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A Novel Role for Osteopontin in Facilitating West Nile Virus Neuroinvasion

Amber M. Paul
University of Southern Mississippi

A NOVEL ROLE FOR OSTEOPONTIN IN FACILITATING WEST NILE VIRUS NEUROINVASION

by

Amber M. Paul

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Approved:

_____
Dr. Fengwei Bai, Committee Chair
Assistant Professor, Biological Sciences

_____
Dr. Glenmore Shearer Jr., Committee Member
Professor, Biological Sciences

_____
Dr. Mohamed O. Elasri, Committee Member
Professor, Biological Sciences

_____
Dr. Yanlin Guo, Committee Member
Professor, Biological Sciences

_____
Dr. Faqing Huang, Committee Member
Professor, Chemistry and Biochemistry

_____
Dr. Janet Donaldson
Chair, Department of Biological Sciences

_____
Dr. Karen S. Coats
Dean of the Graduate School

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ABSTRACT

A NOVEL ROLE FOR OSTEOPONTIN IN FACILITATING

WEST NILE VIRUS NEUROINVASION

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West Nile virus (WNV) is a positive-sensed, single-stranded RNA *flavivirus* that can cause human neuroinvasive diseases, including encephalitis, meningitis, and flaccid paralysis. The mechanisms by which WNV enters the central nervous system and the host-factors that are involved in WNV-neuroinvasiveness are not completely understood. Osteopontin (OPN), a multifunctional glycoprotein, has been implicated as a bio-marker for a number of neuroinflammatory diseases. In particular, secreted (s)OPN has been implicated to participate in recruitment of polymorphonuclear neutrophils (PMN) to sites of its expression, while PMNs have been suggested to act as WNV reservoirs. Therefore, sOPN recruitment of PMNs may contribute to neuroinvasive WNV infection via the 'Trojan horse' mechanism of viral entry into the brain. Therefore, we hypothesize brain-infiltration of PMNs during neuroinvasive WNV pathogenesis is in part mediated by sOPN. Our results show that sOPN expression was significantly increased in human sera, human neuronal cells line, murine plasma, brain homogenates and primary neuronal supernatant following WNV infection, indicating a role for OPN in WNV pathogenesis. In addition, after challenge with WNV *in vivo*, $Opn^{-/-}$ mice exhibited a higher (70%) survival rate than wild-type (WT) mice (30%). Consistent with this, qPCR analysis between WT and $Opn^{-/-}$ mice demonstrated comparable levels of viremia; yet, reduced viral burden in the brains of $Opn^{-/-}$ mice compared to WT controls. Analysis of brain-

infiltrating leukocytes displayed reduced PMNs and PMN-chemokine expression levels in *Opn*$^{-/-}$ mice brains. Importantly, intracerebral supplement of recombinant OPN (rOPN) into *Opn*$^{-/-}$ mice resulted in increased PMN-brain infiltration, increased viral load and reduced overall survival. Together, these data suggest OPN facilitates WNV neuroinvasion in a mouse model.

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DEDICATION

This dissertation is dedicated to my late father Edward J. Paul and mother Valerie Paul. Your strength has inspired me to work hard, to never give up and to maintain focus on completing my career goals. You are my inspirations and I hope to make you proud. I would also like to acknowledge my grandfather Charles Corby for your endless support and encouragement, and my late grandmother Valerie Corby whom provided me with wisdom and guidance. I would like to thank my sister Nicole Smith for teaching me what dedication and strength truly mean, and my fiancé Steve Preite whom has constantly challenged me to question everything and has been my biggest fan throughout my time in Graduate school. Finally, I would like to extend my gratitude to Lily and Bobby Orr for always easing me through my challenges with endless affection and jollity; I dedicate my dissertation to you all.

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LIST OF ABBREVIATIONS

APC	Antigen presenting cell
β-ACTIN	Beta Actin
BBB	Blood brain barrier
BSL3	Biosafety level 3
cDNA	Complementary deoxyribonucleic acid
CNS	Central nervous system
CSF	Cerebral spinal fluid
CTRL	Control
CXCL1	C-X-C motif chemokine ligand 1
CXCL2	C-X-C motif chemokine ligand 2
DAMP	Danger-associated molecular pattern
DC	Dendritic cell
DMEM	Dulbecco's modified Eagle medium
DMSO	Dimethyl sulfoxide
d.p.i.	Days post infection
EBD	Evans blue dye
ELISA	Enzyme-linked immunosorbent assay
EMEM	Eagle's minimal essential medium
FBS	Fetal bovine serum
HBS	HEPES buffered saline
HR	Hour
i.c.	Intracerebral

ICAM-1	Intercellular adhesion molecule 1
IFN-α	Interferon alpha
IFN-β	Interferon beta
IL-1β	Interleukin 1 beta
IL-6	Interleukin 6
iOPN	Intracellular osteopontin
i.p.	Intraperitoneal
L-GLU	L-glutamine
MIN	Minute
MOI	Multiplicity of infection
NET	Neutrophil extracellular traps
NFκB	Nuclear factor kappa B
NK	Natural killer cell
OPN	Osteopontin
$Opn^{-/-}$	Osteopontin knockout
PAMP	Pathogen-associated molecular pattern
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PEN/STREP	Penicillin-streptomycin
PFA	Paraformaldehyde
PFU	Plaque forming units
p.i.	Post infection
PMN	Polymorphonuclear neutrophils

PNS	Peripheral nervous system
PRR	Pattern recognition receptor
qPCR	Quantitative polymerase chain reaction
RFC	Relative fold change
rOPN	Recombinant osteopontin
SEM	Standard error of the mean
sOPN	Secreted/soluble osteopontin
ssRNA	Single-stranded ribonucleic acid
STAT	Signal transducer and activator of transcription
TLR	Toll-like receptor
TNF-α	Tumor necrosis factor alpha
WNV	West Nile virus
WNV-E	West Nile virus envelope
WNV-F	West Nile virus fever
WNV-NA	West Nile virus neuroinvasive acute
WNV-NR	West Nile virus neuroinvasive recovered
WT	Wild-type
ZO-1	Zonula occludens 1

CHAPTER I - BACKGROUND

1.1 West Nile Virus Discovery and Epidemiology

West Nile virus (WNV) is a single-stranded (ss)RNA, arthropod-borne virus, belonging to the family *Flaviviridae*. WNV was first isolated in Uganda, near the West Nile district in 1937, from a female patient that presented febrile-like illness (Chancey, Grinev, Volkova, & Rios, 2015). Since, it has spread to various countries within and outside of Africa, causing serious public health concerns for many parts of the globe. What was once endemic within tropical countries, has since spread to subtropical and even temperate climates, primarily due to the spread of its primary vector the *Culex* species of mosquito (Paz, 2015). The reasons for spread of this mosquito, and in such the virus, are still not completely understood. Some reports have indicated global warming climate changes, increased human-mosquito-bird habitat interaction, and/or fitness of the mosquito and the virus (Paz, 2015).

From 1950-1980, mild WNV febrile-like illness cases were reported in the countries of Israel, Egypt, India, France, and South Africa (Chancey et al., 2015; Paz, 2015). In 1957, the first neuroinvasive case of WNV was reported in Israel (Chancey et al., 2015; Paz, 2015) and in the 1990s, a dramatic geographic spread of WNV throughout the globe began to reach countries such as, Budapest (1996), Russia (1999) and the United States (1999) (Chancey et al., 2015). The frequency and severity of the virus was also developing, as neuroinvasive symptoms of encephalitis, meningitis, and flaccid paralysis were becoming apparent. In the United States, it has spread to all States, including other North American countries, such as Canada and Mexico (Paz, 2015). Additionally, some South American countries and the vast majority of the Asian

continent have also reported WNV cases. It has been regarded as the most causative agent of viral encephalitis (Chancey et al., 2015) and currently, there are no anti-viral vaccines or therapeutics for WNV infection.

1.2 West Nile Virus Transmission

WNV is classified as a blood-borne virus, as it is transmitted via blood-to-blood contact, primarily by the bite of an infected mosquito. WNV is also transmitted by human blood transfusions and organ transplantations (Lanteri et al., 2014). However, the Centers for Disease Control and Prevention (CDC) have instituted screening of donated blood using nucleic-acid based testing (NAT's), which have successfully prevented spread of the virus (CDC, 2004). Additionally, although rare, transmission of the virus has also been documented to occur from mother to baby through pregnancy, delivery or breastfeeding (Hinckley, O'Leary, & Hayes, 2007). Mammalian species, including humans and horses, are considered dead-end hosts of the virus, as the hosts immune system can develop an adequate response to control the virus (Bowen & Nemeth, 2007). However, avian species, are intermediate hosts and are unable to mount an effective immune response against the virus, therefore are considered reservoirs for viral amplification (Bowen & Nemeth, 2007). Not all avian species carry WNV, however, some species such as the house sparrow, the American crow, and the American robin are highly susceptible to WNV infection (Colpitts, Conway, Montgomery, & Fikrig, 2012). Therefore, regions that are highly populated with these particular bird species are more prone to increased frequencies of viral transmission (Brault, 2009). The primary vector for transmission of WNV is the *Culex* mosquito; however, other species of mosquito have been noted to carry WNV, including the *Aedes* species (Colpitts et al., 2012). In addition,

the virus can survive overwintering due to the process of vertical transmission between female mosquitos and their eggs during oviposition (Baqar, Hayes, Murphy, & Watts, 1993). In brief, transmission of the virus is dependent on a variety of factors that enable survival and success of the virus in endemic countries.

1.3 West Nile Virus Structure, Viral Genome, and Life Cycle

Structurally, WNV is approximately 50 nm in diameter and icosahedral in symmetry. The virus has a multi-layered organization, including its outer membrane containing the envelope and membrane proteins and an inner lipid membrane that encompasses the viral genome, called the nucleocapsid, containing the structural capsid protein (Chancey et al., 2015). WNV has a ssRNA genome with positive polarity, which can be directly translated into a polyprotein. Its genome has approximately 11, 000 base pairs, that contains one open reading frame (ORF) encoding a single polyprotein that is subsequently cleaved by viral and host proteases. The viral genome also contains 5' and 3' untranslated regions (UTR), three structural genes, seven non-structural genes, and it is devoid of a 3' poly-adenylated tail, which makes it distinguishable from host RNA (Brinton, 2014).

There are currently seven different WNV lineages that have evolved throughout different regions of the globe, making WNV a genetically diverse virus. In addition to their geographic differences, these different WNV lineages have evolved to cause more human disease outbreaks, including lineages 1, 2 and 5 (Chancey et al., 2015; Ciota & Kramer, 2013). For example, Lineage 1 clade (a) is the principle lineage of WNV that caused the outbreak in North America in 1999 (Chancey et al., 2015).

At the cellular level, WNV binds to host membrane receptors and enters the cell via receptor-mediated endocytosis. Proposed cellular membrane receptors include integrins, which are highly expressed on neutrophils (Burg & Pillinger, 2001) and DC-SIGN (Davis et al., 2006), which are dendritic cell-specific receptors. Through a pH dependent process, the virus fuses its enveloped membrane with the host's endosomal membrane to uncoat its capsid containing the positive-sensed, ssRNA into the cytoplasm. Here, the viral RNA genome is readily translated into functional proteins, such as RNA-dependent RNA polymerase, and it directly copies itself into multiple negative-sensed ssRNA stands to be used as templates for further positive-sensed ssRNA replication. At the rough endoplasmic reticulum (ER) membrane, structural viral proteins and positive-sensed ssRNA are assembled and packaged into infectious virions in the Golgi apparatus, which are subsequently released from the cell via exocytosis (Suthar, Diamond, & Gale, 2013).

1.4 West Nile Virus Disease and Pathogenesis

Following inoculation within the skin, WNV actively replicates itself within skin-resident dendritic cells (DC) called Langerhans cells. This initial infection triggers the innate immune response to initiate an antiviral program, proinflammatory cytokines and inflammatory mediators to inhibit the virus from replicating and secretes chemokines to recruit leukocytes, such as neutrophils, to sites of infection to destroy the virus. However, in some instances, the virus can successfully disseminate to secondary lymphoid organs, including the lymph nodes and spleen where the virus is amplified (Lazear & Diamond, 2014; Suthar et al., 2013).

Most human cases are asymptomatic (80%), while approximately 19% show febrile illness-like symptoms and less than 1% of individuals develop severe neurological illnesses, including meningitis, encephalitis and flaccid paralysis (Lazear & Diamond, 2014; Suthar et al., 2013). WNV is a neurotropic virus because it can actively infect neurons within the central nervous system (CNS). Although the mechanisms of viral entry into the CNS are not completely understood, three primary mechanisms have been proposed; (a) Direct entry of the virus across the blood-brain barrier (BBB); (b) retrograde transport of the virus via the peripheral nervous system (PNS); and/or (c) the "Trojan horse" mechanism, whereby the virus hijacks immune cells to gain entry into the CNS (Lazear & Diamond, 2014; Suthar et al., 2013).

Following infection, the innate and adaptive immune systems participate to control infection. The antiviral immune response, via the generation of type I interferons (IFNα and IFNβ), is initiated within the first few days of infection and is essential to inhibit infection, as mice deficient in the type I IFN response are susceptible to viral infection (Samuel & Diamond, 2005). Following antiviral initiation, innate immune cells such as polymorphonuclear neutrophils (PMN), natural killer (NK) cells, macrophages, and other granulocytes contribute to pathogen clearance through both cell intrinsic mechanisms of phagocytosis and the release of enzymes and reactive oxygen species that kill the virus. For instance, PMNs perform phagocytosis, degranulate and release neutrophil extracellular traps (NETs) to kill invading pathogens (Kolaczkowska & Kubes, 2013). While innate immunity is critical for rapid, non-specific killing of foreign microbes, the adaptive immune system participates in generation of memory against the foreign microbe, to rapidly control infection if encountered a second time. Adaptive

immune cells include $CD4^+$ and $CD8^+$ T cells and antibody-producing B cells. In terms of viral infection, the adaptive immune response does not take full force until approximately a week post infection. At this time cytotoxic $CD8^+$ T cells and helper $CD4^+$ T cells begin to communicate with antigen presenting cells, such as macrophages and DCs that have ingested and processed viral antigen in the context of major histocompatibility proteins I or II (Vono et al., 2017). This interaction initiates the generation of antigen-specific adaptive immunity and is the basis of producing T cells that can distinguish self- from foreign- antigens. WNV infection in mice requires functional $CD8^+$ cytotoxic T cells to successfully control (Acharya et al., 2017) and clear the virus (Shrestha & Diamond, 2004). Therefore, proper participation with both the innate and adaptive immune responses are necessary for effective control of WNV, while defects in any of these pathways result in more severe infection and disease.

It is important to note that viral kinetics and disease pathogenesis is different between human and mice. For instance, more than 80% of mice develop severe neuroinvasive infection with 10^4-10^6 plaque-forming units (PFU) inoculation, which is the infective dose identified during mosquito inoculation (Styer et al., 2007), however, not all humans develop neurodisease. This may be due to different leukocyte population percentages between humans and mice, the robustness of the immune responses generated, the architecture of the blood brain barrier or many other physiological differences between the two species (Mestas & Hughes, 2004; Suen, Prow, Hall, & Bielefeldt-Ohmann, 2014). Nonetheless, control of viral infection in both species involves specific commination with the innate and adaptive immune responses.

1.5 Innate Immune Mediators Against WNV

1.5.1 Pattern Recognition Receptors and Intracellular Antiviral Immune Mediators Against WNV

WNV is an intracellular pathogen that is recognized by pattern recognition receptors (PRRs) of the innate immune system. PRRs are essential mediators of the innate immune response as they are responsible for distinguishing different pathogen- or danger- associated molecular patterns (PAMPs or DAMPs) from self-antigens and ultimately shape the adaptive immune response. Some notable PRRs include mannose-binding lectins (MBL) that are involved in the complement cascade, intracellular PRRs, such as Retinoic inducible gene-1 (RIG-I), Melanoma differentiation-associated protein 5 (MDA-5) and NOD-like receptors (NLRs), including NALP3 and CIITA. Toll-like receptors (TLR) are type I transmembrane PRRs that are located on the plasma membrane and within endosomal membranes (Akira & Takeda, 2004; Chow, Franz, & Kagan, 2015). TLRs are highly expressed on professional antigen presenting cells (APCs), such as DCs. As mentioned DCs are one of the first cells infected by WNV following skin-inoculation and in fact, most intracellular TLR pathways examined are studied using DCs because these cells are involved in linking innate and adaptive immunity, respond well against viral infections and highly express intracellular TLRs 3, 7, 8 and 9 (Lipscomb & Masten, 2002). Aside from APCs, other innate immune cells, including PMNs, mast cells, eosinophils, and basophils all express TLRs, which are necessary to quickly identify various molecular structures and engage immunity against them. In addition, epithelial cells, fibroblasts, and endothelial cells express TLRs in order to elicit their own form of non-specific, innate immunity. Collectively, all cells express

some ability to recognize self from non-self, suggesting the importance of PRRs in restricting various PAMPS or DAMPs that can cause disease (Akira & Takeda, 2004).

