Comparative evaluation of single-site mutations in the West Nile virus envelope protein using a mouse model

by

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Abstract

For many years, West Nile virus (WNV) has been a leading cause of viral encephalitis across the globe. However, there are no human vaccines licensed for use and only four veterinary vaccines available. This thesis aims to investigate the similarities across WNV and other flaviviruses with the goal of developing a common attenuation mechanism. This mechanism could aid in the development of a vaccine candidate that could be used for WNV as well as other viruses in the *Flaviviridae* family. Viruses in the *Flavivirus* genus share significant homology among genomes, including around 40% homology in the envelope (E) protein. The E protein of WNV functions as a class II fusion protein, facilitating viral entry and acting as the main target of neutralizing antibodies. This makes this protein an ideal target for vaccine development technology. The E protein contains 500 amino acid residues and consists of three domains: EDI, EDII, and EDIII. Between two of these domains, EDI and EDII, lies the hinge region. This region appears to be necessary for the pH-dependent conformational changes necessary for endosomal fusion. In this study, eight residues located in the hinge region were investigated: E-A54, E-I130, E-I135, E-I196, E-Y201, E-A269, E-V272, and E-L281. These highly conserved hydrophobic residues were selected to analyze single-site mutations for the development of a flavivirus common attenuation mechanism for candidate live-attenuated vaccine creation. In this thesis, it was determined that one selected single-site mutation, E-Y201P, results in a fully attenuated, immunogenic phenotype, while the mutation E-A54S results in a partially attenuated, immunogenic phenotype. With both mutation sites being highly conserved among flaviviruses, this study lays the foundation for further studies to examine alternate mutations, combinations of these mutations or other attenuating

mutations, and/or candidate live-attenuated vaccine development using this attenuation mechanism.

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Chapter 1 - An Introduction to Flaviviruses and West Nile Virus

Flaviviruses

Flaviviruses are enveloped positive-sense single-stranded RNA viruses that belong to the *Flavivirus* genus in the *Flaviviridae* family. Over 50% of flaviviruses discovered to date are pathogenic in humans and/or animals, causing tens of thousands of deaths annually throughout the world. Some examples of the diseases caused by these viruses include: yellow fever (YF), dengue (DEN), West Nile (WN), St. Louis encephalitis (SLE), and Japanese encephalitis (JE) (Gould & Solomon, 2008). All flaviviruses are evolutionally related and share similar genome organization and virion structure (Araujo et al., 2020).

The *Flaviviridae* family consists of three genera: *Flavivirus*, *Pestivirus*, and *Hepacivirus*. The *Flavivirus* genus contains more than 70 viruses and is the largest of the three genera. *Flavivirus* is derived from the Latin word *flavus*, meaning yellow, which refers to the jaundice caused by yellow fever virus (YFV), the prototype flavivirus (Mukhopadhyay et al., 2005). Pathogenic flaviviruses are also arthropod-borne viruses or arboviruses, meaning they are transmitted by arthropod vectors such as mosquitoes and ticks. Hematophagy of these arthropod vectors is central to the spread of flaviviruses, especially mosquitoes and ticks that have co-evolved with human civilization. Flaviviruses in different serocomplexes often utilize different but specific vectors. For example, *Aedes* spp. mosquitoes are the principal vectors for flaviviruses in the dengue virus (DENV) and YF serocomplexes (Souza-Neto et al., 2019); whereas encephalitic flaviviruses in the JE serocomplex such as West Nile virus (WNV) are mainly transmitted by *Culex* spp. mosquitoes (Vogels et al., 2016).

Flaviviruses cause severe disease in a small proportion of the human population, including viral hemorrhagic fever and encephalitis (Gould & Solomon, 2008). While the majority of

flavivirus infections present as asymptomatic or mild disease, several of these viruses are considered major public health problems due to the severe disease they can cause and are classified as pathogens with high biodefense research priority by the National Institute of Allergy and Infectious Diseases (NIAID) within the National Institutes of Health (NIH) (Gould & Solomon, 2008; Weaver & Barrett, 2004). As of 2021, the *Flaviviridae* family is one of seven virus families targeted by the NIAID pandemic preparedness program.

Countermeasures against pathogenic flaviviruses

While no antiviral therapy has been developed for any flavivirus, vaccines are available for the control of five different flaviviruses, including vaccines for Japanese encephalitis virus (JEV), tick-borne encephalitis virus (TBEV), and yellow fever virus (Carpio & Barrett, 2021). In addition, there are four licensed veterinary vaccines in use for equines for West Nile, and six WNV vaccines in clinical trials for human use. While there is this large push for a West Nile vaccine, all four veterinary vaccines require multiple initial doses as well as annual boosters, which is less than ideal for a human vaccine that should be both protective and cost effective (Kaiser & Barrett, 2019).

All vaccines currently on the market for flaviviruses are inactivated, attenuated, or virus-vector vaccines. The most effective vaccines for flaviviruses are the yellow fever and Japanese encephalitis live-attenuated vaccines (LAVs), which elicit long-lasting protective immunity with one single immunization (Araujo et al., 2020). Other licensed flavivirus vaccines require multi-dose immunization regimen, including the two-dose dengue LAV and inactivated vaccines for JEV and TBEV.

The YFV 17D LAV was developed in 1936 by the empirical attenuation process through the serial passage in embryonated chicken eggs. The 17D vaccine has an excellent track record for safety and immunogenicity. Over 800 million doses of the 17D vaccine have been administered in human medical history and very few cases of vaccine-associated adverse events have been reported. Available evidence suggests that neutralizing antibody responses elicited by the 17D vaccine can be detected up to 35 years after the immunization (Yu et al., 1981). Hence, the protective immunity against wild-type (wt) YFV is considered life-long (Araujo et al., 2020).

The JEV SA14-14-2 LAV was derived from the wt SA14 strain after the serial passage in primary hamster kidney cells and suckling mice (Yu et al., 1981). To date, the SA14-14-2 vaccine is used in the routine pediatric immunization in ten JEV endemic countries, making human JE a vaccine preventable disease.

With vaccination being the most effective countermeasure for flavivirus prevention, there is growing interest in the design of other candidate flavivirus LAVs, but development can be difficult. Preventing a reversion to virulence in a live-attenuated model is of primary concern. However, there continues to be a growing interest in using mutations that balance attenuation with immunogenicity, creating a safe candidate LAV for flaviviruses. In this study, we use WNV as a model virus to investigate the attenuation of the virulence phenotype of flaviviruses. The objective of this project is two-fold: firstly, to generate the knowledge for rational design of the candidate LAV for WNV, and secondly, to identify mutations that attenuate the virulence phenotype of different flaviviruses.

West Nile virus

WNV is one of the most important and widespread of the flaviviruses (Ciota & Kramer, 2013). It was first isolated in Uganda in 1937 and has spread significantly since its discovery. It has since been determined to be endemic in Europe, Africa, the Middle East, and parts of Asia, and has been found on every continent apart from Antarctica (Rossi et al., 2010; WHO, 2017). Since its discovery, the largest outbreaks have occurred in Greece, Israel, Romania, Russia, and the United States (WHO, 2017).

WNV was first detected in the United States in August 1999 in the Queens area of New York City. At the time it was discovered in New York, health officials could only tell that it was an encephalitic disease accompanying muscle weakness and axonal neuropathy. It was originally believed to be St. Louis encephalitis virus as WNV had not yet been seen in North America (Jordan et al., 2000). In the United States alone, WNV has caused over 51,000 human clinical cases and over 2,300 deaths since its introduction, displaying the immense public health importance of this pathogen (Ronca et al., 2021).

WNV lineages

There are two primary lineages of WNV, lineage I and lineage II. Each lineage can be further classified into as many as five genetically different lineages with as much as 20-25% genomic variation (Bialosuknia et al., 2020). A tree detailing the separation of lineages can be seen in **Figure 1-1**. Lineages 1, 2, and 5 have been associated with outbreaks in humans (Chancey et al., 2015).

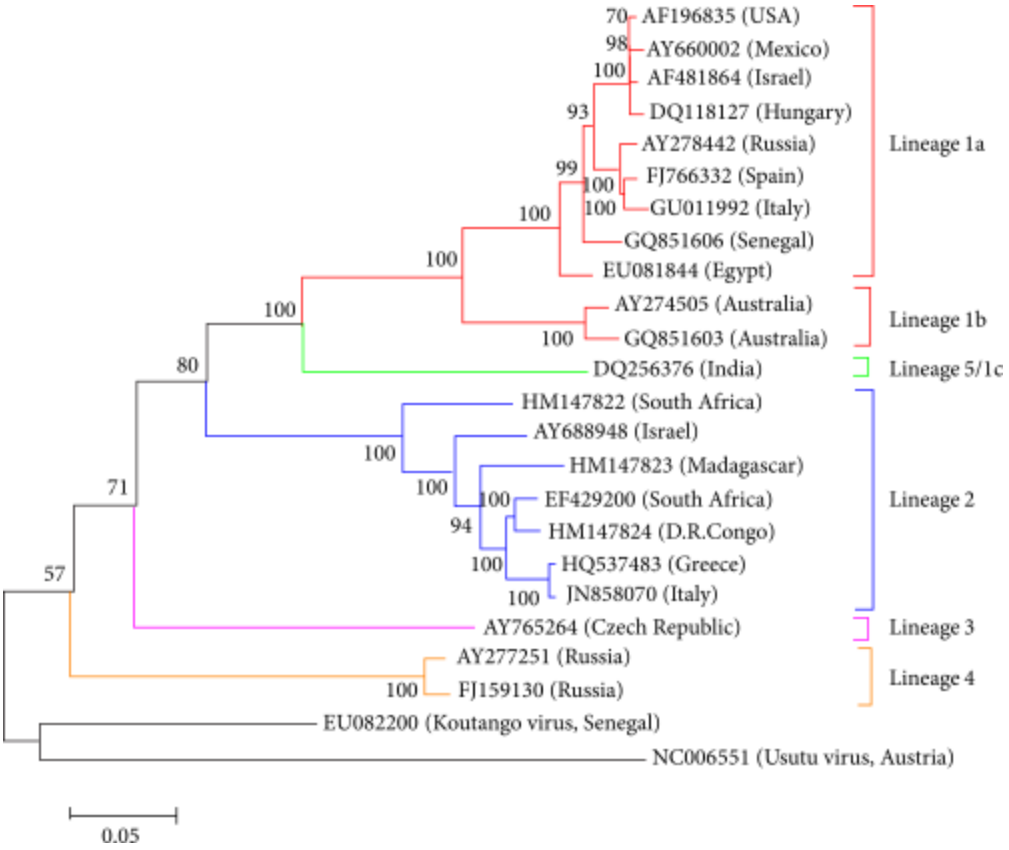

Figure 1-1. West Nile virus lineage tree.

Image taken from Chancey et al., 2015; published under the Creative Commons Attribution License.

Lineage 1 can be broken into 2 clades; clade 1a encompasses isolates from Africa, Europe, the Middle East, Asia, and the Americas, and clade 1b includes the Australian Kunjin virus (KUNV) (Chancey et al., 2015). There was previously clade 1c, but this has been reidentified as Lineage 5, and consists primarily of isolates from India, dating from 1955 to present (Khan et al., 2014; May et al., 2011). Lineage 2 viruses are historically endemic to sub-Saharan Africa and Madagascar and have been associated with the more recent avian and human outbreaks in Europe (Burt et al., 2002). Lineage 3 includes viruses only shown to experimentally infect mosquitoes and mosquito cells and are from isolates collected from mosquitoes on the Czech Republic border in 1997 and 1999 (Aliota et al., 2012). Lineage 4 contains viruses found circulating Russia since

1988; these include a tick isolate from southwest Caucasus and isolates collected from mosquitoes and reptiles near the Volga River (Chancey et al., 2015).

With WNV being an emerging flavivirus, there are increased numbers of clinical cases. 2018 saw a large increase of WNV cases within European countries, with over 1,500 human cases compared to the previous year, which saw just over 200 human cases (Rizzo et al., 2020; European Centre for Disease Prevention and Control, 2017). One of the most important factors to virulence is the continuous evolution of WNV which leads to the emergence of new genetic lineages that are transmitted more efficiently in nature. The replication of these viruses is based on RNA-dependent RNA polymerases that are prone to error, creating diversity within the virus (Yu & Cheng, 2022).

Simple amino acid substitutions can increase virulence in specific hosts. An amino acid substitution of T249P increased virulence in the American crow and replacing the V159A in the NA/WN02 strain shortened extrinsic incubation in *Culex* mosquitoes, which created a higher prevalence of the virus among mosquitoes. Such mutations likely led to both a rapid spread and prevalence within North America (Yu & Cheng, 2022). Another strain, NY10, saw amino acid substitutions of R1331K and I2513M which paralleled increased WNV cases in humans, as well as a higher prevalence of virus seen in mosquitoes. The NY10 genotype displaced the original WN02 genotype in the United States around 2010. The higher prevalence of virus seen in mosquitoes is likely associated with both increased infectivity and transmissibility of this genotype within *Culex pipiens* mosquitoes (Bialosuknia et al., 2022).

