}\). However, low ganglionic levels of VZV DNA are not consistent with the high fraction of neurons reported as positive for IE62 and 63 immunostaining. Hasnie et al reported 72-76% of NeuN staining neurons as positive for IE62, while Garry et al reported that 82% of peripherin staining neurons were IE62 positive \(^\text{117,118}\). We consider the values seen by others may be the result of staining problems similar to that encountered with the analyses of human ganglionic sections for viral latent proteins \(^\text{24,145}\). Our recent re-assessment of IE62 using highly absorbed antibodies suggests IE62 immunoreactivity only occurs in tens to a few hundred neurons per DRG \(^\text{30}\). The IE63 fLuc reporter VZV in the rat
shows little to no expression in any tissue *in vivo* (data not shown). We conclude that VZV can infect axonal endings at the periphery, gains access to the DRG, and likely undergoes restricted gene expression. This may nevertheless be sufficient to induce the changes associated with the development of nocifensive behaviors.

While the mechanisms underlying the development of prolonged nocifensive behaviors are not yet clear, axonal VZV infection has consequences on neuronal biology. We report here the findings that both peripheral and ganglionic changes develop as a result of VZV inoculation. At the periphery, we found a significant decrease in PGP9.5+ staining neurites extending from the dermis past the stratum basale layer of the epidermis in VZV-infected (14.73±1.10/mm stratum basale) compared to rats inoculated with control cells (18.92±1.26/mm stratum basale) (Fig 5). These findings echo similar findings in human patients, where there was significantly reduced peripheral innervation at the skin of the affected dermatome in zoster patients, and even further reduced innervation when the patients are experiencing PHN.

Normal human innervation levels of around 2000 neurites/mm² were reduced in zoster patients to mean innervation density of 1569±230 neurites/mm², while innervation densities in PHN patients were further reduced to 337±92 neurites/mm². Thus the rat model appears to mirror aspects of human zoster and PHN associated changes in peripheral neuron physiology. However it remains to be resolved whether such changes are the result of viral infection at the periphery affecting neurites directly, or neurite retraction is a consequence of VZV induced changes exerted at the sensory ganglia.

We documented changes in host gene expression to establish the platform upon which nocifensive indicators of pain might develop. Using gene array analysis on 10 dpi whole L4,5 dorsal root ganglia from animals exhibiting hypersensitivity to mechanical stimuli, we observed
200 genes that were differentially regulated upon VZV infection (Fig 7 and 8). While altered levels of expression reporting from the arrays were not high, they likely reflect a small number of neurons in a large pool of unaffected cells of the ganglia, and were statistically significant based on multiple parallel samples. Of the genes affected, we found evidence of alterations in the TNF signaling cascade, especially TRADD and TNFsr21 similar to that observed in the SCID-hu-VZV infection mouse model (Fig 8) 147. In addition, we also observed changes in the expression of several genes previously shown to play a role in nociception consistent with other chronic pain models, such as the HIV gp120-induced neuropathic pain model 148. Maratou et al observed an increase in TRPV1, NTRK2 (TrkB) isoforms and down-regulation in CALCA gene expression in animals experiencing HIV gp120-induced pain compared to sham rats 148. We observed a similar increase in TRPV1, NTRK2, and a down-regulation of CALCA in VZV-infected compared to control cell-injected animals (Fig 8). Our data fits with Maratou et al and suggests that virally induced neuropathic pain may activate similar pathways or at least result in similar downstream changes in nociceptive genes. Thus, the changes seen are consistent with a VZV induced pain state.

Lastly we tested an HSV vector that expresses soluble TNF receptor for its effect on VZV-induced hypersensitivity in rats. Such vectors have been shown to be effective in other pain models including the rat L5 spinal nerve ligation (SNL) model 161, the T11-T12 laminectomy spinal cord injury (SCI) model 162, the resiniferatoxin (RTx)-induced model of bladder nociception 163 as well as HIV gp120-induced neuropathic pain 164. VZV inoculated animals that had established mechanical hypersensitivity and were inoculated with a vector expressing TNF soluble receptor had immediate and sustained relief from mechanical hypersensitivity and, while it took longer, thermal stimuli. Interestingly mean area under the curve plots suggested that not
all animals responded to treatment. These data suggest that some PHN patients may be predicted to respond to biologics that target TNFα, a key inflammatory component. However, some severe complications due to reactivated VZV seen in rheumatoid arthritis (RA), inflammatory bowel disease (IBD), Crohn’s disease, ankylosing spondylitis, plaque psoriasis, and psoriatic arthritis patients receiving anti-TNF treatments to reduce the host immune response suggests that whole-body treatment by anti-TNF injectable biologics may not be an optimal therapy for all PHN patients.\textsuperscript{165-172}

Taken together with our previous data, we conclude that VZV footpad inoculation results in a limited, non-productive infection of rat tissue that is not capable of spreading throughout the host by productive amplification. Rather, the self-limiting infection results in a limited gene expression program \textit{in vivo}, which appears sufficient to induce the nocifensive responses. However, such infections probably include infection of axonal termini, and ganglionic delivery of low levels of viral DNA. However, VZV infection of the host DRG results in changes in host gene transcription for genes involved in immune recognition and nociception that may also be involved in the corresponding neurite retraction that is observed. We have also shown that site directed interference of TNF signaling results in quantifiable relief of VZV-induced pain in rats. What remains to be determined is the length of time needed for the neurons to properly re-innervate the periphery, if at all, which subtypes of neurons are affected, and which specific VZV genes are involved in these processes.
5.0 ORF47 IS NECESSARY AND SUFFICIENT TO GENERATE HYPERSENSITIVITY IN A RODENT MODEL OF POSTHERPETIC NEURALGIA

This chapter will be used as the basis of a manuscript for submission, authored by: Jean-Marc G. Guedon, William F. Goins, Michael B. Yee, Emily A. Scott, Mingdi Zhang, Paul R. Kinchington

This chapter was written by JGG and PRK. All animal data was collected and analyzed by JGG with the help of WFG, MZ, and PRK. MBY, EAS, and JGG generated the mutant viruses reported here. JGG performed immunohistochemistry and microscopy along with all the analysis. EAS and JGG performed western blot, sequence confirmation of mutants, and in vitro growth curve analysis.
5.1 INTRODUCTION:

Varicella Zoster Virus (VZV) is the causative agent of chickenpox upon primary infection and herpes zoster upon reactivation from latency that was established in neurons in sensory ganglia along the entire neuraxis. Unlike primary infection, zoster (or shingles) is almost always associated with pain, with greater than 90% of patients seeking medication to alleviate pain. Between 10-30% of patients go on to develop a chronic, long lasting pain state known as Post Herpetic Neuralgia (PHN). PHN patients generally describe three forms of pain, with the most common and distressing being allodynia, or pain from otherwise innocuous stimuli. VZV reactivation and its consequences have proved difficult to study as VZV is highly species specific and there is no small animal model of zoster or reactivation. However, there exists a rat model of VZV-induced pain that appears to recapitulate the allodynia observed in PHN patients. This model of VZV-induced pain has been established in rats and manifests as a chronic hypersensitivity similar to that observed during PHN \(^{111}\). Viral transcription/infection, but not DNA replication, appears to be required for VZV-induced hypersensitivity \(^{117,119,120}\). We have found that viral gene expression appears to be limited to immediate early and early genes, and expression of late genes is undetectable \textit{in vivo} (Chapter 4). These data suggest that protein(s) from immediate early or early expressed gene may be responsible for inducing pain. However, viral infection does affect host gene transcription in whole ganglia and reduces peripheral innervation in the rat. This loss of peripherally innervating neurites has been documented in Zoster and is markedly lower in PHN patients when compared with either zoster, which have reduced innervation compared to healthy controls.
Here we evaluate the two virally encoded, early expressed, serine/threonine kinases ORF47 and ORF66 for their role in VZV-induced pain. Both virally encoded protein kinases are dispensable for viral growth \textit{in vitro}, but both are known to be necessary for different stages of \textit{in vivo} spread. The ORF47 kinase is acidophilic with a consensus phosphorylation motif similar to human casein kinase II. A deduced motif of: S/T-X-D/E-D/E has been identified with a preference for acidic amino acids -1 or +1 \textsuperscript{173}. The kinase appears to have orthologs in all herpesviruses, and is similar to the UL13 kinase of HSV1. The targets for ORF47 have not been fully elucidated, but it is known to phosphorylate several viral proteins including: itself, IE62 (the major transactivators of viral gene expression), ORF9 (a tegument protein), and ORF32 \textsuperscript{131,174,175}. The only known host target is IRF3, but it seems likely there are others\textsuperscript{176}.

The second viral kinase, encoded by ORF66, is a serine/threonine kinase that is the VZV ortholog to HSV1 and PRV US3, a kinase that is conserved among the alphaherpesviruses. ORF66 is a basophilic kinase with a target serine preceded by lysines and/or arginines \textsuperscript{177}. ORF66 has several known targets including itself, IE62, Matrin 3, and HDAC 1 and 2 \textsuperscript{128,178-180}. The target motif of ORF66 overlaps with protein kinase A. Viruses deficient in ORF66 exhibit reduced growth in T cells and corneal fibroblasts \textit{in vitro} \textsuperscript{128,181}.