TLR7 recognizes WNV, as *Tlr7*$^{-/-}$ mice have reduced survival and increased viral load following WNV challenge (Town et al., 2009). In addition, TLR3 can also recognize WNV, whereby TLR3 activation induced TNFα disruption of the BBB and initiated WNV entry into the CNS (T. Wang, Town, Alexopoulou, et al., 2004). The latter report highlights the importance of tightly restricted TLR activation, in order to induce appropriate immune responses against foreign antigens while preventing non-specific tissue damage. WNV signaling via TLRs initiates downstream adaptor proteins to work together to regulate innate and antiviral immunity. Majority of TLR intracellular signaling cascades merge onto the MyD88-dependent pathway, a pathway that is important in controlling WNV infection, as *MyD88*$^{-/-}$ mice are highly susceptible to WNV infection and neurons isolated from *MyD88*$^{-/-}$ mice have increased viral load (Szretter et al., 2010). Interestingly, previous studies have indicated that intracellular osteopontin (iOPN), physically associates with MyD88 following TLR9 and TLR7 stimulation with Herpes Simplex virus and following TLR7/9 ligation in plasmacytoid DCs, activating the interferon response factor-7 (IRF-7) and transcription of IFNα (Cao & Liu, 2006; Inoue & Shinohara, 2011; Shinohara et al., 2006). However, the association between iOPN and WNV has not yet been studied.

In addition, IRF proteins can induce the expression of additional antiviral proteins, including the interferon-induced tetratricopeptide repeat (IFIT) proteins. IFIT proteins have been implicated in controlling WNV infection (Cho, Shrestha, Sen, & Diamond, 2013; Daffis et al., 2010; Paul et al., 2016), Japanese Encephalitis virus

(Kimura et al., 2013) and vesicular stomatitis virus (Fensterl et al., 2012). IFIT proteins are highly conserved among various species (Fensterl & Sen, 2014) and work in concert to recognize and bind to the 5'-PPP end of viruses, host mRNA or to the translation initiation factor eIF-3 to inhibit the initiation of viral/host protein translation (Fensterl & Sen, 2014; Wacher et al., 2007). IFIT molecules have been instrumental in controlling WNV infection, as *Ifit2*$^{-/-}$ mice are susceptible to WNV infection (Cho et al., 2013) and *Ifit1*$^{-/-}$ neurons are highly permissive to WNV infection (Daffis et al., 2010). It has been suggested that IFIT molecules may work in concert to inactivate viruses (Lazear & Diamond, 2014), therefore understanding how multiple innate immune pathways communicate is necessary to tailor specific antiviral therapeutics against WNV. In brief, PRRs and downstream intracellular antiviral programs are important mediators of innate immunity against WNV.

1.5.2 Cytokines, Chemokines and Inflammatory Mediators Against WNV

Following PRR engagement and intracellular signaling cascades, various transcriptional programs are induced to produce copious amounts of cytokines, chemokines, and inflammatory mediators in response to WNV infection. Some notable cytokines that have been induced following WNV infection, including IL1β (Ramos et al., 2012), IL-6 (Pena et al., 2014), TNFα (Shrestha, Zhang, Purtha, Klein, & Diamond, 2008), IL-12 (Town et al., 2009), IL-22 (P. Wang et al., 2012) and IL-23 (Town et al., 2009). Generally, cytokines bind to their associated receptor on target cells to prompt a functional output, which results in the destruction of the virus, apoptosis/necrosis of the host cell, and/or cellular differentiation, which are all mechanisms that inhibit viral amplification.

The chemokines CXCL1 (Bai et al., 2010), CXCL2 (Bai et al., 2010), CXCL10 (Klein et al., 2005) and RANTES (Hussmann & Fredericksen, 2014) have also been important innate immune mediators in the control of WNV. Chemokines play a particularly important role in recruitment of leukocytes following infection to the sites of their expression. Chemotaxis occurs via a concentration gradient and interestingly, secreted osteopontin (sOPN), plays a vital role in neutrophil (Koh et al., 2007; J. Sodek, Ganss, & McKee, 2000), dendritic cell (Weiss et al., 2001) and macrophage recruitment (Giachelli & Steitz, 2000). However, sOPN expression following WNV infection has not yet been studied.

In addition, inflammatory mediators, such as the complement receptor-1 (CR1) (Mehlhop et al., 2005) and nitric oxide (Getts et al., 2012) are also induced following WNV infection. Although in the peripheral immune system, inflammation is an instrumental part of the innate immune response, the induction of overt inflammation in the brain can cause detrimental effects on neurons causing neuropathy, neurodegeneration and/or axonal transduction inhibition (Mrak, 2009). Indeed mice pre-treated with anti-very late antigen (VLA)-4 integrin antibodies to inhibit the infiltration of nitric oxide producing monocytes into the brain following WNV infection, resulted in long-term survival of infected mice (Getts et al., 2012). While, macrophages treated with aminoguanidine, mediated the inhibition of inducible nitric oxide synthase (iNOS) and prolonged survival in WNV infected mice, suggesting nitric oxide plays a negative role for neuroinflammation (Getts et al., 2012). In brief, innate immune mediator involvement in WNV infection is critical, as it is necessary to control the virus, yet, bystander

immunopathology is possible during neuroinvasive WNV-induced inflammation in the CNS.

1.5.3 Innate Immune Cells and WNV

Recognition and clearance of WNV requires a tightly controlled activation cascade, including the production of type I IFNs, proinflammatory cytokines, inflammatory mediators, leukocyte-recruiting chemokines and the induction of phagocytosis. As mentioned above, professional APCs, such as DCs, are among the first innate immune cell type that encounters foreign or immunogenic self-molecules. Recognition receptor engagement triggers these APCs to become rapidly activated to elicit both an innate immune response and promote phagocytosis. Additionally, APCs can shape the adaptive immune response by engaging antigen-specific cellular memory to protect the host from future repeated exposure.

DCs have been studied extensively within the context of WNV infection, as they are the principle cell type that first come into contact with the virus following skin-inoculation (Suthar et al., 2013) and WNV can preferentially bind to the DC-specific cellular receptors called DC-SIGN (Martina et al., 2008). Interestingly, DC function has also been linked to age, which is a determining factor in susceptibility to viral infection. For instance, DCs cultured from older donors (approximate age 73 years) compared to younger donors (approximate age 26 years) had reduced type I IFN production following WNV infection *in vitro* (Qian et al., 2011). In addition, the significance of myeloid DCs production of CXCL10 has been implicated as a biomarker for WNV susceptibility, whereby reduced production of this chemokine resulted in higher prevalence for infection (Qian et al., 2015).

There are four main types of mature DCs; follicular DCs, lymphoid DCs, myeloid DCs and plasmacytoid DCs, which are all capable of producing different innate immune mediators in response to viral challenge (Lipscomb & Masten, 2002). Plasmacytoid DCs (pDCs) are among the primary cells involved in active immunity against WNV infection, as they can secrete copious amounts of type I IFN following infection, compared to myeloid DCs (M. C. Silva, Guerrero-Plata, Gilfoy, Garofalo, & Mason, 2007). In healthy adults, total DC populations comprise of approximately 0.46% of peripheral blood leukocytes (Haller Hasskamp, Zapas, & Elias, 2005), therefore only a very small proportion of these cells types are capable of producing a remarkable response against viral infection, highlighting the importance of these cells in controlling viral infection. Importantly, reactivation of circulating lymphocytes in the CNS by $CD11c^+$ DCs were instrumental in controlling viral infection, suggesting an additional role for DCs in the brain (Durrant, Robinette, & Klein, 2013).

1.5.4 Neutrophils and WNV Persistence

Polymorphonuclear neutrophils (PMN) are classified as granulocytes because they contain multiple granules/lysosomes that package antimicrobial proteins, proteases and reactive oxygen species, which can deactivate pathogens via extracellular secretion or during phagocytosis (Mayer-Scholl, Averhoff, & Zychlinsky, 2004). In addition, they are one of the first cellular responders in the innate immune response against foreign pathogens and are the most abundant immune cell in humans, as they constitute between 50-70% of white blood cells in circulation (Mocsai, 2013) and 10-25% in mice circulation (Mestas & Hughes, 2004). In addition, the number of neutrophils present at sites of infection has been shown to be essential in clearing infection (Zeidler,

Germeshausen, Klein, & Welte, 2009). The lifespan of neutrophils in mice without activation is 12 hours and when activated their lifespan increases seven-fold (Summers et al., 2010), indicating a role for cellular persistence and possible bystander injury within tissues where infection is present. The most unique feature of neutrophils is the release of neutrophil extracellular traps (NETs). NETs are comprised of intracellular chromatin DNA, histones, and granular proteins that actively immobilize pathogens, facilitate phagocytosis of trapped pathogens and kill pathogens by way of antimicrobial histones and proteases (Kolaczkowska & Kubes, 2013; Phillipson & Kubes, 2011).

The role of PMNs in WNV infection has been somewhat of a paradox, whereby a previous study has shown that neutrophils can both respond against WNV challenge by inducing phagocytosis and producing antimicrobial mediators, but are also capable of serving as WNV reservoirs (Bai et al., 2010). In particular, when neutrophils were depleted in mice prior to infection, they survived WNV challenge, however, when PMNs were depleted post-infection this resulted in moribund mice, suggesting neutrophils also protect mice from WNV infection (Bai et al., 2010). Therefore, PMNs are important innate immune cells that respond to infection, yet they may play an important role in the "Trojan horse" mechanism of WNV dissemination into the CNS.

Entry of PMNs into the brain parenchyma is termed leukocyte extravasation or rolling-leukocyte transmigration, which requires several steps in order to anchor the PMNs to the endothelial cells and promote transmigration of PMNs through tight junctions. During PMN (leukocyte) extravasation three steps are performed; first rolling of PMN is initiated by E-selectins on endothelial cells that are upregulated following a stimulus, and bind to carbohydrate molecules on PMNs; second, chemokines bind to

receptors on PMNs sending an activation signal that; three, induces a conformation change in the integrins on the PMN to bind to cell adhesion molecules (CAMs) located on the endothelium, promoting transendothelial migration of the PMN into the parenchyma.

PMNs have multiple killing mechanisms, however why PMNs switch from a defensive immune cell to a viral reservoir is an interesting question. A possible reason for this is the immunosuppressive chemicals within mosquito vector saliva that is delivered into the host during transmission, may inhibit PMN function. Indeed this is the case with lymphocyte function and the yellow fever vector *Aedes aegypti* (Wasserman, Singh, & Champagne, 2004), which is also a primary vector for WNV transmission. However, in the laboratory, viral isolates inoculated in mice do not contain mosquito saliva, so this may not be the principle cause of PMN immunosuppression in laboratory-based settings. As mentioned above, the life span of PMNs increase when activated by cytokines, such as TNF-α and IL-1β, while transmigration also supports PMN survival, by delaying apoptosis though Akt activation (Elbim, Katsikis, & Estaquier, 2009; Pluskota, Soloviev, Szpak, Weber, & Plow, 2008), therefore inflammatory conditions may paradoxically contribute to PMN persistence as viral reservoirs. Additionally, PMNs acting as viral reservoirs have been implicated in other viral infection, such as H5N1 influenza (Gu et al., 2007; Hartshorn et al., 1995) and Epstein-Barr Viral (EBV) infections (Drescher & Bai, 2013; Gosselin et al., 1991; Savard & Gosselin, 2006). In one report, H5N1 influenza was shown to directly decrease the function of PMNs and monocytes by binding the viral surface protein hemagglutinin to the host sialic acid bearing surface receptors CD43 and CD45. This interaction cross-linked these receptors, deactivating the

oxidative burst responses of PMNs and monocytes (Hartshorn et al., 1995). Briefly, viral interaction with host-cell mediators can immunomodulate the function of PMNs through prevention of apoptosis and promotion of cellular persistence (Elbim et al., 2009); therefore, the role of PMNs as viral reservoirs during WNV pathogenesis is possible.

In line with this, human WNV cases with neurological impairment, have exhibited neutrophilia in their cerebral spinal fluid (CSF), indicating a possible role for PMNs in the development of the neurological symptoms of WNV infection (Crichlow, Bailey, & Gardner, 2004; Tyler, Pape, Goody, Corkill, & Kleinschmidt-DeMasters, 2006). Moreover, IL-22 has been shown to be an important molecule in facilitating PMN recruitment during WNV infection, as *Il-22*$^{-/-}$ mice had defective PMN migration into the brain and were more resistant to WNV infection (Wang et al., 2012). In brief, PMNs have been implicated to play a potential role in neuroinvasive WNV infection in humans, whether acting in favor of the virus or the host remains elusive.

1.6 The Central Nervous System and West Nile virus

1.6.1 WNV Neuroinvasive Infection and Mechanisms of Viral Entry into the Brain

WNV causes neuroinvasive symptoms in a small percentage (<1%) of infected individual, however the root cause for neuroinvasiveness that is observed in some patients but not others is still not completely understood. Deficits in the interferon response has been described to play a potential role (Cho & Diamond, 2012). However, another overlooked possibility may be that chronic inflammatory conditions, which are notable within the elderly (the most at risk for infection), may contribute to age-related disease and has recently been termed inflammaging (Franceschi & Campisi, 2014). Low-grade inflammation has been linked to immune senescence, neuronal dysfunction and

changes in body composition (Franceschi & Campisi, 2014), such as in blood-brain barrier integrity (Elahy et al., 2015). During inflammaging cytokines including IL-6 and TNF-α, which can contribute to BBB disruption, are slightly increased (Franceschi & Campisi, 2014) and consistent, low-grade immune stimulation with these proinflammatory cytokines can lead to immune senescence (Deeks, 2011), leaving the host susceptible to WNV. In addition, differential immune cell profiles have been identified between WNV fever and neuroinvasive patients. Some reports have identified that although immune compromised individuals' have poor viral control and higher potential of neuroinvasiveness (Diamond et al., 2003; Kumar, Belcaid, & Nerurkar, 2016; Samuel & Diamond, 2005), there is also the potential for immune-mediated damage causing neurological symptoms. For instance, blood collected from neuroinvasive patients had higher numbers of WNV-responsive cells ($CXCR3^{+}CCR4^{+}CCR6^{-}$T cells) with more potent responses (IFNγ production) and reduced immune suppressive (Treg) cell populations, suggesting uncontrolled immunity against WNV is present in neuroinvasive WNV infections (James et al., 2016).

Furthermore, different strains of WNV may also contribute to neuroinvasiveness (Lanciotti et al., 2002). For instance, NY99 (pathogenic) and WNV Eg101 (non-pathogenic) stains displayed differential host gene expressions, resulting in increased IL-1β and CXCL1 expression following infection with the NY99 pathogenic strain of WNV, but not with WNV Eg101 non-pathogenic infection (Kumar et al., 2016), suggesting unchecked inflammation can contribute to pathogenicity. Adding complexity to this, although induction of unchecked inflammation following infection appears to contribute to pathogenicity, for example increased Il-1β and TNF-α, both of these proinflammatory

mediators have also been described to be necessary to protect mice from WNV by promoting immune trafficking into the brain to control infection (Ramos et al., 2012; Shrestha et al., 2008). Therefore, although the immune response is necessary to clear infection in the brain, it is well-accepted that perturbed, prolonged and robust immune cell trafficking into the brain can also be detrimental to brain architecture through bystander damage (Aarli, 1983; Getts et al., 2012; Varatharaj & Galea, 2017), (Sankowski, Mader, & Valdes-Ferrer, 2015). Therefore, proper WNV immunity must be tightly regulated to only control infection, without causing bystander damage and minimize pathogenic responses, while timing of immune cell infiltration into the brain following WNV infection may also be important to consider. The best example of this is in relation to previous work with PMNs as viral reservoirs, as PMNs depleted from mice prior to WNV infection resulted in better survival, while depletion of PMN post-WNV infection resulted in rapid morbidity (Bai et al., 2010). These results not only identified an important immune cell type that is responsible for WNV clearance in the brain but also provided insight into the persistence of WNV within PMNs, acting as viral reservoirs.