Transmission of WNV

WNV has caused the most widespread arthropod-borne viral disease in the world as well as the largest outbreaks of neuroinvasive disease ever seen. This is partially due to its transmission

cycle, shown in **Figure 1-2**. The enzootic cycle is primarily maintained between *Culex* species mosquitoes and viremic birds. A total of almost 60 species of mosquitoes and almost 300 avian species are known to be susceptible to WNV, giving rise to its ability to adapt to ecological conditions in various geographic regions (Ciota & Kramer, 2013).

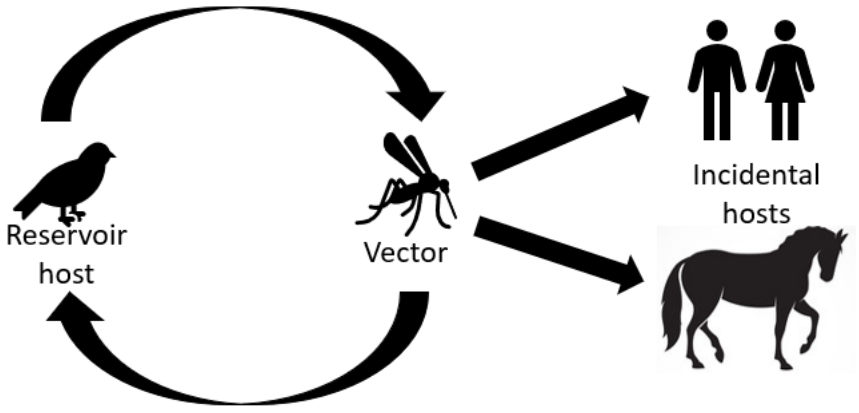

Figure 1-2. Transmission cycle of WNV.

The determination that birds act as a reservoir host in the transmission cycle is fairly new knowledge. In 1953, the virus was isolated from bird populations in the Nile delta region, supplying the awareness that WNV could be isolated from birds, but it was not thought to be pathogenic at this time (Hayes, 2006). In November 1997, a highly virulent strain of WNV became prevalent in Israel, resulting in a significant number of bird deaths from encephalitis and/or paralysis, linking WNV to avian mortality and morbidity (Banet-Noach et al., 2003). This information allowed for further studies which determined that avian species acted as a reservoir host and therefore, outbreaks and spread of disease could commonly been found around migratory bird routes (Rappole et al., 2000). Migratory patterns alongside the adaptability of vector

transmission highlighted by the numerous known species of mosquitoes and birds that can transmit this virus emphasize the ability of range expansion for WNV.

The spread of WNV by competent mosquitoes begins when a mosquito feeds on an infected bird or other competent host. Virus will then disseminate within the mosquito for several days before it enters the mosquito salivary glands. Transmission of WNV occurs when a mosquito with disseminated virus bites a human or other susceptible vertebrate host (Hayes et al., 2005). Mosquitoes tend to feed on a large range of avian and mammalian hosts with variable levels of WNV competence, and feeding behavior varies by mosquito species. The typical reservoir host, avians, can develop neurological disease and/or have varying levels of morbidity and mortality, dependent on species. There are a few mammals not seen in the primary transmission cycle that have been shown to have viremia levels adequate for infecting mosquitoes in laboratory settings, namely small mammals such as tree squirrels, eastern gray squirrels, eastern chipmunks, and easter cottontail rabbits. It remains unclear if these are truly natural, competent hosts (Ciota & Kramer, 2013).

The primary transmission cycle between mosquitoes and birds leaves humans and horses as dead-end hosts in the epizootic spillover. This understanding of the transmission cycle is widely accepted, but it is important to note that a very small portion of human infections that have occurred through organ transplants, blood transfusions, breast milk, and there has been one reported case of transplacental transmission (Sejvar, 2016). WNV and Zika virus (ZIKV) are the only flaviviruses to be tested for during blood transfusions in the United States (Araujo et al., 2020). There is currently no evidence of human-to-human contact transmission (WHO, 2017).

Human disease caused by WNV infection

Epizootic outbreaks of WNV can cause significant human disease and large economic loss. Upon its initial introduction into North America in 1999, WNV infected 62 individuals, resulting in seven deaths of elderly individuals. Along with this, numerous horse and bird deaths were also reported.

Incubation for this virus is 3-14 days and infected individuals may develop influenza-like symptoms such as high fever, chills, malaise, headache, joint pain, muscle and body pain, anorexia, nausea, vomiting, diarrhea, cough, sore throat, flushed face, swollen lymph nodes, and retro-orbital pain (Gould & Solomon, 2008). Only about 20% of infected individuals develop symptoms, leaving 80% of the infected population with asymptomatic infections (Sejvar, 2016). Young and elderly patients are more at risk for developing severe disease and mortality. In about 50% of infected individuals, a rash is observed, occurring most often in children (Gould & Solomon, 2008). Upon more severe infection, which occurs in about 1% of the infected population, enlarged and inflamed organs have been observed, as well as encephalitis, meningitis, and West Nile poliomyelitis (Rizzo et al., 2020; Sejvar, 2016). Patients experiencing neurological symptoms have a fatality rate of 5-10% (Gould & Solomon, 2008). In addition to human West Nile neurological disease, WNV is an important veterinary pathogen.

WNV vaccines

The human and veterinary public health importance of WNV makes vaccine development a top priority for disease control. To date, no human vaccines for WNV are licensed, but veterinary vaccines are available for the control of West Nile neuroinvasive disease (WNND) in horses within the United States. Although horses are a dead-end host and not involved in the transmission cycle,

they are still quite susceptible to the virus; there have been over 25,000 cases of WN equine encephalitis reported to the USDA since 1999, making horses 97% of all reported non-human cases of WNV in the United States (*West Nile virus*, 2022). Veterinary diagnosis for WNV is challenging due to the short viremic phase that supports virus isolation or detection of viral genome (Rizzo et al., 2020).

Currently there are four licensed WNV veterinary vaccines available: three that use the whole inactivated virus and one that is a live chimeric virus that combines the WNV prM/E into a canarypox backbone. While all veterinary vaccines are protective in horses, they currently require two primary doses as well as an annual booster. It is important to mention that while the chimeric vaccine includes a live virus, there is no evidence of canarypox-based vaccines replicating in mammals. This means this vaccine will not elicit as strong of an immune response when compared to a live attenuated vaccine (Kaiser & Barrett, 2019).

Development of WNV vaccines will facilitate the control of human WNND. An ideal candidate WNV vaccine must be immunogenic and elicit the protective immune response with one single immunization. Importantly, LAVs are the desired format for WNV as the elderly population that are immunoquiescent is at the highest risk of WNND in the United States (Piras-Douce et al., 2021). However, the empirical attenuation process has repeatedly failed to attenuate the virulence phenotype of most flaviviruses, including WNV. The development of a live-attenuated WNV vaccine will likely need to be rationally designed and potentially involve multiple mutations to avoid reversion to the virulent phenotype.

WNV replication

WNV belongs to the Japanese encephalitis virus serocomplex, which includes other members such as JEV, SLEV, and Usutu virus (Rossi et al., 2010). WNV is further grouped into two primary genetic lineages (I and II) that are based on the sequence of the envelope protein. The genome of WNV (**Figure 1-3**) is 11k nucleotides in length and contains a single open reading frame (ORF), which encodes three structural and seven non-structural (NS) proteins (Rossi et al., 2010). The genome is further modified to contain a 5´cap structure, be encapsidated, and be packaged into the icosahedral virion that is approximately 50 nanometers in size (Brinton, 2002).

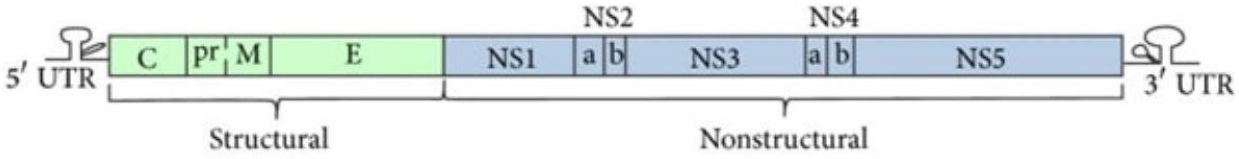

Figure 1-3. Genome of West Nile virus.
Image taken from Chancey et al., 2015; published under the Creative Commons Attribution License.

Flaviviruses all have a similar replication cycle within a host. The envelope (E) protein of flaviviruses interact with host cell receptors, triggering virus internalization. Once the virus enters the endosome, low pH induces a conformational change. This change exposes the fusion loop of the E protein, leading to fusion of the viral protein with endosome membranes. Viral RNA is released, allowing for translation of the viral polyprotein. The polyprotein is cleaved to generate the structural proteins (E, prM/M [premembrane or membrane], and C [capsid]) and non-structural proteins. Newly synthesized RNA and C protein are packaged by the prM and E protein to assemble immature viral particles. These particles bud into the endoplasmic reticulum, where they

are glycosylated. These immature virions are transported through the trans-Golgi network where prM is cleaved by protease furin into a pr peptide and M protein. These trimeric prM-E are rearranged to dimeric M-E heterodimers, which form mature virion particles which are excreted to infect other cells (Araujo et al., 2020).

WNV genome

Structural proteins

Three structural proteins are encoded in the viral genome, including the capsid (C), membrane (M), and envelope (E) proteins. Structural proteins determine the conformation of the virion and hence play an important role in viral entry and virion assembly (Rossi et al., 2010). The E protein and M protein are transmembrane proteins inserted into the lipid bilayer envelope taken from the host cell (Kanai et al., 2006). The viral envelope surrounds the ribonucleocomplex that contains the RNA genome and C protein.

Capsid protein

The C protein is 13 kDa in size and has the major function of encapsidating the viral genome (Zhang et al., 2021). The capsid protein is the first protein reproduced in infected cells. While originally thought to be purely structural, studies have shown the capsid protein also exhibits the autocleavage function involved in the posttranslational processing of the polyprotein (Urbanowski & Hobman, 2013).

Membrane protein

The membrane (M) protein is 75 amino acids in length after the cleavage of the pr peptide from the prM precursor protein by the host furin protease. During the virion maturation, the pr peptide of the prM protein protects the fusion loop of the E protein to prevent the premature membrane fusion in the secretory pathway. In the mature virion, the M protein acts as a chaperone protein that facilitates the folding of the E protein.

Envelope protein

The E protein is the major structural protein in the virion and is anchored to the lipid-bilayer viral envelope via the transmembrane domain, hence, the name E protein. The structure and function of WNV E protein resembles the E protein of other flaviviruses and acts as a class II fusion protein to facilitate viral entry. As a class II fusion protein, the E protein contains 500 amino residues and consists of three domains (EDI, EDII, EDIII) (Nybakken et al., 2006). The three domains are a beta-barrel domain I (EDI), a finger-like dimerization domain II (EDII) which contains the fusion loop, and the immunoglobulin-like domain III (EDIII) (**Figure 1-4**). The E protein interacts with the host cell receptors to initiate the viral entry process. Cell entry of WNV takes place via the fusion of virus and cell membranes. The membrane fusion process is driven by the conformational change of the E protein from dimer arranged in parallel to the virion surface to trimer inserted into the host cell and releases viral genome into the cytoplasm allows for replication (Araujo et al., 2015). Specifically, the membrane fusion activity of the E protein is due to the fusion loop at the EDII, which acts as the molecular anchor to insert the E protein trimer into the host cell. The role of the E protein in viral entry makes it an important component for WNV

pathogenesis. Vertebrate hosts of WNV evolve to develop neutralizing antibody responses mainly targeting the structure-function of the E protein. Therefore, the E protein is a major component for vaccine development (Rizzo et al., 2020; Araujo et al., 2015).