Both kinases are necessary for growth in certain cells \textit{in vivo}. ORF47 is necessary for productive viral growth in T cells and skin in the SCID-hu model and an \textit{ex vivo} model of human T cell VZV infection, as well as for transferring infection from dendritic cells to susceptible fibroblasts \textsuperscript{158,181,182}. Loss of ORF47 kinase function results in aberrant capsid formation that results in decreased cell free spread \textit{in vivo} \textsuperscript{67,182,183}. ORF66 is needed for efficient viral replication in T cells in a SCID-hu model but has only a slight reduction in growth in skin \textsuperscript{184,185}.
Therefore, while both kinases are dispensable for viral growth in vitro, but clearly affect virulence in vivo.

ORF9 is a late expressed, tegument-associated gene that is essential for viral growth\(^\text{186,187}\). During infection, transcripts from the ORF9 gene are readily detected and when quantified, are the most abundant viral transcripts\(^\text{188,189}\). ORF9 is the ortholog of HSV1 UL49 and shares homology with the UL49 encoded protein, VP22 of HSV1. ORF9 interacts extensively with viral proteins including IE62, gE, gI, gH, gN, ORF15, ORF23, ORF38, and ORF47\(^\text{186,190-193}\). These extensive interactions likely make ORF9 important in proper tegument and virion formation, as ORF9 hyperphosphorylation (mediated by ORF47 at Serine 84) influences virion formation and egress\(^\text{193}\).

Here we demonstrate the ORF47 gene product is both necessary and sufficient for the induction of hypersensitivity in animals. Particularly, mutants in the kinase domain affect the induction of pain by VZV. We also investigated mechanisms for this hypersensitivity and show that infection of rats with wild-type VZV reduces peripheral nerve innervation but this trend is reversed when ORF47 is not present. Interestingly, animals inoculated with an ORF9 phosphorylation mutant that alters the consensus site for ORF47 mediated phosphorylation at position 85 only generates partial hypersensitivity. Together these data suggest that ORF47 is required for pain induction, which is partly due to its role in phosphorylation of ORF9.
5.2 RESULTS

5.2.1 ORF47 kinase activity is necessary for induction of hypersensitivity.

Protein kinases play an important role in cell signaling and many are known to be involved in propagating pain signals; therefore we focused on identifying the role of the two VZV encoded protein serine/threonine kinases in VZV-induced pain. Varicella zoster virus encodes two serine/threonine kinases, open reading frames 47 and 66, and VZV deficient for either gene product have been previously described. We first assessed VZV with mutations for either ORF47 or ORF66 to inoculate animals and compare the hypersensitivity that develops to wild type VZV and controls. Rats received 2x10^5 pfu or control uninfected cells into the right hind footpad and were tested for behavioral hypersensitivity to mechanical and thermal stimuli. Animals inoculated with wild type VZV developed markedly lower tolerances to both mechanical and thermal stimuli, when compared to baseline or control cell inoculated animals (Figure 10). Rats inoculated with ORF66stop exhibited hypersensitivity similar to wild type VZV beginning at 5dpi and self-resolving at 53dpi (Fig 10A and D). However, animals injected with two different ORF47 mutants did not develop hypersensitivity to either mechanical or thermal stimuli (Figure 10B and E). The two ORF47 mutants represented one detailed previously (Cohen 47), and one that we generated in which ORF47 was disrupted by a kanamycin resistance cassette. However, animals receiving VZV in which the ORF47 gene was restored by removal of the kanamycin resistance cassette, induced responses similar to wild type VZV and ORF66stop. Together these data suggest that ORF47 is necessary for induction of VZV-induced hypersensitivity.
Figure 10: ORF47 but not ORF66 is necessary for VZV induced pain in rats. Animals were tested for baseline responses at -1 and 0 days post infection, inoculated with control cell equivalents, VZV (pOka), ORF66Stop, ORF47Stop, ORF47Kan, or ORF47Rescuent (n=5). Animals were tested for sensitivity to mechanical (A-C) and thermal (D-F) sensitivity. Data presented is representative of two independent experiments and the Mean±SEM plotted. Lines that overlap were shifted slightly up to be visible. Mean Area under the curve plotted for all animals Mean±SEM. Statistics used: (A,B, D, E) One-Way ANOVA with Dunnett’s multiple comparison test comparing MeWo to all groups (C and F) One-Way ANOVA with Dunnett’s multiple comparison test comparing pOka to all other groups. *=p<0.01, ***=p<0.001.
5.2.2 VZV with Mutations in the ORF47 kinase domain also do not develop hypersensitivity in rats.

To further delineate the functions of ORF47 that are necessary for pain induction, we tested a series of previously detailed mutants that are either deleted most of the C-terminal kinase domain (ΔCterm), or contain a point mutation in the ATP-binding domain (D-N), or a tertiary structural motif (P-S) 67. All three mutants, along with the parental vaccine Oka were used to inoculate animals to evaluate their effects on VZV-induced hypersensitivity. Animals inoculated with vaccine Oka developed mechanical hypersensitivity by 7dpi that spontaneously resolved by 31dpi. The shortened duration of hypersensitivity differs from previous studies (Figure 10) and may have been due to the use of an attenuated strain. Animals that received viruses with any mutation in the kinase domain did not develop mechanical hypersensitivity at any point during the experiment (Figure 11). Thermal hypersensitivity followed a similar trend but was not statistically different between the groups (data not shown). These data suggest that kinase activity necessary for the induction of pain, and that proper folding of the kinase domain may also be necessary.

Figure 11: ORF47 kinase domain mutants do not induce nocifensive behaviors in VZV inoculated rats. Animals were evaluated for baseline responses at 0dpi and then inoculated with either vOka, vOka 47ΔCterm
(truncated after amino acid 267), vOka 47D-N (at amino acid 283), or vOka P-S (at amino acid 333) (n=4). Animals were subsequently evaluated for mechanical sensitivity (A and B). This data is representative of two independent experiments and shows Mean±SEM plotted. Lines that overlap were shifted up to be visible. Mean Area under the curve plotted for all animals Mean±SEM. Statistics used (A) One-Way ANOVA with Dunnett’s multiple comparison test comparing vOka 47ΔCterm to all groups (B) One-Way ANOVA with Dunnett’s multiple comparison test with vOka GFP being the control column. *=p<0.01, ***=p<0.001.

5.2.3 HSV vector delivered VZV ORF47 is sufficient to generate nocifensive behaviors.

To establish whether ORF47 is sufficient to induce hypersensitivity, we utilized an HSV vector to express ORF47. The coding sequence from VZV ORF47 was engineered into a replication incompetent HSV vector at both of the ICP4 loci in a vector background shown to deliver and express genes to ganglia and that we have used to treat VZV-induced pain (Chapter 6). Animals injected with the control HSV vector did not develop nocifensive behaviors, but animals injected with HSV47VZV developed both mechanical and thermal hypersensitivity within 4 and 8 days post infection, respectively (Figure 12). Animals exhibited significantly reduced withdrawal thresholds at several time points (indicated by asterisks) and differences between mean area under the curve were also significantly reduced in HSV47VZV infected animals compared with control-injected animals. This suggests that expression of ORF47 outside the context of VZV infection is sufficient to generate hypersensitivity.

To further confirm the role of ORF47 kinase activity in the induction of pain, we developed an HSV vector which expressed ORF47 of VZV with a point mutation at amino acid 283 changed the aspartate to an asparagline (D-N) in the ATP binding domain of the kinase that ablates kinase activity. When administered to animals, HSV47VZVD-N did not induce nocifensive behaviors and animals exhibited no change in mechanical or thermal sensitivity.
when compared with parental vHG control (Figure 12). These data support our conclusion that VZV ORF47 is both necessary and sufficient to induce nocifensive behaviors in animals, and that kinase activity is necessary for the development of hypersensitivity.

Figure 12: HSV replication incompetent vectors expressing VZV ORF47 generate nocifensive behaviors when inoculated into rats. Baseline behavioral measurements were taken 3 days before inoculation and then at intervals after. Animals were injected with 10⁵ pfu at day 0 with either vHG, HSV 47VZV or HSV 47D-N VZV (n=4) (A and C). Data is representative of two independent experiments and the Mean±SEM plotted (B and D). Lines that overlap were shifted up to be visible. Mean Area under the curve plotted for all animals Mean±SEM. Statistics (A and C) One-Way ANOVA with Dunnett’s multiple comparison test comparing HSV GFP to all groups, (B and D) One-Way ANOVA with Dunnett’s multiple comparison test with HSV GFP being the control column.