As described above, there are three main mechanisms for WNV entry into the CNS; (a) direct entry of the virus across the BBB, albeit through tight junctions or through direct infection of endothelial cells, termed the hematogenous route (Suen et al., 2014); (b) axonal retrograde transport of the virus via the PNS (Samuel, Wang, Siddharthan, Morrey, & Diamond, 2007); and/or (c) the "Trojan horse" mechanism, whereby the virus hijacks immune cells to gain entry into the CNS (Lazear & Diamond, 2014; Suthar et al., 2013) (Diamond & Klein, 2004; Garcia-Tapia, Loiacono, &

Kleiboeker, 2006; Hunsperger & Roehrig, 2006; Kramer-Hammerle, Rothenaigner, Wolff, Bell, & Brack-Werner, 2005; Monath, Cropp, & Harrison, 1983; Samuel & Diamond, 2006; Samuel et al., 2007; Suen et al., 2014; Verma et al., 2009). As mentioned above, lamins and DC-SIGN are two types of receptors that promote WNV infection of cells. Other host receptors on endothelial cells including glycoaminoglycans (GAGs) or c-type lectins can also permit infection, which enables viral entry into the brain parenchyma. For instance, attenuation of GAG expression in mice have reduced BBB invasion of group B streptococcus (Chang et al., 2011). Aside from viral entry through direct infection of endothelial cells, the BBB is designed to only allow small molecules less than 1 nm in diameter (Jain, 2012). WNV has a diameter of 50 nm and therefore a disrupted BBB vasculature would be required for viral entry between endothelial cells. Another mechanism of viral entry into the CNS is via the peripheral nervous system (Samuel et al., 2007). In this model of infection, peripheral WNV infects axons of the PNS near the site of viral inoculation and retrograde transports the PNS into the CNS (Samuel et al., 2007).

The third and most relevant to this study is the Trojan horse mechanism of viral entry, which is considered a "second wave" of viral entry (Roe et al., 2012) because inflammation in response to WNV establishment in the brain precedes recruitment of leukocytes to clear infection. As mentioned, PMNs have been identified as major carriers of WNV (Bai et al., 2010), while infected T cells and macrophages have also been identified to harbor WNV into the CNS (Cardosa, Porterfield, & Gordon, 1983; Samuel & Diamond, 2006; Wang et al., 2008), albeit at much lower levels than PMNs (Wang, Town, Alexopoulou, et al., 2004). Leukocyte recruitment is mediated through induction

of endothelial cell surface proteins, such as ICAM and VCAM that initiate leukocyte extravasation into the CNS (Roe, Orillo, & Verma, 2014) and development of inflammatory cytokines that promote BBB permeability (Roe et al., 2012). The adherence proteins ICAM-1, VCAM-1, and E-selectin are all involved in PMN extravasation and are induced in human brain microvasculature following WNV infection (Roe et al., 2014). In addition, reduced tight junction proteins, between endothelial cells can contribute to WNV disease severity (Roe et al., 2012). For example, tight junction proteins ZO-1, Occuldin, JAM-1 and Claudin-1 showed reduced expression following WNV starting at day 4 post-infection, while leukocyte infiltration was observed in brains at day 8 p.i. (Roe et al., 2012), suggesting expression of tight junction proteins are also necessary to prevent WNV Trojan horse entry of leukocytes, which can contribute to neurodisease.

1.6.2 CNS Resident Cells and WNV Infection

Under non-pathogenic conditions, a robust and activated immune system is absent in the nervous system primarily because presence of high numbers of activated immune cells could cause detrimental effects by way of bystander brain tissue damage (Aarli, 1983; Getts et al., 2012). Unchecked neuroinflammation as a results of atypical, active immune cell infiltration in the brain occurs in many neuroinflammatory diseases, such as multiple sclerosis and Alzheimer's disease (Comi et al., 2010; Shimizu et al., 2013), while regulated, steady state leukocytes in the brain can also contribute to repair and healing (Schwartz, Kipnis, Rivest, & Prat, 2013). Indeed the brain is in constant surveillance and in communication with the immune and circulatory systems (Shimada & Hasegawa-Ishii, 2017), however the CNS has also adapted its own immune strategy

within CNS-resident cells (Hanamsagar, Hanke, & Kielian, 2012; Lampron, Elali, & Rivest, 2013; Ransohoff & Brown, 2012) through microglia and astrocytes. Both of these brain-resident immune cells derive from the myeloid cell lineage (similar to macrophages) and function to survey the CNS for foreign microbes and respond to infection by producing inflammatory mediators, cytokines and chemokines (Giulian, Baker, Shih, & Lachman, 1986; Hayes, Woodroofe, & Cuzner, 1988; Zucker-Franklin, Warfel, Grusky, Frangione, & Teitel, 1987). In line with this, CNS diseases are possible if microglia responses are not tightly regulated (Czirr & Wyss-Coray, 2012; Ransohoff & Brown, 2012). For instance, lipopolysaccharide (LPS) and IFNγ primed macrophages and microglia can induce the similar levels of nitric oxide (NO) at 48 and 74 hour post activation (Brantley et al., 2010) and produce similar cytotoxic response against tumor cells (Eue, Kumar, Dong, Killion, & Fidler, 1998), suggesting bystander tissue damage is possible within the CNS through brain-resident immune cell activation in the absence of peripheral leukocyte infiltration. It has also been described that, although a mechanism for immunity is present in the brain, these responses are not capable of clearing infection alone and must recruit circulating immune cells for aid (Schwartz et al., 2013), therefore communication between the brain and the circulatory and immune systems are highly interconnected.

Neurons can express TLRs on their surface and within endosomal compartments (Lee, Lee, Cho, & Lee, 2013); therefore, they possess the ability to recognize and respond to WNV challenge. Antiviral mechanisms in neurons are in place to inhibit viral replication, for example, IL1β and IFNγ have been described to synergize within neurons to inhibit WNV replication (Ramos et al., 2012). As mentioned above, IFIT molecules

within the CNS, in particular within neurons, play an important role in controlling WNV infection (Cho et al., 2013; Daffis et al., 2010); therefore, antiviral programs are initiated following WNV challenge. In line with this study, cortical neurons were found to be the most prominent producers of OPN in HIV-associated neurocognitive disorders, compared to microglia and astrocytes (Silva et al., 2015). However, the role for OPN production in WNV neuroinflammation has not yet been studied. WNV is classified as a neurotropic virus that can cause both direct neuronal damage via induction of CNS-intrinsic mediators and indirect neuronal damage via infiltrating immune cell production of innate immune mediators. Acute and chronic inflammation can lead to long-term neuropathological effects including flaccid paralysis, neuropathy, chronic fatigue, and/or possible life-threatening clinical symptoms. Therefore, understanding WNV neuropathology is of critical importance, not only to inhibit viral transmission but also to develop new therapeutics against WNV-induced neurodisease.

1.6.3 Disruption of the Blood-Brain Barrier

The brain was previously thought to be an immune privileged organ, primarily because grafting tissue in the brain results in prolonged tissue survival in comparison to grafting outside of the brain (Shimada & Hasegawa-Ishii, 2017). Since it has been identified that the brain is not immune privileged, communication between the immune system and the CNS is constant (Shimada & Hasegawa-Ishii, 2017). Very recently, a lymphatic system has been identified in the brain and functions to drain interstitial fluid and provide immune surveillance (Iliff, Goldman, & Nedergaard, 2015). Another communication interface between the peripheral immune system and the CNS is via the BBB, which serves as an interface between the circulatory system and brain parenchymal

cells (Shimada & Hasegawa-Ishii, 2017). The BBB consists of blood vessels that are comprised of endothelial cells interconnected with transmembrane tight junction proteins such as, occludins and claudins, which are linked to adaptor proteins anchored to actin filaments, called zo-1. There are also additional adherens junction proteins, such as cadherins that are linked to cytoskeleton catenins (Larochelle, Alvarez, & Prat, 2011). Surrounding these cells includes the endothelial basement membrane, contractile cells called pericytes and the parenchymal basement membrane, which directly communicates with astrocytic endfeet within the brain (Sofroniew, 2015).

The main function of this highly organized barrier is to prevent neurotoxic compounds from entering the brain parenchyma (Alyautdin, Khalin, Nafeeza, Haron, & Kuznetsov, 2014) and is designed to prevent passage of materials that are greater than 1 nm is size (Jain, 2012). Indeed, nanomedicine therapeutics struggle to determine how to transport molecules across the BBB, while literature in this field is of great importance to study BBB architecture. It has been describe that although permissive to molecules less than 1 nm in size, additional factors such as receptor anchoring and hydrophobicity play a role in transport of molecules across the BBB (Jain, 2012; Siegel, 1999). This would account for the neuroinvasive potential of WNV (50 nm in diameter) early in infection, which can bind to GAGs, c-type lectins and laminins that are expressed on endothelial cell surfaces (Perera-Lecoin, Meertens, Carnec, & Amara, 2013) in the absence of BBB compromise. On the other hand, leukocytes (7-30 μm in diameter) gain access into the brain following receptor anchorage that is observed during immune cell extravasation only after inflammation induces expression of these proteins on the surface of endothelial cells. Therefore, under normal conditions, circulating immune system cells have limited

access into the brain, while under disease conditions; immune cells are recruited into the brain, through leukocyte extravasation with the goal of clearing toxic mediators. However, timing and duration of immune cell infiltration is key to preventing aberrant responses. For example, GFP-tagged peripheral monocyte-derived macrophages in mouse spinal cord injury showed delayed entry and enhanced clearing potential following BBB breached-injury, while they were not detected in non-lesion spinal cords (Shechter et al., 2009). Therefore, highlighting the precise regulation of immune "intensity", i.e. via the level of proinflammatory mediators produced, leukocyte extravasation timing and the type of infiltrating leukocytes distinguishes healthy from compromised BBBs.

Indeed the BBB is vulnerable to proinflammatory mediators, such as NO and matrix metalloproteinases (MMPs) (Kempuraj et al., 2016; Larochelle et al., 2011; Zhang et al., 2015), and following WNV infection brain-resident cells produce TNF-α and IL-6 (Kumar, Verma, & Nerurkar, 2010; Pena et al., 2014). As a mechanism of viral entry, penetration of WNV into the CNS is associated with a transient increase of BBB permeability, suggesting WNV enters the brain initially through the hematogenous route (Wang, Town, Alexoupoulou, et al., 2004). Other molecules such as TNF-α receptor-1 and ICAM-1 are required for BBB permeabilization following WNV infection (Dai, Wang, Bai, Town, & Fikrig, 2008; Wang et al., 2008; Wang, Town, Alexoupoulou et al., 2004), therefore many different mechanisms are engaged following WNV infection and BBB disruption, resulting in immune cell extravasation into the brain parenchyma. Therefore, controlled immune surveillance and regulated inflammation during viral infection is indispensable to prevent neurodegeneration (Kempuraj et al., 2016).

1.7 Osteopontin

1.7.1 Intracellular Osteopontin

Osteopontin (OPN) is a negatively charged, glycoprotein originally named for its origin within bone, in particular for its expression within osteoclasts and osteoblasts (Butler, 1989; Oldberg, Franzen, & Heinegard, 1986). It was first described by Hynes and colleagues, as a phosphorylated protein that is secreted from transformed cells (Senger, Wirth, & Hynes, 1979). OPN has been implicated in a variety of cellular processes including bone remodeling, tumorigenesis and immune cell chemotaxis (Sodek et al., 2000). OPN is secreted by cells of the immune system, such as activated T cells and has also been called early T-lymphocyte activation 1 (Eta-1) (Patarca et al., 1989; Wang & Denhardt, 2008). This multifaceted protein has been induced in different neurological pathologies, including multiple sclerosis (Shimizu et al., 2013), Alzheimer's disease (Comi et al., 2010), frontotemporal dementia (Mattsson et al., 2008), HIV-associated dementia (Brown et al., 2011; Burdo, Ellis, & Fox, 2008), malignant gliomas (Lu et al., 2012) and brain tumor associated metastases (Weber & Ashkar, 2000). However, the role of OPN during WNV infection has not yet been studied.

There are two isoforms of OPN, one is expressed exclusively inside cells downstream of the TLR7/9 signaling cascade, called intracellular OPN (iOPN), which lacks a 16 amino acid signal sequence, targeting OPN to the cytoplasm and not the Golgi (Shinohara, Kim, Kim, Garcia, & Cantor, 2008). Initial identification of iOPN was a result of two primary observations; one, treatment with recombinant (r)OPN in *Opn*$^{-/-}$ mice did not always reverse the phenotypes, and two, OPN depletion with antibodies in WT mice did not always reproduce *Opn*$^{-/-}$ phenotypes, suggesting an intracellular role for

OPN (Bourassa, Monaghan, & Rittling, 2004; Sato et al., 2008; Shinohara et al., 2006; Zhu et al., 2004). Its expression is cell-specific, as APCs for example, produce high levels of iOPN compared to T cells (Cantor & Shinohara, 2009). The expression of iOPN is also involved in cytoskeletal rearrangements (Zhu et al., 2004) and has been a major area for cancer research, as it has been actively involved in uncontrolled proliferation of tumor cells (Bourassa et al., 2004). However, the role for iOPN has yet to be elucidated during WNV infection.

1.7.2 Secreted Osteopontin

The other isoform is full-length, secreted OPN (sOPN) (Shinohara et al., 2008; Zhao et al., 2010), which contains a 16 amino acid signal sequence that targets sOPN to the Golgi for packaging and secretion out of the cell (Sodek et al., 2000). sOPN participates in leukocyte recruitment (Ashkar et al., 2000), DC emigration to lymph nodes from the epidermis (Weiss et al., 2001) and recruitment of PMNs to sites of its expression (Koh et al., 2007; Sodek et al., 2000). In line with this, previous literature has indicated reduced PMN extravasation and tissue injury is observed in *Opn*$^{-/-}$ mouse models of hepatic disease (Yang et al., 2014) and OPN expression precedes innate immune cell infiltration in mouse models of hepatic disease (Diao et al., 2004). While, following myocardial infarction OPN is induced resulting in increased migration of PMNs and monocytes into the infarct tissue (Singh, Foster, Dalal, & Singh, 2010). Moreover, a recent report has indicated that inflammatory cells infiltrated into the CNS in response to OPN expression and caused localized inflammation following i.p. injection of pertussis toxin (Marcondes et al., 2014). This migration was a result of increased expression of the OPN receptor CD44v6 on peripheral immune cells (Marcondes et al.,

2014). In line with this, PMNs can express high levels of CD44 (Takazoe et al., 2000) and the integrin receptor $\alpha_v\beta_3$, both of which are important for neutrophil migration in transepithelial migration across the intestinal epithelium in human intestinal epithelial cells (Brazil et al., 2013; Kim, Skokos, Myer, Agaba, & Gonzalez, 2014); suggesting not only does increased sOPN, but increased PMN cell receptors for OPN binding, results in PMN migration.

1.7.3 Osteopontin in Central Nervous System Diseases

OPN expression within the CNS has been linked to disease development. As mentioned above, OPN has been induced in many different neurological pathologies, including multiple sclerosis (Shimizu et al., 2013), Alzheimer's disease (Comi et al., 2010), frontotemporal dementia (Mattsson et al., 2008), HIV-associated dementia (Brown et al., 2011; Burdo et al., 2008), malignant gliomas (Lu et al., 2012) and brain tumor associated metastases (Weber & Ashkar, 2000). During multiple sclerosis (MS), OPN expression is linked to the severity of the disease, secondary progressive patients had significantly higher OPN expression compared to relapsing-remitting MS patients in plasma (Shimizu et al., 2013). During MS pathogenesis myelin-reactive T-cells infiltrate into the CNS and cause neuroinflammation and ensuing neurodegeneration and since increased OPN is found in more severe cases, sOPN may be a major contributor to increased brain-infiltrating, reactive leukocytes in MS pathogenesis. Alzheimer's disease (AD) is another neuropathological disease that has indicated a role for OPN, whereby AD patients have increased OPN expression in their CSF early in disease progression (Comi et al., 2010). It has also been suggested that PMNs migrate toward amyloid plaques in a mouse model of AD and upon arrival, they accumulate and create senile plaques, which

are implicated in pathophysiology of AD (Baik et al., 2014). Therefore, after initial Aβ plaque deposition ensues in the brain, innate immune cells (i.e. PMNs) migrate into the brain to non-specifically clear the underlying condition, however, can paradoxically cause bystander inflammation and tissue destruction, increasing disease progression. This may also occur during WNV neuroinvasion, whereby innate immune cells traffic into the brain to clear infection, yet carry more virus into the brain, contributing to increased inflammation and neuronal death. In addition, frontotemporal dementia has been described as a progressive, neuropathological disease that is characterized by axonal degeneration and induced OPN expression, that is only induced during rapid progression of disease (Mattsson et al., 2008). In brain tumor models, OPN involvement has been implicated as a prognostic biomarker, as it has been associated with metastatic brain tumors and malignant gliomas. In one report, application of rOPN enhanced migration of glioma cells and the expression of heme oxygenase (HO)-1, by signaling the transcription factor Nrf-2, downstream the ERK and Akt signaling pathways (Lu et al., 2012). In addition, ERK, Akt and HO-1 inhibitors reduced cellular migration of glioma cells in brain metastasis. Therefore, sOPN can act on OPN receptors to activate downstream ERK and Akt signaling pathways to promote the translocation of the transcriptional promoter Nrf-2 and subsequent HO-1 production (Lu et al., 2012). Furthermore, CD44v6 receptors work through the Akt pathway of activation (Herishanu et al., 2011) that is involved in cellular migration and proliferation, which may be the cause of increased PMN survival during neuroinvasive WNV infection. In brief, understanding the role of OPN within the brain, along with the peripheral immune system is critical and may serve as an important target to prevent neurological disorders.