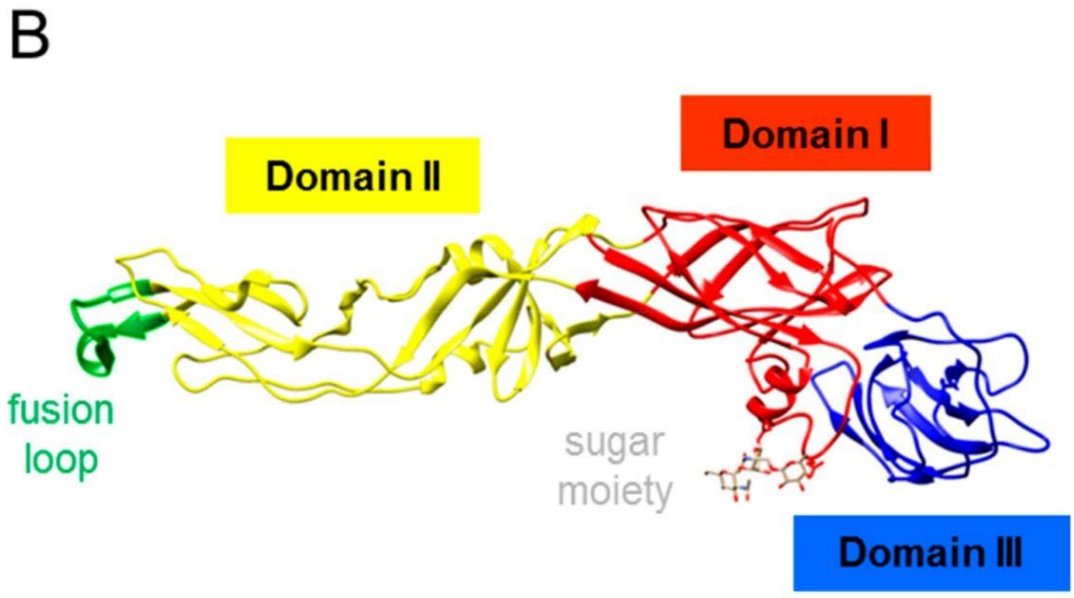

Figure 1-4. Crystal structure of West Nile virus E protein.
Image taken from Jiménez de Oya, et al., 2019; published under the Creative Commons Attribution License.

Non-structural proteins

WNV contains seven non-structural (NS) proteins: NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5. NS1 is multifunctional as there are two forms; a cell-associated form that is part of the replication complex, and a secreted form that works to modulate the host innate immune response. NS2A has been reported to be involved in viral RNA replication, modulation of the host-antiviral interferon response, and is part of virus particle assembly/secretion. The NS2B protein is a cofactor complexed with the serine protease domain of the NS3 protein by assisting the folding

and catalytic activity. In addition to the N-terminal protease domain, the NS3 protein also contains a helicase domain at the C-terminus, which exhibits nucleoside 5´-triphosphatase activity. NS4A participates in virus replication complex formation, induces autophagy to prevent cell death and assist viral replication, and regulates NS3 helicase ATPase activity. NS4B acts similarly, interacting with the NS3 helicase domain and dissociating it from single-stranded RNA. It also has multiple immunomodulatory functions, including the induction of the unfolded protein response in the host cells and the inhibition of type I interferon signaling. NS5 is the largest non-structural protein, and it is directly involved in capping and replication of the viral genome, as well as immunomodulation. Two domains give rise to the enzymatic activity of the NS5 protein; the N-terminal S-adenosylmethionine-dependent methyltransferase domain which adds the cap structure in the 5´ end of the viral genome and the C-terminal RNA-dependent RNA polymerase domain (Araujo et al., 2015). The 5´-cap structure is crucial for the translation and replication of the viral genome and prevents the degradation by host nucleases, which is part of the innate antiviral immune responses.

Attenuating mutations targeting the structure-function of WNV proteins

Gaining more knowledge of the structure and function of each viral protein allows research to follow a more logical pathway to rationally design a candidate LAV. With this knowledge, mutations within the viral proteins can be engineered to allow for an attenuated phenotype of the target virus, in this case, WNV.

The majority of attenuating effects have been reported due to mutations in the NS proteins. It has been shown that mutations can interfere with the function of non-structural proteins NS1, NS3, NS4B, and NS5 to fully attenuate the virulence phenotype of WNV. During WNV infection,

antibodies against the E protein as well as NS1 protein have been shown to be important in the protective immune response. There are three important sites along the NS1 protein, all asparagines, that are highly conserved among all WNV strains due to their importance for the structure and function of the protein: $NS1_{130}$, $NS1_{175}$, and $NS1_{207}$. Generally, studies have shown that the removal or alteration of these sites can impair viral replication and decrease neurovirulence (Whiteman et al., 2010). Mutations within NS3 have shown genome replication reduction, reduced antigen accumulation, decreased cytopathic effect, and an increased ability to persist within cell culture and/or attenuation *in vivo* (Rossi et al., 2007). It is believed the NS4B protein plays in important role in virulence phenotype determination. In previous studies, substitution of cysteine at position 102 with serine led to a temperature-sensitive phenotype as well as attenuation of the neuroinvasive and neurovirulent phenotypes in mice, and an adapted mutation (E249G) showed reduced RNA synthesis in host cells (Botha et al., 2008). Like the other non-structural proteins that have proven useful in virus attenuation, the use of NS5 for attenuation involves a mutation at specific amino acids. One particular study examined mutations on NS5 at K61A and E218A, both mutations when used alone, seem to decrease enzyme activity of the protein and attenuate the virus in a mouse model (Kaiser & Barrett, 2019).

Similar to many RNA viruses, a safe level of attenuation requires multigenic mutations in the RNA genome of WNV. Most attenuating mutations identified to date are located in the NS proteins. There have been very few attenuating mutations identified within the structural proteins, particularly the E protein, giving rise to the necessity to investigate this protein further.

Targeting the E protein for WNV LAV design

The WNV E protein facilitates viral entry into the host cell and is the main target of neutralizing antibodies, making it an ideal target for vaccine design (Araujo et al., 2015). Finding mutations within one or more of the domains in the E protein that do not allow for the conformational change needed to enter the host cell could create an attenuated phenotype.

During the structural change for viral entry, a dimer-to-trimer transition of WNV takes place due to the movement of EDII and EDIII in relation to the structurally central EDI. The movement of EDII has the utmost importance for the membrane fusion process by inserting the fusion loop into the host cell and initiating the formation of E protein trimer. Importantly, four motifs located at the interface of EDI and EDII exert the hinge effect to govern the EDI-EDII interdomain movement, hence, the name EDI-EDII hinge region (Nybakken et al., 2006). Looking at mutations within these domains or between these domains may alter the ability to finalize this conformational change and could attenuate the virus to make a candidate LAV.

For this thesis, eight residues within the EDI and EDII regions were selected for further study: E-A54, E-I130, E-I135, E-I196, E-Y201, E-A269, E-V272, and E-L281 (**Figure 1-5**). This region between EDI and EDII functions as a "molecular hinge" to facilitate viral fusion with cell membranes. In previous studies, alterations in or near this region have impacted cell infectivity as well as mouse virulence when compared to parental viruses. However, compensatory mutations are common (Butrapet et al., 2011). Identifying individual mutations that are causing an attenuated phenotype must be prioritized. Additionally, these eight residues lined up across the sequencing of 20 flaviviruses, and to date, only three of these residues, E-A54, E-L135, and E-I270, have been investigated using an infectious clone of wt DEN-2 (Butrapet et al., 2011). All of these conserved residues are hydrophobic amino acids which form a hydrophobic pocket in the crystal structure of

the E protein (Kanai et al., 2006). Previous studies have shown that placing a hydrophobic amino acid or a hydrophilic portion of the E protein into various areas of the protein where these were not previously present can disrupt protein function following host cell entry (Goo et al., 2006). Potentially, this means exchanging these amino acids for amino acids with lower hydrophobicity may also interfere with cell entry, therefore creating an attenuated phenotype of WNV in mice.

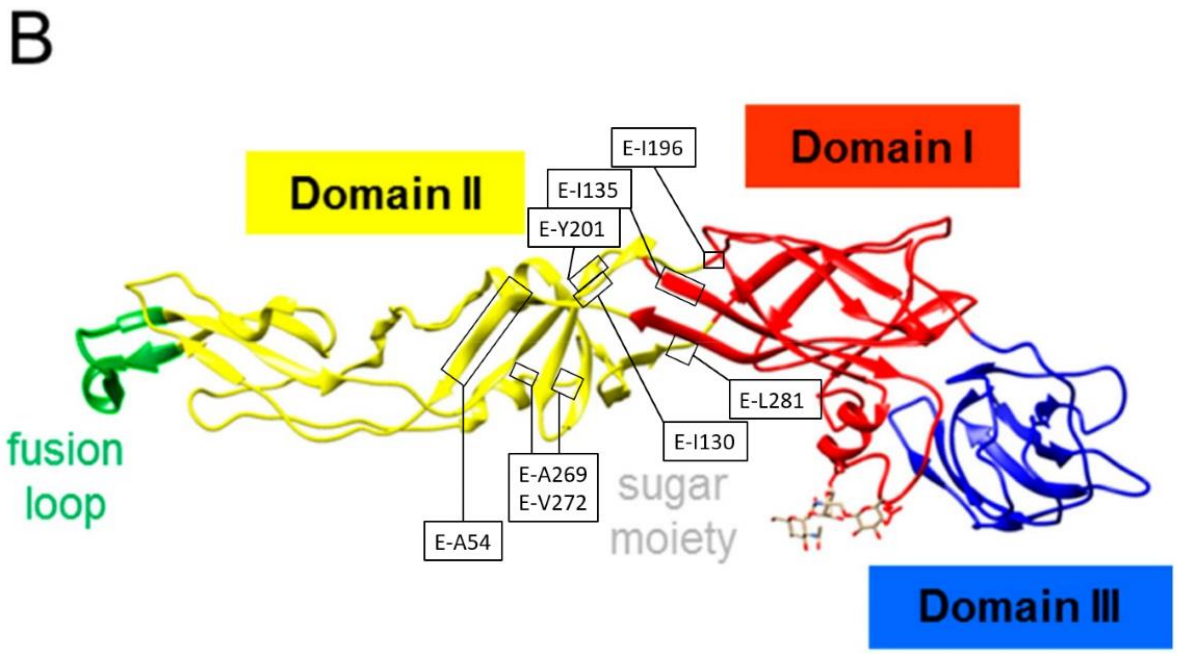

Figure 1-5. Labeled proteins on the WNV E protein crystal structure.
Image taken from Jiménez de Oya, et al., 2019; published under the Creative Commons Attribution License. Labels of generalized protein locations added for the purpose of this thesis.

Mouse models with WNV

Mouse models have been used for WNV studies and flavivirus vaccine development for many years. The mouse model has provided important details pertaining to the innate and adaptive immune responses to viral infection in numerous viruses (Graham et al., 2015). Mice are commonly used for WNV studies due to the availability of mouse models that mimic features of

human disease, which makes them a great model for zoonotic pathogens (Lazear & Diamond, 2015). With using a mouse model, you may also see different routes of infection and viremia, based on the method of inoculation, making them a versatile model (Nagata et al., 2015). The mouse model is one that does not take much space, remains relatively cost-effective, and they serve as a robust model for WNV infection, making them an appropriate animal model for this study.

Justification for research

The overarching goals of this thesis are to broaden the breadth of knowledge pertaining to rational design of a candidate LAV for WNV, and to pinpoint the mutations within the hinge region between EDI-EDII of the E protein which attenuate the virulent phenotype of WNV as well as other flaviviruses. The residues selected for use in this study are highly conserved across 20 flaviviruses and all present as hydrophobic. Knowing these residues are hydrophobic may play a large role in LAV design; this thesis replaced these residues with slightly less hydrophobic residues, potentially impacting the stability of the mutant viruses to varying degrees.

With no antiviral therapies for any flaviviruses and human vaccines only existing for a small number of flaviviruses including Japanese encephalitis virus, tick-borne encephalitis virus, and yellow fever virus, the need for preventatives or treatments remains (Carpio & Barrett, 2021). Despite years of research behind WNV, there continues to only be veterinary vaccines for the prevention of WNV. With WNV being one of the most widespread flaviviruses, it is important to continue researching potential preventative measures and to continue increasing the general knowledge base surrounding the E protein of WNV and vaccine design.

Chapter 2 - Materials and Methods

Introduction

This study aims to develop an attenuation process for WNV using single-site mutations in the E protein. The cDNA infectious clone of WNV NY99 strain (WNV-NY99ic) was used to introduce individual mutations and each mutant proven to retain the engineered mutation was characterized using the WNV neuroinvasive model in the outbred Swiss Webster mice. Mutants were created by exchanging single amino acids for an amino acid possessing a lower hydrophobicity. Previous studies have shown alterations to these hydrophobic residues and their hydrophobic properties could disrupt protein function following cell entry, consequently attenuating the neuroinvasive phenotype of the virus (Goo et al., 2006).

Cell lines

African green monkey Vero cells were propagated and maintained in minimum essential media-α supplemented with 10% FBS, 100 U/mL penicillin, 100 ug/mL streptomycin, 2 mM L-glutamine, and MEM vitamin solution at 37°C with 5% CO_2.

Design and creation of mutant viruses

The site-directed mutagenesis method for engineering mutations from the cDNA infectious clone of WNV NY99 strain has been previously described (Kinney et al., 2006).

The two-plasmid system codes for the genomic region between the 5´ untranslated region (UTR) and the *NgoMIV* site of the NS1 gene in the 5´ plasmid and the genomic region between the *NgoMIV* site of the NS1 gene and the 3´ UTR in the 3´ plasmid. PCR site-directed mutagenesis

was conducted to introduce substitutions in the EDI-EDII hinge region of eight flavivirus highly conserved hydrophobic residues using the In-Fusion cloning method (Takara) (**Figure 2-1**). In-Fusion cloning requires three steps: amplification, combination, and transformation. Chosen template fragments are amplified with primers and PCR to create a product where primers are attached to the template. This product is combined with the In-Fusion master mix and a linearized vector in a cloning reaction. The mixture is then transformed using competent cells to create the desired plasmid.