*=p<0.01, **=*p<0.001.
5.2.4 **Loss of peripheral neurite innervation upon VZV inoculation is reversed in animals inoculated with ORF47 mutants.**

Humans with zoster and PHN have been reported to have reduced peripheral innervation of the affected skin\textsuperscript{141,160}. This observation is replicated in rats inoculated with VZV, which also have a significant reduction in peripheral innervation of PGP9.5+ neurites at day 10-post infection (Chapter 4). We therefore investigated whether this reduction also occurred in animals inoculated with ORF47 mutant viruses. Animals inoculated with control cells, VZV, 47Kan, or 47Rescuant were allowed to develop hypersensitivity and at 13dpi, animals were sacrificed, ipsilateral glabrous skin was removed, preserved, cryosectioned, and stained for the pan-neuronal marker, PGP9.5. Neurites stained by PGP9.5 that project past the stratum basale layer of the epidermis were counted and are plotted here as PGP9.5+ neurites/mm of the stratum basale. As expected, animals inoculated with wild type VZV (pOka) (mean ± standard error = 9.53±0.69) and ORF47Rescuant (9.72±0.88) which both developed pain had a significant reduction in PGP9.5+ neurites/mm compared to control cell inoculated animals (12.97±0.75) (Figure 13). Importantly, animals inoculated with ORF47Kan (15.41±0.92) did not develop a reduction in peripheral neurites. Thus reduction in peripheral innervation correlates with hypersensitivity in animals, and in the converse, a lack of reduction in peripheral innervation correlates with no change in sensitivity to stimuli.
Figure 13: Peripheral neurite retraction induced by VZV and 47Rescuant but not ORF47KO virus.

Animals were inoculated with control cells, pOka, pOka 47Kan, or 47Rescuant. At 13dpi, when animals developed hypersensitivity to mechanical and thermal stimuli, animals were sacrificed and glabrous paw skin was removed. Skin was cryosectioned in 30µm slices and stained with PGP9.5 (green) and Hoechst (blue) (A). White line represents the border between the stratum basale and stratum spinosum layers of the epidermis. White arrows denote neurites that innervate past the stratum basale layer of the epidermis. (B) Quantification of neurite density (n=4 per group total of 20 sections counted, 5 sections per animal) Statistics One-Way ANOVA with Tukey’s multiple comparison test comparing all columns. *=p<0.01, ***=p<0.001.
5.2.5 **ORF47 and ORF9 mutant virus generation and growth kinetics *in vitro.*

An important viral target of ORF47 is ORF9, which has been shown to interact with several tegument associated proteins and glycoproteins. This interaction is direct, and leads to the phosphorylation of ORF9, which is important in viral assembly and egress. Interestingly, mutant viruses that do not express the ORF47 kinase appear morphologically similar to viruses that express a mutant ORF9 that has an altered ORF47 phosphorylation target site at amino acid 84. It has been shown that ORF47 mediated phosphorylation of ORF9 is necessary for proper virion formation and both exhibit defects in virus assembly and egress \(^{193}\). We therefore asked if the lack of hypersensitivity in animals inoculated with ORF47 mutant viruses is the result of phosphorylation of ORF9 protein by the ORF47 kinase. To address this question, we generated two VZV mutants that disrupted ORF9 protein phosphorylation at position 84 (ORF9 E85R and ORF9 S84A E85R). Both mutants were VZV BAC derived and verified to be correct by sequencing and restriction enzyme mapping (Fig. 14A). Mutant viruses were analyzed for their growth characteristics in MeWo cells (Figure 14B) and while we observed a slight reduction in the growth of ORF47Kan and ORF9 S84A E85S, compared to wild type VZV (pOka), all replicated robustly in this cell type.

We subsequently analyzed ORF47 kinase function and mutation of the ORF9 phosphorylation sites by immunoblot. Cells were infected with 500 pfu of wild type VZV (pOka), 47Rescuent, 47Kan, ORF9 E85R, or ORF9 S84A E85R and lysates were made at 24-hour post infection, and probed for ORF9 protein and its phosphorylation species. Analysis revealed that wild type pOka and 47Rescuent have a substantial portion of ORF9 that is hyperphosphorylated and migrates slower in the gel,. However 47Kan and both ORF9 mutants have little to no hyperphosphorylated ORF9 species. Mutations in ORF47 and ORF9 did not drastically affect
viral growth in vitro and both mutations, as expected, result in decreased hyperphosphorylation of ORF9.

**Figure 14: ORF47 knock out mutant growth characteristics and expression.** ORF47 was disrupted by the insertion of a kanamycin resistance cassette into the open reading frame of ORF47 (A). Growth curve analysis of ORF47 knock out and ORF9 phosphorylation mutant viruses in MeWo cells (B). Western blot analysis of ORF9
phosphorylation (using an polyclonal ORF9 specific antibody) as a surrogate for ORF47 activity and to confirm the mutation of the ORF9 phosphorylation site at position 84 abrogated ORF9 phosphorylation by ORF47 (C).

5.2.6 ORF9 phosphorylation mutants exhibit modulated pain response in vivo.

We then tested whether the VZV mutations that affect hyperphosphorylation of ORF9 by ORF47 induced hypersensitivity in animals. Animals were inoculated with control cells, wild type VZV, or mutant viruses, subcutaneously, under the glabrous skin of animals and were subsequently tested for sensitivity to mechanical and thermal stimuli. Wild type VZV inoculated animals developed the characteristic hypersensitivity to mechanical and thermal stimuli by 14 and 17 dpi. As before, 47Kan inoculated animals did not develop signs of hypersensitivity at any point either to mechanical or thermal stimuli (Fig 15A-D). Interestingly, mutants that altered ORF9 hyperphosphorylation showed two different effects. Animals inoculated with ORF9 E85R, a more conservative alteration to the consensus ORF47 phosphorylation site at ORF9, exhibited transient mechanical hypersensitivity at 9dpi that resolved and did not return (Figure 15). However, animals inoculated with a more extensive disruption of the phosphorylation site, ORF9 S84A E85R that mutates the serine at position 84, developed hypersensitivity at 7dpi that lasted in only one animal for the duration of the experiment while the other animals displayed hypersensitivity at 7dpi that resolved, but then returned (at 29 and 35dpi) (Figure 15A). Thermal hypersensitivity, while less robust, does not show any transient hypersensitivity at 7dpi, but does show the same trend with mean area under the curve of ORF9 E85R more similar to 47Kan, and ORF9 S84A E85R displaying an intermediate phenotype (Figure 15D). These data imply that the ORF9 phosphorylation, which is necessary for proper virus assembly, may be a contributing factor to VZV-induced hypersensitivity in animals. At this stage we cannot fully explain the
intermediate phenotype seen in VZV ORF9 S84A E85R inoculated animals. This is preliminary work that will be repeated.

**Figure 15: ORF9 phosphorylation mutants affect VZV-induced pain in animals.** Animals (n=5 per group) were inoculated with VZV, 47Kan, ORF9 E85R, or ORF9 S84A E85R at 0dpi. Animals were tested for sensitivity to mechanical (A) and thermal (B) stimuli. Mean±SEM plotted (C and D). Mean area under the curve plotted for each animal. Statistics used: Statistics (A and B) One-Way ANOVA with Dunnett’s multiple comparison test comparing 47Kan to all groups, (C and D) One-Way ANOVA with Tukey’s post test comparing all columns. *=p<0.01, **=p<0.001.
5.3 DISCUSSION

ORF47 is one of two viral serine/threonine kinases and has a consensus phosphorylation target site sequence similar to Casein Kinase II. The kinase is homologous to that of HSV UL13, which is conserved among α, β, and γ herpesviruses. ORF47 is not necessary for viral growth in vitro, and appears to not be required for the establishment of latency in rodents. However, ORF47 is necessary for in vivo infection of T cells and skin. The kinase is also required for infection of immature dendritic cells (DC) and in the transfer of infection from these DCs to permissive melanoma cells. Here we show that ORF47 is necessary to generate pain in the rat model of PHN. This is not a general feature of viral kinases since the other viral protein kinase, ORF66, is not necessary for VZV induction of pain. Two different ORF47 mutants were utilized: ORF47Kan that was derived in a BAC using Red-mediated recombination to insert a kanamycin resistance cassette into ORF47, while the second virus is ORF47stop (a kind gift from J. Cohen), which was cosmid derived and has two stop codons inserted into ORF47. Both viruses showed the same reduction in hypersensitivity, confirming the necessity for ORF47 for the induction of hypersensitivity. This is the first report of a Varicella Zoster Virus gene involved in the induction of nocifensive behaviors and pain, and is only the second viral protein discovered to be involved in pain, the other being HIV gp120. Unlike ORF47, gp120 is a surface protein that is essential for HIV binding and entry into susceptible cells. When gp120 is injected into the footpad or applied epineurally or intrathecally, animals become chronically hypersensitive to stimuli. This phenomenon is at least partially derived from neuronal injury and death, resulting in the release of proinflammatory cytokines (interleukin 1, interleukin 6, and tumor necrosis factor α) and chemokines (chemokine C-C ligand 5 also known as RANTES and monocyte-chemoattracting protein 1) as shown in chapter 4. VZV may
also instigate pain, through injury and maintain pain through a neuro-immune interaction. We have shown previously that VZV infection reduces peripheral innervation, increases TRADD transcription, and treatment of VZV-hypersensitive animals with an HSV vector expressing soluble TNF receptor alleviates hypersensitivity (Chapter 4). This hypothesis of immune mediated pain during VZV infection is intriguing but requires further study.