1.7.4 Osteopontin in Central Nervous System and Non-Central Nervous System Viral Infections

OPN expression has also been linked to viral-related pathologies. For instance, Dengue virus, another arthropod-borne virus, showed marked increase in sOPN and iOPN in plasma isolated from first-time infected Dengue fever and dengue hemorrhagic fever patients (Chagan-Yasutan et al., 2014). OPN levels were only observed during the critical phase of infection (day 4-5), when symptoms are most severe, while reduced OPN expression was observed in the recovery phase (day 7-8) of infection, suggesting an important role for this protein in response to acute infection. Chronic hepatitis B virus (HBV) causes liver disease and hepatocellular carcinoma and marked increase in sOPN expression has been reported in plasma isolated from hepatocellular carcinoma patients, as well as is a prognostic biomarker for the development of the disease following HBV infection (da Costa et al., 2015). Moreover, hepatitis C virus (HCV) also induces chronic liver injury and disease and increased sOPN expression was observed in the liver and serum of patients infected with chronic HCV (Choi et al., 2014). In this report, treatment of Huh7.5 cells with sOPN resulted in increased HCV replication, while neutralizing sOPN with OPN specific aptamers repressed HCV levels (Choi et al., 2014). Respiratory syncytial virus (RSV) infection in mice was shown to induce OPN expression after intranasal inoculation and studies with older (19-21 months) versus younger (2-3 months) mice displayed expression of sOPN was delayed but prolonged in older mice, which are more susceptible to RSV infection (Wong et al., 2014). In addition, human T-cell lymphotropic virus type-1 (HTLV-1) infection results in neuroinflammatory disease that is associated with CD4+ T cell infiltration into the CNS and myelopathy/tropical spastic

paraparesis (M/TSP) and increased sOPN, IL-17 and IL-22 expression were observed in M/TSP patients but not asymptomatic carriers of HTLV-1 (Sarkis et al., 2013). These results further suggest other factors independent of the virus (i.e. immune cell infiltration into the brain), contribute to neurological disease symptoms observed in HTLV-1 infections. Moreover, elevated OPN levels in the brain and plasma of simian immunodeficiency virus (SIV) infected macaques, along with increased CD44v6 receptor expression within monocytes, positively correlated with viral-induced CNS disease (Brown, 2012). Additionally, OPN is elevated in the brain, CSF and plasma of patients with human immunodeficiency virus (HIV)-associated dementia (Burdo et al., 2008). Interestingly, HIV-infection induced OPN in brain tissue and CSF, yet HIV-associated dementia patients exhibited OPN induction in plasma, as well as in the brain and CSF (Burdo et al., 2008), suggesting multi-organ involvement for sOPN may contribute to disease progression.

CHAPTER II – SIGNIFICANCE & HYPOTHESIS

2.1 Significance of the Study

West Nile virus (WNV) is a neurotropic virus that can cause encephalitis, meningitis, flaccid paralysis and/or possible death in humans. Recent clinical disease case data indicates 56% of all WNV reported cases in the United States in 2016 were neuroinvasive (1,140 / 2,038) (CDC, 2017), therefore controlling neuroinvasive WNV infection is of critical importance.

Understanding the mechanisms involved in the hosts' innate immune response against WNV are necessary to control viral-induced neuropathogenesis. It is well established that unchecked, aberrant or chronic inflammation leads to bystander tissue damage, which becomes of particular concern in relation to the CNS (Kempuraj et al., 2016; Ransohoff, 2016). The antiviral chemokine OPN, has a dual role in innate immunity, whereby intracellular OPN (iOPN) is involved in the production of type I IFNs and secreted OPN (sOPN) is a potent chemokine that recruits dendritic cells (DC) (Weiss et al., 2001) and polymorphonuclear neutrophils (PMN) (Koh et al., 2007; Sodek et al., 2000) to sites of its expression, preceded by viral infection (Roe et al., 2012). Additionally, sOPN induction has been observed in multiple CNS-viral infections (Chimparlee et al., 2015; Choi et al., 2014; Silva et al., 2015) and is robustly induced in neurons following infection with HIV (Silva et al., 2015). Therefore, early OPN expression following infection is instrumental in generating immunity within the CNS, however prolonged OPN may contribute to aberrant inflammation. Moreover, since sOPN can recruit leukocytes to sites of its expression, it may play a role in the Trojan horse mechanism of WNV entry. In brief, the focus of this dissertation is to identify the

mechanisms involved during neuroinvasive WNV and the role that OPN plays in this process.

2.2 Hypothesis

Understanding the immune mechanisms against WNV is critically important to control WNV-induced neurodisease. Innate immune responses against WNV include, recognition of the virus via various PRRs within both the PNS and the CNS, induction of intracellular signaling cascades that produce cell-intrinsic molecules to directly inhibit WNV and soluble extracellular proteins to recruit leukocytes to aid in the defense against the virus (Lazear & Diamond, 2014; Suthar et al., 2013). Importantly, it is only after CNS-infection or injury is established in the brain that peripheral immune cells traffic into the brain with hope to clear the underlying problem (Wilson, Weninger, & Hunter, 2010), which has been suggested to be the case in neuroinvasive WNV infection (Roe et al., 2012). First, WNV establishes itself within neurons, as it is a neurotropic virus, prior to the induction of various chemokines, disruption of tight junction proteins and induction of leukocyte extravasation markers, which then recruits innate immune cells into the brain to clear the virus (Roe et al., 2012). However, when immune cells traffic into the brain they may be carrying virus, as proposed by the 'Trojan horse' mechanism of WNV neuroinvasion (Suthar et al., 2013); paradoxically contributing to viral pathogenesis.

OPN is a multifaceted protein that participates in the intracellular innate antiviral program as it is involved in the amplification of type I IFNs in plasmacytoid DCs (Cao & Liu, 2006; Inoue & Shinohara, 2011). It also works as a secreted chemokine to recruit various leukocytes such as DCs (Weiss et al., 2001) and PMNs (Koh et al., 2007) to sites

of its expression. PMNs are important cells involved in innate immunity as they are the first responder to sites of infection and the most abundant leukocyte in the human immune system. However, they have also been described as viral reservoirs for WNV (Bai et al., 2010). sOPN has been implicated in many different CNS pathologies, including multiple sclerosis (Shimizu et al., 2013), Alzheimer's disease (Comi et al., 2010), frontotemporal dementia (Mattsson et al., 2008), HIV-associated dementia (Brown et al., 2011; Burdo et al., 2008), malignant gliomas (Lu et al., 2012) and brain tumor associated metastases (Weber & Ashkar, 2000). From these reports, we can hypothesize that viral reservoirs, such as PMNs are recruited into the brain by the induction of sOPN signaling from infected neurons, in the attempt to respond to infection. However, PMN recruitment plays a negative role in neuroinvasive WNV infection, as it traffics more virus into the brain causing increased neuroinvasive disease. Therefore, we hypothesized that soluble OPN recruits WNV-infected PMNs into the brain early in the course of infection, contributing to neuroinvasive WNV infection. In brief, a better understanding for the role of sOPN within the CNS following WNV infection can offer insight into necessary treatments that prevent neurodisease early in infection.

CHAPTER III – METHODS

3.1 Ethics Statement, Biosafety, and Animal Studies

All animal experimental procedures were reviewed and approved by the Institutional Animal Care and Use Committee at The University of Southern Mississippi (USM) (protocol # 12041201/15101601). 7-week-old, sex-matched (C57BL/6J background) WT and *Opn*$^{-/-}$ mice (Jackson Laboratories) were intraperitoneal (i.p.) inoculated with 2,000 plaque forming units (PFUs) of WNV (isolate CT2741) in 100 µl of 2% gelatin in phosphate buffered saline (PBS) and mice were monitored daily up to day 30 post infection (p.i.). Brain, spleens, and blood were collected from WT and *Opn*$^{-/-}$ mice at days 2, 4, and 6 p.i. for subsequent analyses. For intracerebral (i.c.) injections of WNV (100 PFU/mouse in 20 µl 2% gelatin) and recombinant mouse OPN (rOPN; 50 ng/mouse in 20 µl in PBS) were performed on 8-week-old, sex-matched mice anesthetized with 30% isoflurane in isopropanol. For rOPN studies, one-hour post i.c. rOPN supplement, i.p. inoculation with 2,000 PFU of WNV per mouse was performed and mice were monitored up to 30 days p.i.. In additional studies, mock WT mice were i.p. injected with high dose rOPN (200 ng/ml) 4 hr prior to blood and brain collections.

Written informed consent was obtained from all human volunteers and human WNV cases prior to inclusion in this study. The protocol for human subject has been reviewed and approved by the University of Southern Mississippi (USM) Institutional Review Board (protocol # CH-R11120601). All the *in vitro* experiments and animal studies involving live WNV were performed by certified personnel in the biosafety level 3 (BSL3) laboratories following standard biosafety protocols approved by the USM Institutional Biosafety Committee.

3.2 Cell Culture

WNV isolate (CT2741) kindly provided by John F. Anderson, was propagated one time in Vero cells (ATCC CCL-81) and titered by using a Vero cell plaque assay, as previously described (Paul et al., 2014). Vero cells were grown in Dulbecco's modified Eagle medium (DMEM) containing 1% l-glutamine (L-glu), 1% penicillin-streptomycin (Pen/Strep) and 10% fetal bovine serum (FBS). SHSY5Y cells (ATCC CRL-2266) were grown in Eagle's minimal essential medium (EMEM) and F12 medium (1:1) containing 10% FBS.

Primary mixed neuronal cells were isolated from adult (6- to 12-month-old) WT and *Opn*$^{-/-}$ mice, as previously described with some modifications (Eide & McMurray, 2005; Paul et al., 2016). Briefly, whole brains were isolated in ice-cold HEPES-buffered saline (HBS), minced and triturated in HBS containing Papain (2 mg/ml) and incubated for 15 minutes at 37 °C. Following incubation, cell suspensions were counted and 6 x 10^5 cells/ml were plated on poly-ornithine pre-treated 12-wells plates for 20 minutes at 37°C, followed by a gentle wash with HBS to remove cellular debris. Complete media consisting of DMEM:F12 (1:1) medium (Thermo Scientific) supplemented with 1% Pen/Strep, 10% FBS, 1% L-glu, and glucose (4.5 g/l) was added to the cells. On day 11 of culture, supernatant was removed and replaced with neuronal feeding medium consisting of Neurobasal®-A media (Life Technologies) supplemented with 2% B-27 (50X, Life Technologies), 10% FBS, 1% L-glu and 1% Pen/Strep and cells were infected with WNV (MOI = 5) for 24 hrs.

Human peripheral blood mononuclear cells (PBMC) were isolated from whole blood and isolated following Ficoll-Paque PLUS (GE Healthcare) manufactures

instructions. Briefly, whole blood was collected in heparin-coated tubes and were diluted into RPMI 1640 (1:4 dilution, Life Technologies) media. 15 ml of Ficoll-Paque PLUS (GE Healthcare) was added to 50 ml conical tubes and the diluted blood was slowly layered on top of Ficoll-Paque PLUS, followed by density gradient centrifugation at 400 xg for 40 minutes, without brake. The PBMC layer was isolated from the diluted plasma/Ficoll interface and washed with RPMI 1640 twice, to remove any residual Ficoll or plasma proteins. Cells were counted and plated at $1x10^6$ cells/ml and additional infection studies were performed.

Neutrophils were isolated from bone marrow, as previously reported (Swamydas & Lionakis, 2013). Briefly, neutrophils were flushed from WT and *Opn*$^{-/-}$ femurs and tibias with RPMI-1640 supplemented with 10% FBS and a 27-gauge needle. Cell were centrifuged at 1400 rpm for 7 minutes and resuspended in 0.2% NaCl followed by 1.6% NaCl, to lyse the red blood cells. Cells were washed with RPMI+10% FBS and were resuspended in 1 ml of ice-cold PBS. In a 15 ml conical tube, 3 ml of Histopaque ®-1119 (density: 1.119 g/ml, Sigma-Aldrich) was added, followed by a 3 ml layer of Histopaque ®-1077 (density: 1.077 g/ml, Sigma-Aldrich) on top. PMNs were carefully layered on top of the Histopaque layers and a density gradient centrifugation was performed at 834 xg for 30 minutes, without brake. Following centrifugation, neutrophils within the 1119/1077 interface (80% purity) were isolated, washed twice and resuspended in appropriate concentrations and media for subsequent assays.

3.3 Quantitative Polymerase Chain Reaction (qPCR)

Total RNA was extracted from all cells with TRIreagent (Molecular Research Center, Inc.) and converted into the first strand cDNA using the iSCRIPT™ cDNA

synthesis kit (Bio-Rad). qPCR assays were performed using iTAQ™ Polymerase Supermix for probe-based assays (Bio-Rad) or iQ™ SYBR® Green Supermix polymerase (Bio-Rad). WNV-envelope (*WNV-E*) gene, human OPN and mouse cellular gene primers and probes sequences were adapted according to previous publications: *WNV-E* (Town et al., 2009), *OPN* (Wu et al., 2012), *β-Actin* (Bai et al., 2005), Opn (Chapman, 2010), *Zo-1* (Yu, 2013), *Claudin 5* (Hamers, 2014), *Occludin* (Dalessandri, 2016), *Cxcl1* (Bellet, 2012), *Cxcl2* (Liu, 2015), *Tnf-α* (Sun et al., 2016), *Il-1β* (Alderson, Pearsall, Lindsay, & Wong, 1999), *Il-6* (D'Angelo et al., 2017) and *Icam-1* (D'Angelo et al., 2017). Analyses were performed using either the ΔΔcT method, normalized to *β-Actin*, and represented as relative fold change (RFC) or the ratio of the absolute gene copy number of *WNV-E* to *β-Actin*. All the primers and the *WNV-E* probe were synthesized either by Integrated DNA Technologies or Applied Biosystems.

3.4 Flow Cytometry

Whole mouse brains were isolated on day 4 p.i., as previously described (Bai et al., 2010). Briefly, whole brains were mechanically homogenized and separated into single cell suspensions using a 70 μm cell-strainer. Infiltrating leukocytes were isolated using Percoll gradient (70-37-30) centrifugation, followed by two washes with RPMI-1640. Cells were then fixed in 2% paraformaldehyde (PFA) and resuspended in PBS containing 2% FBS. Cells (1×10^6 cells/ml) were blocked with Fc block (5 μg/ml) in PBS + 2% FBS for 20 minutes at room temperature and probed with antibodies against CD45, Ly6G, CD11b, CD4, CD8, CD19 and F4/80 and analyzed with a flow cytometer (LSRFortessa™, BD Bioscience) using version 6.0 FACSDiva™ software (BD Biosciences) for acquisition and analysis. All antibodies were purchased from

eBioscience. Unstained and single-color compensation controls were used as controls. Anti-WNV primary antibody (Abcam) and secondary anti-mouse IgG conjugated to FITC antibody (eBioscience) were used for viral detection.

3.5 Enzyme-Linked Immunosorbent Assay (ELISA)

Levels of OPN were quantified in human and murine plasma samples by using OPN ELISA kits (ENZO Life Sciences) following manufactures recommendations.

3.6 Evans Blue Assay

To assess blood-brain barrier integrity, WT and *Opn*$^{-/-}$ mice were i.p. infected with 2,000 PFU of WNV and on day 4 p.i., 800 µl of 2% Evans Blue dye (EBD) in PBS was i.p. injected. One hr after EBD injection, mice were transcardially perfused with ice-cold PBS. EBD absorbance (610 nm) in whole brain tissue was quantified using a spectrophotometer after DMSO homogenization (Bio-Rad Smart Spec 3000^{TM}). All samples were normalized to control brains (with EBD, without WNV infection) for each genotype group (WT or *Opn*$^{-/-}$).