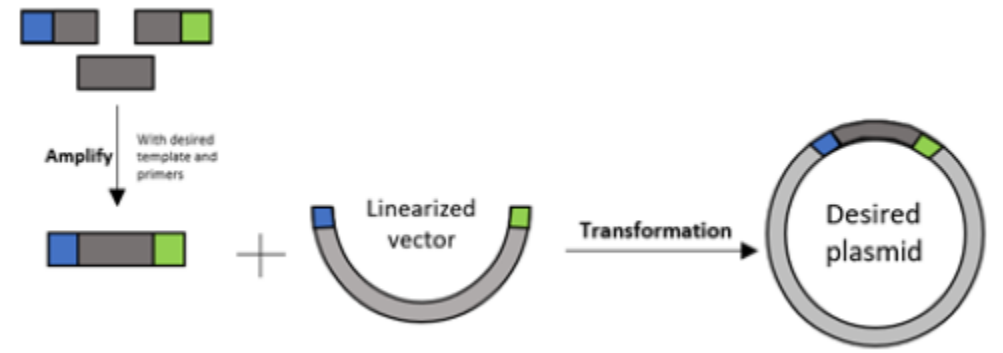

Figure 2-1. In-Fusion cloning method.

The E-A54 and E-A269 residues, both with a hydrophobic index of 41, were changed to serine with a hydrophobic index of -5, using G1126T and G1771T nucleotide substitutions, respectively. A table of hydrophobicity can be seen in **Table 2-1**, and a table detailing specified codon changes can be seen in **Table 2-2.**

Table 2-1. Hydrophobicity of amino acids.

Higher sidechain hydrophobicity is symbolic of higher hydrophobicity.

Amino Acid	Sidechain hydrophobicity	Amino Acid	Sidechain hydrophobicity
Ala	41	Leu	97
Arg	-14	Lys	-23
Asn	-28	Met	74
Asp	-55	Phe	100
Cys	49	Ser	-5
Gln	-10	Thr	13
Glu	-31	Trp	97
Gly	0	Tyr	63
His	8	Val	76
Ile	99		

Table 2-2. WNV codon changes to create amino acid substitutions.

This table details the codon changes that occurred to create the amino acid substitutions detailed by the E protein residue mutations. The nucleotide substitution for each mutant is detailed in red in this table.

E protein mutation	Original codon	Mutated codon
A54S	GCA	TCA
I130N	ATC	AAC
I135N	ATC	AAC
I196N	ATT	AAT
Y201P	TAC	CCC
A269S	GCC	TCC
V272T	GTG	ACG
L281N	TTG	AAC

Residues E-I130, E-I135, and E-I196, all with a hydrophobic index of 99, and residue E-L281 with a hydrophobic index of 97 were replaced with asparagine which has a hydrophobic index of -28. The E-I130N, E-I135N, and E-I196N mutations were created through a single nucleotide substitution of threonine to asparagine. This occurred at genome position 1355 in E-I130N, 1370 in E-I135N, and 1553 in E-I196N.

Creating a mutation at E-L281N required three entire codon changes: T1807A, T1808A, and G1809C.

The E-V272 residue with a hydrophobic index of 76 was mutated to threonine, which has a hydrophobic index of 13. This was done by two nucleotide substitutions at the first and second codon, G1780A and T1781C.

The E-Y201 residue with a hydrophobic index of 63 was changed to a proline. This specific change was made due to a lack of amino acids that contain an aromatic ring in the side chain of this residue, and there being a lower hydrophobicity than tyrosine. This mutation maintains the ring structure in the side chain and exhibits lower hydrophilicity in physiological pH based on previous findings (Monera et al., 1995; Kovacs et al., 2005). Furthermore, the E-Y201P mutation involved change of the first and second codons, T1567C and A1568C.

Engineered nucleotide substitutions were confirmed by Sanger sequencing for each plasmid. Plasmids were prepared using 500 mL of overnight XL-1Blue strain *Escherichia coli* competent cells (Stratagene) cultured and propagated in 2x YT broth which was supplemented with ampicillin at 50 ug/mL. These were then purified using the alkaline lysis method (Nucleobond® Xtra Maxi kit, Takara).

Approximately 5 μg of 5′ plasmid was digested with *AscI* and *NgoMIV,* followed by the treatment with calf intestinal alkaline phosphatase (CIP). Approximately 20 μg of 3′ plasmid was first digested with *MluI-HF* then treated with CIP. Digested 5′ and 3′ plasmids were then purified using a Nucleospin® column. Following this, the purified 3′ plasmid was digested using *NgoMIV* and purified once more. These digested 5′ and 3′ plasmids were ligated with T4 DNA ligase at room temperature for two hours then inactivated at 70°C for 50 minutes. The ligated fragment that was created was then digested using *XbaI* and protease K prior to purification by phenol-

chloroform extraction and ethanol precipitation method. The viral genome was produced by *in vitro* transcription using the Ampliscribe T7 High Yield Transcription kit (Lucigen) which was incubated at 37°C for two hours.

Roughly 10 μL of RNA transcripts were transfected into 4 million Vero cells and suspended in 400 μL of Dulbecco's phosphate buffer saline with no Mg^{2+}/Ca^{2+}, to be kept on ice. Transfections were conducted at one pulse of single wave condition, set to 225 volts for 25 milliseconds in a 4 mm cuvette using a BioRad GenePulser® system. The electroporated cells were then allowed to recover while on ice for approximately 15 minutes before being seeded in a T75 flask with pre-warmed media. Cells were harvested four days post-transfection due to apparent cytopathic effects in 70-80% of the cells. All following *in vitro* and *in vivo* experiments were conducted using virus stocks harvested from these transfected cells with no additional passages.

Nucleotide sequencing analysis

Confirmation of the retention of selectively engineered amino acid substitutions within the viable WNV mutants was completed using Sanger.

RNA was extracted from 250 μL of virus stock using the Direct-zol RNA Miniprep Plus kit (Zymo Research). Three fragments which overlap and correspond to part of the prM and E genes were added to the extracted RNA. This mixture was amplified using one-step reverse transcription-polymerase chain reaction (RT-PCR), then separated using agarose electrophoresis, and purified for Sanger sequencing using a procedure which has been previously described (Beasley et al., 2003). cDNA libraries of the wt WNV NY99ic strain and the created mutants were prepared using random hexamers before being sequenced on the NextSeq550 platform for NGS analysis. The sequencing files were randomly down sampled to an average of 2000

reads/nucleotide position to determine the consensus sequence as well as identify any single nucleotide variants (SNVs) that comprised at least 1% of the total RNA population. This was used to calculate Shannon Entropy using previously described methods (Kaiser et al., 2019[a]; Kaiser et al., 2019[b]).

Infectivity of WNV mutants

Plaque assays were conducted in Vero cells to demonstrate infectivity of virus stocks that were harvested from transfected cells. Plaque assays were also used to determine the genome copy-to-plaque-forming units (PFU) ratios. These ratios allow us to measure the amount of viral particles in each sample that can complete an infectious cycle.

50 μL of differing 10-fold serial dilutions of virus were inoculated into 24-well plates before being placed into an incubator at 37°C for 60 minutes to allow for adsorption. Plates were maintained in culture media which was supplemented with 1.5% carboxymethylcellulose and maintained at 37°C with 5% CO_2 for five days. Following incubation, plaques were fixed using 10% formol saline and stained with 1% crystal violet solution.

Neuroinvasive phenotype of WNV mutants in mice

Groups of four-week-old outbred Swiss Webster mice (Charles River) were used to determine the neuroinvasive phenotype of the engineered WNV mutants. 3- to 4-week-old Swiss Webster mice have been used in previous studies using WNV and have been shown to be an effective animal model. Outbred Swiss Webster mice are highly sensitive to WNV-NY99ic and succumb to neuroinvasive disease induced by intraperitoneal challenge [LD_{50} = 0.5 plaque forming unit (pfu)] (Beasley et al., 2002; Beasley et al., 2005). Therefore, a high dose of infectious viruses

(1000x LD_{50}) was chosen to determine the attenuating effect of respective mutations in the E protein. Five male and five female mice were inoculated with 500 PFU of a WNV mutant or the wt WNV NY99ic via the intraperitoneal (i.p.) route and monitored for 14 days. Mice were euthanized if 20% or greater weight loss or signs and symptoms of neurotropic disease occurred (**Figure 2-2**). All animal experiments were conducted in compliance with the *National Institute of Health Guide for the Care and Use of Laboratory Animals* and approved by the Institutional Animal Care and Use Committee within Kansas State University under protocol number 4322.

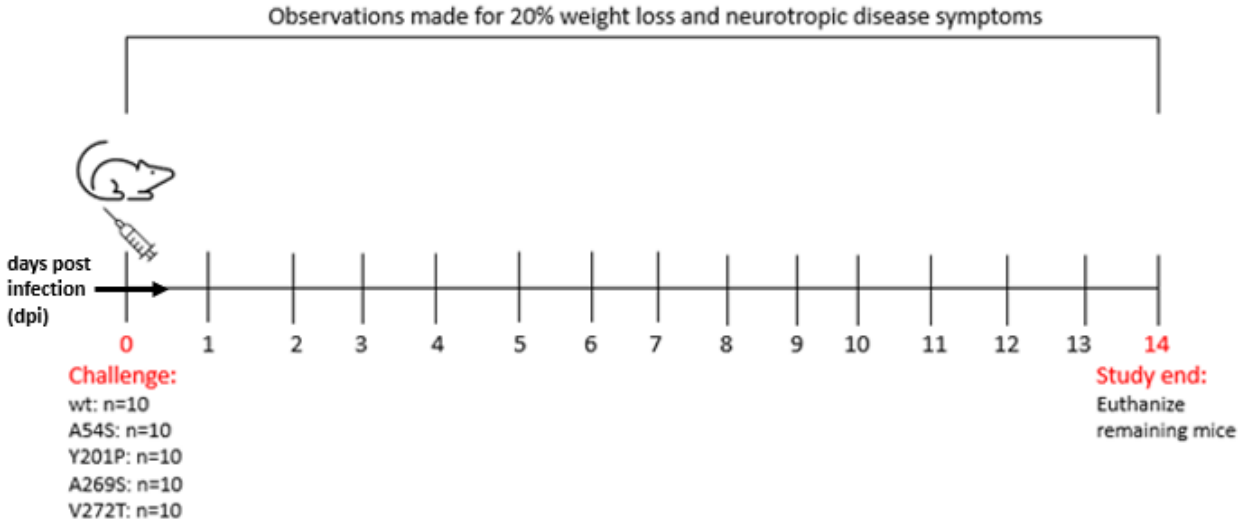

Figure 2-2. Mouse study schedule.

This graphic details the study design of this project. Days post infection (dpi) is shown on the axis, with neuroinvasive challenge of mice via i.p. injection on day 0 and study end at day 14. Mice were euthanized premature to the end of the project if they began to show signs of neurotropic disease. Daily checks in the morning (8am), afternoon (4pm), and night (11pm) were conducted to ensure this process was completed properly.

The brain of each mouse was collected following euthanasia to quantify tissue viral load using quantitative RT-PCR (RT-qPCR). RNA was extracted from a separate aliquot by use of the Direct-zol miniprep plus columns (Zymo Research).

Some extracted viral RNA was used to confirm that the virus had not reverted to virulence *in vivo*. RT-PCR was used on extracted RNA to create cDNA fragments of the mutated regions. Sanger sequencing was conducted, and results were compared to plasmid maps created in SnapGene software to confirm mutations had remained.

Remaining viral RNA from each sample was analyzed through RT-qPCR using the pan-flavivirus mFU1 and CFD2 primer set, and a WNV-specific probe which was conjugated with 6-carboxyfluorescein at the 5′ terminus (5′-TGCGTGAAGTTGGCACCCGGCCT-3′) which has been described previously (Chao et al., 2007). 2 μL of RNA extract was amplified in 20 μL reaction using cycling parameters: reverse transcription at 50°C for 30 minutes, hot-start activation at 95°C for 15 minutes, 45 cycles of denaturation at 95°C for 15 seconds and annealing and amplification at 48°C for 3 minutes with continuous fluorescence detection. Viral RNA quantity of each sample was calculated using the standard curve generated from serially diluted RNA transcripts.

From this analysis, ct values above 33 were considered negative detection as previously described (Lyons et al., 2018). In addition to the brain, serum samples were collected from the mice that were euthanized at the endpoint of the study to determine serum neutralizing activity using 50% plaque reduction neutralization test ($PRNT_{50}$) as described previously (Roehrig et al., 2008). Serum samples were heat-inactivated, and 2-fold serially diluted before mixing with 100 PFU of the biological isolate of WNV NY99 strain. These samples were incubated at 37°C for 60 minutes before being inoculated into 24-well plates. Plaques were stained at 5 days post infection (dpi) as described above. $PRNT_{50}$ titer was defined by the reciprocal of the highest dilution that created a 50% reduction in plaque number when compared to the virus control.