There are two main functional domains of ORF47, C terminal kinase activity and N terminal domain involved in protein binding to the viral regulatory protein IE62 67. Here we utilize previously published C terminal mutants to discern the role of kinase activity for the induction of pain 67. 47ΔCterm expresses a truncated form of ORF47 with all amino acids after 226 removed and 47D-N has a mutated ATP binding site at amino acid 283 (from DYS to NYS). Both of these mutants affect ORF47 kinase activity and pathogenesis in skin and T cells 67,183. The 47P-S mutant has a substitution in the ORF47 APE tertiary folding motif at amino acid 333 (from PPE in VZV to SPE), but this does not affect kinase activity or pathogenesis in skin 67. We show that mutations in the kinase domain that affect kinase activity do not induce hypersensitivity in animals. Animals inoculated with HSV 47D-N VZV also do not develop nocifensive behaviors, whereas animals inoculated with HSV 47 VZV did. Together, these data imply that ORF47 kinase function is necessary for pain induction. Unexpectedly, animals inoculated with a 47P-S mutant virus do not exhibit nocifensive behaviors. Intriguingly, 47P-S mutation does not affect either kinase activity or skin pathogenesis because this mutation was designed to alter a tertiary folding motif in the kinase domain, but not directly ablate kinase function. This perturbation may result in a folding defect that slightly changes the kinase in a way that it no longer phosphorylates the host and viral targets that mediate VZV-induced pain.
responses. 47P-S may also affect some yet unknown function of the C terminus of ORF47 that requires correct folding.

Peripheral nerve retraction in VZV inoculated animals replicates a hallmark of PHN and zoster in human patients \(^{141,142,160}\). It is clear that both hypersensitive rats and PHN patients have a significant retraction of peripheral nerve fibers. This retraction is not present in animals inoculated with an ORF47Kan virus, but is in ORF47Rescuant infected animals. The retraction of peripheral neurites may help explain the long-term hypersensitivity observed in rats. This hypothesis requires further investigation in animals that have had their hypersensitivity resolve naturally. Intriguingly, each virus would be expected to induce a localized immune response at the peripheral site of injection, similar to wild type VZV. As such this may exclude peripheral immune responses as contributing to the hypersensitivity.

Lastly, we investigated one possible mechanism of ORF47 mediated hypersensitivity in rats by examining a known target of ORF47, the tegument protein ORF9. We generated two mutants that alter the ORF47 phosphorylation target site in ORF9: one alters the phosphorylation site from SEDD to SRDD by replacing the glutamate at position 85 with an arginine (ORF9 E85R); the other alters both the serine and glutamate to an alanine and arginine (ORF9 S84A E85R). The viruses generated were reported to be morphologically similar to those generated by ORF47 deletion mutants, as ORF9 E85R increases defective virus formation and reduces extracellular viruses \(^{193}\). It was proposed that the phosphorylation of ORF9 affects viral tegument assembly at the transgolgi network \(^{193}\). Here we show that both mutations result in a reduction in hyperphosphorylation and that the ORF9 S84A E85R mutant virus has only a slight reduction in growth \textit{in vitro}. We hypothesized that VZV-induced hypersensitivity depends on ORF47 through its phosphorylation of ORF9. Affecting ORF9 protein incorporation into virions, altering cell
free virus spread. Interestingly, we saw two different affects when ORF9 phosphorylation mutant viruses were administered to animals. The published ORF9 mutant, ORF9 E85R, resulted in no thermal hypersensitivity and only transient mechanical hypersensitivity in animals that did not last past 14 days post infection and never returned. A mutant that directly changes the serine at position 84 (ORF9 S84A E85R) also generated a burst of mechanical hypersensitivity early, which resolved in 2-3 animals, but eventually reappeared in all animals at 29dpi. Thermal hypersensitivity showed the same trend for both viruses with ORF9 S84A E85R inoculated animals developing an intermediate sensitivity that is not different from either wild type VZV or 47Kan (as shown in mean area under the curve (Figure 15 D)). While we cannot fully explain these results, it is unlikely that the phenotypic change in capsid morphology due to ORF9 hyperphosphorylation by ORF47 renders the virus unable to infect neurons or is deficient in transport back to the soma. Studies with ORF47KO inoculated animals have suggested similar numbers of genomes to wild type infected animals in cotton rats and in our model (144 and unpublished findings). Altering the serine at position 84 may have unintended consequences on the virus and pain responses to it. This mutation may block not only ORF47 phosphorylation but also casein kinase II mediated phosphorylation as ORF9 E85R does not completely remove the serine at position 84 which can be phosphorylated by casein kinase II, in the absence of ORF47193. The double mutant may represent a slower infection that lasts longer, as its growth is slightly reduced in vitro, and yet this smoldering infection may result in more pain with a prolonged immune response and damage to neurons and their support cells. While this data is intriguing, the experiment needs to be repeated.

We further show that ORF47 is necessary for VZV-induced hypersensitivity in animals and is sufficient to generate hypersensitivity when expressed outside of the context of VZV
infection. Interestingly, if ORF47 is expressed in replication defective HSV, animals exhibit nocifensive behaviors but when the ATP binding domain was mutated, animals did not develop hypersensitivity. This suggests that kinase activity of ORF47 can directly contribute to pain induction. This may not be surprising given the expression of a viral kinase may result in extensive changes in host signaling pathways and phosphorylation events that can affect pain.

In conclusion we show that ORF47 is necessary and sufficient to induce pain and infection with a 47Kan virus does not induce the peripheral neurite retraction seen in wild type VZV infected animals. The effects of ORF9 phosphorylation on hypersensitivity is intriguing but requires further study. ORF47 would be a good target for novel anti-herpesvirus pain drugs.
6.0 NOVEL HERPESVIRUS VECTORS EXPRESSING PAIN MODULATORY GENES AND THEIR EFFECTS ON HYPERSENSITIVITY IN A RAT MODEL OF VZV-INDUCED PAIN

This chapter is a reprint of a manuscript published in the journal Gene Therapy authored by Jean-Marc Guedon, William Goins, Mingdi Zhang, Joseph C. Glorioso, and Paul Kinchington.

All animal work was performed by JGG, MZ, and WFG. WFG and JCG generated the HSV vectors expressing human preproenkephalin and its parent (vHG). JGG performed nucleic acid extraction and analysis as well as all data analysis.
6.1 INTRODUCTION

Varicella zoster virus (VZV), a ubiquitous human herpesvirus, causes herpes zoster (“shingles”) following its reactivation from a neuronal latent state that was established during the primary disease, varicella (“chickenpox”). Herpes zoster is associated with considerable morbidity as a result of debilitating acute and chronic pain, with incidence increasing with rising age and/or declining immune status. Zoster will eventually occur in approximately 1/5th to 1/3rd of the population, usually occurring in those over age 60. While vaccines for both varicella and zoster are available, the zoster vaccine is only partially effective in preventing the occurrence of zoster and pain associated with it.27

Pain may occur before, during and/or after the skin disease of zoster and even occurs in its absence. Up to 90% of zoster patients experience acute pain, which may be alleviated by timely antiviral administration to limit viral replication. However, 1/3rd of patients progress to develop chronic, more difficult to treat pain states known as Post-Herpetic Neuralgia (PHN) that usually fail to respond to antiviral treatments. The most common and debilitating pain experienced by PHN patients is moderate to severe mechanical allodynia and/or thermal hypersensitivity. These may become so severe that they lead to disparate secondary consequences such as depression, withdrawal from society and loss in the quality of life. Current treatment strategies for PHN include tricyclic antidepressants, topical lidocaine or capsaicin patch treatments, opioids and gabapentinoids, but these are often ineffective and associated with moderate to severe side effects, poor patient compliance, or abuse. PHN remains a significant public health concern in urgent need for improved treatment strategies.
Although there is no small animal model of VZV latency, reactivation, zoster-like disease and subsequent pain, a rat model of VZV-induced pain has been described. Animals inoculated at the footpad with VZV-infected cells develop long-term chronic nocifensive behaviors similar to those exhibited by PHN patients, including mechanical allodynia (MA), thermal hyperalgesia (TH), and anxious-like behaviors. VZV-infected animals show a viral dose-dependent increase in sensitivity with expression of some VZV proteins in neurons colocalizing with peripherin, Neurofilament 200, and Neuropeptide Y in ipsilateral but not contralateral ganglia. It has been established that pain behaviors developing in the VZV inoculated rat model do not respond to acyclovir blockade of viral replication, which mirrors the observations that pain in the majority of human PHN patients is not alleviated by antiviral therapy. While the pain indices that develop in the rat differ from human PHN in that it follows an acute primary infection rather than a reactivation from latency, the rat model has proved highly useful for preclinical assessment of many current and novel drug treatment strategies, and many treatments in the rat echo the response of some PHN patients. Animals treated with Gabapentin, Morphine, sodium channel blockers (mexiletine and lamotrigine), or tricyclic antidepressant (Amitriptyline) showed significant reduction in hypersensitivity. However, many drug treatments show only short-term relief, and some of the treatment strategies evaluated in the rat require administration routes that are impractical for PHN patients.