3.7 Neutrophil Migration Assay

PMN migration assays were performed as previously described (Koh et al., 2007) with additional modifications. Briefly, WT and *Opn*$^{-/-}$ PMNs in RPMI-1640 were added to inserts of transwells (3-µm pore, Corning), while RPMI-1640 with or without rOPN (2 µg/ml, R&D Systems) was added to the bottom of each well. The cells were incubated for 8 hr in a 37 °C, 5% CO2 incubator. Migrated PMNs were quantified by counting the number of DAPI positive cells that were fixed with 4% PFA in the interface of the cells using a confocal LSR510 microscope (Zeiss) at 63 × magnification.

3.8 Statistical Analyses

Data were compared with a Student t test or a two-way analysis of variance with Bonferroni post hoc analysis. Survival curves were analyzed using Kaplan-Meier analysis. All statistical analyses were done using GraphPad Prism software (version 6.0).

CHAPTER IV – RESULTS AND DISCUSSION

4.1 Osteopontin expression is Induced Following WNV Infection.

4.1.1 Osteopontin is Induced in Human Peripheral Blood Mononuclear Cells and Sera from Patients Previously Exposed to WNV.

Human peripheral blood mononuclear cells (PBMC) were isolated from healthy human volunteers without a history of WNV infection, were infected *in vitro* with WNV at a multiplicity of infection (MOI) of 1 for 24 hr and the expression of *Opn* was measured by quantitative real-time PCR (qPCR). The qPCR results showed that WNV infection induced *Opn* expression in human PBMCs (Figure 4.1A). OPN has intracellular and soluble forms, therefore, we sought to measure the production of secreted OPN (sOPN) by an ELISA in a panel of human patient sera, including acute and chronic WNV fever (WNV-F) cases, neuroinvasive acute WNV (WNV-NA) cases, and cases recovered from neuroinvasive WNV infection WNV (WNV-NR). The ELISA results showed that protein levels of sOPN were higher in all WNV-infected cases compared to control sera obtained from humans with no history of WNV infection (Figure 4.1B).

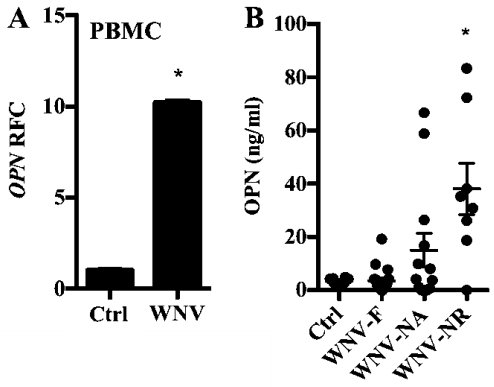

Figure 4.1 Osteopontin is induced in human blood and sera following WNV infection.

(A) Human peripheral blood mononuclear cells (hPBMCs) were isolated from whole blood healthy controls (n=4) and infected with WNV at a multiplicity of infection (MOI) 1 for 24 hours. The relative fold change (RFC) of Osteopontin (*Opn*) normalized to *β-Actin*

was measured by qPCR. (B) Human volunteer patient sera from healthy controls (Ctrl, n=4), WNV-fever (WNV-F, n=8), WNV-neuroinvasive acute infection (WNV-NA, n=8) and WNV-neuroinvasive recovered infection (WNV-NA, n=7) was collected and an ELISA was performed to determine the expression of soluble OPN. All experiments were performed twice and analyzed using a two-tailed, Student t test (* denotes $p < 0.05 \pm 1$ SEM).

Chronic inflammation has been linked to increased OPN levels in plasma/sera (Burdo et al., 2008; Silva et al., 2015), while increased HIV pathogenesis is dependent on sOPN (Brown et al., 2011; Burdo et al., 2008) and patients that suffer from secondary progressive MS have higher expression of sOPN compared to relapsing-remitting patients (Shimizu et al., 2013). Collectively these reports suggest a role for sOPN in chronic inflammation and possibly during inflammaging. In our studies, it is interesting to observe increased expression of sOPN in patient samples that have recovered from neuroinvasive WNV (WNV-NR) infection as compare to acute WNV (WNV-NA) infection, which may contribute to increased fatigue and issues in cognition that was observed in this cohort. In line with this, increased fatigue has been associated with neuroinvasive WNV infection (Garcia et al., 2014) while, RRMS patients treated with Natalizumab showed reduced sOPN levels and improved cognition and fatigue (Iaffaldano, Ruggieri, Viterbo, Mastrapasqua, & Trojano, 2014), suggesting increased sOPN can contribute to fatigue and cognition. Future studies include identifying additional patient symptoms, with the goal to better delineate the role for sOPN in recovered neuroinvasive WNV infections. In addition, it would be important to test if there are increased levels of sOPN in WT mice that have recovered from viral infection, and if so, assess the behavioral phenotypes observed between WT and *Opn*$^{-/-}$ mice, as this would indicate an additional role for OPN in possible promotion of fatigue and/or reducing cognition. In addition to fatigue, reactivation of neurological symptoms

including paralysis and neuropathic pain were also reported in our recovered neuroinvasive WNV infected cohort, therefore additional testing in our WT mouse model could also delineate these pathways as well.

4.1.2 Increased Soluble Osteopontin in Mouse Plasma and Brain Homogenates Early in WNV Infection.

To determine if sOPN was also increased following acute WNV in our murine model, plasma and brain homogenates were collected at indicated time points post intraperitoneal (i.p) infection with 2,000 PFU of WNV, which showed increased sOPN levels were observed early in WNV infection in plasma and brain tissue homogenates (Figure 4.2). Since the expression of sOPN is increased in the brain early in the course of infection (D2 p.i.), this expression may play an important role in terms inflammation and the "Trojan-horse" mechanism of viral infection, as sOPN can recruit PMNs to sites of its expression (Koh et al., 2007; Sodek, Ganss, & McKee, 2000), which harbor WNV (Bai et al., 2010). Since whole brain homogenates showed increased sOPN levels, the type of cells that secrete sOPN following WNV in the brain is of interest. In line with this, neurons are permissive to WNV infection and have been previously described to secrete sOPN following other viral infections (Silva et al., 2015). In fact, neurons may be responsible for the majority of secreted OPN in the brain (Silva et al., 2015). Therefore, identifying the production of sOPN in neurons following WNV is necessary.

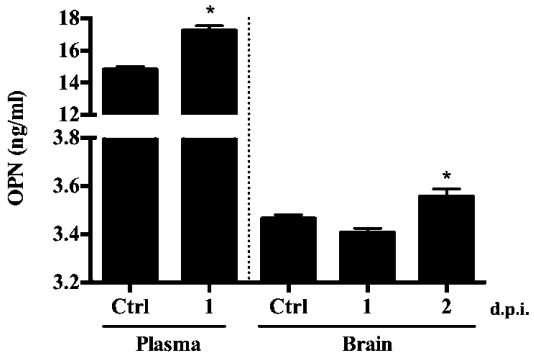

Figure 4.2 Mouse plasma and brain homogenates secrete osteopontin early during WNV infection.

ELISA was performed to determine the concentration of sOPN within samples on 1-day post infection (d.p.i.) in plasma (n=6) and on 1 and 2 d.p.i. in brain homogenates (n=3) post intraperitoneal (i.p.) infection with 2,000 PFU of WNV in WT mice. Ctrl denotes uninfected WT controls. Experiments were performed twice and analyzed using a two-tailed, Student *t* test (* denotes $p < 0.05 \pm 1$ SEM).

4.1.3 Neurons Secrete Osteopontin Following WNV Challenge *In Vitro.*

As mentioned, WNV is a neurotropic virus (Verma et al., 2009) and sOPN is involved in the recruitment of immune cells to sites of its expression (Sodek, Ganss & McKee, 2000), our next aim was to determine if WNV-infected neurons express OPN. For this, human neuroblastoma cells (SH-SY5Y) and murine primary neurons were infected with WNV (MOI = 5) for 24 hr and both *Opn* transcripts and sOPN were measured by qPCR and ELISA, respectively. The results showed that Opn transcripts (Figure 4.3A) and sOPN (Figure 4.3B) expressions were induced following WNV infection in human and murine neuronal cells. Collectively, these results demonstrate that permissive cells to WNV infection, including neurons produce OPN following infection, suggesting a possible role for OPN in the pathogenesis of WNV. In addition, the production of sOPN occurred in culture within a 24 hr time frame, suggesting rapid and robust production of sOPN occurs following WNV infection.

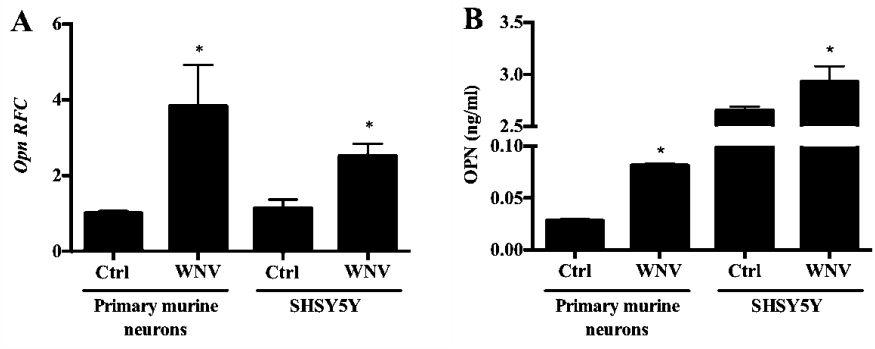

Figure 4.3 Osteopontin is induced in neurons following WNV infection.

The relative fold change (RFC) of *Opn* normalized to *β-Actin* was measured by qPCR (A) and sOPN was measured by ELISA (B), in primary murine neurons and in a human neuronal cell line (SHSY5Y) that were infected with WNV (MOI = 5) for 24 hours. All experiments were repeated one time and analyzed using a two-tailed, Student t test. Data represent mean ± SEM, *$p < 0.05$ (n=3 per group).

4.2 Osteopontin Knockout (*Opn*$^{-/-}$) Mice are Protected against WNV Infection.

4.2.1 Survival Curve Analysis of WT and *Opn*$^{-/-}$ Mice Following WNV Infection.

To determine the putative role of OPN in the pathogenesis of WNV *in vivo*, we intraperitoneally (i.p.) infected OPN deficient (*Opn*$^{-/-}$) mice with WNV (2,000 plaque forming units (PFU) per mouse) and monitored morbidity and mortality daily for 30 days. The survival results showed that nearly 70% of *Opn*$^{-/-}$ mice survived after WNV infection, compared to approximately 30% survival of the same genetic background wild-type (WT) controls, suggesting the role for OPN in WNV infection in mice is detrimental (Figure 4.4).

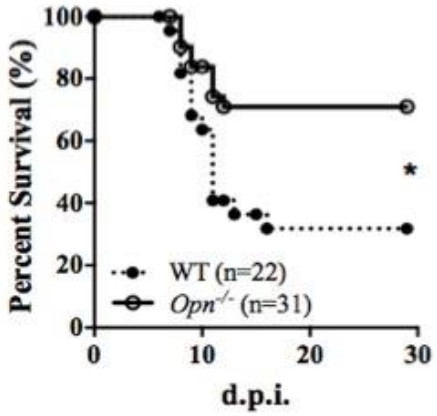

Figure 4.4 *Opn*$^{-/-}$ mice infected with WNV have increased survival.

WT (black circles) and *Opn*$^{-/-}$ (white circles) mice were infected with 2,000 PFU / mouse of WNV (i.p.) and monitored for survival for 30 days. Survival experiments were performed three times with combined samples indicated for WT (n=22) and *Opn*$^{-/-}$ (n=31) and analyzed using Kaplan-Meier analysis (* denotes $p < 0.05 \pm 1$ SEM).

4.2.2 Peripheral Viremia and Immunity against WNV is No Different Between WT and *Opn*$^{-/-}$ Mice.

To further delineate the role of OPN during WNV infection the expression of WNV-envelope (E) (*WNV-E*) in blood and tissues was measured by qPCR, which indicated comparable viral load in blood and spleens between WT and *Opn*$^{-/-}$ mice at D2 and D4 p.i. (Figure 4.5A and 4.5B). Since OPN has been suggested to regulate type I interferon production (Cao & Liu, 2006; Zhao et al., 2016), we measured expression of *Ifn-β* by qPCR in spleens, which was not significantly different between WT and *Opn*$^{-/-}$ mice at D2, D4 and D6 p.i. (Figure 4.5C). Additional cytokines were also analyzed to determine if OPN contributed to induction of key proinflammatory cytokines involved in innate immunity, however, there was no significant difference between WT and *Opn*$^{-/-}$ mice splenocytes with *Il-6* and *Tnf-α* (Figure 4.5C). Since proinflammatory genes *Tnf-α* or *Il-6* have been implicated in disruption of the BBB (Zhang et al., 2015), (Franceschi &

Campisi, 2014) and no significant differences were observed with these cytokines we can conclude that OPN expression does not directly enhance antiviral or proinflammatory immunity against WNV in the periphery.

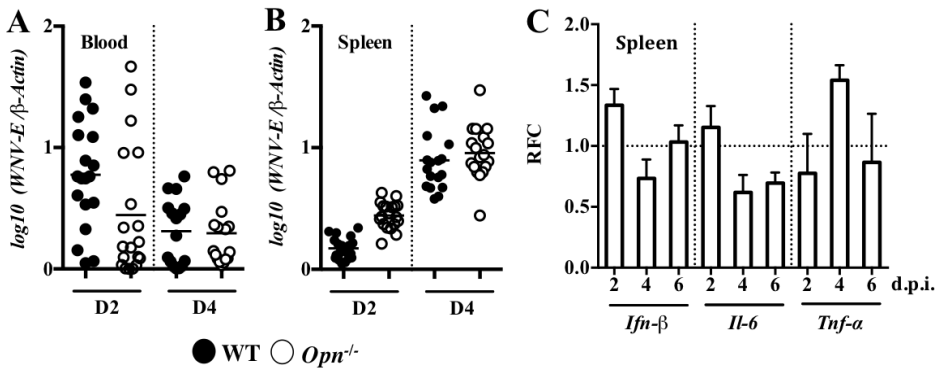

Figure 4.5 WT and *Opn*$^{-/-}$ mice infected with WNV display no difference in viremia.

WT (black circles) and *Opn*$^{-/-}$ (white circles) mice were infected with 2,000 PFU / mouse of WNV (i.p.) blood **(A)** and spleens **(B)** were collected on D2 and D4 p.i. and *WNV-E* and cellular *β-actin* were measured by qPCR and plotted as log10 (RNA expression) (n=16-22). **(C)** Spleens were collected at 2, 4 and 6 days post infection (d.p.i.) and qPCR was performed to measure *Ifn-β*, *Il-6*, and *Tnf- α* normalized to *β-actin*. Data are presented as relative fold change (RFC) with WT-infected controls set at 1 (horizontal, dotted line) and *Opn*$^{-/-}$ mice data is depicted in white bars. All experiments were performed twice and analyzed using a two-tailed, Student *t* test (* denotes $p < 0.05 \pm 1$ SEM).

4.2.3 Brain Tissue Isolated from *Opn*$^{-/-}$ Mice Display Reduced Viral Load.

In contrast to peripheral blood and tissue, viral burden in the brain tissues of *Opn*$^{-/-}$ mice was significantly lower than in WT control mice at D4 and D6 p.i., while WNV RNA was minimally detected in brain tissues collected on D2 p.i. (Figure 4.6). These results suggest that WNV can enter WT and *Opn*$^{-/-}$ mice brain parenchyma at the same rate (D2), however following WNV establishment in the brain, viral load increased significantly in WT mice that was not present in *Opn*$^{-/-}$ mice brains (D4 and D6). Therefore, two scenarios are possible in *Opn*$^{-/-}$ mice; one, *Opn*$^{-/-}$ brains are more resistant to viral infection, or two, defective entry of additional virus occurs in *Opn*$^{-/-}$ mice brains.

In brief, viral burden in brain tissues agree with the survival results (Figure 4.4), suggesting that OPN may contribute to WNV neurodisease by enhancing infection in the brain.

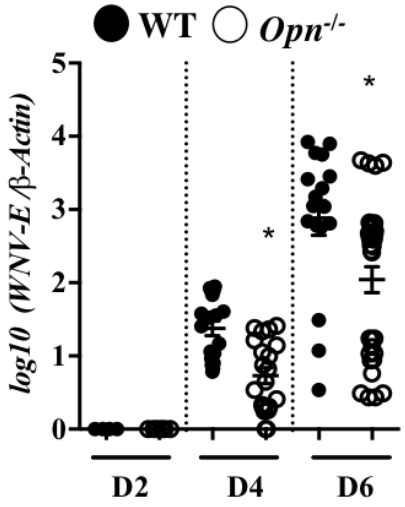

Figure 4.6 *Opn*$^{-/-}$ mice infected with WNV have reduced viral burden in brain tissue.