Statistical analysis

Statistical analysis was completed in a few different ways for the variety of assays conducted. Infectivity and mouse brain viral load of WNV mutants were compared to WNV NY99ic using one-way analysis of variance (ANOVA). Survival outcomes of mice challenged with WNV mutants were compared using the Kaplan-Meier survival curve. All statistical analysis was performed using the Prism 9 program (GraphPad Software).

Chapter 3 - Neuroinvasive Phenotype of WNV in the Mouse Model

Introduction

We hypothesized that mutations of flavivirus-conserved residues in the EDI-EDII hinge region would attenuate the virulent phenotype of flaviviruses. To test our hypothesis, the virulence phenotypes of the A-E54S, E-Y201P, E-A269S, and E-V272T mutants were investigated in the neuroinvasive disease model in outbred mice. An additional mutant was engineered for use in the mouse model, E-L281N, but reverted to virulence *in vivo*, so data is not shown.

Infectivity of engineered WNV mutants

Before being placed in the mouse model, these mutants were investigated in cell culture to observe infectivity. The respective mutants were proven to produce infectious viruses detectable by plaque assay and retain the engineered nucleotide substitutions(s). The replicates of these plaque assay experiments can be seen in **Figure 3-1** and for easier visualization, the average of the replicates for these plaque assay experiments can be seen in **Figure 3-2**.

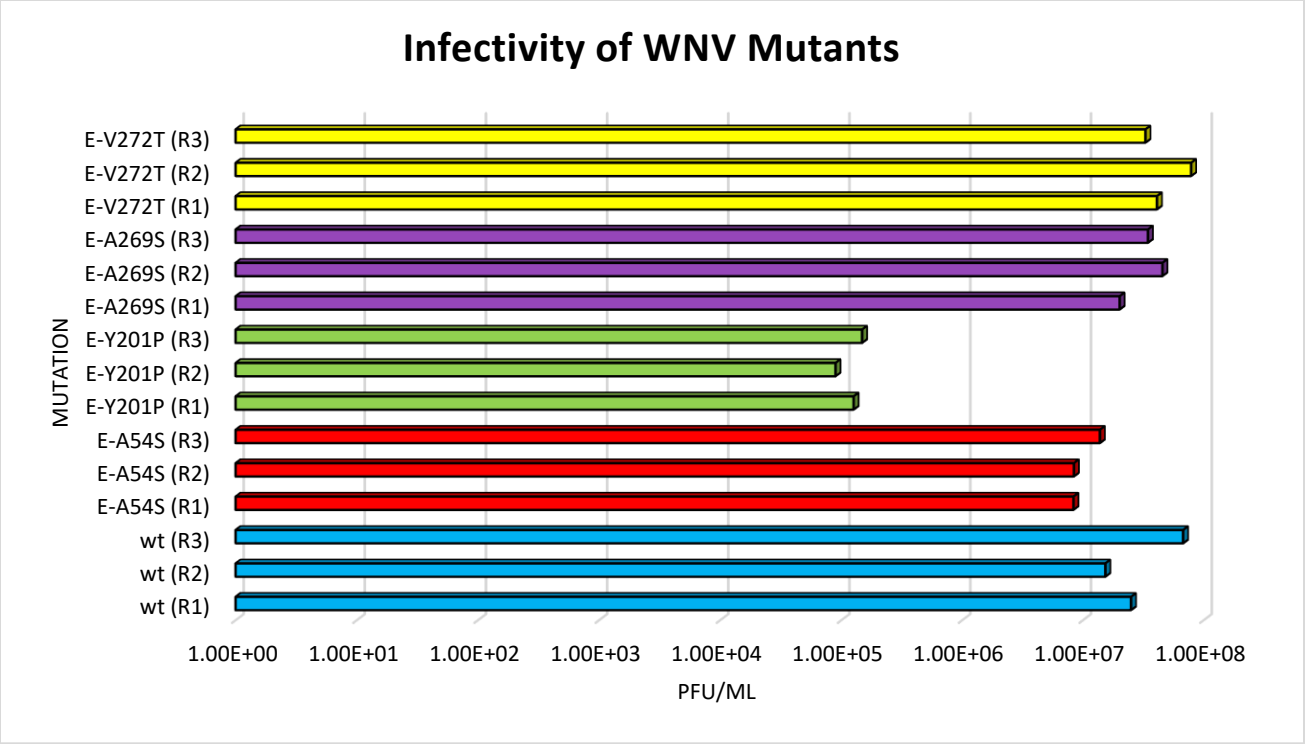

Figure 3-1. Replicate infectivity of WNV mutants.

Analysis was done in triplicate (R1, R2, R3) to look at infectivity of WNV mutants based on PFU/mL using plaque assay.

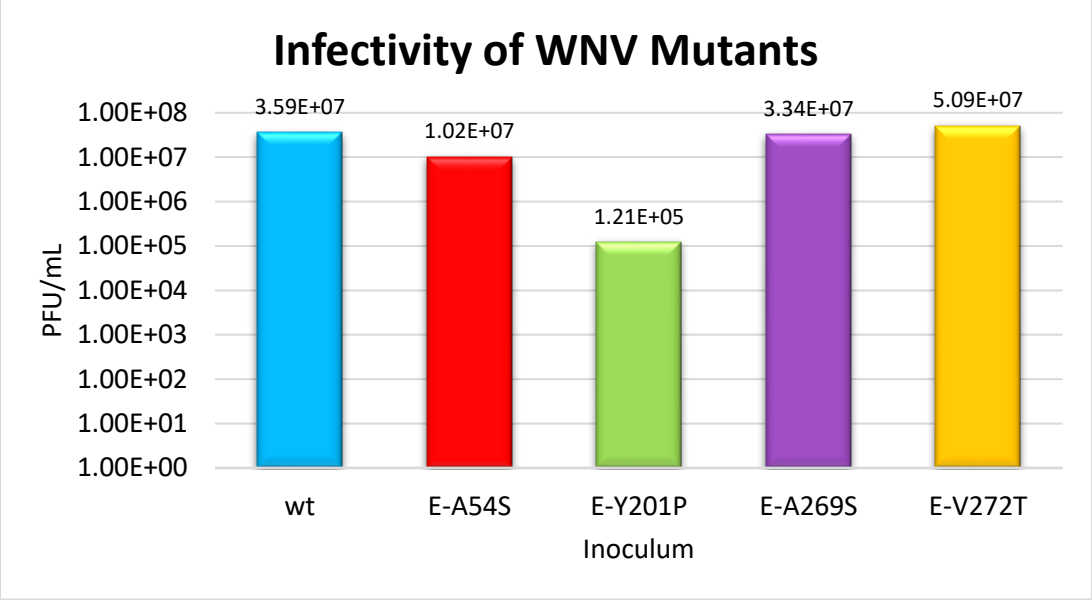

Figure 3-2. Average infectivity of WNV mutants.

This figure shows the average PFU/mL value for each mutant group as determined by plaque assay. Average PFU/mL for the respective group can be seen at the top of each bar in the graph.

This study utilized four-week-old outbred Swiss Webster mice as the selected mouse model. Swiss Webster mice have previously been used to investigate WNV infection (Beasley et al., 2003; Beasley et al., 2005). Animals were divided into groups of five males and five females (total n = 10) for each mutant or wt control group. All mice were inoculated via the intraperitoneal (i.p.) route at 500 PFU per mouse and observed for up to 14 days. Weights were recorded for each mouse every morning to observe for 20% or greater weight loss between days, and mice were checked 3 times daily to observe for signs of neurotropic disease including quiescence, paralysis of limbs, hunched posture, ruffled fur, and difficulties in mobility. Throughout the course of the study, mice were humanely euthanized 24 hours after the onset of neurotropic disease in compliance with the IACUC protocol.

Single mutations can attenuate the neuroinvasive phenotype of WNV

Numerous factors were considered when looking at the neuroinvasive phenotype, with the most important factor being mortality. **Table 3-1** details how many mice survived to the study endpoint, and details when mortality was experienced for mice which did not survive to the study endpoint. During this study, there was no observed mortality in the E-Y201P mutant group, leading to the conclusion that this single-site mutation likely attenuates the neuroinvasive phenotype. Notably, there was a 20% survival rate in the group challenged with E-A54S mutant, potentially pointing to a partially attenuated phenotype. Mutants E-A269S and E-V272T exhibited similar uniform lethality to the parental wt WNV control group.

Table 3-1. Mortality time of mice.

Mice were checked three times daily (morning at 8AM [M], late afternoon at 4PM [A], and night at 11PM [N]). Time of mortality in this table is denoted by days post infection (dpi) and respective check time. dpi is determined by what day post infection the mortality occurred and is not influenced by whether the mouse was euthanized at any specific check throughout the day. This graphic is to showcase when and how quickly inoculated groups succumbed to disease.

Virus	Mouse	dpi	Time of mortality	Virus	Mouse	dpi	Time of mortality
WNV NY99 - Cage 1	1	7	M	WNV NY99 - Cage 2	1	7	A
	2	6	M		2	8	M
	3	6	M		3	7	N
	4	7	M		4	7	M
	5	7	M		5	7	N
E-A54S - Cage 1	1	9	M	E-A54S - Cage 2	1	9	N
	2	8	A		2	10	N
	3	14	End of Study		3	11	M
	4	8	A		4	14	End of Study
	5	8	A		5	7	A
E-Y201P - Cage 1	1	14	End of Study	E-Y201P - Cage 2	1	14	End of Study
	2	14	End of Study		2	14	End of Study
	3	14	End of Study		3	14	End of Study
	4	14	End of Study		4	14	End of Study
	5	14	End of Study		5	14	End of Study
E-A269S – Cage 1	1	7	M	E-A269S- Cage 1	1	8	A
	2	7	M		2	7	A
	3	7	M		3	7	A
	4	7	N		4	7	A
	5	7	M		5	7	N
E-V272T- Cage 1	1	7	N	E-V272T- Cage 2	1	6	M
	2	7	M		2	7	M
	3	7	M		3	6	N
	4	6	A		4	7	M
	5	7	A		5	7	M

E-A54S and E-Y201P mutants exhibit significantly extended average survival time

Average survival time (AST) of each group was determined by dpi when mortality occurred. The AST of mouse groups exhibiting signs of neurotropic disease averaged between 6 and 8 dpi (**Table 3-2**). The exception to this were the groups inoculated with the fully attenuated E-Y201P mutant and the partially attenuated E-A54S mutant in which we saw increased average survival times. To accompany AST, a graphic was created to detail the percent of mice in each group which survived to end of study (**Figure 3-3)**.

Table 3-2. Average survival times.

This table details the average survival time post infection for each group of inoculated mice as well as the standard deviation seen within the respective group.

Group	Average Survival Time	Standard deviation
WNV NY99 (wt)	6.9	0.567646
E-A54S	9.8	2.485514
E-Y201P	>14	0
E-A269S	7.1	0.316228
E-V272T	6.7	0.483046

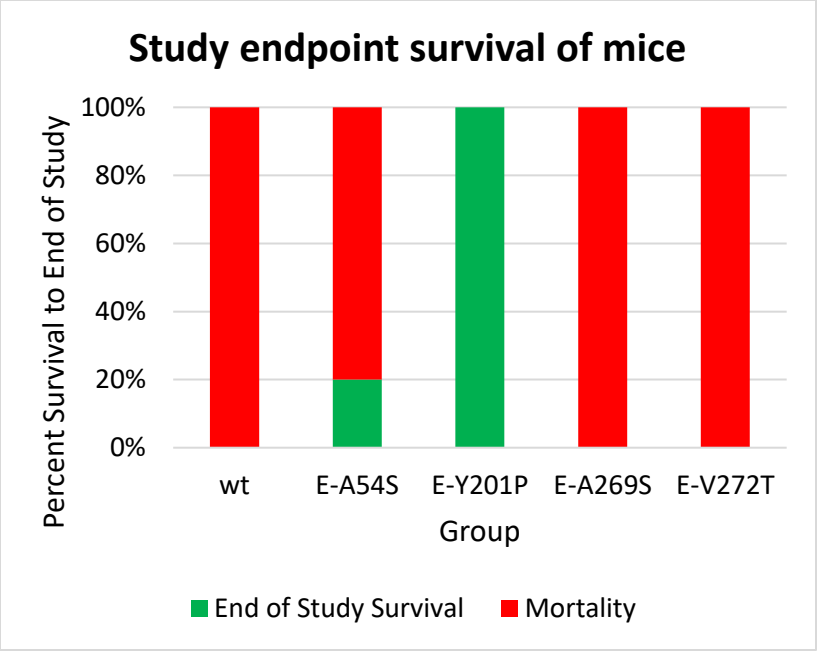

Figure 3-3. Study endpoint survival of mice.