Here, we show that nocifensive behaviors developing in VZV footpad-inoculated Sprague-Dawley rats are effectively treated and prevented with HSV vector-delivered expression of human preproenkephalin (vHPPE). Preproenkephalin gives rise to natural opioids that modulate pain perception, and can be found in interneurons that synapse onto primary and
second order neurons in the dorsal horn of the spinal cord. Release of vesicle-stored Met- and Leu-enkephalin opioids bind and activate $\delta$, and to a lesser extent, $\mu$-opioid receptors on both primary and second order neurons. This results in the lowering of cyclic AMP production and a hyperpolarization of the neuronal membrane, thereby activating inwardly rectifying $K^+$ channels with concurrent inhibition of voltage-sensitive $Ca^{2+}$ channels.

vHPPE is based on replication-defective herpes simplex virus type 1 (HSV-1), a neurotrophic herpesvirus that establishes latency (and expresses) in sensory neurons and is not associated with chronic pain. The effective long-term relief of VZV induced pain in the rat promotes the exciting potential for long-acting treatment of the PHN that follows Herpes Zoster.

**Figure 16: Schematic of HSV vectors used in this study.** The top line represents the vHG control vector, which has the essential IE gene ICP27 and both copies of essential IE ICP4 gene deleted (bold black lines). The virus is also altered to render the ICP22 and ICP47 gene promoters to be expressed as early (or $\beta$-) genes. The minimal human cytomegalovirus Immediate Early gene promoter (HCMV IEp) -eGFP expression cassette (black box) is inserted into both copies of the ICP4 locus. The lower line represents the vHPPE vector, derived from the vHG control vector, which contains the insertion of the cDNA for human preproenkephalin (HPPE, grey box), driven by the HCMV IEp, followed by the bovine growth hormone polyadenylation signal (BGHpA), inserted in place of eGFP.
6.2 RESULTS

6.2.1 Nocifensive behaviors induced in Sprague-Dawley rats inoculated with VZV

We first established the model of VZV-induced hypersensitivity in Sprague-Dawley rats, which have been used extensively in many inflammatory and neuropathic pain models. Previous reports on VZV induced pain used Wistar rats. Nocifensive behaviors were induced by VZV pOka, a wild-type varicella isolate of Japanese origin that was the basis for subsequent attenuation for use in the current VZV vaccines. We employed this viral strain since it has been used in most genetically manipulatable systems for VZV. Animals were inoculated with live cell-associated VZV, because VZV infectivity remains highly cell-associated and cell-free virus cannot be obtained at the titers required to induce pain. All animals receiving VZV in multiple studies developed markedly different behavioral responses to mechanical and thermal stimulation compared to animals receiving uninfected cell equivalents (Figure 17a,b) or sham-inoculated animals (data not shown). No difference was observed between control cell-inoculated animals and sham-inoculated animals. Chronic hypersensitivity to mechanical stimuli developed only in the VZV-inoculated paw, resulting in a biased ipsilateral/contralateral ratio lasting several weeks. The contralateral paw of animals receiving VZV did not respond to most von Frey filament stimulations, as seen in the ipsilateral or contralateral paws of animals receiving uninfected cells. Significant hypersensitivity to thermal stimuli (Figure 17b) also only developed in VZV-injected footpads as compared to the un-inoculated contralateral paw or uninfected cell inoculated paws. VZV-induced nocifensive behaviors persisted in this study for approximately 5 weeks post infection (wpi), whereupon hypersensitivity responses reduced, and by 6-8 wpi, were similar to untreated animals. Timing of spontaneous recovery showed
consistency between groups of animals within the same experiment, although rats in other studies showed variation in the time at which spontaneous resolution occurred. We also evaluated MA and TH responses in rats receiving VZV preparations that were UV-inactivated just prior to inoculation. These showed no significant mechanical or thermal hypersensitivity (Figure 17). This indicates that the VZV-infected cell inoculum used in these studies did not contain pre-existing factors inducing the hypersensitivity, and that live virus with \textit{de novo} transcription and gene expression are required for the induction of pain.
Figure 17: Mechanical and thermal hypersensitivity induced by VZV in Sprague-Dawley rat requires de novo transcription. Animals were pretested 2 days prior to infection for baseline MA and TH responses, and then inoculated (n=4 per group) at day 0 with 2x10^5 PFU of pOka or uninfected cell equivalents. Animals were evaluated for (a) mechanical allodynia (MA) scores using von Frey filaments and for (b) thermal hyperalgesia using a Hargreaves apparatus, as detailed in the methods with the graphs depicting the ratio of ipsilateral to contralateral responses. Mean +SEM is plotted. This study is representative of three similar studies. Statistics used One-Way ANOVA between groups and Dunnett’s multiple comparison test comparing groups to MeWo. Mean area under the curve is plotted for each animal; line represents the mean and bars are SEM (c). Mean area under the curve with
One-Way ANOVA between groups and Tukey’s multiple comparison test, with *\(=P<0.05\), ***\(=P<0.001\) indicating significance.

### 6.2.2 HSV vector PPE expression modulates VZV-induced nocifensive behaviors

While novel drug treatments have been evaluated in the rat PHN model, most provide only a short-lived relief of pain and often require administration routes that would be impractical for the treatment of human PHN. Given human PHN can be prolonged, a long-term treatment strategy would be desirable. Therefore, we evaluated treatment using a replication-defective HSV-1 vector expressing human PPE (Figure 16). Rats inoculated with VZV and showing significant mechanical and thermal hypersensitivity by 19 dpi (Figure 18) were then inoculated at the same footpad with PBS or \(10^8\) infectious units of HSV vector (vHPPE or vHG control). Animals receiving PBS or vHG continued to show significantly biased MA and TH ipsilateral/contralateral responses after vector administration (Figure 18a,b). Remarkably, VZV-induced MA and TH nocifensive responses showed prolonged relief following a single inoculation of \(10^8\) vHPPE (Figure 18b,d). Neither HSV vector induced changes in behavioral responses of uninfected cell inoculated rats (Figure 18c). These results strongly suggest that HSV-1 vector-mediated expression of human PPE could effectively alleviate VZV-induced nocifensive responses in the rat model for extended periods.
Figure 18: Administration of vHPPE provides prolonged relief of VZV-induced hypersensitivity.

Animals were injected on day 0 with 2x10^5 PFU of pOka or equivalent number of uninfected MeWo cells (n=5 per group) and assessed for MA and TH at times shown. At 19 dpi, animals were injected with 10^8 PFU of vHPPE, vHG or equivalent volume of PBS and subsequently evaluated for (a, b) MA using von Frey filaments and (c, d) TH using a Hargreaves apparatus. All results are presented as a ratio of the score of the ipsilateral to contralateral sides. Mean ±SEM is plotted. Arrowheads indicate when HSV vectors were administered. The study shown is representative of two studies with similar results. Statistics used One-Way ANOVA between groups and Dunnett’s multiple comparison test comparing groups to pOka + PBS. Mean area under the curve is plotted for each animal; line represents the mean and bars are SEM (e). Mean area under the curve with One-Way ANOVA between groups and Tukey’s multiple comparison test, with *=P<0.05, ***=P<0.001 indicating significance.
To evaluate whether alleviation of nocifensive behaviors by HSV delivered PPE was dose dependent, incremental lower doses of the HSV vectors were examined. All rats injected with VZV-infected cells developed nocifensive behaviors. On day 19, VZV-infected and control animals were inoculated with either PBS, $10^4$ or $10^6$ PFU per footpad of vHPPE or vHG control, respectively. Rats receiving PBS treatment or HSV vHG at $10^4$ or $10^6$ PFU continued to show a biased MA footpad response to the VZV-inoculated paw (Figure 19a,b). In contrast, animals with a VZV-induced hypersensitivity that received vHPPE at $10^4$ or $10^6$ PFU developed obvious relief from MA within 14 days of administration. However, the MA response in rats receiving the lower dose ($10^4$ PFU/footpad) showed only short-term alleviation, and biased MA responses returned in the VZV-infected paw at 42 dpi of VZV. This result indicated that relief of the MA responses by vHPPE was dose-dependent. However, the short-term relief by vHPPE could be re-initiated by re-administration of $10^8$ PFU of vHPPE at 53 dpi (Figure 19a). Such animals showed an immediate relief from VZV-induced nocifensive behaviors that then persisted for the length of the study. Thermal responses were concurrently evaluated and while they showed a similar trend, statistical difference could not be established between the groups (data not shown). We conclude that vHPPE can modulate VZV-induced nocifensive responses in a dose-dependent manner that affects duration of analgesia and does not preclude potential for re-administration and extension of relief.
Figure 19: VZV-induced nocifensive behaviors respond to reduced vHPPE dosing and vector re-administration. Animals were evaluated for MA at day 0 and then injected with $2 \times 10^5$ PFU of pOka or equivalent number of uninfected MeWo cells (n=5 per group), followed by MA evaluation at day 7 and 16. At 19 dpi, animals were injected with either (a) $10^4$ or (b) $10^6$ PFU of vHPPE, vHG or PBS. Animals were evaluated for sensitivity by MA calculated using von Frey filaments and the ipsilateral to contralateral ratios calculated. All animal experiments were repeated with similar results. Animals that were originally dosed with (a) $10^4$ PFU of vHPPE or vHG were given a later dose of $10^6$ PFU of vHPPE at 53 dpi. Mean +SEM plotted. Arrowheads indicate when HSV vectors were administered. Statistics used included one-way ANOVA between groups and Dunnett’s multiple comparison test comparing groups to pOka + PBS. Mean area under the curve is plotted for each animal; line represents the mean and bars are SEM (c), was analyzed by One-Way ANOVA between groups and Tukey’s multiple comparison test, with *=P<0.05, ***=P<0.001 indicating significance.
6.2.3 Prophylactic vHPPE administration prevented VZV induced hypersensitivity