WT (black circles) and *Opn*$^{-/-}$ (white circles) mice were infected with 2,000 PFU / mouse of WNV (i.p.) and *WNV-E* and cellular *β-actin* were measured in brain tissue by qPCR and plotted as log10 (RNA expression) (n=4-36) at selected time points post infection (p.i.). Experiments were performed twice and analyzed using a two-tailed, Student *t* test (* denotes $p < 0.05 \pm 1$ SEM).

4.2.4 *Opn*$^{-/-}$ Brain Tissues Display Reduced *Icam-1*, but No Difference in Proinflammatory or Antiviral Gene Expression Early in WNV Infection.

To determine the antiviral and inflammatory response with the brain following WNV between WT and *Opn*$^{-/-}$ mice, brains were collected on days 2 and 4 p.i. following infection with 2,000 PFU of WNV i.p.. The qPCR results showed that there are no significant differences between WT and *Opn*$^{-/-}$ mice proinflammatory gene expression, including *Tnf-α*, *Il-1β*, *Il-6* and antiviral gene *Ifn-β* early in disease (D2 and D4 p.i.) (Figure 4.7). Both *Tnf-α*, *Il-1β*, and *Il-6* have been shown to contribute to BBB disruption (Franceschi & Campisi, 2014; Zhang et al., 2015) however, these cytokines are not

increased in WNV-infected *Opn*$^{-/-}$ mice early after WNV establishment in the brain (D2), suggesting these cytokines do not contribute to BBB permeability in *Opn*$^{-/-}$ mice. Interestingly, *Icam-1* (intracellular adhesion molecule-1) an endothelial cell marker involved in leukocyte extravasation, was reduced on D2 p.i., but was no different on D4 p.i. (Figure 4.7), suggesting reduced leukocyte infiltration may also be inhibited early in viral infection (D2) in *Opn*$^{-/-}$ mice, but is not dependent on ICAM-1 expression alone. In line with this, *Icam-1* expression is dependent on inflammatory mediators (Wung, Ni, & Wang, 2005). Therefore, we propose since major proinflammatory genes *Tnf-α*, *Il-1β* and *Il-6* are no different between WT and *Opn*$^{-/-}$ mice reduced OPN expression may be the primary contributor to reduced *Icam-1* expression that is observed early in infection of *Opn*$^{-/-}$ mice brains, which may affect leukocyte extravasation into the brain parenchyma.

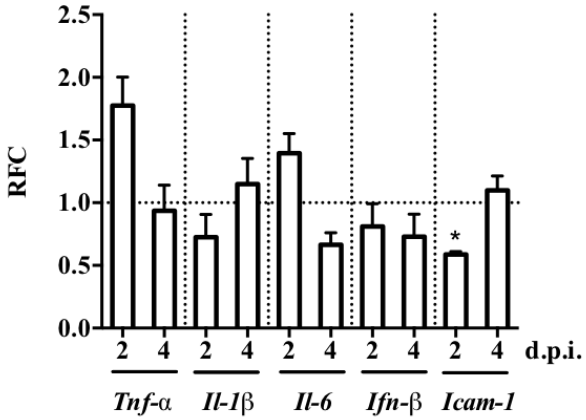

Figure 4.7 Brain tissue isolated from *Opn*$^{-/-}$ mice display no significant difference in proinflammatory and antiviral gene expression early in WNV infection.

WT and *Opn*$^{-/-}$ mice (white bars) brains were collected as indicated days post infection (d.p.i.) and qPCR for *Tnf-α*, *Il-1β*, *Il-6*, *Ifn-β* and *Icam-1* normalized to *β-Actin* was performed. Data are expressed as relative fold change (RFC) with WT-infected controls set at 1 (horizontal, dotted line, n=4-12). Experiments were performed two times and analyzed using a two-tailed, Student t test (* denotes $p < 0.05 \pm 1$ SEM).

4.2.5 *Opn*$^{-/-}$ Mice Lack Brain-Intrinsic Antiviral Function Against WNV.

To determine if brain-intrinsic antiviral immunity was involved in *Opn*$^{-/-}$ mice, we directly injected 100 PFU of WNV into the brains of *Opn*$^{-/-}$ and WT control mice via an intracerebral (i.c) inoculation route, and brain tissue was collected on day 6 (D6) p.i. to measure viral burden by qPCR (Figure 4.8). The results showed increased WNV RNA in the brains of *Opn*$^{-/-}$ mice compared to WT controls, which excludes the possibility that brain tissue of *Opn*$^{-/-}$ mice might be resistant to WNV. In fact, these results describe just the opposite, as there is significantly more virus in *Opn*$^{-/-}$ brains following direct inoculation into the brain parenchyma. Therefore, providing additional insight into the pathogenesis of WNV and the importance of a cohesive BBB. Since peripheral infection in *Opn*$^{-/-}$ mice results in reduced viral load in the brain, while i.c. infection results in increased viral load in the brain, there must be a defect in trafficking of virus into the brain. In addition, the integrity of the BBB following viral infection may be tighter in *Opn*$^{-/-}$ mice, which can inhibit the Trojan horse mechanism of viral entry. In brief, these results suggested that OPN might facilitate WNV brain invasion in mice.

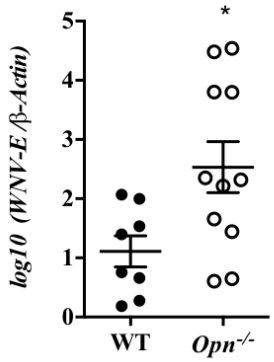

Figure 4.8 Intracerebral WNV infection results in increased viral burden in *Opn*$^{-/-}$ mice brains.

The ratio of *WNV-E* to *β-actin* in brain was determined by qPCR and plotted as log10 (RNA expression) in WT (black circles, n=8) and *Opn*$^{-/-}$ (white circles, n=11) mice following intracerebral (i.c.) infection with 100 PFU of WNV per mouse. Experiments were performed twice and analyzed using a two-tailed, Student t test (* denotes $p < 0.05 \pm 1$ SEM).

4.3 *Opn*$^{-/-}$ Mice Have Uncompromised Blood-Brain Barriers (BBB) Following WNV Infection, While Osteopontin Supplement Does Not Affect BBB Integrity.

4.3.1 *Opn*$^{-/-}$ Mice Have Tighter BBB Following WNV Infection.

The blood-brain barrier (BBB) is an important physiological barrier for protecting the brain against various insults, including WNV and other neuroinvasive pathogens (Dahm, 2016; Verma et al., 2009). Since we found a similar level of viremia, in *Opn*$^{-/-}$ and WT mice following i.p. infection (Figure 4.5), but *Opn*$^{-/-}$ mice displayed a lower viral burden in brain tissue (Figure 4.6) that was not due to intrinsic *Opn*$^{-/-}$ brain tissue resistance (Figure 4.8), we next sought to determine if the BBB is tighter in *Opn*$^{-/-}$ mice than WT mice following WNV infection. We used an Evans blue assay to determine the permeability of the BBB and found that *Opn*$^{-/-}$ mice had reduced Evans blue dye integration in their brain tissues as compared to WT controls, suggesting that *Opn*$^{-/-}$ mice have a tighter BBB following WNV infection (Figure 4.9). In comparison to uninfected controls that displayed no difference in Evans blue absorbance, only upon WNV infection does the BBB become compromised. Moreover, inhibition of Evans blue dye in *Opn*$^{-/-}$ mice brain parenchyma following infection indicates that there may be reduced neuronal damage associated with WNV infection in these mice.

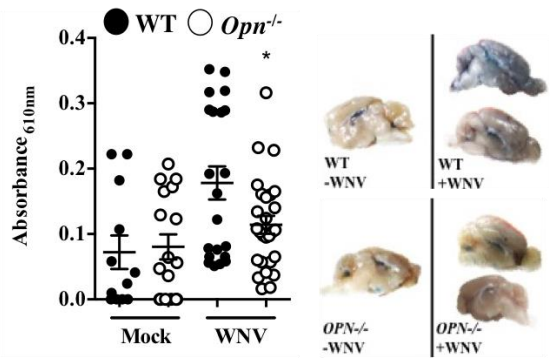

Figure 4.9 *Opn*$^{-/-}$ mice have reduced Evans blue dye integration in brain parenchyma.

WT (black circles, n=21) and *Opn*$^{-/-}$ (white circles, n=27) mice were infected with WNV (2,000 PFU i.p.) and mock WT (black circles, n=12) and *Opn*$^{-/-}$ (white circles, n=15) treated (PBS i.p., 100 µl) mice were used as absorbance controls. (Left panel) Brain infiltrated Evans blue dye was measured with a spectrometer (610 nm) on day 4 p.i.. (Right panel) Visual representation of brain tissues post-Evans blue injections. Experiments were performed twice and analyzed using a two-tailed, Student *t* test (* denotes $p < 0.05 \pm 1$ SEM).

4.3.2 *Opn*$^{-/-}$ Mice Have Increased Expression of Tight Junction Genes Following WNV Infection.

To further confirm *Opn*$^{-/-}$ mice have a tighter BBB, we evaluated the expression of BBB tight junction-related genes *Zo-1*, *Claudin 5* and *Occludin* and found that the expression of *Zo-1* and *Claudin 5* were significantly higher in brain tissue of *Opn*$^{-/-}$ mice on D4 p.i. (Figure 4.10). These results further highlight the importance of leukocyte extravasation during neuroinvasive WNV infection, since changes in tight gene expression were only observed at a time point when leukocyte extravasation is active (D4 p.i.), with no difference in tight junction gene expression at the time of initial viral entry into the brain (D2 p.i.) (Figure 4.10). Therefore, increased % of leukocytes carrying WNV into the brain may be a major contributor to neuroinvasive WNV infection. These results collectively suggest that *Opn*$^{-/-}$ mice are protected from BBB damage-associated

with WNV infection that occurs in WT brains, and further confirmed that *Opn*$^{-/-}$ mice have a tighter BBB than WT mice following WNV infection.

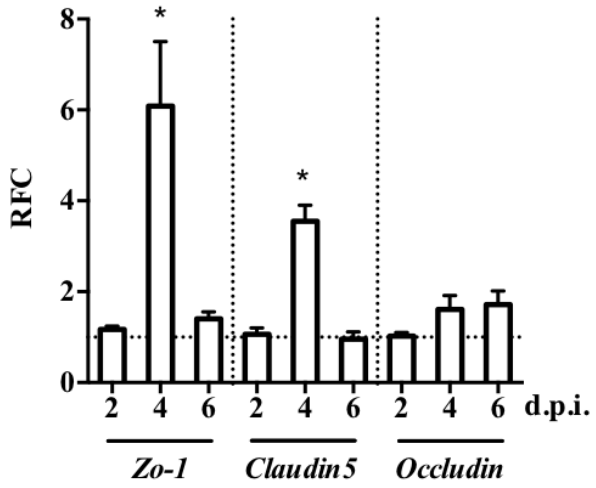

Figure 4.10 *Opn*$^{-/-}$ mice have tighter blood brain barriers.

Relative fold change (RFC) of tight junction marker genes *Zo-1*, *Claudin 5* and *Occludin* normalized to *β-actin* were measured in brains on 2, 4, and 6 days post infection (d.p.i) by qPCR (n=4-6). The horizontal dotted line represents WT-infected controls at each time point set as 1 and *Opn*$^{-/-}$ mice data is depicted in white bars. All experiments were performed twice and analyzed using a two-tailed, Student *t* test (* denotes $p < 0.05 \pm 1$ SEM).

Since WT mice have increased BBB compromise as compared to *Opn*$^{-/-}$ mice (Figure 4.9), but do not display difference in proinflammatory mediator expression at similar time points (D4) (Figure 4.7) following infection, we sought to determine if in the absence of WNV infection, overexpressed OPN could disrupt the integrity of the BBB directly or possibly induce proinflammatory mediators including *Tnf-α, Il-6,* and *Il-1β* that can cause BBB compromise (Franceschi & Campisi, 2014; Kempuraj et al., 2016; Zhang et al., 2015).

4.3.3 Increased Osteopontin Does Not Induce Proinflammatory Cytokines or Inhibit BBB Tight Junction Gene Expression in Uninfected Mice.

BBB compromise is a result of heightened inflammation, via increased expression of *Tnf-α*, *Il-6*, and *Il-1β* (Franceschi & Campisi, 2014; Kempuraj et al., 2016; Zhang et al., 2015). While *Opn*$^{-/-}$ mice had tighter BBB (Figure 4.9) and increased tight junction gene expression (Figure 4.10) during infection, it is not known if OPN directly caused BBB compromise. Therefore, in the absence of infection, high-dose OPN (rOPN, 200 ng/ml) was supplemented into WT mice for 4 hr via i.p. injection to determine if overexpressed OPN, in the absence of infection, contributed to BBB compromise. High dose concentration of rOPN was determined by comparing plasma sOPN concentrations that were well above levels observed in WNV infected mice (Figure 4.2), indicating a possible pathophysiological concentration of OPN. Blood was collected and qPCR analyzed for expression of proinflammatory genes *Tnf-α*, *Il-6* and *Il-1β* (Figure 4.11A), which indicated no difference in inflammation following rOPN treatment. To determine if high levels of OPN can affect the BBB compromise directly, brain tissues were also collected 4 hr post treatment and qPCR analyzed for the expression of BBB tight junction genes *Zo-1*, *Claudin 5* and *Occludin* (Figure 4.11B). The results indicate that at this time point of blood and tissue collection, supplementing high dose concentrations of rOPN to uninfected WT mice does not induce a pro-inflammatory response or directly contributes to BBB compromise. Moreover, these results highlight the important contribution of active leukocyte extravasation (carrying virus) into the brain, being a principle mechanism of WNV neuroinvasive infection.

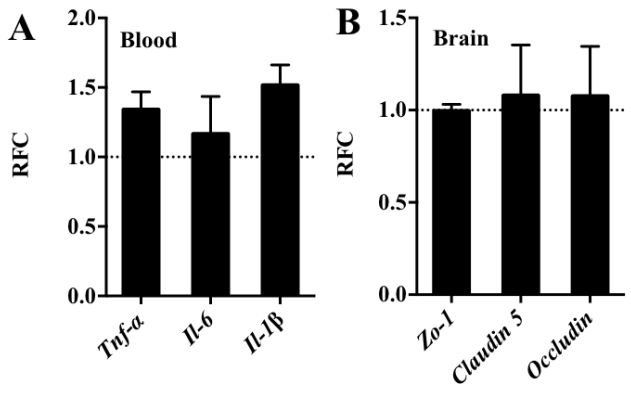

Figure 4.11 Recombinant osteopontin supplement in uninfected mice does not result in inflammation or blood-brain barrier compromise.

WT uninfected mice were i.p. treated with high dose rOPN (200 ng/ml) (black bars) or PBS (dotted line) as a vehicle control. Proinflammatory gene expression in blood for *Tnf-α*, *Il-6* and *Il-1β* (A) and brain tight junction markers *Zo-1*, *Claudin 5* and *Occludin* (B) were measured by qPCR 4 hr post-treatment. PBS vehicle controls are set at 1 (horizontal, dotted line) and all data were normalized to *β-Actin* and expressed as relative fold change (RFC). Experiment was performed one time (n=3 mice per group) and analyzed using a two-tailed, Student *t* test (* denotes $p < 0.05 \pm 1$ SEM).

4.4 Viral Resistance in *Opn*$^{-/-}$ Mice is Dependent On Reduced Polymorphonuclear Neutrophil (PMN) Brain Infiltration.

4.4.1 PMNs are Primary Carriers of WNV into the Brain during Neuroinvasive Infection in Mice.

To alluded to the importance of leukocyte extravasation contributing to neuroinvasive WNV infection and to determine the principle cell type that contributes to neuroinvasive trafficking of the virus into the brain. Total leukocytes isolated from WT-infected mice (i.p. 2,000 PFU) on day 4 p.i. were profiled, as previous studies have identified PMN-infiltration into the brain following WNV infection at this time point (Brehin et al., 2008). The results showed that approximately 80% of $Ly6G^{+}CD11b^{+}/CD45^{high}$ (PMNs) harbored WNV, as compared to other brain-infiltrating leukocytes including $CD4^{+}/CD45^{high}$ (~20%), $CD8^{+}/CD45^{high}$ (~30%), $CD19^{+}/CD45^{high}$

(~5%) and LY6G^{-}CD11b^{+}/CD45^{high} (myeloid cells, ~40%) (Figure 4.12). Therefore, PMN are the most permissive leukocyte to WNV infection and are the primary vehicles for WNV delivery into the brain during neuroinvasive infection.