This figure details the percent of mice within each group which survived to the study endpoint. These values are expressed as percentages with green bars indicating the percent of each respective group that survived to the end of the study, and red indicating the percent of each respective group which did not survive to the end of the study.

In the wild-type control group, male mice survived an average of 6.6 days with females surviving an average of 7.2 days. This makes for an average survival time of 6.9 days.

The group inoculated with E-A54S had two outliers, one in each sex as previously mentioned. With this understanding, including in that data gives the males an average survival time of 9.4 days and females an average survival time of 10.2 days for a combined average survival time of 9.8 days ($p<0.01$). With the exclusion of the surviving mice, the males and female mice that succumbed to the neuroinvasive disease had an average survival time of 8.25 and 9.25 days, respectively. The AST for all mice succumbed to i.p. challenge with E-A54S mutant is 8.75 days.

There was no euthanasia needed during the study period in the male and female group inoculated with E-Y201P as there was no signs of neurological disease observed. Average survival time for both groups exceeded the duration of this study, i.e., 14 days.

The E-A269S groups were very consistent among individuals. All males were euthanized by 7 dpi, generating an average of 7 days. Females had slight variation, producing an average of 7.2 days. Overall, this group had an average survival time of 7.1 days.

The final group, the E-V272T mutant group, was very similar to the control group. Males in this group had an average survival time of 6.8 days while females had an average survival time of 6.6 days, creating an overall survival time of 6.7 days.

Attenuated mutants failed to cause significant weight loss in mice compared to other mutants tested

One of the most important indicators used for the onset of WN neurotropic disease in mice is weight. Weights of all mice were measured during the first daily animal check of the morning (**Table 3-3**); these weights have been averaged for male cages and female cages within each inoculated group. These can be found in **Figure 3-4**.

Table 3-3. Individual mouse weights.

Weights of each mouse (measured in grams) were taken every morning during the 8AM check. This image details the starting weight, the peak weight, and the final weight for each mouse; starting weight indicates weight on day 0 of the study, peak weight indicates the heaviest weight each mouse acquired during the study, and final weight is the weight taken at time of euthanasia. This graphic is used to showcase the partially attenuated effects of the E-A54S mutant and the fully attenuated effects of the E-Y201P mutant by highlighting weights of the mice that survived to the study endpoint. Male mice are denoted by a number and "M" while female mice are denoted by a number and "F".

Group	Mouse	Starting wt (g)	Peak wt (g)	Final wt (g)	Mouse	Starting wt (g)	Peak wt (g)	Final wt (g)
WNV NY99 wt	1M	15.9	21.2	18.2	1F	9.3	11.9	10.3
	2M	12.9	17.5	14.5	2F	11.0	14.2	10.9
	3M	14.2	20.0	16.5	3F	9.3	12.1	10.0
	4M	15.5	21.3	17.7	4F	9.3	11.1	9.0
	5M	20.5	26.5	21.5	5F	8.5	10.2	8.7
E-A54S	1M	18.0	25.2	22.3	1F	10.7	14.6	12.4
	2M	16.0	23.2	21.0	2F	10.3	16.1	14.8
	3M	15.7	28.7	28.7	3F	10.5	12.9	11.2
	4M	18.3	25.3	20.4	4F	11.8	14.1	13.8
	5M	14.6	22.5	17.0	5F	12.2	14.2	11.7
E-Y201P	1M	14.6	30.3	30.3	1F	6.6	17.2	17.2
	2M	16.2	29.4	29.4	2F	8.5	21.8	21.8
	3M	15.4	29.0	29.0	3F	9.3	19.6	19.6
	4M	15.1	28.6	28.6	4F	10.8	23.0	23.0
	5M	15.6	25.7	25.7	5F	10.1	19.8	19.8
E-A269S	1M	15.0	20.4	17.0	1F	11.3	15.6	15.5
	2M	16.2	22.6	19.8	2F	7.5	10.3	9.5
	3M	17.0	24.2	20.7	3F	9.0	12.4	10.2
	4M	15.1	21.6	19.2	4F	8.8	10.9	8.7
	5M	14.6	20.4	16.0	5F	7.7	9.9	8.2
E-V272T	1M	15.7	21.9	19.9	1F	7.6	11.2	9.3
	2M	18.0	25.6	21.0	2F	8.3	12.4	10.4
	3M	14.8	21.4	19.8	3F	9.0	15.2	13.3
	4M	14.9	20.6	16.3	4F	8.9	14.1	11.5
	5M	14.0	18.8	15.7	5F	11.6	17.4	14.5

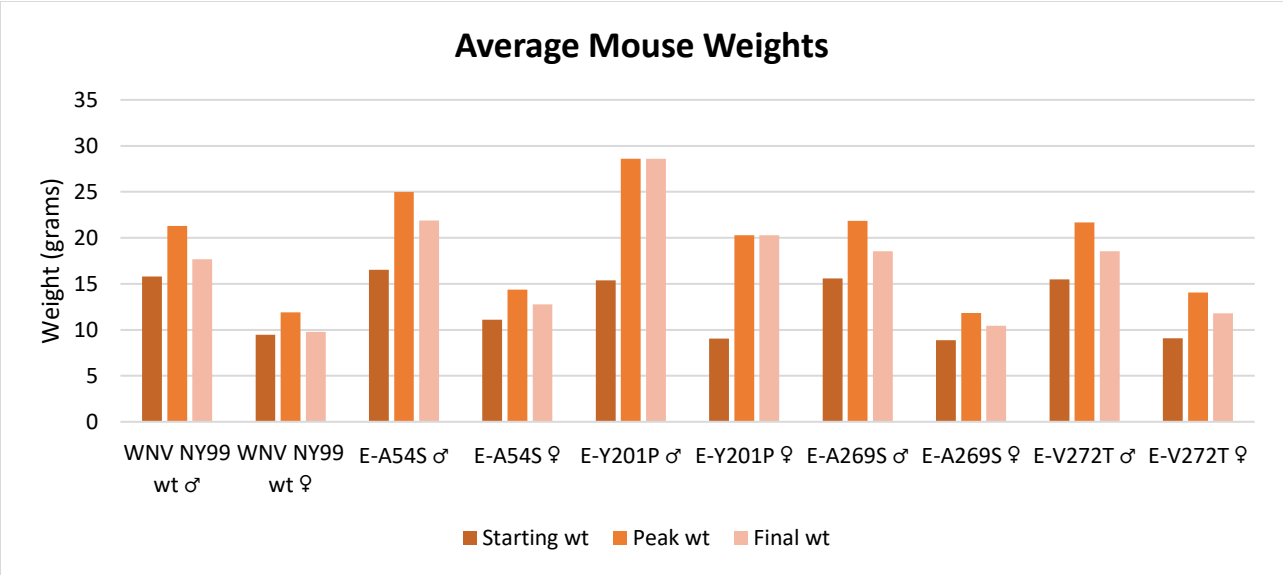

Figure 3-4. Average mouse weights.

Weights of each mouse (measured in grams) were taken every morning during the 8AM check. Averages for each group and each sex can be found in this figure. This image details the starting weight, the peak weight, and the final weight for all mice; starting weight indicates weight on day 0 of the study, peak weight indicates the heaviest weight mice acquired during the study, and final weight is the weight taken at time of euthanasia. This graphic is used to showcase the weight loss that was observed in all cages except the fully attenuated mutant E-Y201P cages.

The kinetics of weight loss caused by the i.p. challenge with the wt control virus represents the progression of WN neurotropic disease. All male mice were gaining weight up until 5 dpi, during which WNV likely began to multiply and disseminate from the peritoneal cavity. At this point, three mice began showing signs of weight loss. By 6 dpi, all male mice in this group began showing weight loss, with two being euthanized. The females of the wt group lost weight initially following inoculation, potentially due to the configuration of ventilated cages that can be challenging for young female mice to access water. However, weight gain within this group of

females was consistent following cage configuration resolution until 5 dpi when two began to show weight loss. Similar to the males, all females had begun to show weight loss by 6 dpi.

In the group inoculated with the E-Y201P mutant, none of the males lost weight during the entire study. All gained between 65% to 108% of their initial weight measured at 0 dpi. Female mice exhibited transient weight loss resembling those in the wt control group, once again likely due to cage configuration, prior to gaining weight and ending the study at 96% to 161% of their initial weight taken at 0 dpi.

Two mice inoculated with the E-A54S mutant survived to the end of the study, indicating the single E-A54S mutations can partially attenuate the neuroinvasive phenotype of WNV. All male mice within this group began showing weight loss at 5 to 6 dpi, but the only surviving male mouse began gaining weight again at 9 dpi. As for the females, following the initial weight loss, all females began gaining weight until 7 to 9 dpi, prior to succumbing to the neurotropic disease. The only surviving female mouse began to gain weight again at 12 dpi.

In the E-A269S mutant inoculated group, weight loss was robust and consistent. The males in this group began showing weight loss at 6 dpi, with females showing weight loss consistently at 7 dpi.

In the group inoculated with the E-V272T mutant, males began showing weight loss at 6 dpi, and females consistently showed weight loss at 6 dpi.

Neurotropic disease

A wide range of neurotropic disease was seen within the groups of mice, ranging from hunched posture to severe neurological signs.

Along with the observation of weight loss, male mice within the group inoculated with wild-type virus also began showing signs of neurotropic disease on day 6 dpi. Signs of disease that were observed in this group first were hunched spinal posture, inhibited motor movements, a lack of escape attempt from human hands, quiescence, and leaning to one side. Signs seen later in infection in this group included trembling and uncoordinated movements.

Female mice in the wild-type group also showing signs of disease 6 dpi, with the first symptoms observed being tremoring and uncoordinated movements. Notably, there was a rapid progression in the development of neurotropic disease. The later check on 6 dpi identified mice that developed symptoms of paralysis onset, ruffled fur, and hunched spinal posture. Signs of disease continued to be observed and ranged from mice leaning to one side to discovering mice on their side and unable to move during checks.

Male mice in the E-A54S inoculated group began showing symptoms a day after the wt group at 7 dpi. Symptoms started out as quiescence, hunched spinal posture, tremors, and uncoordinated movements. This progressed quickly; some mice had succumbed to neurotropic disease between two daily checks on the same day. The mouse in this group that survived to 14 dpi was bright, alert, and responsive (BAR) most of the study, but did seem to slow in movements on 7 dpi, with a return to baseline the following day.

Female mice in the E-A54S group also began showing symptoms at 7 dpi, with the first symptom seen being limb paralysis. This mouse found with paralysis was BAR at the previous check. Other symptoms seen in this group were quiescence, hunched spinal posture, ruffled fur, uncoordinated movement, and mice found on their side during checks. The mouse in this group that survived to 14 dpi, did show signs of disease starting at 8 dpi. This mouse was found to be BAR but had ruffled fur and a slightly hunched posture from 8 dpi to 14 dpi.

Male and female mice in the E-Y201P mutant groups were BAR for the entirety of the study, showing no signs of disease at any point during the study. The lack of symptoms of WN neurotropic disease provides evidence that the E-Y201P mutation can fully attenuate the neuroinvasive phenotype of WNV in mice.

Male mice in the E-A269S mutant groups began showing signs of disease starting at 6 dpi with the observation of quiescence and uncoordinated movements in two mice. From there, signs of disease advanced to hunched posture, depression, and hind limb paralysis.

Female mice in the E-A269S inoculated group had a similar experience to the males in the group. Signs of disease started on 6 dpi with one mouse showing signs of being slow to escape human touch. All mice in this group began showing signs of disease by 7 dpi including ruffled fur, quiescence, hunched posture, and/or uncoordinated movements. Symptoms in this group increased to severe neurological disease including leaning to one side, seizures, tremors, and limb paralysis.

Male mice of the E-V272T inoculated group began showing signs like the other infected groups around 6 dpi, with one mouse going from BAR on day 5 dpi to displaying limb paralysis during the afternoon check on day 6 dpi. Other mice in this group began displaying similar symptoms the following day, ranging from depression and quiescence to limb paralysis.

Females of the E-V272T group also began showing symptoms at 6 dpi like the males of this group. The first mouse to display symptoms was quiescent with limb paralysis. Another mouse succumbed to neurotropic disease during the night check, displaying symptoms of inability to move off its side and tremors. By 7 dpi, observations of disease included depression, limb paralysis, and discharge from nasal and oral orifices.

Discussion

Overall, the mortality, average survival time, weight loss, and kinetic for the onset of neurotropic disease are helpful in identifying single mutations that have the attenuating effect on the mouse neuroinvasive phenotype of WNV. The E-Y201P mutation has the most robust attenuating effect and led to the complete loss of neuroinvasive phenotype of WNV in outbred Swiss mice challenge via the i.p. route at 500 PFU. To a much less extent, the attenuated phenotype of the E-A54S mutant is partial, based on the 20% survival rate. It is immediately apparent that neither the E-A269S mutant nor the E-V272 mutants are attenuated in the outbred Swiss mice receiving i.p. challenge. Further analysis on the brain viral load discussed in Chapter 4 provides further insight on the attenuating effect of the respective mutants.