Zoster is often associated with prodromal signs that predict the development of disease,\textsuperscript{212,213} so it is feasible that prophylactic treatment could prevent VZV-induced pain. To evaluate this, animals were first inoculated with PBS or $10^8$ PFU of control vHG or vHPPE, followed by VZV inoculation at the same site. Nocifensive behaviors were not induced by the HSV vectors alone (data not shown), mirroring previously reported absence of pain from replication-competent HSV \textsuperscript{214-221} or replication-defective HSV \textsuperscript{132,222-229}, even when expressing reporter genes or other proteins. However, VZV-induced MA nocifensive behaviors only developed in animals pre-inoculated with PBS or vHG vector, and did not develop in vHPPE treated animals (Figure 20). As such, we conclude that prophylactic administration of vector vHPPE can block the development of VZV-induced pain.
Figure 20: Prophylactic administration of vHPPE blocks development of VZV-induced Mechanical hypersensitivity. Animals were injected 7 days before VZV infection with either $10^8$ PFU of vHPPE, vHG or PBS. On day 0, animals were inoculated with $2 \times 10^5$ PFU of VZV pOka or uninfected MeWo cell equivalents (n=5 per group). MA scores were determined at the indicated times using von Frey filaments and are presented as a ratio of ipsilateral to contralateral paw responses. All animal experiments were repeated with similar results. Mean +SEM is plotted. Statistics used One-Way ANOVA between groups and Dunnett’s multiple comparison test comparing groups to pOka + PBS and Mean area under the curve is plotted for each animal; line represents the mean and bars are SEM (b) was analyzed for significance using One-Way ANOVA between groups and Tukey’s multiple comparison test, with *=P<0.05, **=P<0.001 indicating significance.
6.2.4  **VZV-induced hypersensitivity and its relief by PPE do not act at the periphery**

VZV-induced hypersensitivity and its relief by peripheral administration of vHPPE were suspected to act at the ganglia, but it is possible that vHPPE-expressed enkephalin acts on sensory nociceptor termini at the periphery. Direct quantification of enkephalin expression was not sufficiently sensitive to reveal differences between treated and untreated controls, as seen previously. Global quantification of innervating ganglionic transcripts of human PPE mRNA revealed a 3-5 fold $2^{\Delta\Delta Ct}$ increase in levels over that detected in control animals (Figure 21).

![Graph showing the expression of human PPE in animals injected with vHPPE](image)

**Figure 21:**  **Ganglionic expression of human PPE in animals injected with vHPPE.** Animals were inoculated with $10^8$ PFU of vHPPE or vHG and sacrificed at 1 and 2 weeks post inoculation (n= 6 per group). The L4-6 DRGs were removed and the extracted RNA was converted to cDNA. Transcripts for human PPE were quantified by Taqman RT-PCR and are presented using the $2^{\Delta\Delta Ct}$ compared to rat GAPDH as a surrogate for normalized gene expression against control animals. Mean ±SEM is plotted.

To further probe for effects at the periphery, we analyzed whether peripheral administration of DAMGO or naloxone affected VZV-induced pain or its relief by vHPPE
administration. Animals showing VZV-induced nocifensive MA behaviors at 3 wpi were subcutaneously injected at the footpad with 1 or 10µg of DAMGO, a synthetic µ-opioid receptor agonist with high specificity for the µ-opioid receptor. We reasoned that the peripheral administration of DAMGO would alter VZV-induced pain responses if a mechanism of vHPPE relief acted at the periphery (Figure 22). However, VZV-induced biased MA responses showed no detectable relief immediately following DAMGO administration into the footpad. Higher dosing with 10µg DAMGO did not lead to any relief of nocifensive behaviors over a period of 1 h. We then assessed if peripheral administration of naloxone to the footpad would negate relief of VZV-induced MA responses by 10^8 vHPPE. Peripherally delivered naloxone, a competitive antagonist of the opioid receptors, would be expected to block enkephalin interaction with any peripherally located opioid receptors, but not with DRG axons terminating within the dorsal horn of the spinal cord. VZV-infected animals showing nocifensive behaviors that were then relieved by 10^8 HSV vHPPE showed no biased MA responses, while control animals continued to show a strong ipsilateral to contralateral bias (Figure 23). Animals receiving either 5 or 50µg of naloxone in the same paw showed no change in MA responses, either short term (up to 60 min) or after 24 h post administration (data not shown). Furthermore, peripheral naloxone administration did not affect vHPPE relief of VZV-induced nocifensive behaviors, suggesting that HSV vector-mediated expression of enkephalin within the DRG acts centrally within the spinal cord. The latter has been strongly implied from prior studies using similar pain relieving vectors, in which blockade of the effects of enkephalin were obtained following intrathecal administration of antagonists 164,223,230-232. We conclude from this data that it is unlikely that VZV-induced pain and its relief induced by vHPPE vector were due to effects mediated at the periphery.
Figure 22: Effect of peripheral delivery of DAMGO on VZV-induced MA hypersensitivity. Animals inoculated with 2x10^5 PFU of pOka at the footpad were housed to allow the development of MA hypersensitivity. Animals were evaluated to determine baseline responses and then injected with 1 or 10µg/20µL DAMGO or equivalent volume of PBS at 9 dpi (n=4). Animals were subsequently tested for mechanical sensitivity by von Frey filaments and presented as an ipsilateral to contralateral ratio. Times of MA assessment were 10 min before injection for baseline determination and 5, 20 and 60 min post injection. Mean ±SEM is plotted.

Figure 23: Effect of peripheral delivery of naloxone on VZV-induced MA hypersensitivity treated with vHPPE or vHG vector. Animals were inoculated with 2x10^5 PFU of pOka, and then received at 9 dpi either 10^5 PFU of vHPPE, vHG or PBS. Animals received a third footpad injection at 23 dpi with 5 or 50µg/20µL naloxone or the equivalent volume of PBS (n=4). Animals were tested for mechanical hypersensitivity by von Frey filaments, 10 min before injection and 5, 20 and 60 min post injection. The ratio of ipsilateral to contralateral paw responses is shown. Mean ±SEM is plotted.
6.3 DISCUSSION

The goal of this study was to further develop a rat model of VZV-induced pain to evaluate the therapeutic and prophylactic administration of human enkephalin using an HSV vector ganglionic delivery system. The efficacy of this strategy strongly promotes the potential for use of enkephalin gene delivery as a long-acting treatment for the pain and PHN associated with zoster. The model appears robust and reproducible, particularly with respect to induced mechanical hypersensitivity, but it is not yet clear exactly how VZV induces nocifensive behaviors in rats. The length of hypersensitivity in rats was found to vary somewhat between different experiments. We postulate that this could be the consequence of the out-bred strain and subtle differences in the genetics of these animals. It is also possible that subtle variations in the experimental procedures and even subtle changes in housing conditions could contribute to this variation, although every step was taken to minimize these experimental differences. We note that the duration of chronic nocifensive behaviors were also variable in previously reported studies using the Wistar rat model. However, the variation reflects the duration of extended pain in PHN patients, which may be short or last for years. Differences may also be the consequence of virus strain used. Each lab has evaluated different strains in the rat model that differ from the virus we employed here, parent Oka (pOka). However, we point out that the pOka strain is the standard virus most employed in the field of VZV genetics and should be useful for future genetic studies to evaluate pain-inducing VZV genes in the rat model. Most rodents are not permissive for VZV replication although some early phases of infection may occur. Two separate studies have reported that the development of VZV-induced pain behaviors in rats is not prevented by the viral DNA replication inhibitor, acyclovir. However, given that some viral gene products can be detected in neurons of the innervating ganglia, but not in
contralateral ganglia (including VZV immediate early proteins IE62 and IE63\textsuperscript{30,117,118}), it seems likely that an abortive type of infection with some viral gene expression is required for pain development. Our data showing that rats receiving UV-inactivated VZV failed to develop hypersensitivity behaviors is consistent with this hypothesis, since it indicates infectivity and/or \textit{de novo} VZV gene transcription are required. We suspect that transcription of VZV genes within virus-infected neurons may themselves lead to the chronic pain state either through induction of inflammatory mechanisms or through expression of proteins, such as transcriptional regulators, that may alter host cell expression patterns.\textsuperscript{118,233,234} Studies are ongoing to determine if viral transactivators, IE62 and IE63, are required for the induction of the chronic pain state.