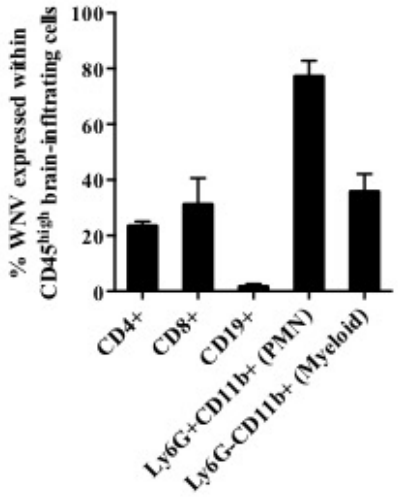

Figure 4.12 WNV infected brain-infiltrating leukocyte populations during WNV neuroinvasion.

WT mice were i.p. infected with 2,000 PFU of WNV and brains were collected on D4 p.i. for flow cytometric profiling of brain-infiltrated leukocyte populations that harbored WNV (% WNV). Subpopulations were gated within total CD45^{high} brain infiltrating cells. Experiment was performed twice (n=3 mice per group).

4.4.2 *Opn*$^{-/-}$ Mice Have Reduced PMN Brain Infiltration Following WNV Infection.

To further investigate the mechanism by which OPN contributes to WNV-neuroinvasion, we used flow cytometry to examine immune cell infiltration following WNV infection. Since immune cells can play a dual role, either limiting or facilitating pathogenicity during WNV infection (Bai et al., 2010; Drescher & Bai, 2013), we isolated and profiled the brain infiltrating immune cells in WNV-infected (2,000 PFU/mouse i.p) WT and *Opn*$^{-/-}$ mice on day 4 p.i. by flow cytometry. The results showed *Opn*$^{-/-}$ mice had similar level of brain-infiltrating leukocytes (CD45^{high}), CD4^{+} cells (CD4^{+}/CD45^{high}) and myeloid (Ly6G^{-}CD11b^{+}/CD45^{high}) cells, while slightly increased B-

lymphocytes ($CD19^+/CD45^{high}$) and $CD8^+$ cells ($CD8^+/CD45^{high}$) were observed, along with significantly reduced PMNs ($Ly6G^+CD11b^+/CD45^{high}$). These results identified PMNs as the major cell type that was reduced (~10%) compared to WT controls in *Opn*$^{-/-}$ brains following WNV infection (Figure 4.13), and since PMNs have been previously identified to harbor WNV into the brain (Bai et al., 2010) (Figure 4.12), these cell types are an attractive cell to study. To further identify the contribution of OPN during PMN-WNV neuroinvasion, we next examined PMN recruitment and viral permissiveness compared to WT controls.

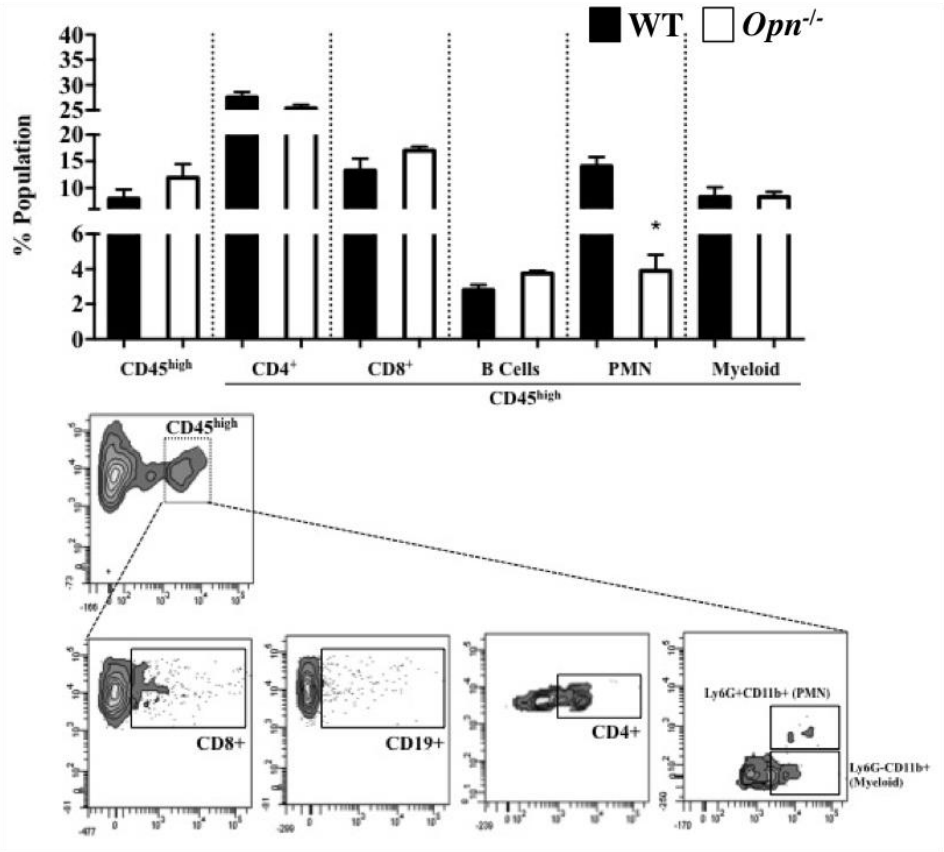

Figure 4.13 *Opn*$^{-/-}$ mice have reduced PMN brain infiltration following WNV infection.

WT (black bars) and *Opn*$^{-/-}$ (white bars) mice were infected with 2,000 PFU (i.p.). (Top panel) Brain-infiltrating leukocytes were quantified by flow cytometry on day 4 p.i., including total infiltrating leukocytes ($CD45^{high}$), $CD4^+$ cells ($CD4^+/CD45^{high}$), $CD8^+$ cells ($CD8^+/CD45^{high}$), B cells ($CD19^+/CD45^{high}$), PMNs ($Ly6G^+CD11b^+/CD45^{high}$) and myeloid cells ($Ly6G^-Cd11b^+/CD45^{high}$); n=4-6 mice

per group. (Bottom panel) Representative flow cytometric plots for used in gating of subpopulations. Experiments were performed twice and analyzed using a two-tailed, Student *t* test (* denotes $p < 0.05 \pm 1$ SEM).

4.4.3 *Opn*$^{-/-}$ Mice Have Reduced PMN-Specific Chemokine Expression Following WNV Infection.

Since chemokines are responsible for PMN recruitment (Kobayashi, 2008), we next measured expression of chemokines in the brain tissue of WT and *Opn*$^{-/-}$ mice and found that the expression of the two major PMN chemoattractant genes *Cxcl1* and *Cxcl2* were significantly reduced in *Opn*$^{-/-}$ mice brain tissue compared to WT mice (Figure 4.14), which may be attributed to less viral infection in the brain of *Opn*$^{-/-}$ mice (Figure 4.6). Interestingly, the reduction of PMN chemokines did not gain significance until D6 p.i., while reduced brain-infiltrating PMNs were observed before this time point at D4 p.i. (Figure 4.13), suggesting other recruitment molecules are probable early in infection, i.e., sOPN. Collectively, these results suggest that while reduced expression of PMN-specific chemokines may contribute to reduced PMN brain infiltration later during the course of infection (D6 p.i.), PMN infiltration in the brains earlier in infection (i.e. D2-4) may be more dependent on sOPN chemotactic properties.

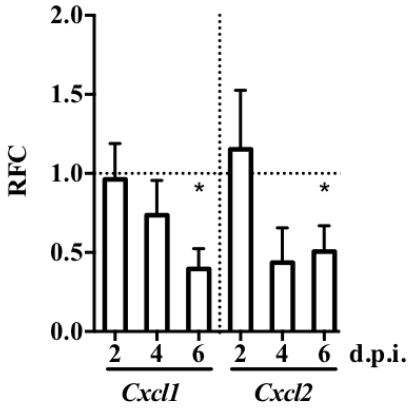

Figure 4.14 *Opn*$^{-/-}$ mice have reduced PMN-specific chemokine expression profiles in the brain following WNV infection.

WT (dotted line) and *Opn*$^{-/-}$ (white bars) mice (n=4-6) were infected with 2,000 PFU (i.p.) and whole brains were collected and subjected to qPCR on 2, 4 and 6 days post infection (d.p.i.). Relative fold change (RFC) of *Cxcl1* and *Cxcl2* normalized to *β-actin*, with WT-infected controls set at 1 (horizontal, dotted line). Experiments were performed twice and analyzed using a two-tailed, Student t test (* denotes $p < 0.05 \pm 1$ SEM).

4.5 Soluble Osteopontin Directly Recruits PMNs, While WT and *Opn*$^{-/-}$ PMNs Display Similar Viral Permissiveness.

4.5.1 PMNs Can Migrate to Sites of Osteopontin Expression.

Since reduced PMN infiltration was observed in brains of *Opn*$^{-/-}$ mice (Figure 4.13) and OPN has been shown to recruit PMNs *in vitro* (Koh et al., 2007), and we also wanted to confirm that recombinant OPN (rOPN) could attract PMNs in a transwell migration assay (Figure 4.15). For this, rOPN (2 μg/ml) was added to the bottom chamber with bone marrow derived PMNs added to the top chamber of a transwell plate, and cells were incubated for 8 hr. Following incubation, the top layer of PMNs were removed and the interphase layer of the transwell chamber was fixed and counted to determine the number of migrating PMNs, as previously described (Koh et al., 2007). These results confirmed that along with reduced expression of *Cxcl1* and *Cxcl2* chemokine expression (Figure 4.14), reduced PMN infiltration in brains of *Opn*$^{-/-}$ mice might be due to lack of sOPN.

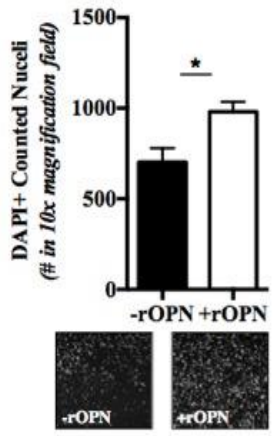

Figure 4.15 Recombinant osteopontin recruits PMNs *in vitro*.

A transwell migration assay using bone marrow derived PMNs isolated from WT mice (black bar) with recombinant mouse OPN (white bar, rOPN, 2 μg/ml), n=3 per group. Experiments were performed twice and analyzed using a two-tailed, Student *t* test (* denotes $p < 0.05 \pm 1$ SEM).

4.5.2 WT and *Opn*$^{-/-}$ PMNs are Equally Permissive to WNV Infection.

As reduced PMNs were observed in *Opn*$^{-/-}$ mice brains following infection (Figure 4.13) and PMNs can harbor WNV (Figure 4.12), it was important to describe if PMNs from WT and *Opn*$^{-/-}$ had similar permissiveness to WNV infection. Along with our results (Figure 4.12), previous studies have demonstrated that PMNs are very permissive to WNV infection and could facilitate WNV invasion into the brain (Bai et al., 2010; Wang, Zenewicz, Dai, Gate, Cheng, Yang, Qian, Yuan, Montgomery, Flavell, Town, Fikrig, 2012). Therefore, to determine if deficiency of OPN affects the permissiveness of PMNs to WNV, we compared WNV replication in bone marrow-derived PMNs from WT and *Opn*$^{-/-}$ mice by qPCR (Figure 4.16). The results indicated that PMNs from *Opn*$^{-/-}$ mice are similarly permissive to WNV infection compared to WT PMNs, which suggests although there is defective recruitment into the brain following WNV in *Opn*$^{-/-}$ mice, *Opn*$^{-/-}$ PMNs display similar viral permissiveness to WNV. These results further suggest

similar antiviral function between WT and *Opn*$^{-/-}$ PMNs. Therefore, we hypothesized that the primary role for sOPN following WNV infection is to recruit PMNs into brain, which act as viral reservoirs, paradoxically enhancing WNV neuroinvasion.

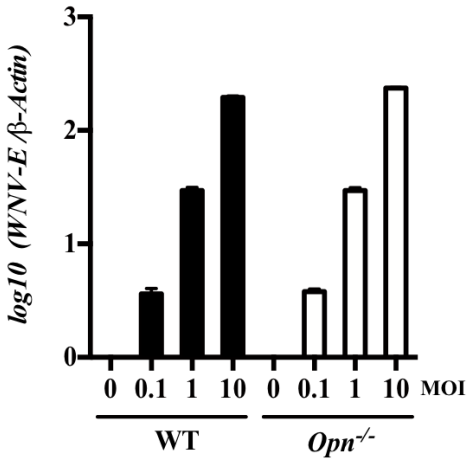

Figure 4.16 WT and *Opn*$^{-/-}$ PMNs are similarly permissive to WNV infection.

Bone-marrow derived PMNs from WT (black bars) and *Opn*$^{-/-}$ (white bars) mice (n=4 per group) were infected in vitro with WNV at a multiplicity of infection (MOI) of 0.1, 1 and 10 for 24 hr, followed by qPCR analysis to measure *WNV-E* relative to *β-Actin* and plotted as log10 (RNA expression). Experiments were performed twice and analyzed using a two-tailed, Student t test (* denotes $p < 0.05 \pm 1$ SEM).

4.6 Intracerebral Treatment With Recombinant Osteopontin (rOPN) Results in Increased Morbidity in *Opn*$^{-/-}$ Mice.

4.6.1 Intracerebral Treatment of rOPN in *Opn*$^{-/-}$ Results in Increased PMN Infiltration into the Brain Parenchyma Following WNV Infection.

Since sOPN can recruit PMNs *in vitro*, to test this *in vivo*, we injected rOPN (50 ng in 20 µl / mouse, i.c.), or an equal volume of PBS as a vehicle control, into the brains of *Opn*$^{-/-}$ mice and 1 hr later infected them with WNV (2,000 PFU, i.p.). The mice were euthanized and their brains were isolated on D4 p.i., a time point when the BBB was compromised, as indicated by Evans blue BBB permeability assay (Figure 4.9). The results showed that that infiltrated PMN population ($Ly6G^{+}CD11b^{+}/CD45^{high}$) were

significantly increased following rOPN supplement in *Opn*$^{-/-}$ mice brains, while the infiltration of CD4^{+} cells, CD8^{+} cells and macrophage populations (F4/80^{+}) were not significantly altered (Figure 4.17), suggesting that rOPN can selectively recruit PMNs into brain following WNV infection.

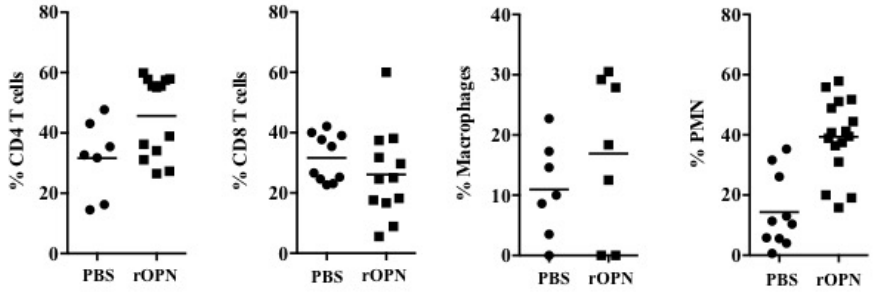

Figure 4.17 Intracerebral recombinant osteopontin supplement recruits PMNs in *Opn*$^{-/-}$ mice.

Opn$^{-/-}$ mice were pretreated with rOPN (squares, 50 ng, i.c.) or PBS (circles, as a vehicle control) and 1 hr later were infected with WNV (2,000 PFU, i.p.). Brain-infiltrating leukocytes (n=7-16) were quantified by flow cytometry on day 4 p.i.; CD4^{+} cells (CD4^{+}/CD45^{high}), CD8^{+} cells (CD8^{+}/CD45^{high}), macrophages (F4/80^{+}/CD45^{high}) and PMNs (Ly6G^{+}CD11b^{+}/CD45^{high}). Experiments were performed three times and analyzed using a two-tailed, Student *t* test (* denotes $p < 0.05 \pm 1$ SEM).

In addition, i.c. injection with PBS in *Opn*$^{-/-}$ mice resulted in slightly increased infiltrating populations of CD8^{+} cells (~35%) and PMNs (~15%) into the brain (Figure 4.17), compared to *Opn*$^{-/-}$ mice without i.c. PBS injections (CD8^{+} cells (~18%) and PMNs (~4%), Figure 4.13), indicating that direct brain parenchyma disruption, via i.c. PBS injections, contributes to recruitment of leukocytes, suggesting immune cell brain-infiltration is in response to direct brain insult. In line with this, it has been shown that following brain infarction there is increased brain leukocyte infiltration, including PMNs and CD8^{+} T cells (Gronberg, Johansen, Kristiansen, & Hasseldam, 2013), while % infiltration was dependent on the time of occlusion, whereby PMNs peaked early in the brain (D1-2) following long-term occlusion (120 min), resulting in death versus short-

term occlusion (30 min time, D3 peak infiltration) (Gronberg et al., 2013). Although models for stroke and WNV are different, their relationship with immune cell infiltration causing brain disruption is recognized between the two. Therefore, a better understanding of the population kinetics of leukocyte infiltration into the brain parenchyma following WNV neuroinvasion, are of interest as well.