Chapter 4 - Assessment of Brain Viral Load in Mice Challenged with the Respective WNV Mutant

Introduction

In addition to the high mortality induced by WN neurotropic disease, we hypothesize that the single mutations that exhibit the attenuating effect on the neuroinvasive phenotype of WNV can reduce the brain viral load in mice. Following the euthanasia of each mouse, the brain was collected to allow for viral load detection. Along with the brain, mice surviving to 14 dpi had serum collected by cardiac puncture for further assessment.

Attenuating effect of E-A54S and E-Y201P mutants resulted in a reduced brain viral load in mice

The neuroinvasive phenotype of the four selected mutants were compared with the parental wt WNV NY99ic. **Figure 4-1** details the brain viral load observed in each individual mouse, and **Figure 4-2** details the brain viral load of each inoculated group as an average. As noted, wt WNV NY99ic led to lethal disease in all mice between 6 and 8 dpi. However, no mortality was observed in the group challenge with the E-Y201P mutant, and no viral RNA was detected in the brains of these mice either (limit of detection = $4.8x10^4$ genome copies/gram of tissue). From this information, we can further assess that the E-Y201P mutation fully attenuated the neuroinvasive phenotype of WNV in the chosen mouse model when challenged with 500 PFU.

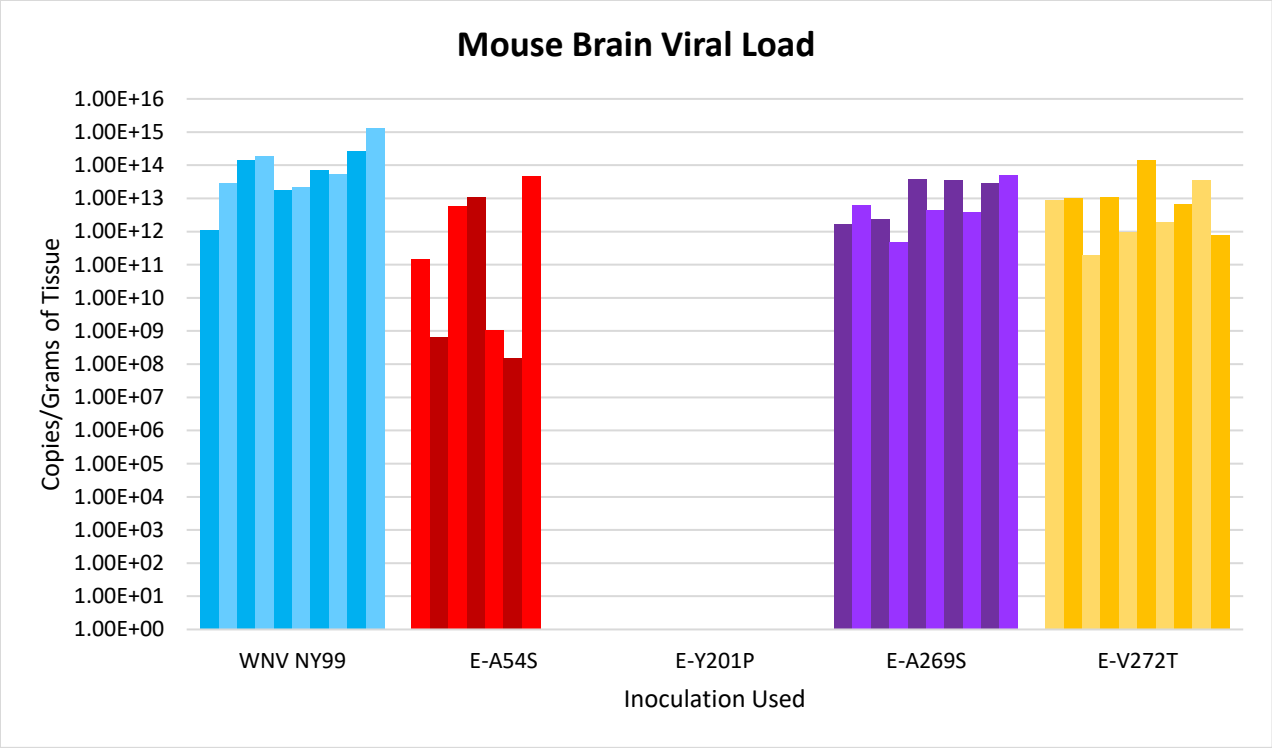

Figure 4-1. Mouse brain viral load.

This figure details the mouse brain viral load of every individual mouse from each respective group following euthanasia. Load is quantified by viral copies/gram of tissue.

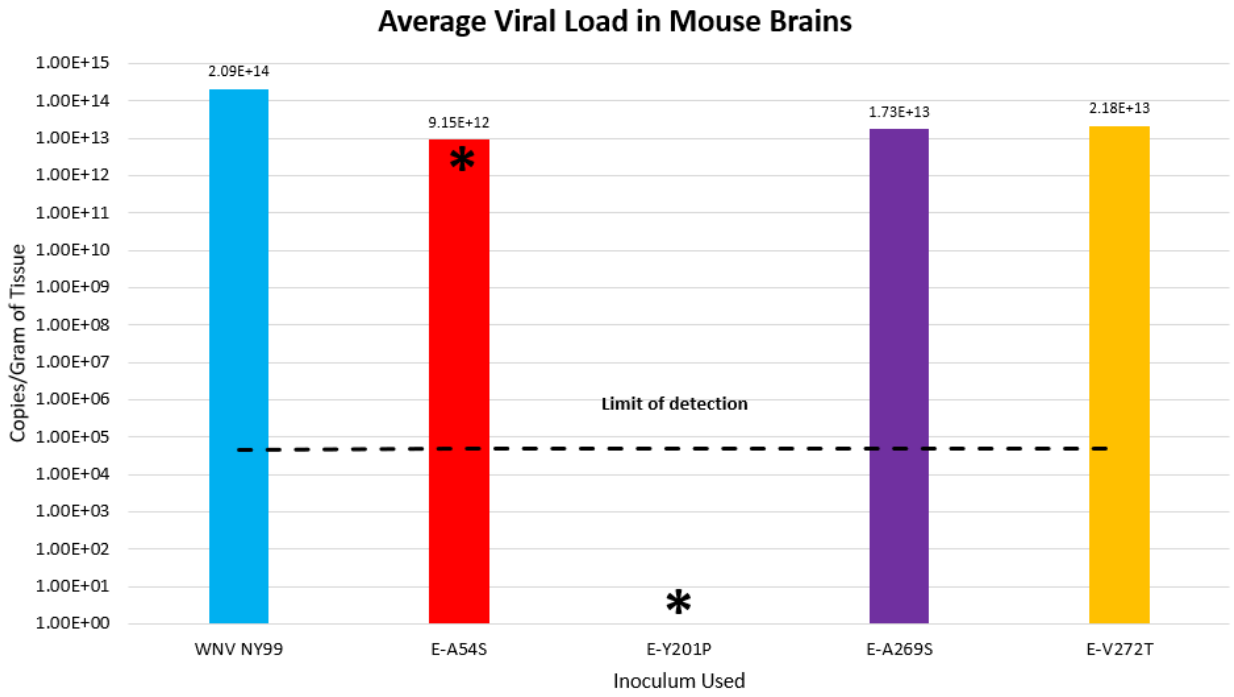

Figure 4-2. Average mouse brain viral load.

This figure details the mouse brain viral load of all mice in each respective group as an average. This image depicts the limit of detection for this assay as well as significantly different values (*) as compared to the WNV NY99 inoculum.

The E-A54S mutation was observed to have a partially attenuated phenotype that led to 80% mortality rate (8/10) but a significantly increased average survival time. Overall, the brain viral load of mice challenged with the E-A54S mutant was significantly lower than those challenged with the wt WNV NYic. Notably, three mice challenged with the E-A54S mutant had no detectable viral load in the brain, including the two animals that survived to 14 dpi.

While the i.p. challenge with the E-A269S and E-V272T mutants led to 100% mortality between 6 and 8 dpi, there was still a significant reduction of viral load within the brain when compared to the WNV NY99ic control group. This suggests that both the E-A269S and E-V272T mutations still have a minimally attenuating effect on the neuroinvasive phenotype of WNV. Despite the minimally attenuating effect, neither the E-A269S mutation nor the E-V272T mutation

alone is sufficient to cause demonstrable impact on the lethal disease caused by the i.p. challenge at 500 PFU.

Serum neutralizing activity

An important criterium for the selection of the attenuating mutations for the rational design of candidate WN LAVs is the immunogenicity, i.e., the ability to elicit immune responses that protect against wt WNV. To gain a full understanding of the immunogenicity of each mutant tested in this study, serum neutralizing activity was examined for the mice surviving to day 14 dpi (**Table 4-1**). In the group of mice inoculated with mutant E-Y201P, all but one mouse (90%, 9/10) developed serum neutralizing activity above 1:10 $PRNT_{50}$ titer (geometric mean: 43.2). A serum neutralizing activity above 1:10 $PRNT_{50}$ titer is the protection threshold of flaviviruses. Notably, although it was a very small sample size in comparison, there was no significant difference in serum neutralizing activity between surviving mice in the E-A54S mutant inoculated group (n=2, geometric mean of $PRNT_{50}$ titer: 160) and the E-Y201P mutant inoculated group.

Table 4-1. Serum neutralizing activity.

Virus	$PRNT_{50}$ Titers	Percentage of Mice with Serum Neutralizing Activity	Geometric mean $PRNT_{50}$ Titer
E-A54S	320	2/2 (100%)	160
	80		
E-Y201P	<10	9/10 (90%)	43.2
	40		
	80		
	160		
	40		
	10		
	20		
	20		
	80		
	80		

Discussion

In summary, there is a correlation between the attenuated phenotype observed with the mortality rate and the attenuated phenotype observed with brain viral load among the four mutants evaluated in this study. It demonstrates that all four mutations have varying degrees of attenuating effect on the neuroinvasive phenotype of WNV. This finding has important implications for the relationship between the structure-function of the E protein and WNV pathogenesis; it highlights that flavivirus-conserved hydrophobic residues in the EDI-EDII hinge region are crucial for the neuroinvasive phenotype of WNV. With data supplied from the serum neutralizing activity, it can also be determined that these two mutations are not only showing attenuating effects but also immunogenicity, giving this compilation of data a strong foundation for vaccine development.

Chapter 5 - Discussion, future directions, and concluding remarks

Introduction

In this study, we examined the attenuating effect of four flavivirus-conserved hydrophobic residues in the WNV E protein EDI-EDII hinge region; E-A54S, E-Y201P, E-A269S, and E-V272T. Each mutant containing the respective single amino acid substitution was tested in the outbred Swiss mouse model for neuroinvasive disease. The observed attenuated phenotype of the E-A54S and E-Y201P mutants generates the knowledge for the rational design of candidate WN LAVs.

The attenuation process of WNV is a proof of concept for the development of a flavivirus common attenuation mechanism targeting flavivirus-conserved amino acids that are equivalent to the WNV E-A54 and E-Y201 residues. There is a significant likelihood that mutations of these flavivirus-conserved hydrophobic residues could lead to an attenuated phenotype translatable between flaviviruses. The residue found at E-A54 in WNV is highly conserved among all flaviviruses, showing the importance of this specific amino acid location and its partial attenuation for LAV development (Butrapet et al., 2011; Nybakken et al., 2006). As for WNV E-Y201, this residue, or its equivalent position, is highly conserved across various flavivirus serocomplexes. For example, other members of the JEV serocomplex possess an aromatic ring side chain at this position: the E-F202 residue in the JEV E protein and E-Y201 of both the St. Louis encephalitis virus and Murrey Valley encephalitis virus E protein. Additionally, in the DENV serocomplex, the comparable location of the E protein possesses a hydrophobic methionine, which is consistent across all four serotypes (de Wispelaere et al., 2018). With the discovery of these two attenuating mutations, there could be additional research to combine these two mutations and observe

compatibility, especially due to the proximity. Creating dual mutations that are compatible, produce an attenuated phenotype, and prevent the virus from reverting to virulence could create an even more robust and safe LAV design.

Alterations to the hinge region for candidate vaccine design

The E protein is a class II fusion protein that exhibits up to 40% sequence homology across different flaviviruses (Rey et al., 1995). Therefore, the conserved structure-function of the E protein engages in host cell attachment, membrane fusion, and viral assembly, which are all critical processes for flavivirus pathogenesis. With the careful consideration of the structural and functional homology of this protein, it is likely that the attenuation process of WNV described in this project can be expanded to a broad range of flaviviruses.

Within the E protein there are three domains and between these domains there are peptide strands functioning as hinges. EDIII is connected to EDI by a single peptide strand and EDI is connected to EDII by four peptide strands. These hinges are in use during low pH-induced protein reorganization which facilitates the viral fusion with endosome membranes. The EDI-EDII hinge region controls the conformational change of 90 copies of E protein homodimer in the virion to a trimer, a conserved mechanism of action driving the fusion of virus and cell membranes (Butrapet et al., 2011). These hinges also assist the virus through conformational changes related to maturation (Goo et al., 2006). Previous studies have shown E protein monomers of both mature and immature virions demonstrating rotation around the hinge region, which suggests hinge motions are not only assisting in viral maturation, but essential to it (Butrapet et al., 2011).