We show that VZV-induced pain behavioral indicators can be effectively reversed and even prevented with sustained effect by HSV vector-mediated delivery of preproenkephalin. We exploited replication-defective HSV-1 vectors, which are engineered to enter a latent-like state in sensory neurons. In contrast to VZV, human HSV recurrent infections are not associated with more than slight acute pain. Indeed, Wistar rats inoculated with high titers of replicating HSV-1 show only momentary behavioral pain responses.\textsuperscript{116} Consistent with this we found no pain indicators developing in Sprague-Dawley rats inoculated with either vHG control vector or vHPPE vector (data not shown) as has been previously described in replication-competent HSV\textsuperscript{214-221} or replication-defective HSV\textsuperscript{132,222-229} vectors expressing genes other than enkephalin in various pain models. This supports the fact that while HSV and VZV are genetically related, they have very different consequences on pain induction. Additionally, this is consistent with a variety of HSV vectors that do not induce pain in preclinical animal models and human clinical trials.\textsuperscript{214,235} This supports the safety for the potential use of such vectors in humans. Our study extends the application of this vector–gene delivery combination to a model of a common and
significant clinical problem, PHN. HSV-1 vectors expressing human preproenkephalin have been used in various other pain animal models including: pain associated with pancreatitis\textsuperscript{216,227}, formalin-injection\textsuperscript{223}, spinal nerve ligation\textsuperscript{226}, complete Freund’s adjuvant (CFA)-induced arthritis\textsuperscript{215,219,221}, chronic constriction injury (CCI)\textsuperscript{220,229}, bone cancer pain\textsuperscript{222}, pertussis toxin induced pain\textsuperscript{217}, and in a bladder nociception model.\textsuperscript{132,224,225} In these studies, HSV vector-mediated enkephalin produced abrogation of nocifensive behaviors to varying extents. The long-term effects of such vectors and their peripheral administration are particularly attractive for application to PHN, which may last weeks to years. Evaluation of morphine, amitriptyline, gabapentin, ibuprofen and the Win55212-2 2 compound in the rat PHN model\textsuperscript{117} all provide only temporary relief lasting 4-6 days or less, and some human treatments such as Morphine are associated with tolerance and abuse issues. Oral administration of gabapentin, or the sodium channel blockers mexiletine and lamotrigine were also found to last for hours or less in the rat model.\textsuperscript{118} Short-term effects on VZV pain in the rats by agents such as the astrocyte toxin LAA, as well as iNOS inhibitors (L-NIL), NO scavengers (PTIO), IL1 receptor antagonist, the cytokine inhibitor pentoxifylline, NMDA receptor antagonists (AP5 and (R)-CPP), and the non-competitive NMDA receptor antagonist MK801 have also been reported, but these require intraperitoneal or intrathecal administration, routes not particularly suited to human treatment.\textsuperscript{117,118,120} The single peripheral administration of vHPPE abrogated VZV-induced nocifensive behaviors completely at high doses (Figures 18b,d). We argue this is the first strategy to show a prolonged effect on VZV-induced pain behaviors with single dosing and relatively simple administration. The potential for vHPPE re-administration (Figure 19) was also demonstrated. A similar replication-defective HPPE expressing HSV vector has been evaluated in a Phase-I trial for patients with intense chronic cancer related pain, and reduced pain scores.\textsuperscript{235}
We believe our data establishes a basis for evaluating the efficacy of this vector in human PHN trials.

The presumed mechanism(s) of action in the enkephalin treatment strategy is that following HSV infection of sensory nerves, ganglionic expression of enkephalin probably results in Leu- and Met-enkephalin incorporation into secretory vesicles and release at synapses of sensory nerve axons terminating within the dorsal horn of spinal cord, where binding to opioid receptors prevents synaptic transmission of pain signals. Our studies with the peripheral administration of enkephalin agonists and antagonists suggest that enkephalin is acting centrally. Previous reports\textsuperscript{222,223} indicate that intrathecal administration of naltrexone, an opioid receptor antagonist, reversed HSV-ENK vector-mediated relief, suggesting a central acting role for enkephalin in those models. In our VZV PHN model peripheral injection of DAMGO or naloxone had no influence on hypersensitivity or effect on vHPPE-mediated relief, respectively. All time points had similar effects, suggesting local delivery of opioid receptor agonist or antagonist does not alleviate hypersensitivity or block analgesia. This further supports the hypothesis that HSV vector-mediated enkephalin acts centrally.

Taken together, we have established that VZV induces a robust mechanical and quantifiable thermal hypersensitivity in Sprague-Dawley rats, which is dependent upon \textit{de novo} VZV gene expression. These pain behaviors can be effectively relieved for prolonged periods by peripheral administration of an HSV vector expressing human PPE, and can be prevented from developing by prophylactic vector administration. These studies suggest further development of these or similar vectors as prolonged treatment strategies for pain associated with Herpes Zoster.
7.0 DISCUSSION AND FUTURE DIRECTIONS

7.1.1 Viral expression is necessary for hypersensitivity in the rat

Here we demonstrate that VZV gene expression/infectivity is necessary for the establishment of hypersensitivity in rats (Figure 17). Others have shown that treatment with the viral DNA synthesis inhibitor, acyclovir or its derivatives, does not affect hypersensitivity in animals \(^{117,120}\). Taken together these data suggest that full viral replication is not needed to induce hypersensitivity in animals.

We have observed very little viral replication in animals particularly in innervating ganglia. Therefore, we sought to identify the block in viral replication utilizing rat primary tissue as a surrogate for VZV infection. Primary cultures infected with VZV reporter viruses showed luciferase expression. However, both kinetic classes are not expressed to the levels as seen in permissive cells, with late gene expression at or below input cell-associated virus luciferase expression (Figure 4). Viral RNA obtained from infected ganglia confirmed the lack of late gene expression, but immediate early and early genes were detected (Figure 5). These data suggest there is a block in viral replication that occurs after immediate early gene and before late gene expression. Our data suggest that in rats, the block in VZV replication appears before DNA replication, as DNA copies do not increase in DRGs and late genes are not expressed. This could be the result of a lack of compatible host gene products or a lack of VZV gene products needed.
for DNA replication. The latter could be established by comparing the expression of VZV genes involved in DNA replication between permissive and nonpermissive cells. These genes include the large and small subunits of the viral DNA polymerase (ORF28 and 6), DNA binding protein (ORF29, which we have shown to be expressed in vivo (Figure 5)), origin binding protein ORF5, and the primase-helicase complex (ORFs 6, 52 and 55). A more intensive and expansive detailing of host and viral gene expression in permissive and nonpermissive cells would elucidate if there are any obvious changes between the two, though this would be resource and time intensive.

7.1.2 Host Changes upon VZV infection

The host changes that are incurred upon VZV infection have to this point been largely unexplored. One group reported changes in microglial activation in the spinal cord and another group looked at immunoreactivity of proteins indicative of neuropathic pain, but we are the first to report analysis of host transcriptional changes upon VZV infection in the rat (Figure 7)\textsuperscript{118,120}. We have confirmed some of the data by qPCR (Figure 8). The changes we have thus far described include TRADD, Ntrk2, TRPV1, and CALCA. It will be important to determine if these changes extend to protein expression. Whole DRG were used for the above analysis, but the vast majority of cells in the DRG are likely uninfected. Dissociating DRG and obtaining only the VZV infected cells would be helpful to determine the full extent of viral infection on the various cell types present in DRG. Of course excising and dissociating DRG will likely induce changes to host and viral gene expression that may confound our findings.

A major question remains: what neurons do VZV infect and how do they contribute to PHN? In order to answer this question we would need to mark infected neurons. We could
accomplish this in two ways: using a Cre responsive animal or a betagalactosidase-expressing virus. Unfortunately, Cre responsive rats are not available; however, a betagalactosidase-expressing virus would circumvent this limitation. Removal of innervating ganglia and assessing infection with X-gal staining will allow us to analyze, histologically, which size neurons VZV infects. In addition, more careful analysis of neurons infected with VZV could be accomplished using fluorescent VZV viruses. Analyzing viral infection of neurons using this method has proved difficult due to low fluorescent signal. Tagging a more abundantly expressed gene, such as ORF29 (DNA binding protein), may provide adequate signal to be detected. Once marked, we could analyze VZV infected neurons for expression of activated transcription factor 3, galanin, neuropeptide Y, and activated caspases. Furthermore, Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay could be used to determine if VZV infected neurons are dying.