4.6.2 rOPN-Treated *Opn*$^{-/-}$ Mice Have Increased Viral Load in Brain Parenchyma.

While increased PMNs were observed in rOPN-treated *Opn*$^{-/-}$ mice brain (Figure 4.17), and PMNs can harbor WNV (Figure 4.12), the viral burden in brain tissue of PBS- and rOPN-treated *Opn*$^{-/-}$ mice was measured. In complement with the PMN-infiltration results (Figure 4.13), we found that i.c. rOPN supplemented into *Opn*$^{-/-}$ mice resulted in increased viral burden as compared to i.c. PBS-supplemented mice (Figure 4.18), suggesting sOPN recruits WNV-infected PMNs into the brain and further identifies OPN as an important contributor to neuroinvasive WNV disease.

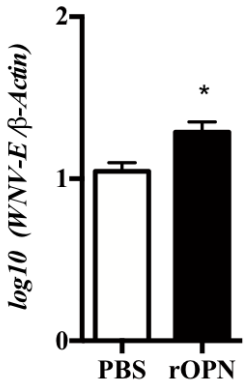

Figure 4.18 Intracerebral recombinant osteopontin supplement in *Opn*$^{-/-}$ mice results in increased viral burden in brain tissues.

Opn$^{-/-}$ mice were pretreated with PBS (white bars) or rOPN (black bars, 50 ng, i.c.) and were infected 1 hr later with WNV (2,000 PFU i.p.). Brains (n=4 per group) were collected on day 4 p.i. and a qPCR of *WNV-E* gene to *β-actin* was performed and plotted as log10 (RNA expression). Experiments were performed three times and analyzed using a two-tailed, Student *t* test (* denotes $p < 0.05 \pm 1$ SEM).

4.6.3 rOPN-treated *Opn*$^{-/-}$ Mice Have Increased Morbidity Following WNV Infection.

As mentioned, increased viral load is associated with neuroinvasive disease (Diamond et al., 2003), therefore to determine if increased WNV burden in the brains of rOPN-supplemented *Opn*$^{-/-}$ mice (Figure 4.18) resulted in increased neuroinvasive disease survival tests were performed, which showed dramatically reduced survival in *Opn*$^{-/-}$ mice supplemented with rOPN compared to *Opn*$^{-/-}$ mice supplemented with PBS (Figure 4.19). In fact, rOPN supplemented *Opn*$^{-/-}$ mice closely resembled the survival curve of WT mice (~30% survival, Figure 4.4 compared to ~40% survival Figure 4.19). In addition, death of WT mice occurred between days 9-14 (Figure 4.4), while rOPN treated *Opn*$^{-/-}$ mice death occurred between days 9-12 (Figure 4.19), suggesting similar PMN kinetics in neuroinvasive disease between these two groups. Collectively, these results confirmed that sOPN could recruit WNV-infected PMNs into the brain, facilitating WNV neuroinvasive disease.

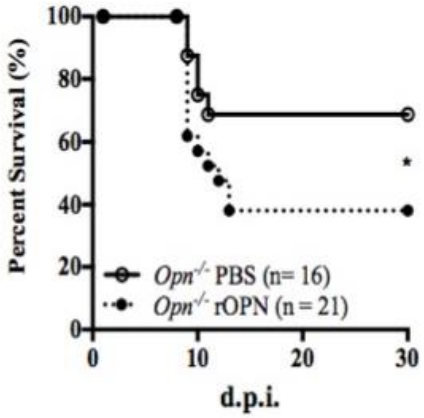

Figure 4.19 Intracerebral recombinant osteopontin supplement in *Opn*$^{-/-}$ mice results in reduced survival.

Survival of 8-week-old *Opn*$^{-/-}$ mice either pretreated i.c. with rOPN (black circles, 50 ng/mouse n=21) or PBS (white circles, n=16) for 1 hr, followed by i.p. infection with 2,000 PFU of WNV and were monitored for 30 days p.i.. Experiments were performed three times and analyzed using Kaplan-Meier analysis (* denotes $p < 0.05 \pm 1$ SEM).

CHAPTER V – CONCLUSIONS AND FUTURE DIRECTIONS

This study has identified that OPN promotes neuroinvasion in a mouse model of WNV infection. More specifically, *Opn*$^{-/-}$ mice in comparison to WT mice have more than twice the higher rate of survival after WNV infection, which was associated with a tighter BBB, decreased PMN infiltration and reduced viral burden in the CNS early in the time course of infection. To confirm the effects of OPN on WNV neuroinvasive disease, supplementing recombinant OPN into brains of *Opn*$^{-/-}$ mice enhanced WNV neuroinvasion, complementary to WT infection. The overall findings support the hypothesis that OPN promotes WNV neuroinvasion via a 'Trojan-horse' mechanism of viral CNS entry (Verma et al., 2009) through recruitment of WNV-infected PMNs into the CNS, enhancing disease severity.

In this study, the mouse immune system and the innate immune mediator OPN was thoroughly analyzed to determine if this protein could be involved in neuroinvasive WNV infection. Understanding the role of OPN, and its contribution to neuroinvasive WNV offered some additional insight into the pathogenesis of neuroinvasive WNV in mice. For instance, soluble OPN acts as a chemokine to attract PMNs into the brain following infection. This study identified WNV must first establish itself in the brain, through an unknown mechanism of viral entry, prior to triggering a specific brain-intrinsic inflammatory cascade and recruitment of leukocytes to clear infection. Paradoxically, in *Opn*$^{-/-}$ mice following WNV infection there was reduced recruitment of a specific immune cell type referred to as PMNs, early in disease (D4). While no additional brain collections were made post D4, we suspect later in disease course (D6-D8) additional immune mechanisms would develop to clear the virus. Therefore, future

studies could identify the subpopulations of brain-infiltrating leukocytes between WT and *Opn*$^{-/-}$ mice that occur later in disease progression. For instance, CD8^{+} T cells (Shrestha & Diamond, 2004) and PMNs (Bai et al., 2010) also contribute to viral clearance later during infection and therefore, these additional studies may provide insight into the viral resistance that is displayed in *Opn*$^{-/-}$ mice.

Additionally, the inflammatory response is a complex pathway that is triggered in response to infection or injury in order to commit to controlling foreign challenge, yet overt inflammation has contributed of many brain-related pathologies (Franceschi & Campisi, 2014). Therefore, in the brain, the timing, propensity, and resolve of inflammation is critical to prevent neurodegeneration. Following WNV infection in *Opn*$^{-/-}$ mice there was no difference in inflammatory mediator gene expression *Tnf-α*, *Il-1β,* and *Il-6*, however, these are just three of a wide variety of inflammatory mediators that contribute to inflammation, including molecules such as IL-12 and nitric oxide, to name a few. Therefore, future directions for this project would be to run a microarray on the brain tissue isolated from WT and *Opn*$^{-/-}$ mice, at different time points p.i. to identify the immune profiles and inflammatory mediators against WNV that are altered between these two groups, which may aid in a better understanding of the host immune kinetics against the virus.

Interestingly, *Icam-1* was reduced in *Opn*$^{-/-}$ mice compared to WT controls at D2 p.i., which suggests reduced inflammation and antiviral immunity may have contributed to reduced *Icam-1* expression, as its expression is induced by NFκB and STATs (Wung et al., 2005), following inflammation. Since ICAM-1 has been implicated to facilitate WNV entry into the brain (Dai, Wang, Bai, Town, & Fikrig, 2008), identifying the mechanism

for *Icam-1* expression is of great interest in the Trojan horse mechanism of viral entry and neuropathogenesis, which requires further studies. Additional other endothelial receptors such as selectins also require attention, as their expression is also inflammation-dependent and participate in leukocyte extravasation. In line with leukocyte extravasation, PMN activation prior to binding to endothelial cells requires a conformational change in their cell surface integrins. Interestingly, sOPN binds to integrins and this interaction may aid leukocyte migration into brain parenchyma. Indeed this has been previously described with embryo implantation into the endometrium (Kang, Forbes, Carver, & Aplin, 2014). In addition, while OPN can also bind to an additional receptor called CD44v6, which enables cell survival. This mechanism has been previously characterized in various cancer models (Lee et al., 2007; Maeda et al., 2015). In line with our studies, CD44v6 is expressed on PMNs and binding of OPN to this receptor may promote PMN survival, which is of interest in terms of viral hijacking of immune cells and viral dependence on the host cell for survival. It would be interesting to look at the survival kinetics of WNV-infected PMN in the presence or absence of rOPN, to determine if OPN contributes to PMN survival, which could contribute to the Trojan horse mechanism of WNV neuroinvasion.

Furthermore, the induction of tight junction molecules in $Opn^{-/-}$ mice is still an open question that requires further studies. To address this in future studies, nitric oxide and MMP-9 has been shown to be major contributors to tight junction protein cleavage (Roe et al., 2012), and therefore additional studies into the expression of these proteins, or other mediators that are devoid in $Opn^{-/-}$ immunity is essential. Additional protein

assays, such as immunohistochemistry of tight junction protein expression between WT and *Opn*$^{-/-}$ brain tissue could contribute to these studies as well.

While OPN may be an attractive target for therapeutic intervention of disease development, practicality of designing a therapeutic to target this protein is complex. Following infection of neurons, sOPN is highly upregulated and is then secreted out of the brain into blood circulation to recruit PMNs. However, inhibiting sOPN with anti-OPN antibodies or specifically designed drugs may be difficult since large molecules cannot pass the BBB. In addition, targeting sOPN and or PMNs during the course of infection must be carefully timed and varies from patient to patient depending on the time course of WNV dissemination into the brain. This is critical since PMNs serve to carry the virus early in infection and clear the virus later during infection (Bai et al., 2010), suggesting intervention targeting sOPN must be done early in viral establishment, which must be standardized in humans. The same holds true for other therapeutic design, for example, PMNs can enter the brain parenchyma in response to infection through binding to endothelial receptors, such as ICAM-1, and reduced *Icam-1* in brains of *Opn*$^{-/-}$ early in disease, may have contributed to reduced overall immune cell extravasation into the brain parenchyma. Therefore, blocking leukocyte adhesion receptors early in disease progression may prevent the Trojan horse mechanism of neuroviral infection. However, this blockage must be reversible and performed very early following infection establishment to prevent the Trojan horse entry of infected leukocytes, but as mentioned above, inhibiting immune cell infiltration in the brain later during infection may contribute to disease progression (Bai et al., 2010).

In summary, OPN has been implicated in many pathological conditions in humans and this study has identified its role during neuroinvasive WNV infection in mice. While offering insight into the mechanism of neuroinvasive WNV infection, to translate these studies to humans, it is imperative to determine if PMNs indeed act as Trojan horse vehicles for viral delivery into the brain. Although neutrophilia has been associated with neurocognitive impairment in human WNV infection (Crichlow et al., 2004; Tyler et al., 2006), the contribution of these cells as viral carries in humans is still an open question. Nonetheless, this study successfully identified PMNs as principle cells involved in viral hijacking and Trojan horse transmigration into the brain parenchyma in mice and the role for OPN in this process. In brief, if sOPN or PMNs are inhibited early during viral pathogenesis, a better prognosis for viral-induced neurodisease may be possible.

APPENDIX A – IACUC Approval letters

THE UNIVERSITY OF
SOUTHERN MISSISSIPPI

INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE

118 College Drive #5116 | Hattiesburg, MS 39406-0001
Phone: 601.266.6791 | Fax: 601.266.4377 | iacuc@usm.edu | www.usm.edu/iacuc

NOTICE OF COMMITTEE ACTION

The proposal noted below was reviewed and approved by The University of Southern Mississippi Institutional Animal Care and Use Committee (IACUC) in accordance with regulations by the United States Department of Agriculture and the Public Health Service Office of Laboratory Animal Welfare. The project expiration date is noted below. If for some reason the project is not completed by the end of the approval period, your protocol must be reactivated (a new protocol must be submitted and approved) before further work involving the use of animals can be done.

Any significant changes should be brought to the attention of the committee at the earliest possible time. If you should have any questions, please contact me.

PROTOCOL NUMBER:	15101601 (Replaces 12041201)
PROJECT TITLE:	Immunotherapeutics Against Flaviviruses and Alphaviruses
PROPOSED PROJECT DATES:	10/2015 -09/2018
PROJECT TYPE:	Renewal
PRINCIPAL INVESTIGATOR(S):	Fengwei Bai
DEPARTMENT:	Biological Sciences
FUNDING AGENCY/SPONSOR:	Wilson Research Foundation
IACUC COMMITTEE ACTION:	Full Committee Approval
PROTOCOL EXPIRATON DATE:	September 30, 2018

Frank Moore, PhD
IACUC Chair

10/01/2015
Date

The University of
Southern Mississippi

Institutional Animal Care and Use Committee

118 College Drive #5147
Hattiesburg, MS 39406-0001
Tel: 601.266.6820
Fax: 601.266.5509
www.usm.edu/spa/policies/animals

INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE NOTICE OF COMMITTEE ACTION

The proposal noted below was reviewed and approved by The University of Southern Mississippi Institutional Animal Care and Use Committee (IACUC) in accordance with regulations by the United States Department of Agriculture and the Public Health Service Office of Laboratory Animal Welfare. The project expiration date is noted below. If for some reason the project is not completed by the end of the three year approval period, your protocol must be reactivated (a new protocol must be submitted and approved) before further work involving the use of animals can be done.

Any significant changes (see attached) should be brought to the attention of the committee at the earliest possible time. If you should have any questions, please contact me.

PROTOCOL NUMBER:	**12041201**
PROJECT TITLE:	**Immunotherapeutics against flaviviruses**
PROPOSED PROJECT DATES:	**08/01/2012-07/31/2015**
PROJECT TYPE:	**New**
PRINCIPAL INVESTIGATOR(S):	**Fengwei Bai**
DEPARTMENT:	**Biological Sciences**
FUNDING AGENCY/SPONSOR:	
IACUC COMMITTEE ACTION:	
PROTOCOL EXPIRATON DATE:	**7/31/2015**

Jodie M. Jawor, Ph.D.
IACUC Chair

10 September 2012
Date

APPENDIX B – IRB Approval Letter

THE UNIVERSITY OF
SOUTHERN MISSISSIPPI

INSTITUTIONAL REVIEW BOARD
118 College Drive #5147 | Hattiesburg, MS 39406-0001
Phone: 601.266.5997 | Fax: 601.266.4377 | www.usm.edu/research/institutional-review-board

NOTICE OF COMMITTEE ACTION

The project has been reviewed by The University of Southern Mississippi Institutional Review Board in accordance with Federal Drug Administration regulations (21 CFR 26, 111), Department of Health and Human Services (45 CFR Part 46), and university guidelines to ensure adherence to the following criteria:

- The risks to subjects are minimized.
- The risks to subjects are reasonable in relation to the anticipated benefits.
- The selection of subjects is equitable.
- Informed consent is adequate and appropriately documented.
- Where appropriate, the research plan makes adequate provisions for monitoring the data collected to ensure the safety of the subjects.
- Where appropriate, there are adequate provisions to protect the privacy of subjects and to maintain the confidentiality of all data.
- Appropriate additional safeguards have been included to protect vulnerable subjects.
- Any unanticipated, serious, or continuing problems encountered regarding risks to subjects must be reported immediately, but not later than 10 days following the event. This should be reported to the IRB Office via the "Adverse Effect Report Form".
- If approved, the maximum period of approval is limited to twelve months.
  Projects that exceed this period must submit an application for renewal or continuation.

PROTOCOL NUMBER: **CH-R11120601**
PROJECT TITLE: **The Role of Neutrophic Virus Pathogenesis**
PROJECT TYPE: **Change to a Previously Approved Project**
RESEARCHER(S): **Fengwei Bai, Ph.D.**
COLLEGE/DIVISION: **College of Science & Technology**
DEPARTMENT: **Biological Sciences**
FUNDING AGENCY/SPONSOR: **N/A**
IRB COMMITTEE ACTION: **Expedited Review Approval**
PERIOD OF APPROVAL: **04/21/2014 to 04/20/215**

Lawrence A. Hosman, Ph.D.
Institutional Review Board

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