Within the E protein domains, EDI, EDII, and EDIII, there are also molecular determinants of virulence that can be found in distinct clusters. The first cluster is found in the hinge of EDI and

EDII, at the base of EDII, which is a necessary area of the E protein that affects pH-dependent conformational changes for endosomal fusion (Goo et al., 2006). This region is where the mutations chosen for this thesis occur. Mutations introduced into the second cluster, found at the tip of EDII in the highly conserved fusion peptide area, appear to interrupt fusion. The third and final cluster can be found on the lateral face of EDIII. In this cluster, there is an integrin-binding motif: disruptions in this cluster appear to interrupt receptor binding. It is important to note that this region is also very hydrophilic. Introducing a hydrophobic amino acid into this cluster or another hydrophilic portion of the E protein has been previously shown to disrupt protein function at a stage following host cell entry (Goo et al., 2006).

The hinge region between EDI and EDII provides the defined molecular basis for an attenuation platform; disruption of interdomain movement in this region will effectively impair the membrane fusion process. Finding mutations within this region that could be used across several flaviviruses gives a solid platform for a common attenuation method and LAV development.

Current comparable vaccines and vaccine candidates

In the United States, WNV continues to be a leading cause of viral encephalitis, yet no licensed human WN vaccine is available. The E-A54S and E-Y201P mutations used in this thesis exhibit an attenuating effect that supports the rational design of candidate WN LAVs.

The mutations at residues E-A54 and E-Y201 attenuate the mouse neuroinvasive phenotype of WNV with an intraperitoneal challenge, but another important criterion for the selection of attenuating mutations for the development of candidate WN LAVs is attenuation of the neurovirulence phenotype in mice receiving intracerebral challenge. Previous research has

shown that the mouse neurovirulence phenotype is important for the safety of flavivirus LAVs. The JEV SA14-14-2 LAV, which has an excellent safety profile, is fully attenuated for the mouse neurovirulence phenotype, and has been used in humans for many years now (Kaiser & Barrett, 2019). In contrast, the ChimeriVax-WN LAV was introduced to the market in 2006 but had to be withdrawn in 2010 due to unacceptable reactogenicity in horses (Iyer and Kousoulas, 2013); therefore, this platform will require additional mutations to achieve full attenuation and be allowed back to market. Additionally, neither the chimeric candidate WN LAV based on the DENV-2 PDK-53 strain nor the chimeric candidate WN LAV derived from the DENV-4 Δ30 strain is fully attenuated for the mouse neurovirulence phenotype, indicating that existing platforms of chimeric flavivirus candidate LAVs may have limited utility for the development of safe candidate WN LAVs (Huang et al., 2005; Pletnev et al., 2006). The discovery of attenuation at E-A54 and E-Y201 in this study is an introductory benchmark for a safe and efficacious candidate vaccine design and it is imperative to investigate the impact of mutations are residues E-A54 and E-Y201P further.

Investigating the immunogenicity of attenuated mutants

In addition to reaching a safe level of attenuation, attenuating mutations must also maintain the antigenicity of the E protein and consequently the immunogenicity of candidate WN LAVs. Both the highly effective YF 17D and JE SA14-14-2 LAVs use neutralizing antibodies as the correlate of protection (Yu et al., 1981). Hence, it is likely that an effective WN LAV would also use neutralizing antibodies as the immune marker for the correlate of protection.

As detailed earlier, the EDI-EDII hinge region is the focus of this project in part due to its importance in the structure function of the protein. Additionally, the hinge region holds significance in the human neutralizing antibody response. Type-specific neutralizing epitopes

against different flaviviruses often involve residues in the EDI-EDII hinge region, including WNV, YFV, and DENV. Type-specific neutralizing antibodies are the most protective amongst distinct types of neutralizing antibodies raised against flaviviruses (Diamond et al., 2008; Volk et al., 2009). This knowledge lends credence to evaluating the mutants outlined in this thesis further; both mutations, E-A54S and E-Y201P, did not compromise the ability of WNV to elicit the neutralizing antibody response. This was demonstrated through analysis of the serum neutralizing activity of all mice from the two mutant groups that survived to day 14 dpi.

Mutations used for the development of a flavivirus common attenuation mechanism

The structure-function of the E protein domains have been characterized for years, yet few attenuating mutations have been discovered or engineered within the EDI-EDII hinge region. With current technologies, available mutations typically occur at amino acids which are unique to the respective flavivirus or to a respective serocomplex (Hurrelbrink & McMinn, 2001). These current technologies leave us with limited capabilities to use mutations across numerous flaviviruses.

To date, there are only two flavivirus-conserved hydrophobic amino acids in the EDI-EDII hinge region that have been thoroughly studied and shown to support the engineering of attenuating mutations: E-I130 and E-I135. These mutations have been found to significantly reduce the infectivity of DENV-2 in cell culture and in intrathoracically injected mosquitoes (Butrapet et al., 2011). However, it is important to note that these residues have been looked at specifically in DENV and neither the E-I130 residue nor the E-I135 residue is suited for the rational design of candidate flavivirus LAVs because mutations of either residues can lead to the lethal phenotype following transfection of Vero cells, the recommended cell type for the manufacturing of human vaccines (Butrapet et al., 2011; Kiesslich and Kamen, 2020). This further highlights the

importance of our discovery as both the attenuated E-A54S and E-Y201P mutants were recovered from the transfection of Vero cells with viral RNA transcripts.

Additional residues in flavivirus genomes have been studied, however, these residues have received less attention. In a study conducted by Goo et al. in 2006, it was determined that a single mutation at residue 198 in the WNV E protein resulted in a 70-fold increase to neutralizing affects by monoclonal antibodies simply by targeting a hidden epitope in this fusion loop. This introduced mutation caused the hidden epitope to become exposed and the conformation change reduced viral stability. When this study was replicated in DENV, the same result occurred. These results suggest that this one single residue can modulate the structural ensemble in flaviviruses, and further proves that a single mutation conserved among many flaviviruses could potentially be used for technology of flavivirus common attenuation methods (Goo et al., 2006).

In the Goo et al. study, they examined multiple residues in the EDI and EDII hinge region but determined residue 198 to have significance in structure modulation. When residue 198 was mutated in WNV and observed *in vivo*, there was a significantly lower mortality rate in mice infected with mutant T198F (2/15) compared to the wild-type WNV (13/15). In fact, they determined the mutated virus was suppressed early in infection and therefore was attenuated for neuroinvasion and neurovirulence. It was determined that this mutation caused a rapid decay of virions, likely from a conformational alteration that led to irreversible changes on the E protein which were incompatible with infectivity. Furthermore, when examining naturally occurring isolates, residue 198 was a threonine in WNV and a phenylalanine in the corresponding residue of DENV and ZIKV, displaying conservation among these flaviviruses (Goo et al., 2006). However, while this mutation attenuated the WNV phenotype *in vivo*, it was ineffective for DENV, highlighting that would not be a useful residue for a flavivirus common attenuation mechanism.

Another study conducted by Butrapet et al. in 2011 examined numerous mutations in the E protein of flaviviruses, some of which overlap the study discussed in this thesis. The Butrapet et al. study found substitutions at E-Q52, E-A54, and E-E133 reduced infectivity within mammalian cells and even altered the fusion pH threshold. Similar effects were observed when looking at mutations at E-F193, E-G266, and E-G281, where they saw an affected fusion pH threshold as well as decreased replication in cells. By the end of the study, they concluded that E-Q52, E-A54, E-E133, E-L135, E-F193, E-G266, E-I270, E-T280, and E-G281 were determinants for viral infection (Butrapet et al., 2011). This study provided additional basis for the investigation of E-A54, E-I135, and E-L281 for this thesis.

While it has been determined that just a single residue can have large impacts on infectivity, overall, it is accepted that mutations in the hinge region may disrupt the receptor-ligand interaction. In another study looking at regions in the E protein rather than singular mutations, it was determined that a range of mutations within region E52-E54, region E129-E136, region E191-E200, and region E266-E284 can cause loss of neurovirulence or neuroinfectivity. Furthermore, looking specifically at JE, mutations in similar regions (E270 and E138) and in JE/YF chimeras (E138) cause similar effects (Hurrelbrink and McMinn, 2001). However, the mutation E138K, does not attenuate the virulent phenotype in WNV, displaying that it could not be used for a common attenuation mechanism (Kaiser et al., 2019 [b]). Discovery of these conserved residues and how mutations impact them are an integral part of creating a candidate LAV. All residues investigated for the purpose of this thesis are conserved among twenty flaviviruses, creating the foundation for a candidate flavivirus common attenuation method.

Future directions

The attenuated phenotype of WNV evaluated in this study provides the basis for rational design of candidate WN LAVs. However, additional studies should be conducted to further assess the safety and efficacy of any LAV design, as well as the immunogenicity.

We recognize single or double mutations are unlikely to prove a complete and safe level of attenuation, and mutations of the E-A54 and E-Y201 residues will inevitably be evaluated with other mutations in the non-structural genes. Previous research has shown that WNV NS1 in conjunction with the E protein are particularly important to the immune response (Whiteman et al., 2010). Therefore, combining the mutations in the E protein with mutations to the NS1 protein, perhaps in the three highly conserved residues of NS1, could further attenuate and stabilize the candidate LAV. Additionally, mutations in the NS4B and/or NS5 proteins could also be used to create a candidate LAV; previous studies have shown a single substitution in the NS4B protein attenuated the neuroinvasive and neurovirulent phenotype in mice and a single mutation in the NS5 protein has shown an attenuated phenotype in mice as well (Botha et al., 2008; Kaiser & Barrett, 2019). Furthermore, the JEV SA14-14-2 strain has 19 mutations that are attributed to the attenuated phenotype, including a combination of non-structural protein and structural protein mutations (Huang et al., 2019). Combining non-structural protein mutations with E protein mutations will likely enhance the robusticity and safety of a candidate LAV for WNV.

Additional studies to characterize the immunogenicity of these attenuated WNV mutants will be necessary as well. A future investigation could use a mouse model which has been immunized with the discovered attenuated mutants. Sera from these immunized mice could then be transferred to immunologically naïve mice to evaluate if protection is demonstrable and

consistent. Collecting sera from the inoculated naïve mice would allow for serum neutralizing antibody analysis, allowing for a further analysis of immunogenicity *in vivo*.

Finally, future studies would look at these mutations in other flaviviruses, likely starting with other viruses in the JEV serocomplex. This study aimed to identify a common attenuation mechanism using highly conserved residues. The residues selected for use in this study are conserved across twenty different flaviviruses, meaning they are important to viral structure function, therefore, translating these mutations to other flaviviruses has significant potential to cause an attenuated phenotype and further investigate this mechanism as a common attenuation system.

Concluding remarks

The attenuation of the virulent phenotype of WNV in intraperitoneally challenged outbred Swiss mice at 500 PFU provides the basis for the application of mutations at the E-A54 and E-Y201 residues for the rational design of candidate WN LAVs. While a preliminary *in vivo* study predicated the use of 500 PFU inoculum for the mice in this thesis, a separate previous study conducted by Pletnev et al. in 2006 identified that lethal wt WNV PFU for 5-day old Swiss Webster mice is 10 PFU, and 3-day old Swiss Webster mice have a much lower threshold of 1 PFU (Pletnev et al., 2006). The mice used for this study were 4 weeks old, which could explain the higher PFU requirement. With the chosen 500 PFU, this study showed a mortality average of 6.9 dpi. A previous study performed by Huang et al. in 2005 identified significant neurovirulence for newborn mice by 6 dpi (Huang et al., 2005).

By looking specifically at mutations in the E protein, our work builds on published studies that have previously identified determinants of virulence in the flavivirus E protein (Huang et al.,

2005). Overall, the attenuation of the mouse neuroinvasive phenotype is important for the safety of LAVs for these encephalitic flaviviruses. The SA14-14-2 vaccine, which is a safe and highly effective LAV for flaviviruses in the JEV serocomplex, has been shown repeatedly to be attenuated for the mouse neurovirulence phenotype. Therefore, the further evaluation of the attenuated phenotype will require additional demonstration of attenuation of the mouse neurovirulence phenotype.

This thesis outlines mutations that could lead to the rational design of not only a WN LAV but potentially LAVs for other flaviviruses. Data gathered during this study proves that E-Y201P is a fully attenuated mutation for WNV and that E-A54S is a partially attenuated mutation, both of which are highly conserved flavivirus genome residues. The compilation of knowledge and discovery culminated into the creation of this thesis gives a solid foundation for future work that looks to study these attenuated mutants further and presents the groundwork for development of candidate LAVs for WNV and/or other flaviviruses.

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