We have also observed a reduction in peripheral neurite innervation due to VZV infection. This retraction does not occur in control cell or UV-inactivated VZV inoculated animals. This aspect of the rat model mimics what is seen in zoster and PHN patients, with PHN patients having more severe denervation than zoster patients \(^{141,160}\). There are still two major questions that remain: which neuron subtype is retracting and does reinnervation correlate to hypersensitivity returning to baseline? To determine which type of neuron is retracting we could stain footpad sections for NF200, IB4, CGRP, and TRPV1. I hypothesize that reinnervation and resolution of hypersensitivity will not correlate, as human skin from PHN patients does not re-innervate by 6 months after the zoster rash has resolved and pain and hypersensitivity have subsided \(^{336}\).
We have yet to determine if the hypersensitivity in the rat model is a result of ganglionic infection or if the hypersensitivity is the result of peripheral effects of viral infection and the associated clearance. Our preliminary data suggest that there is not a large infiltrate of nucleated cells (H&E staining) into glabrous skin from animals inoculated with VZV at 10dpi. We have observed a T cell infiltrate into VZV infected ganglia, with CD45/CD4+ cells significantly increased at three weeks post infection (Figure 24). However, hypersensitivity begins as early as one week post infection, which is shorter than any unprimed adaptive response. The role of innate immune responses in the DRG and skin would likely address whether or not there is an immune component to VZV-induced pain. Before further analysis, the effects of immune depletion should be tested for its effects on VZV-induced hypersensitivity in rats. This could be accomplished by making animals immunodeficient via intraperitoneal injection of high dose steroids. If animals do see relief or blockade of pain behaviors, then analysis of peripheral and ganglionic infiltrates should be pursued, with an emphasis on innate immune cell infiltrate (e.g. CD11b+/Ly-71 macrophages, CD335+ natural killer, and CD11c/CD123+dendritic cells). Interestingly, pain generated by gp120 of HIV appears to be at least partially mediated by increased cytokine and chemokine expression \(^{195,200-203}\). Analysis of cytokine and chemokine expression profiles of DRG, dorsal horn of the spinal cord, and peripheral skin would determine their correlation to VZV-induced pain.
Figure 24: VZV infected ganglia have infiltrating CD45+, CD4 or CD8+ T cells. Animals were inoculated with either control cells or VZV (pOka) a 0dpi and DRG were harvested at 1, 2, 3, 4, and 6 weeks post infection. Ganglia were dissociated with collagenase and stained for CD45, CD4 and CD8. Samples were run on a fluorescence assisted cell sorting machine, gated on live lymphocytes, based on forward and side scatter and then subgated as CD45+ (A) and then quantified as either CD45/CD4+ or CD45/CD8+ (B). Mean±SEM plotted. Two tailed T test used to determine significance. *=P<0.05.

Lastly, we have only investigated VZV-induced pain in young, male rats. An important consideration would be the effects of VZV in aged and/or female animals. Zoster disproportionately affects the aged with the risk of PHN greatly increased in people over the age of 80\(^27\). Aged animals may more accurately reflect PHN. We utilize male animals to avoid the estrous cycle confounding our findings; however, it would be interesting to test hypersensitivity in female Sprague-Dawley rats on a matched estrous cycle to determine if sex plays a role in VZV-induced hypersensitivity.
7.1.3 Viral genes necessary for VZV-induced pain

We have shown that ORF47, a viral serine/threonine protein kinase, plays a role in VZV-induced pain. Animals inoculated with two different ORF47 knock out mutants do not exhibit hypersensitivity to mechanical or thermal stimuli, but animals infected with a rescued virus do (Figure 9). Interestingly, animals inoculated with an HSV vector expressing VZV ORF47 have quantifiable mechanical and thermal hypersensitivity (Figure 11). We further defined the necessity of ORF47 kinase activity for the induction of hypersensitivity in rats, as animals inoculated with kinase active site mutants do not exhibit hypersensitivity (Figure 10, 11). Other mutations that do not affect kinase activity also do not induce hypersensitivity (Figure 10).

ORF9 is a structural protein involved in virion release from the cell surface. This function is partially mediated by phosphorylation of ORF9 by ORF47. Interestingly, animals infected with phosphorylation mutants of ORF9, which are phenotypically similar to ORF47 knock out viruses, have two different effects on hypersensitivity. A conservative mutation that does not alter the serine at position 84 results in no thermal and only transient mechanical hypersensitivity that returns to baseline by 14dpi. A more extensive mutant that alters the serine at position 84 induces hypersensitivity to mechanical and thermal stimuli. Taken together our data suggests ORF47 is necessary and sufficient for the induction of hypersensitivity in rats. This may be a result of ORF47 mediated phosphorylation of ORF9, but this hypothesis requires further investigation.

Our data on the role of ORF9 hyperphosphorylation in VZV-induced hypersensitivity is intriguing. In order to confirm that phosphorylation of the serine at position 84 in ORF9 is necessary for hypersensitivity in rats, we would also need to investigate animals inoculated with a phosphomimetic ORF9 in an ORF47 deficient virus. We could accomplish this by altering the
serine at position 84 to an aspartic acid (ORF9 S84D ORF47Kan, or multiple serines if hyperphosphorylation is needed), which will act as a constitutively phosphorylated ORF9 in the backbone of a virus that does not express ORF47. The *in vitro* characterization of the virus including electron microscopic analysis of virions and viral growth kinetics will need to be reexamined. However, if phosphorylation of ORF9 is involved in VZV-induced pain, animals infected with ORF9 S84D ORF47Kan would still exhibit hypersensitivity.

We also need to address whether ORF47 affects viral infection of neurons and for transport back to the soma. This is unlikely as VZV genomes are detected in ganglia of 47Kan infected rats, and 47Stop virus inoculation did not change the viral genome burden in cotton rats at latency 144. We also need to address if ORF47 deficient viruses affect the rate of transport to the soma of neurons. I have made a fluorescent ORF47 knockout with mCherry tagged to ORF23. This virus would be ideal for testing in human embryonic stem cell derived neurons. If 47Kan VZV travels at a reduced rate or does not reach the soma, it may be an excellent candidate to replace current vaccines, especially for the prevention of varicella, since this mutant would affect the establishment of latency. If the virus does travel back to ganglia, then characterization of its viral gene expression by RNAseq or nanostring would help determine if it has any gross defects affecting its ability to generate pain. An exhaustive study of host and viral gene targets for ORF47 would better our understanding of the biology of the virus and could also lead to new targets for pain therapeutics.

Animals inoculated with ORF47 knock out viruses still exhibit limited sensitivity to mechanical and thermal stimuli. This suggests that ORF47 may not be the only viral gene affecting hypersensitivity. Elucidating other viral genes that affect pain remains an area of active investigation in the laboratory. One candidate could be IE62 as animals inoculated with an HSV
vector expressing IE62 (the homolog of ICP4) exhibit nocifensive behaviors (Figure 25). Animals inoculated with a control vector or a vector expressing another immediate early gene, IE63, do not develop hypersensitivity. This suggests that IE62 is sufficient to generate hypersensitivity. The mechanism for this IE62 mediated hypersensitivity is unknown, but may be related to an epitope in IE62 that produces an immune response to host BDNF. We have made a virus that has the cross reactive epitope removed and would be interested in testing this hypothesis in vivo. It is also possible that expression of IE62, a transcription activator, affects a multitude of cellular processes and influences chronic hypersensitivity during VZV infection.

**Figure 25: VZV genes expressed by HSV vectors generate mechanical hypersensitivity.** HSV vectors expressing VZV ORFs 47, 62, 63, or 66 were inoculated into animals at day 1 and measured for mechanical hypersensitivity. Data plotted as ratio of ipsilateral response / contralateral, Mean±SEM.
7.1.4 New treatments

This model serves as an excellent platform to test novel therapeutics. We have shown here that treatment of VZV-induced pain in animals using herpesvirus vectors that express pain modulatory genes is not only possible but also effective. Recently, we have expanded our efforts to include HSV vectors that express glycine or ivermectin sensitive chloride channels. These channels can be selectively activated through the application of glycine or ivermectin, respectively, and animals inoculated with the vector and administered the appropriate agonist reduced hypersensitivity in a dose dependent manner (Figure 26). However, animals treated with HSV-GlyR and administered various doses of ivermectin, or animals transduced with HSV-GlyRM and administered various doses of glycine remained hypersensitive. The use of HSV vectors to treat chronic pain conditions like PHN remain tantalizing, but require further study.
Figure 26: Treatment of VZV-induced pain with HSV vector expressed glycine or ivermectin sensitive chloride channels. Animals were inoculated (n=5 per group) at day 0 with 2x10^5 PFU of VZV and evaluated for mechanical sensitivity using von Frey filaments (displayed as paw withdrawal threshold in the ipsilateral paw), once VZV-induced hypersensitivity was repeatedly observed (20dpi), animals were inoculated with 10^5PFU of control vector (HSV-GFP), a vector expressing a glycine sensitive chloride channel (HSV GlyR), or a vector expressing a mutant glycine derived chloride channel that responds to ivermectin. After 1-week post transduction, animals were tested for hypersensitivity 15 minutes after administration of vehicle (PBS), glycine, or ivermectin at the indicated concentrations. The Mean ±SEM is plotted.
Another avenue that remains intriguing is the role of TrkB signaling in VZV-induced pain. It has been previously reported that a cross-reactive epitope in IE62 causes an antibody response in mice that also reacts with human/mouse BDNF. The authors show that these cross-reactive antibodies, when administered to cells, result in increased cell body area, dendrites and number of branches in neurons. They also show that a proportion of PHN patients have these cross-reactive antibodies, presumably leading to their chronic pain state. BDNF binds to and activates its receptor TrkB (Ntrk2), which we observe to be increased in our VZV infected animals (Figure 7 and 8). Taken together, these data suggest a role for BDNF/TrkB signaling in PHN and merits further study. Perhaps blocking this pathway can help alleviate the initiation of pain and serve as a new therapeutic for PHN.

This model serves as the only platform for the testing of treatments for PHN. Therefore, combination therapies that include low dose Gabapentin with a BDNF inhibitor would be interesting to pursue in this model.

There are drawbacks to this model as discussed in chapter one, with a major concern being the model’s sensitivity. Many treatments seem to achieve the same reduction in sensitivity while targeting very different processes. It remains unclear the exact mechanism for chronic pain induced by VZV, and if there is only one etiology or a multiple of different processes that result in the same end as has been suggested. Although the model is not perfect, rats are the only small animals known to exhibit nocifensive behaviors.


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